Fusion of the tissue factor extracellular domain to a tumour stroma specific single-chain fragment variable antibody results in an antigen-specific coagulation-promoting molecule

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Solid tumours growing beyond a size of 1–2 mm in diameter induce supporting connective tissue structures, the tumour stroma, comprising activated fibroblasts and newly formed blood vessels, embedded in an extracellular matrix. The selective destruction of this tissue or the inhibition of its function (e.g. tumour neoangiogenesis) may result in the destruction of tumour nodules, thus providing novel opportunities for tumour therapy. Our approach aims at an antibody-mediated induction of coagulation in tumour nodules to cut off their blood supply. As a target structure the fibroblast activation protein (FAP) is used, which is specifically and abundantly expressed on the activated fibroblasts of the tumour stroma. We constructed a fusion protein comprising a single-chain module of a FAP-specific humanized antibody [single-chain fragment variable (scFv) OS4] and the extracellular domain of human tissue factor. The fusion protein, designated TFOS4, was produced in the *Proteus mirabilis* protoplast expression system with a yield of 15 μg/ml. Biochemical characterization of TFOS4 revealed high-affinity binding to cellular FAP. Further, TFOS4 bound to factor VIIa and also exerted allosteric activation of factor VIIa. A complex of TFOS4 and factor VIIa bound to FAP-expressing cells efficiently generated activated factor X. Finally, cell-bound TFOS4 selectively induced plasma coagulation, implying its activity under physiological conditions, notably with relevant concentrations of coagulation factors and their natural inhibitors. These findings suggest that TFOS4 has the potential to increase the procoagulant state in a cell-type-specific fashion. No systemic coagulation or side effects were observed when TFOS4 was injected intravenously into normal mice, indicating the biosafety and specificity of the recombinant protein.

Key words: immunotherapy, L-form bacteria, scFv.

INTRODUCTION

In recent years, encouraging novel anti-tumour strategies have been described, some of which are based on interference with tumour-supplying structures, e.g. by selective destruction of tumour vasculature or tumour stroma, or inhibition of the formation of these tissues [1]. Destruction of the supporting structures should cut off tumour nutrition and oxygen supply and thus result in necrosis of the tumour. A further advantage of targeting the stroma instead of the malignant tumour cells is that the tumour stroma, as a non-transformed tissue component, is genetically stable. Thus development of therapy resistance as a result of somatic mutations is less likely. Various selective markers for tumour endothelium and tumour stroma have been described, such as $\alpha V\beta 3$ integrin and the fibronectin splice variant B-FN [2], which may be used for an antibody-mediated targeting of these tissues [3].

Our current investigations are focused on fibroblast activation protein (FAP), which is selectively expressed on activated fibroblasts of the tumour stroma in a wide spectrum of epithelial tumours [4]. In normal tissues, FAP is only detectable during wound healing and embryogenesis. Because of its abundant, but selective, expression and its tissue location, FAP is considered to be easily accessible to antibodies via the blood circulation, and therefore suitable for diagnostic and therapeutic 'tumour stroma targeting'. This concept has been corroborated by phase 1 *in vivo* targeting studies with a FAP-specific monoclonal antibody (mAb), F19 [5].

For destruction of the tumour stroma, it is conceivable to use antibody-targeted effector functions, e.g. immunotoxins [6], radioisotope-labelled antibodies or cellular cytotoxicity mediated by bispecific antibodies [7]. A promising novel approach makes use of the physiological process of coagulation as an anti-tumour effector function: Huang et al. [8] and Ran et al. [9] reported the induction of tumour regression in two different mouse models (neuroblastoma and Hodgkin tumour) caused by an antibody-mediated induction of coagulation by human tissue factor (hTF), which led to the destruction of tumour vasculature due to microthrombosis. Wild-type hTF is a membrane protein which, in complex with the soluble factor VIIa (FVIIa), can activate factor X (FX) as one of the initial steps in the extrinsic coagulation cascade, leading finally to fibrin deposition and thrombus formation in the vasculature [10] (Figure 1A). hTF needs a close contact with the cell membrane to perform its action. Therefore

Abbreviations used: BCIP, 5-bromo-4-chloroindol-3-yl phosphate; Chromozyme tPA, Chromozyme for tissue plasminogen activator; FAP, fibroblast activation protein; FVIIa (etc.), factor VIIa (etc.); IMAC, immobilized metal affinity chromatography; mAb, monoclonal antibody; PFA, PBS (pH 7.5), 2% (v/v) fetal calf serum and 3 mM sodium azide; scFv, single-chain fragment variable; TF, tissue factor; hTF, human TF; tTF, trunacted TF; VCAM-1, vascular cell adhesion molecule-1; Xase, factor X protease.

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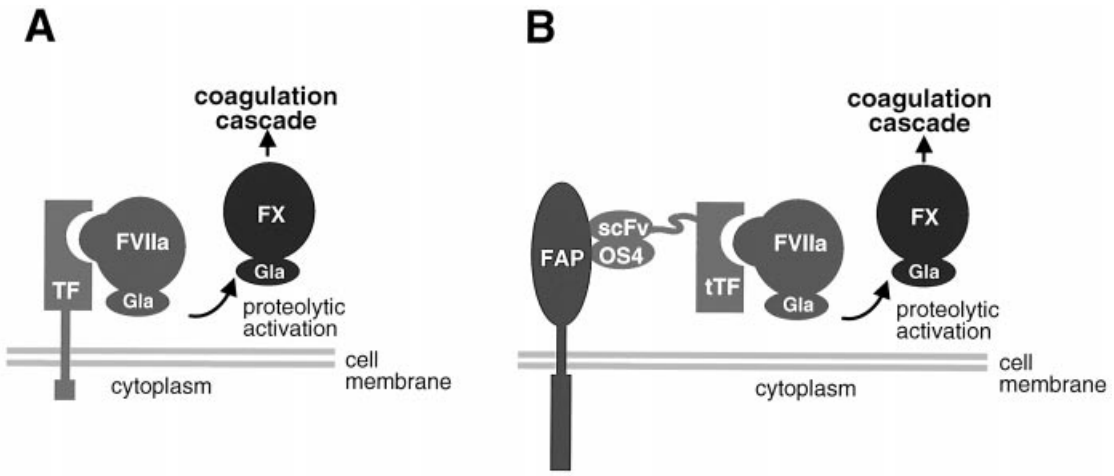

Figure 1 Coagulation induction by (A) wild-type TF and (B) TFOS4

(**A**) The membrane anchor of the wild-type hTF 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 [10]. (**B**) Antibody-mediated targeting and activation of FX by tTF using the scFv–tTF fusion protein TFOS4.

the soluble recombinant form of the extracellular hTF domain [truncated TF (tTF)] is virtually inactive [11]; however, antibody-mediated contact of tTF with the cell membrane restores its activity as a coagulation activator [8,9].

In their experiments, Huang and co-workers [8] used for tumour targeting the experimentally created targeting structure of the MHC II, artificially overexpressed on the tumour and tumour endothelium. In the approach of Ran and co-workers [9] the murine vascular cell adhesion molecule-1 (VCAM-1), a tumour endothelium marker, was targeted with an anti-VCAM-1 murine mAb. For targeting of hTF, different antibody constructs were used: Huang et al. [8] applied a bispecific antibody (anti-MHC II/anti-hTF) consisting of two chemically linked Fab fragments, and Ran et al. [9] employed a complete murine mAb chemically conjugated with hTF.

We have developed these ideas further by applying antibody-mediated tTF targeting and coagulation induction to a clinically relevant target molecule using the tumour stroma marker FAP (Figure 1B). Moreover, a fusion protein (TFOS4) in a single-chain antibody format [(single-chain fragment variable (scFv)] was constructed, which consists of a FAP-specific, humanized version of the mAb F19 engineered by CDR (complementarity-determining region) grafting and the human tTF. The antigen-binding as well as the tTF moiety of TFOS4 are functional, and the biochemical characterization of both activities is described. Moreover, we reveal that binding of TFOS4 to FAP on cell surfaces reconstitutes the activity of tTF and leads to a specific induction of plasma coagulation under physiological conditions.

EXPERIMENTAL

Reagents

All chemicals were obtained from Sigma, unless specified otherwise. Restriction enzymes and chromogenic peptide substrates were from Roche Diagnostics. FVIIa was generously donated by Novo Nordisk Pharma G.m.b.H. FX was purchased from Enzyme Research Laboratories Inc., and the hTF preparation (Thromborel S®) was from Behring Diagnostic G.m.b.H. The full-length cDNA for hTF was a gift from M. B. Taubmann (Mount Sinai School of Medicine, New York, NY, U.S.A.). Human fibrosarcoma cells expressing FAP (HT1080-FAP^{wt}) were generated at Boehringer Ingelheim Pharma KG by J. E. Park. Citrated human plasma was prepared from adult donors.

Construction of the tTF and scFv OS4 fusion protein TFOS4

The eukaryotic expression vector pLXSN-TF [12] was digested with *Xho*I/*Bam*HI, and the 1 kb fragment obtained was used as a template for PCR. TF-linker protein was amplified by *Pwo* polymerase with upstream primer 5′-GTG GCC ATG GCT TCA GGC ACT ACA-3′ (s2022) and downstream primer 5′-CTG AGC CGT GGA TCC GCC GCC ACC CGA CCC ACC ACC GCC AGA GCC ACC GCC TCC TTC TCT GAA TTC CCC-3′ (s1963), increasing the annealing temperature after the first five cycles from 42 °C to 62 °C for a further 25 cycles. A *Bgl*II restriction site was introduced in the N-terminal sequence of scFv OS4 by PCR, using the *Nco*I/*Not*I restriction fragment of plasmid pEA40 as template and 5′-AGG CAG ATC TCA GGT GCA ACT AGT G-3′ (s2023) and 5′-GCG TGC TTC TGA AAA TTC-3′ (Yol-PCR [13]) as upstream and downstream primers respectively. The TFOS4 expression plasmid was generated by ligation of the *Nco*I/*Bam*HI fragment of the first PCR and the *Bgl*II/*Hin*dIII fragment of the second PCR into the *Nco*I/*Hin*dIII-restricted pEA40 plasmid. The sequence of TFOS4 was confirmed by DNA sequencing using the ALF-Pharmacia protocol (Pharmacia).

Expression of recombinant proteins using L-form cells, and protein purification

The generation of expression strains and culture of *Proteus mirabilis* L-form cells was performed as described [14,15]. Briefly, stable protoplast-type L-form cells, carrying the expression plasmid, were grown as a shaking flask culture at 30 °C in 50 ml of brain heart infusion medium (BHI; Difco), supplemented with 5 % (w/v) yeast extract and 50 mg/l kanamycin. Recombinant-protein synthesis was induced with 0.5 mM isopropyl β-D-thiogalactoside at mid-exponential growth phase. After a pro-

duction period of 10 h, the supernatant of the culture was frozen in liquid nitrogen or dialysed against PBS (pH 8.0) prior to chromatographic purification. Proteins with a His_6-tag at the C-terminus were purified by immobilized metal affinity chromatography (IMAC) as described in [15]. Size-exclusion chromatography was optionally performed with a G300PW$_{XL}$ column (model 08021; Tosohaas) at room temperature with PBS (pH 8.0) or HBS/Ca^{2+} [20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$] as elution buffer. The homogeneity of the recombinant proteins in the elution fractions was analysed by SDS/12 %-PAGE and subsequent Coomassie Brilliant Blue staining. Concentrations of the TF derivatives were determined by comparison of their Western blot signal intensities with those of serial dilutions of highly purified tTF and scFv preparations [15]. TF activities of the recombinant derivatives were compared with that of Thromborel S® by characterizing allosteric FVIIa activation towards the peptide substrate Chromozyme tPA (Chromozyme for tissue plasminogen activator) (see below).

Western blot analysis

For Western blot analysis of recombinant proteins, aliquots of L-form cell culture supernatants were separated on an SDS/12 %-polyacrylamide gel. Low-molecular-mass markers were supplied by Pharmacia. Separated proteins were electroblotted on to a nitrocellulose membrane (Sartorius) and unspecific binding was blocked with Tris-buffered saline (pH 7.5) containing 5 % (w/v) milk powder and 0.05 % (v/v) Tween 20. An anti-c-myc specific murine mAb from hybridoma supernatant in blocking buffer was used as a primary detection antibody [16]. Proteins that lack the c-myc tag were detected by a sheep IgG fraction against hTF (CL20150A; Cedarlane Laboratories) in a 20 % (v/v) lysate of *Proteus mirabilis* L-form cell culture in blocking buffer. Secondary antibodies, conjugated with alkaline phosphatase, were either mouse Fc (Jackson Immuno Research Laboratories) or sheep Fc (A-8062; Sigma) specific. Antibody–antigen complexes were detected with NitroBlue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate (BCIP) as enzyme substrates.

Allosteric FVIIa activation and determination of K_D (app) by a direct chromogenic assay

The extracellular domain of hTF acts as allosteric activator of FVIIa [17]. Therefore the increased amidolysis of Chromozyme tPA by the TFOS4–FVIIa complex was used to quantify tTF activity [11,18]. A dilution series of TFOS4 was incubated for 10 min at room temperature with 175 μl of 20 nM FVIIa in FVIIa reaction buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 1 % (w/v) BSA]. Amidolysis of the chromogenic substrate was initiated by the addition of 25 μl of 2 mM Chromozyme tPA solution. Initial rates of substrate amidolysis were determined by monitoring absorbance at 405 nm as a function of time, using a microplate reader (model 3550; Bio-Rad). The concentration of TF reference solution was adjusted to 12 nM by solubilization of Thromborel S® sample in 1 ml of water. Plots of maximum reaction rate (V_{max}) against dilution of TFOS4 solution revealed a typical saturation curve. The TF concentration was calculated from the slopes of the linear region of the plots by comparison with those obtained from dilution series of Thromborel S®.

The same amidolysis assay was used to determine the K_D (app) of the TFOS4–FVIIa complex, as described in [19]. In contrast with the quantification of TF activity, a lower concentration of FVIIa (1 nM) was incubated for 15 min at room temperature with increasing concentrations of IMAC-purified TFOS4. Chromogenic substrate amidolysis was started, and the maximum rate of amidolysis was determined by kinetic protocol of a microplate reader (model 3550; Bio-Rad) at 405 nm, as described above. From four independent experiments with doublet samples, FVIIa activity was measured and plotted against TFOS4 concentration. Using a hyperbolic curve-fitting algorithm (GraphPad Prism), K_D (app) was calculated from the titration curve.

Binding of TFOS4 to eukaryotic cells

Cell-binding studies of scFv OS4 and TFOS4 to stable transfectants of HT1080 cells expressing FAP (HT1080-FAP^{wt}) and to FAP-negative control cells (HT1080) were performed as described [15]. Briefly, IMAC-purified scFv antibody or TFOS4 fusion protein was incubated with blocking solution [5 % (w/v) milk powder/PBS (pH 7.5), 1:2, v/v] for 15 min at room temperature. Samples were centrifuged twice at 10000 ***g*** for 10 min, and the cleared supernatant was further diluted with PFA [PBS (pH 7.5), 2 % (v/v) fetal calf serum and 3 mM sodium azide] and incubated for 1 h at room temperature with 10^5 eukaryotic cells per well (75 μl) in a microtitre plate. In all further steps, 3 mM sodium azide was used to block active internalization of membrane proteins. Subsequently, the cell suspensions were incubated with anti-c-myc mAb and peroxidase-conjugated anti-mouse antibody (Jackson Immuno Research Laboratories) for 30 min at room temperature to detect antigen–antibody complexes on the cell surface. After each incubation, cells were washed three times with PFA. The colorimetric reaction was performed with 2,2′-azidobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) peroxidase substrate, and absorbance at 405 nm was determined after 15 min.

Alternatively, cell-bound TFOS4 was detected by allosteric activation of FVIIa. After antigen binding of TFOS4, cells (10^5 cells/75 μl) were washed three times with FVIIa reaction buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$ and 1 % (w/v) BSA] and incubated in 100 μl of 100 nM FVIIa for 1 h at room temperature. Cells were washed three times and resuspended in 75 μl of FVIIa reaction buffer supplemented with 1 mM Chromozyme tPA. After incubation at room temperature for 3 h, amidolysis of chromogenic substrate was determined using a microplate reader (model 3550; Bio-Rad) at 405 nm. Colorimetric signals from peroxidase or amidolysis reactions were plotted against TFOS4 concentration, and the half-maximum binding of scFv OS4 or TFOS4 was calculated using a curve-fitting algorithm (GraphPad Prism).

Measurement of FX activation

The proteolytic activation of FX by the TFOS4–FVIIa complex was analysed in a coupled amidolytic reaction using the hydrolysis of Chromozyme X (Roche Diagnostics) as an assay of FXa activity [19–22]. As a reference for FX protease (Xase) activity, increasing concentrations of hTF preparations (Thromborel S®) were incubated for 10 min at 37 °C with a saturating concentration of FVIIa (300 nM) in 120 μl of FVIIa reaction buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$ and 1 % (w/v) BSA]. The determination of the Xase activity of the cell-bound TFOS4–FVIIa complex was performed with HT1080-FAP^{wt} cells and HT1080 control cells (10^6 cells/100 μl), saturated consecutively with TFOS4 (100 nM) and FVIIa (300 nM) in 100 μl of PFA or FVIIa reaction buffer respectively. Prior to this treatment, the intrinsic hTF activity of HT1080 cells was optionally blocked by incubation of the cells with 0.8 μg/μl anti-hTF IgG fraction (Cedarlane Laboratories) in 100 μl

of PFA. After each incubation, cells were washed three times with PFA or FVIIa reaction buffer, and increasing cell concentrations in 120 μl aliquots of FVIIa reaction buffer were used to activate FX.

The proteolytic reaction was started by the addition of 40 μl of 2 μM FX in FVIIa reaction buffer, and the mixture was incubated at 37 °C with agitation. Every 2 min, aliquots of 48 μl of the reaction were taken and further FX activation was blocked by the addition of 12 μl of ice-cold 500 mM EDTA and storage on ice until further treatment. The activity of proteolytically activated FX was determined from initial amidolysis rates after the addition of 15 μl of 2 mM chromogenic substrate (Chromozyme X). The FXa concentration was determined by comparison with identical assays using known concentrations of FXa preparations (New England Biolabs). The maximum FXa activation rate (V_{max}) was plotted against the hTF or FAP density of HT1080-FAP^{wt} cells (1.5×10^6 FAP molecules/cell; D. Moosmayer and J. F. Rippmann, unpublished work), and the slopes of the linear regressions of different hTF preparations, which correspond to the k_{cat}, were compared.

The Michaelis–Menten constant (K_m) was determined by incubation of TFOS4 (100 nM)- and FVIIa (300 nM)-saturated cells (4.8×10^4 cells/160 μl), as described above, with increasing concentrations of FX. The rate of FXa generation at a given FX concentration was normalized to the extrapolated maximum rate of FXa generation in a given experiment. The proportion of TFOS4-mediated FX activation was determined from the difference in the FXa generation rate between HT1080-FAP^{wt} and HT1080 control cells. Data were analysed by a curve-fitting algorithm (GraphPad Prism) on the basis of the Michaelis–Menten equation.

Alternatively, a TF preparation (Thromborel S®) with saturating concentration of FVIIa (250 nM) was used as reference for wild-type TF activity.

One-stage clotting assay

The coagulation-promoting activity of TFOS4 was further characterized by the physiological reaction of clot formation [21,22]. HT1080-FAP^{wt} cells and HT1080 control cells (10^6 cells) were incubated optionally with 0.8 μg/μl anti-hTF IgG fraction (Cedarlane Laboratories) in 100 μl of PFA to block intrinsic TF activity. Further, cells were saturated with 100 nM of milk-blocked TFOS4 in 100 μl of PFA as described above. After each step, cells were washed with PFA. After the final binding step, cells were washed three times with FVIIa reaction buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 1 % (w/v) BSA], and 10^6 cells were resuspended in 210 μl of FVIIa reaction buffer. An aliquot of 70 μl was prewarmed at 37 °C with an equal volume of 20 mM $CaCl_2$, and the clotting reaction was started by the addition of 70 μl of citrated standard human plasma. Reactions were performed in triplicate, and clotting time was recorded.

A standard curve of coagulation time was generated using Thromborel S®. Triplicate samples of serial dilutions of Thromborel S® in FVIIa reaction buffer in a volume of 70 μl were incubated at 37 °C with 70 μl of 20 mM $CaCl_2$ for 10 min. Finally, 70 μl of citrated standard human plasma was added, and the clotting times of the samples were recorded.

RESULTS AND DISCUSSION

Construction and expression of recombinant proteins in *Proteus mirabilis* L-form cell cultures

The concept of our antibody–TF fusion protein aims to direct procoagulant activity to tumour sites via the activated fibroblasts of the tumour stroma (Figure 1B). Two formats of a fusion protein were constructed: NH_2-scFv–TF-COOH and NH_2-TF–scFv-COOH. In initial binding experiments the former construct showed unspecific binding to eukaryotic cells (results not shown). This was unexpected, as scFv OS4 has been successfully fused previously with different peptides at its C-terminus without affecting its binding specificity. Therefore subsequent studies were focused on the latter construct (TFOS4), which exerted specific antigen binding which is characterized in detail below. The bacterially expressed soluble TFOS4 fusion protein contained the following domains, as depicted schematically in Figure 2: an N-terminal signal sequence for secretion, the extracellular domain of hTF fused via a $(GGGGS)_3$-linker to the N-terminus of scFv OS4, as well as a c-myc epitope and a His_6-tag for detection and purification respectively. In view of a possible clinical use, the TFOS4 protein is expected to have a low antigenic potential, because it consists mainly of human sequences. Nevertheless the further development of TFOS4 as a clinical reagent requires the generation of a construct without c-myc- and His_6-tags.

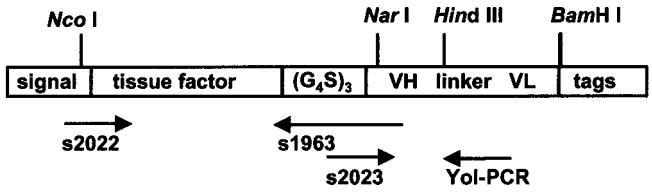

Figure 2 Features of the cDNA encoding the 60 kDa scFv OS4 TF fusion protein TFOS4

Primer numbers, annealing sites and the direction of elongation by *Pwo* polymerase are indicated by the arrows. Restriction sites used for the cloning are indicated. The *OmpA* signal sequence is needed for bacterial secretion of the protein. The extracellular domain (residues 1–219) of hTF is fused by a $(GGGGS)_3$-linker to the N-terminus of the variable heavy region (VH) domain of the scFv OS4. The variable light region (VL) chain of the antibody is linked by the YOL-PCR-linker to the VH domain, and the C-terminus of the protein is tagged by c-myc epitope for detection and with His_6 for purification.

Stable protoplasts from *Proteus mirabilis* (LVI) were used for the production of TFOS4, scFv OS4 and the extracellular domain of hTF (tTF). This strain allows high-production yield and active product secretion into the culture supernatant [15]. TFOS4 and tTF are produced as soluble, active proteins in LVI supernatant (Figure 3), with a production yield of 14.6 ± 0.7 mg/l and 42.7 ± 1.1 mg/l of culture volume respectively. The apparent molecular masses determined by Western blot analyses (Figure 3) were 60 kDa for TFOS4, 31 kDa for tTF and 31 kDa for scFv OS4, which correspond to the values calculated from the deduced amino acid sequences. Active TFOS4 could be partially purified (90 % homogeneity) from 100 ml of supernatant by IMAC with a yield of 300 μg (Figure 3).

Fusion protein TFOS4 shows high-affinity binding to antigen and to FVIIa

To meet more closely the physiological conditions, a cell-binding assay instead of an ELISA on immobilized antigen was chosen to measure the affinity of binding of the antibody moiety of TFOS4 to FAP. HT1080-FAP^{wt} cells and HT1080 control cells were incubated with increasing amounts of TFOS4, or of scFv OS4 as a control. After washing the cells, antigen binding of the recombinant scFv derivatives was detected via the c-myc tag or, in the case of TFOS4, additionally via FVIIa binding and its

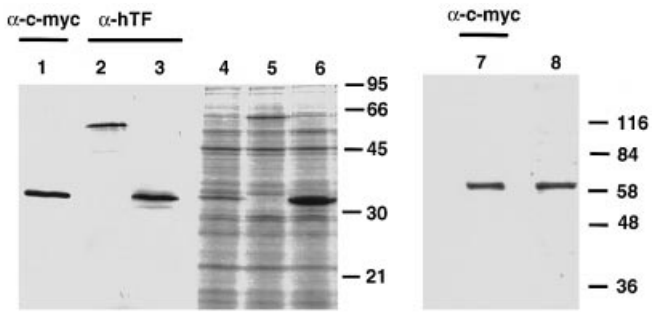

Figure 3 Western blot and Coomassie Brilliant Blue stained SDS/polyacrylamide (10% or 12%) gels of recombinant proteins from L-form cells of *Proteus mirabilis*

Lanes 1–6 were loaded with 10 μl portions of supernatants from induced overnight cultures of *Proteus mirabilis* LVI, producing scFv OS4 (lanes 1 and 4), TFOS4 (lanes 2 and 5) and tTF (lanes 3 and 6). Portions of purified TFOS4 of 100 ng (lane 7) and 900 ng (lane 8) were also applied. After separation of proteins, the gel was electroblotted on to nitrocellulose (lanes 1–3 and 7) or stained with Coomassie Brilliant Blue (lanes 4–6 and 8). Recombinant proteins were detected by anti-c-myc (α-c-myc) murine mAbs (lanes 1 and 7) or by an IgG fraction against hTF (α-hTF; lanes 2 and 3) as marked above the lanes. Antigen–antibody complexes were stained by enzymic reaction of alkaline phosphatase-conjugated secondary antibodies with BCIP and X-Phosphate as substrates. Molecular masses (kDa) of marker proteins are indicated at the right.

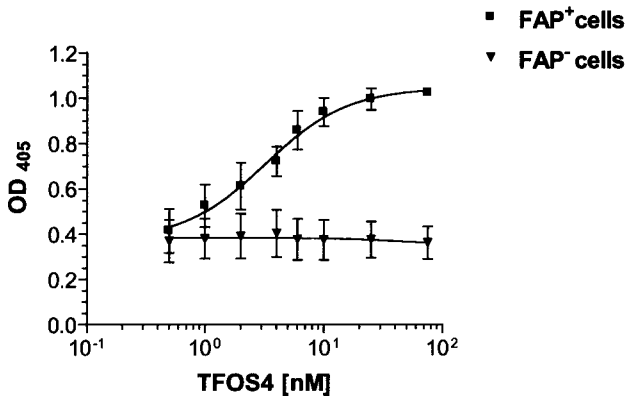

Figure 4 Titration curve of TFOS4 bound to FAP-overexpressing cells or FAP-negative control cells

HT1080-FAP^{wt} cells and HT1080 control cells were incubated in duplicate with IMAC-purified and milk-blocked TFOS4. Cell-bound TFOS4 was saturated by incubation with FVIIa. All incubations were performed for 1 h at room temperature, and after each step cells were washed three times with the corresponding buffer. The chromogenic reaction was initiated by the addition of Chromozyme tPA, and substrate amidolysis was determined using a microplate reader (Bio-Rad) to measure absorbance (OD) at 405 nm after a 3 h incubation at room temperature. The data are means of three independent experiments; bars denote S.E.M. Data were fitted to a binding equation and half-maximal cell binding was calculated (GraphPad Prism).

amidolytic activity towards a chromogenic peptide substrate (Chromozyme tPA).

As expected, the recombinant antibodies bound specifically to FAP-expressing cells and not to control cells. From saturation binding curves (Figure 4), the half-maximal binding concentrations of the recombinant antibody moieties were determined. For TFOS4, nanomolar antigen affinities of 7.0 ± 0.7 nM and 3.2 ± 1.6 nM were measured via c-myc (results not shown) and FVIIa (Figure 4) detection respectively. Therefore TFOS4 possesses an affinity comparable with that of scFv OS4, which has

Table 1 Biochemical characterization of TFOS4

The biochemical constants of the fusion protein TFOS4 are compared with those of scFv OS4, the preparation of wild-type hTF (Thromborel S®) and data from the literature. Data are means $\pm$ S.E.M. of three to five independent experiments (n).

	Half-maximal cell binding (nM)	FVIIa binding: K_D (app) (nM)	FX activation: K_m (nM)	Catalytic efficiency: k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
scFv OS4	9.4 ± 0.9 (3)	–	–	–
Thromborel S®	–	–	77 ± 5 (3)	26
TFOS4	4.7 ± 0.9* (5)	4.0 ± 1.3 (4)	182 ± 31 (3)	0.61
tTF [19]	–	4.0	–	–
hTF [21]	–	–	73	–

* Mean of half-maximal cell binding determined by detection via c-myc-specific antibodies ($n = 2$) or FVIIa activity ($n = 3$).

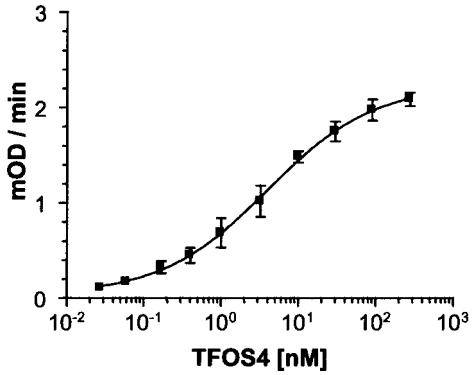

Figure 5 Determination of the K_D (app) of the soluble TFOS4–FVIIa complex

A constant concentration of FVIIa was incubated in duplicate with increasing concentrations of IMAC-purified TFOS4. The maximum rate of amidolysis of Chromozyme tPA was determined using a microplate reader (Bio-Rad) to measure absorbance at 405 nm (mOD, milli-absorbance units). Data are means from four independent experiments; bars denote S.E.M. Using a hyperbolic curve-fitting algorithm, a K_D (app) value of 4.0 ± 1.3 nM was calculated (GraphPad Prism).

half-maximal binding on eukaryotic cells at 9.4 ± 0.9 nM (Table 1). Further, from the saturating concentrations of TFOS4 and scFv OS4, a FAP density of 1.3×10^6 molecules/cell was determined, which confirmed previous results with an iodinated FAP-specific mAb (D. Moosmayer, unpublished work). Therefore the antigen-binding activity of the antibody moiety of TFOS4 remains unchanged after fusion of the extracellular domain of hTF to the N-terminus of scFv. Moreover, TFOS4 binds efficiently and activates FVIIa after association with the membrane protein FAP (Figure 4).

The binding affinity of TFOS4 for FVIIa in solution was determined by measuring the rate of amidolysis of the peptide substrate Chromozyme tPA by the soluble TFOS4–FVIIa complex (Figure 5). From the titration curve of four independent experiments (Figure 5, Table 1), a K_D (app) of 4.0 ± 1.3 nM was calculated, which is in good agreement with a K_D (app) of 4 nM for the soluble tTF reported in [19].

Taken together, both the antigen and the FVIIa affinities of the TFOS4 molecule are within the same nanomolar order of magnitude. The physiological concentration of FVII/FVIIa in plasma is approx. 10 nM [23]. Therefore TFOS4 should be sufficiently associated with FVIIa after incubation with plasma,

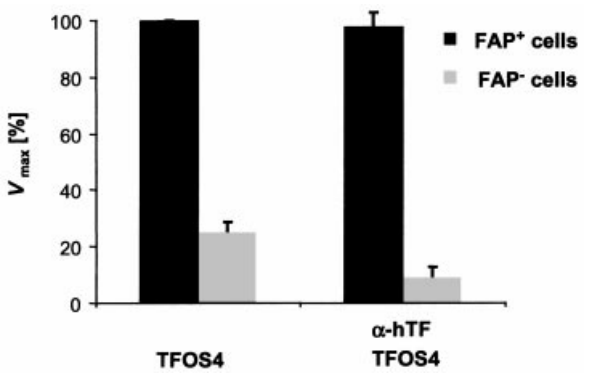

Figure 6 Activation of FX by cell-bound TFOS4–FVIIa complex

HT1080-FAP^{wt} cells and HT1080 control cells were incubated with saturating concentrations of TFOS4 and FVIIa. After each step, cells were washed three times with the corresponding buffer, and increasing cell concentrations were incubated in duplicate with FX. The data are means of three independent experiments with or without pretreatment of HT1080-FAP^{wt} and HT1080 control cells with an IgG fraction against hTF. Data are expressed as a percentage of the maximal FXa activation rate in HT1080-FAP^{wt} cells treated with TFOS4 alone. Bars indicate S.E.M.

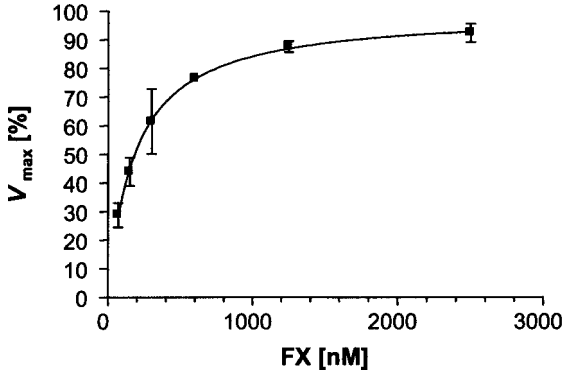

Figure 7 Michaelis–Menten kinetics of FXa generation by TFOS4–FVIIa complex specifically bound to FAP-positive cells

HT1080-FAP^{wt} cells and HT1080 control cells were saturated with TFOS4 and FVIIa, and incubated in duplicate with increasing concentrations of FX. The rate of FXa generation was determined by a coupled amidolysis assay using Chromozyme X as substrate. The rate of FXa generation at a given FX concentration was normalized for the extrapolated maximum rate of FXa generation. In three independent experiments, the mean of the FXa generation rate in HT1080-FAP^{wt} cells minus the mean of the FXa generation rate in HT1080 control cells gave the FAP-specific Xase activity of the cell-bound TFOS4–FVIIa complex for a given FX concentration. A K_m value of 182 ± 31 nM could be determined from the half-maximal rate of activation of FX (v) of the typical Michaelis–Menten equation using a curve-fitting algorithm (GraphPad Prism). Bars indicate S.E.M.

and the amount of active, cell-bound TFOS4–FVIIa complex will be largely determined by the FAP density of the target cells.

Cell bound TFOS4–FVIIa complex is an active protease

FX is the natural substrate of the hTF–FVIIa complex [24]. It has been shown that both the steric orientation of the hTF–FVIIa–substrate complex [25] and its close contact with negatively charged phospholipids are of critical importance for the efficient proteolytic activation of FX ([26]; see Figure 1A). Therefore the Xase activity of the soluble TFOS4–FVIIa complex was compared with the activity of TFOS4–FVIIa complex that was membrane-anchored by binding to FAP. HT1080-FAP^{wt} cells were saturated consecutively with TFOS4 (100 nM) and FVIIa (300 nM) and subsequently washed to remove unbound reagents. FX activation was measured in a coupled amidolytic assay with a saturating concentration of FX. The catalytic-centre activity [$k_{cat} = 0.11 \pm 0.02$ s^{-1} (mean $\pm$ S.E.M., $n = 7$)] of treated cells was 400-fold higher than that of the soluble TFOS4–FVIIa complex [$k_{cat} = 2.5 \times 10^{-4}$ s^{-1} ($n = 2$)], indicating a functional binding and proteolytic activity of the TFOS4–FVIIa complex towards its substrate FX (results not shown). This Xase activity was linearly dependent on the cell concentration in the assay (results not shown), and 4.2 ± 0.9-fold higher ($n = 4$) than the intrinsic Xase activity of the identically treated control cells (Figure 6). The intrinsic activity of the fibrosarcoma cells could be partially blocked by incubation with an IgG fraction against hTF prior to TFOS4 and FVIIa treatment, resulting in a 15.8 ± 7.6-fold ($n = 4$) higher Xase activity of the HT1080-FAP^{wt} cells compared with the control cells (Figure 6).

The biochemical parameters of TFOS4 were analysed in more detail and compared with those of the wild-type hTF molecule by using a hTF/phospholipid preparation from human placenta (Thromborel S®), routinely applied for clinical coagulation assays. Cells saturated with TFOS4 (100 nM) and FVIIa (300 nM) were incubated with different FX concentrations, and the rate of FXa generation (v) was determined by a coupled amidolytic assay (Figure 7). From the titration curve (Figure 7), the Michaelis–Menten constant ($K_m = 182 \pm 31$ nM) for FX activation by the cell-bound TFOS4–FVIIa complex could be calculated, and was approx. 2.4 times that for the Thromborel S® hTF preparation (Table 1). Since this value was close to published K_m values for the wild-type hTF–FVIIa complex [21] (Table 1), the characteristics of substrate binding to the cell-bound TFOS4–FVIIa complex are probably undisturbed.

However, if we consider that every FAP molecule should be associated with TFOS4 and FVIIa, as shown in Figure 4, the catalytic-centre activity (k_{cat}) of the cell-bound TFOS4–FVIIa complex is about 20 times lower than that of the same hTF concentration of Thromborel S® [$k_{cat} = 2.0 \pm 0.4$ s^{-1} ($n = 6$)]. This reduced activity might be caused either by sterical differences in the cell-bound TFOS4–FVIIa complex or by the fusion of tTF to the scFv, which might limit optimum trapping of FVIIa in its active conformation [20]. Additionally, the fusion protein is probably more flexible compared with the membrane-anchored hTF, resulting in a reduced probability of a stable enzyme–substrate complex (TFOS4–FVIIa–FX) at the phospholipid surface. Nevertheless, further experiments revealed that the activity of TFOS4 was sufficient for specific induction of coagulation in human plasma.

Specific plasma coagulation activated by cell-bound TFOS4

The clotting assay with standard human plasma [21,22] was used in order to examine plasma coagulation induced by the cell-bound TFOS4–FVIIa complex in the presence of physiological concentrations of FVIIa, FX, and even coagulation inhibitors such as TF pathway inhibitor [23,27]. Incubation of HT1080-FAP^{wt} cells and HT1080 control cells with plasma in the absence of TFOS4 led to clot formation after 73 s (Figure 8), due to the addition of calcium and the presence of hTF on the cell surface of the HT1080 cells. Pretreatment of HT1080-FAP^{wt} cells with the anti-hTF IgG fraction increased the coagulation time to 132 s. For unknown reasons, in all experiments the HT1080-FAP^{wt} cells showed slightly slower coagulation compared with HT1080 control cells after this pretreatment. Saturation of the

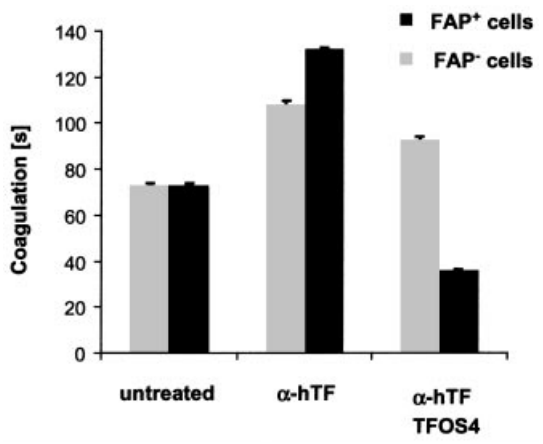

Figure 8 Clotting assays for FAP-overexpressing cells and FAP-negative control cells

HT1080-FAP^{wt} cells and HT1080 control cells were incubated with FVIIa reaction buffer (untreated) or with an IgG fraction against hTF (α-hTF) as controls, or with an IgG fraction against hTF and consecutively saturating concentrations of milk-blocked TFOS4 (α-hTF/TFOS4). After each step, cells were washed three times with FVIIa reaction buffer. The clotting assay was performed at 37 °C by mixing equal volumes of an aliquot of the cell suspension (3×10^5 cells/70 μl) with 20 mM $CaCl_2$ and citrated human plasma. Data are means $\pm$ S.E.M. ($n = 3$).

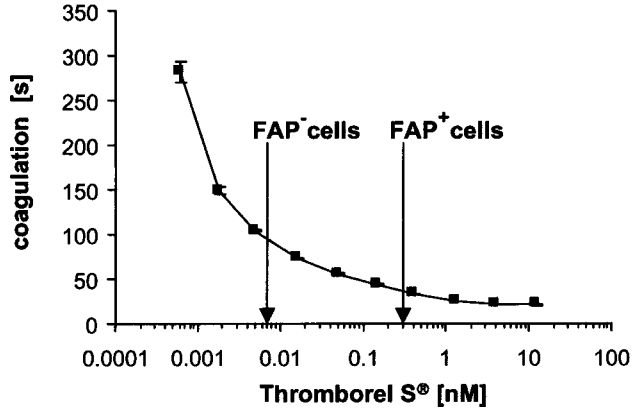

Figure 9 Correlation of TFOS4-induced coagulation of human plasma with a standard coagulation curve with Thromborel S®

A dilution series of Thromborel S® in FVIIa reaction buffer (70 μl) was incubated in triplet with 70 μl of 20 mM $CaCl_2$ for 10 min at 37 °C. After the addition of 70 μl of citrated human plasma, the clotting time was recorded. Data are means $\pm$ S.E.M. ($n = 3$). The coagulation times of HT1080-FAP^{wt} cells and HT1080 control cells (3×10^5 cells/70 μl) treated with anti-hTF IgG fraction and TFOS4, as described in the legend to Figure 8, are also indicated.

pretreated HT1080-FAP^{wt} cells with TFOS4 significantly decreased the coagulation time from 132 to 36 s, which corresponds to a reduction of 73 %. Control cells showed only a slight decrease (14 %) in coagulation time after saturation with TFOS4. Thus our results demonstrate that TFOS4 binding to HT1080-FAP^{wt} cells triggers rapid coagulation in human plasma in an antigen-specific manner.

Additionally, we correlated the TF activity of TFOS4 in combination with FAP-positive and control cells (pretreated with an IgG fraction against hTF) with the coagulation times of different concentrations of Thromborel S® in a standard curve of coagulation (Figure 9). The coagulation activity of TFOS4 on FAP-positive and control cells corresponded to 0.3 nM and 0.007 nM Thromborel S® respectively. This indicates a 40-fold increase in TF activity on the surface of HT1080-FAP^{wt} cells after treatment with TFOS4, compared with control HT1080 cells.

Therapeutic perspective

As FAP is a highly selective tumour-stroma-associated marker in a wide variety of carcinomas, TFOS4 may be considered for use as a novel therapeutic agent. After systemic application, TFOS4 should accumulate selectively in the tumour stroma and induce extravascular coagulation in an antigen-specific manner. This could result in a localized intravascular coagulation in the tumour vessels and, consequently, the interruption of tumour blood supply. The feasibility of this strategy is suggested by the following considerations. First, FAP is highly expressed in the tumour stroma compartment, immediately adjacent to tumour capillaries, and is therefore readily accessible via the bloodstream [5]. Additionally, the detection of fibrin deposits in the tumour stroma *in vivo* demonstrates the accessibility of this compartment for the substrates of TFOS4-mediated coagulation, which is facilitated by the increased permeability of the tumour vasculature [28,29]. Finally, it has been reported that tumour stroma [30] and tumour endothelium are commonly found in a procoagulant state, and hence even incremental changes via TFOS4 could shift the equilibrium towards localized coagulation [31].

Although hTF is known to act on the murine coagulation system [8], the characterization of *in vivo* effects of TFOS4 is not possible in conventional mouse models. These typically involve human cancer cells in immunodeficient mice, where the tumour stroma is host-derived and therefore not recognized by the human FAP-specific TFOS4. An appropriate test system may involve transplantation of normal human tissue into SCID (severe combined immunodeficiency disorder) mice in combination with tumour cells [32]. Alternatively, transgenic mice could be generated in which the endogenous murine FAP is replaced by the human homologue, thus resulting in human FAP expression in murine tumour stroma cells. With regard to *in vivo* safety and specificity, initial experiments indicate that a single intravenous injection of 30 μg of TFOS4 can be applied to mice ($n = 3$) without triggering apparent systemic coagulation or other unwanted side effects (U. Bamberger, unpublished work).

In conclusion, the scFv–tTF format may have several advantages over previously reported [8,9] tTF targeting constructs. First, production, purification and systemic application are readily achieved. Secondly, the scFv–hTF fusion protein format can be used in combination with scFvs of different specificity to target the hTF activity to other antigens, such as tumour endothelial markers (see Introduction), which are also considered to be suitable for the induction of specific, localized coagulation to cause a selective destruction of tumour vessels.

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REFERENCES

1 Folkman, J. (1998) Antiangiogenic gene therapy. Proc. Natl. Acad. Sci. U.S.A. **95**, 9064–9066

2 Zetter, B. (1997) On target with tumor blood vessel markers. Nat. Biotechnol. **15**, 1243–1244
3 Sipkins, D. A., Cheresh, D. A., Kazemi, M. R., Nevin, L. M., Bednarski, M. D. and Li, K. C. (1998) Detection of tumor angiogenesis in vivo by alphaVbeta3-targeted magnetic resonance imaging. Nat. Med. (N.Y.) **4**, 623–626
4 Garin-Chesa, P., Old, L. J. and Rettig, W. J. (1990) Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. Proc. Natl. Acad. Sci. U.S.A. **87**, 7235–7239
5 Welt, S., Divgi, C. R., Scott, A. M., Garin-Chesa, P., Finn, R. D., Graham, M., Carswell, E. A., Cohen, A., Larson, S. M., Old, L. J. and Rettig, W. J. (1994) Antibody targeting in metastatic colon cancer: a phase I study of monoclonal antibody F19 against a cell-surface protein of reactive tumor stromal fibroblasts. J. Clin. Oncol. **12**, 1193–1203
6 Pai, L. H. and Pastan, I. (1998) Clinical trials with Pseudomonas exotoxin immunotoxins. Curr. Top. Microbiol. Immunol. **234**, 83–96
7 Weiner, L. M., Clark, J. I., Davey, M., Li, W. S., Garcia de Palazzo, I., Ring, D. B. and Alpaugh, R. K. (1995) Phase I trial of 2B1, a bispecific monoclonal antibody targeting c-erbB-2 and Fc gamma RIII. Cancer Res. **5**, 4586–4593
8 Huang, X., Molema, G., King, S., Watkins, L., Edgington, T. S. and Thorpe, P. E. (1997) Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. Science **275**, 547–550
9 Ran, S., Gao, B., Duffy, S., Watkins, L., Rote, N. and Thorpe, P. E. (1998) Infarction of solid Hodgkin's tumors in mice by antibody-directed targeting of tissue factor to tumor vasculature. Cancer Res. **58**, 4646–4653
10 Davie, E. W., Fujikawa, K. and Kisiel, W. (1991) The coagulation cascade: initiation, maintenance, and regulation. Biochemistry **30**, 10363–10370
11 Neuenschwander, P. F. and Morrissey, J. H. (1994) Roles of the membrane-interactive regions of factor VIIa and tissue factor. The factor VIIa Gla domain is dispensable for binding to tissue factor but important for activation of factor X. J. Biol. Chem. **269**, 8007–8013
12 Bromberg, M. E., Konigsberg, W. H., Madison, J. F., Pawashe, A. and Garen, A. (1995) Tissue factor promotes melanoma metastasis by a pathway independent of blood coagulation. Proc. Natl. Acad. Sci. U.S.A. **92**, 8205–8209
13 Dübel, S., Breitling, F., Fuchs, P., Braunagel, M., Klewinghaus, I. and Little, M. (1993) A family of vectors for surface display and production of antibodies. Gene **128**, 97–101
14 Kujau, M. J., Hoischen, C., Riesenberg, D. and Gumpert, J. (1998) Expression and secretion of functional miniantibodies McPC603scFvDhlx in cell-wall-less L-form strains of Proteus mirabilis and Escherichia coli: a comparison of the synthesis capacities of L-form strains with an E. coli producer strain. Appl. Microbiol. Biotechnol. **49**, 51–58
15 Rippmann, J. F., Klein, M., Hoischen, C., Brooks, B., Rettig, W. J., Gumpert, J., Pfizenmaier, K., Mattes, R. and Moosmayer, D. (1998) Procaryotic expression of single-chain variable-fragment (scFv) antibodies: secretion in L-form cells of Proteus mirabilis leads to active product and overcomes the limitations of periplasmic expression in Escherichia coli. Appl. Environ. Microbiol. **64**, 4862–4869
16 Moosmayer, D., Dübel, S., Brocks, B., Watzka, H., Hampp, C., Scheurich, P., Little, M. and Pfizenmaier, K. (1995) A single-chain TNF receptor antagonist is an effective inhibitor of TNF mediated cytotoxicity. Ther. Immunol. **2**, 31–40
17 Martin, D. M. A., Boys, W. G. and Ruf, W. (1995) Tissue factor: molecular recognition and cofactor function. FASEB J. **9**, 852–859
18 Shigematsu, Y., Miyata, T., Higashi, S., Miki, T., Sadler, J. E. and Iwanaga, S. (1992) Expression of human soluble tissue factor in yeast and enzymatic properties of its complex with factor VIIa. J. Biol. Chem. **267**, 21329–21337
19 Schullek, J. R., Ruf, W. and Edgington, T. S. (1994) Key ligand interface residues in tissue factor contribute independently to factor VIIa binding. J. Biol. Chem. **269**, 19399–19403
20 Ruf, W., Kalnik, M. W., Lund-Hansen, T. and Edgington, T. S. (1991) Characterization of factor VII association with tissue factor in solution. High and low affinity calcium binding sites in factor VII contribute to functionally distinct interactions. J. Biol. Chem. **266**, 15719–15725
21 Dittmar, S., Ruf, W. and Edgington, T. S. (1997) Influence of mutations in tissue factor on the fine specificity of macromolecular substrate activation. Biochem. J. **321**, 787–793
22 Drake, T. A., Ruf, W., Morrissey, J. H. and Edgington, T. S. (1989) Functional tissue factor is entirely cell surface expressed on lipopolysaccharide-stimulated human blood monocytes and a constitutively tissue factor-producing neoplastic cell line. J. Cell Biol. **109**, 389–395
23 Baugh, R. J., Broze, G. J. and Krishnaswamy, Jr, S. (1998) Regulation of extrinsic pathway factor Xa formation by tissue factor pathway inhibitor. J. Biol. Chem. **273**, 4378–4386
24 Banner, D. W., D'Arcy, A., Chène, C., Winkler, F. K., Guha, A., Konigsberg, W. H., Nermerson, Y. and Kirchhofer, D. (1996) The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. Nature (London) **380**, 41–46
25 McCallum, C. D., Su, B., Neuenschwander, P. F., Morrissey, J. H. and Johnson, A. E. (1997) Tissue factor positions and maintains the factor VIIa active site far above the membrane surface even in the absence of the factor VIIa Gla domain. A fluorescence resonance energy transfer study. J. Biol. Chem. **272**, 30160–30166
26 Ruf, W., Rehemtulla, A. and Edgington, T. S. (1991) Phospholipid-independent and -dependent interactions required for tissue factor receptor and cofactor function. J. Biol. Chem. **266**, 2158–2166
27 Willems, G. M., Janssen, M. P., Salemink, I., Wun, T.-C. and Lindhout, T. (1998) Transient high affinity binding of tissue factor pathway inhibitor-factor Xa complexes to negatively charged phospholipid membranes. Biochemistry **37**, 3321–3328
28 Nagy, J. A., Brown, L. F., Senger, D. R., Lanir, N., Van de Water, L., Dvorak, A. M. and Dvorak, H. F. (1989) Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. Biochim. Biophys. Acta **948**, 305–326
29 Zacharski, L. R., Memoli, V. A., Ornstein, D. L., Rousseau, S. M., Kisiel, W. and Kudryk, B. J. (1993) Tumor cell procoagulant and urokinase expression in carcinoma of the ovary. J. Natl. Cancer Inst. **85**, 1225–1230
30 Kao, J., Fan, Y. G., Haenel, I., Clauss, M. and Stern, D. (1993) Endothelial-monocyte activating polypeptides (EMAPs): tumor derived mediators which activate the host inflammatory response. Behring Inst. Mitt. **92**, 92–106
31 Shoji, M., Hancock, W. W., Abe, K., Micko, C., Casper, K. A., Baine, R. M., Wilcox, J. N., Danave, I., Dillehay, D. L., Matthews, E. et al. (1998) Activation of coagulation and angiogenesis in cancer: immunohistochemical localization in situ of clotting proteins and vascular endothelial growth factor in human cancer. Am. J. Pathol. **152**, 399–411
32 Brooks, P. C., Stromblad, S., Klemke, R., Visscher, D., Sarkar, F. H. and Cheresh, D. A. (1995) Antiintegrin alpha v beta 3 blocks human breast cancer growth and angiogenesis in human skin. J. Clin. Invest. **96**, 1815–1822

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