Targeted Delivery of Tissue Factor to the ED-B Domain of Fibronectin, a Marker of Angiogenesis, Mediates the Infarction of Solid Tumors in Mice

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ABSTRACT

The selective thrombosis of tumor blood vessels, leading to the starvation and subsequent death of tumor cells, is an attractive anticancer strategy. Here we report that a fusion protein, consisting of an antibody fragment specific for the oncofoetal ED-B domain of fibronectin fused to the extracellular domain of tissue factor, selectively targets tumor blood vessels in vivo. Furthermore, this fusion protein mediates the complete and selective infarction of three different types of solid tumors in mice. At the highest doses administered, complete tumor eradication was observed in 30% of the mice treated without apparent side effects. These results are of therapeutic relevance because the ED-B domain of fibronectin, a naturally occurring marker of angiogenesis identical in mouse and man, is expressed in the majority of aggressive solid tumors but is undetectable in normal vessels and tissues.

INTRODUCTION

Angiogenesis, i.e., the proliferation of new blood vessels from preexisting ones, is a characteristic feature of aggressive solid tumors and relevant disorders such as age-related macular degeneration, diabetic retinopathy, and rheumatoid arthritis (1, 2). Molecules capable of inhibiting angiogenesis or of selectively targeting and destroying new blood vessels, would be promising agents for the treatment of angiogenesis-related diseases (3–9).

A novel approach to cancer therapy based on the antibody-directed targeting of the human coagulation-inducing protein TF to tumor vasculature has recently been proposed (10, 11). The approach is based on the concept that thrombosis of tumor vessels may stop the supply of nutrients and oxygen to tumor cells, thereby causing their death.

TF is a cell-surface glycoprotein and a major initiator of blood coagulation (12). 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. tTF consisting of only the extracellular soluble domain (residues 1–219), exhibits an ability to activate the clotting cascade in solution (13–17). B-FN accumulates around neovascular structures in aggressive tumors and other tissues undergoing angiogenesis, such as the endometrium in the proliferative phase and some ocular structures in pathological conditions, but is otherwise undetectable in normal adult tissues.

In a first article, Huang et al. (10) used a bispecific antibody to target tTF to an artificial marker of angiogenesis (MHC-II) experimentally induced on tumor vascular endothelium by grafting in mice neuroblastoma cells that had been transfected with the IFN-γ gene. In that study, the investigators observed extensive intravascular thrombosis of the tumor and complete regressions in 38% of the mice treated (10).

In a second study, immunoconjugates were used to target tTF to a naturally occurring marker of tumor vascular endothelium, VCAM-1 (11). VCAM-1 is expressed by vascular endothelial cells in Hodgkin’s lymphoma and in various solid tumors 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 (11). In this study, the authors observed selective localization of tTF to VCAM-1-expressing vessels in the tumor, causing thrombosis of those vessels, a 50% reduction of tumor growth rate, but no complete remission. The immunoconjugate also localized 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-PS antibody in the mouse showed that the VCAM-1-expressing vessels in the tumor 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 were directed against a naturally occurring marker of angiogenesis that is expressed in the majority of aggressive solid tumors but undetectable in normal vessels and tissues, and if it mediated the selective thrombosis of tumor blood vessels.

A good quality marker for both tumoral and nontumoral neovascularization is the ED-B domain of fibronectin (a sequence of 91 amino acids identical in mouse, rat, rabbit, dog, and man) that can be inserted into the fibronectin molecule by a mechanism of alternative splicing (13–17). B-FN accumulates around neovascular structures in aggressive tumors and other tissues undergoing angiogenesis, such as the endometrium in the proliferative phase and some ocular structures in pathological conditions, but is otherwise undetectable in normal adult tissues.

To date, the production of monoclonal antibodies directly recognizing the ED-B domain in B-FN has not been possible using hybridoma technology because of tolerance. However, this problem has been overcome using antibody phage technology (18) with large synthetic antibody repertoires (19, 20). Several antibody fragments specific for the ED-B domain of fibronectin have been generated recently. These antibody fragments stain vascular structures in tumor sections and selectively target tumor neovascularization, as shown in tumor-bearing mice using IR fluorescence and radioactive techniques (21, 22). Increased binding affinity leads to improved targeting of tumoral angiogenesis, as demonstrated by biodistribution studies performed using the L19 antibody fragment with affinity for the ED-B domain in the pM range and L19 mutants with reduced affinity (23).

In this study we investigated whether the selective antibody-mediated targeting of tTF to B-FN would result in thrombosis of tumor blood vessels. B-FN is a component of the modified extracellular matrix that surrounds tumor blood vessels, and it was therefore not known whether the delivery of a procoagulant agent at this abluminal site would be capable of mediating the complete and selective intraluminal coagulation of tumor blood vessels.

In this article we show that a fusion protein consisting of the L19 fragment and native tTF is a promising candidate to mediate selective thrombosis of tumor blood vessels in vivo.
antibody fragment fused to tTF mediates the complete and selective infarction of three different types of solid tumors (F9, C51, and FE8) in mice. Injection of 35 μg of the fusion protein led to complete tumor eradication in ~30% of the treated animals, whereas tTF-fusion proteins with binding specificity for an irrelevant antigen did not show any therapeutic effect.

MATERIALS AND METHODS

Cell Lines, Genes, and Antibodies. The tumoral cell lines used were F9 murine teratocarcinoma (24), C51 murine colon adenocarcinoma (25), and FE8 rat fibroblast sarcoma (26). The scFv(L19) and scFv(D1.3) have been described elsewhere (22). The cDNA for the TF was obtained from American Type Culture Collection (Rockville, MD).

Cloning, Expression, and Purification of scFv-tTF Fusions. The scFv(L19)-tTF expression vector (pDNS) was constructed by the 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 NolI/EcoRI sites of a derivative of vector pDNS (27) 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:

- The primer TF-banot (5’-GAG TAC TTA GGC GGC GCA GTG GGC GTG GCC 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 NotI. It also introduced a short linker COOH-terminal of the restriction site consisting of four glycines (GGGG);
- The primer TF-fostueco1 (5’-GTC ATC GTC CTT GTA GTC AGG CCT TTC ACG-3’) introduced at the 3’ end of the tTF DNA sequence. The DNA sequence had been restricted at the EcoRI site of the TF, which was removed by PCR with the primers TF-banot and TF-fostueco1 (5’-AGA GAA TTC TTA TTA CTT ATC-3’).

The clonal expression of scFv(L19)-tTF was achieved by transfection of E. coli RA330 using pDNS vector. The colonies with the correct insertion were selected by colony hybridization. The correct clone was identified by sequencing the DNA inserts.

In the case of scFv(D1.3)-tTF, fractions eluted from a lysozyme column were neutralized with 1M Tris-HCl (pH 7.4) and loaded on a Resource S cation exchange column (Amersham Pharmacia Biotech). Typical yields of tripurified therapeutic fusion protein were ~0.2 mg/liter culture, mainly attributable to the low capacity and limited life of the a-Flag M2 resin. Protein yields after the first affinity chromatography step were ~1–2 mg/liter.

In the case of scFv(L19)-tTF, fractions eluted from a lysozyme column were neutralized with 1 M Tris-HCl (pH 7.4) and loaded on a Sigma M2-resin for affinity purification using the FLAG tag (Sigma, St. Louis, MO). 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). The monomeric peak fraction was collected and desalted with a preparative gel filtration column PD-10 (Amersham Pharmacia Biotech). Typical yields of tripurified therapeutic fusion protein were ~0.2 mg/liter culture, mainly attributable to the low capacity and limited life of the a-Flag M2 resin. Protein yields after the affinity chromatography step were ~1–2 mg/liter.

Measurement of Immunoreactivity and Tissue Factor Activity. The immunoreactivity of the scFv-tTF fusion protein was analyzed by ELISA immunoassay, by BIACore, and by affinity chromatography on antigen column, as described (21–23).

The enzymatic activity of the scFv-tTF fusion protein was analyzed using the Spectrzyme FXa assay (American Diagnostica, Pfungstadt, Germany) as described by Ruiz et al. (12).

Tumor Mouse Models. Tumor-bearing mice were obtained by s.c. injection of 10^6 tumor cells of C51 murine colon adenocarcinoma, FE8 ras-transformed rat fibroblasts, or F9 murine teratocarcinoma in female BALB/c nude mice (Laborklinik der Universität Zürich, Zürich, Switzerland). Mice were monitored daily, and tumor volume was measured with a caliper using the following formula: volume, = length x width^2 x π/6. Experiments were performed in agreement with Swiss regulations and under a project license, “Tumor Targeting,” issued to D. N. by the Kantonsale Veterinärämtes des Kantons Zürich (Bewilligung 53/97). In addition to the experiments with nude mice, a set of experiments with F9 and C51 tumors was also performed with immunocompetent mice, yielding results comparable with the ones obtained with nude mice (data not shown). According to our project license, mice had to be euthanized when tumors became too large, if animals lost >20% of body weight, or if they showed signs of pain during the therapeutic experiments.

Biodistribution Experiments. The in vivo targeting performance was evaluated by biodistribution analysis as described by Tarif et al. (22). Briefly, purified scFv(L19)-tTF fusion protein was radioiodinated and injected into nude mice with s.c. implanted F9 murine teratocarcinoma. Mice were sacrificed at 24 h after injection. The organs were weighed and the radioactivity counted. Targeting results of representative organs are expressed as a percentage of the injected dose per gram of tissue (%ID/g).

Treatment of Mice with scFv-tTF Fusion Proteins. Cohorts of mice with tumors of volume ~200–300 mm^3 (n = 4) were injected with 14 μg scFv-tTF fusion protein in 200 μl of saline or injected with saline only. The injection was repeated after 48 and 96 h. Mice with tumors of volume ~1500 mm^3 were injected with a single dose of 20 μg of scFv-tTF fusion protein in 200 μl of saline.

In a dose-escalation investigation, mice with FE8 tumors with volume ~300–500 mm^3 were injected with 9, 15, or 35 μg of scFv-tTF fusion protein in 200 μl of saline or injected with saline only. The injection was repeated after 72 and 144 h. In all experiments, mice were monitored by tumor volume, weight, and photographs.

Histology. To assess the toxicity of treatment by histological analysis, organs (lung, liver, spleen, intestine, kidney, heart, and brain) were collected 24 h after injection of 20 μg of scFv-tTF or saline, fixed in 4% buffered paraformaldehyde and embedded in paraffin. Four-μm sections were cut, stained with H&E, and analyzed. At least one slide was available per 0.5 cm of diameter of the sample.

Mice were sacrificed at different time points after injection of 20 μg of scFv(L19)-tTF (1 h, 4 h, 6 h, 12 h, and 24 h). The tumors were excised, fixed in 4% buffered paraformaldehyde and embedded in paraffin. Sections were cut and stained with H&E. At least one slide was available per 0.5 cm of tumor diameter. Thrombosis of intratumoral vessels was defined according the following criteria: total or incomplete occlusion by closely packed erythrocytes with blurred outline.

RESULTS

Cloning, Expression, and Characterization of Antibody-tTF Fusion Proteins. We used the antibody fragment scFv(L19), which recognizes the ED-B domain of fibronectin with affinity in the pM range (20), for the selective targeting of angiogenesis. ScFv(D1.3), which recognizes hen egg lysozyme but not mouse lysozyme (28), for the selective targeting of angiogenesis. ScFv(D1.3), which recognizes hen egg lysozyme but not mouse lysozyme (28).
purified to homogeneity using a multistep procedure based on affinity chromatography on antigen column, then purification on the M2 anti-FLAG resin, and subsequent dialysis, cation exchange chromatography, and desalting by gel-filtration (Fig. 1b). Both purified fusion proteins were monomeric as assessed by gel filtration.

To check that both moieties of the fusion proteins were functional, immunoreactivity was measured both by affinity chromatography on antigen column (22) and by BIAcore (31, 32). Tissue factor activity was measured as described (12), detecting the cleavage of the FXa fluorogenic peptide mediated by the scFv-tTF/Factor VIIa complex. A half-maximal activity was observed at 45 nM protein concentration, in line with previously published values obtained with tTF (12).

The ability of scFv(L19)-tTF to selectively localize on tumor blood vessels was examined in mice bearing a s.c. grafted F9 murine teratocarcinoma by quantitative biodistribution analysis using a radioiodinated protein sample (22). Fig. 2 shows the results of this analysis expressed in terms of the percentage of injected dose per gram of tissue (%ID/g) 24 h after i.v. injection. At this time point, the %ID/g (tumor) was 16.8, with a tumor-blood ratio of 17:1, which is comparable to the ratio published for radiolabeled scFv(L19) (22). Tumor: blood ratios >2 can be observed as early as 3 h after injection. Considering that L19 localizes to tumoral blood vessels, which represent only a small percentage of the weight of F9 tumors (22), the results of Fig. 2 confirm that scFv(L19)-tTF is able to accumulate at a high density around vascular structures.

ScFv(L19)-tTF Mediates the Infarction of Aggressive Solid Tumors in Mice. To test whether the targeted delivery of tTF to B-FN, a component of the modified extracellular matrix that accumulates around vascular structures in aggressive tumors, was able to promote thrombosis of tumor vessels, we injected tumor-bearing mice with scFv(L19)-tTF, scFv(D1.3)-tTF, or saline. The tumors grafted s.c. in mice were the F9 murine teratocarcinoma (24), the C51 murine colon adenocarcinoma (25), and the FE8 rat sarcoma (26).

Mice bearing small F9, C51, or FE8 tumors (<500 mg) were injected at different time points with three doses of 14 μg scFv-tTF (or saline). A few hours after the first injection, all tumors turned black only with the L19 fusion protein (Fig. 3a), but not with the saline or with scFv(D1.3)-tTF (Fig. 3b), suggesting that scFv(L19)-tTF did mediate a selective intraluminal blood coagulation in tumor blood vessels, as confirmed by histochemical analysis (Fig. 4).

At the doses used, a statistically significant reduction in tumor growth rate was observed for all three tumor models upon injection of scFv(L19)-tTF (Fig. 5a–c). 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 antitumor effect.

The effect of a single injection of 20 mg of scFv(L19)-tTF in mice bearing larger tumors (>1500 mg) was even more dramatic. Fig. 5 shows a plot of tumor volume versus time for the three different tumor models after a single i.v. injection of D1.3-tTF or the L19-tTF fusion protein. Within 1–2 h after injection of scFv(L19)-tTF [but not scFv(D1.3)-tTF], tumors turned black. At 2 days after injection, most of the tumor had been converted into a crusty mass. However, a portion of the tumor typically survived at the border with the peritoneal cavity and would eventually grow back and kill the animal (Fig. 6a). A histochemical analysis of tumors revealed that, at the dose used, only a small fraction of vessels close to the peritoneal cavity was occluded (Fig. 6b) in spite of the fact that all tumor vessels were ED-B-positive. By contrast, blood vessels in other parts of the tumors were completely thrombosed.

Fig. 4. H&E sections of FE8 tumors 1 h after injection of saline (a) or scFv(L19)-tTF (b), which mediates extensive thrombosis of blood vessels. Scale bars: 50 μm.

A Dose Escalation Study with scFv(L19)-tTF in Tumor-bearing Mice. Early experiments with i.v. administration of scFv(L19)-tTF to tumor-bearing mice prevented the administration of fusion protein at doses >20 μg because side effects were observed. In addition to a 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 depositing at the extremities of animal’s body. The antibody preparation did not show any 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. Filtration of the fusion protein before i.v. 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. 7 shows FE8 tumor 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), ~30% of the mice exhibited a complete tumor regression. In these conditions, mice showed a transient loss of ~10% body weight, indicating the onset of some toxicity. Mice regained weight after tumor eradication. Fig. 8 shows pictures of the mice responding to treatment.

No apparent abnormality could be detected in the organs of treated mice (lung, liver, spleen, intestine, kidney, heart, and brain) at autopsy and by histological analysis 24 h after injection. Neither were areas of hemorrhagia, necrosis, or thrombosis observed. By contrast, ~50% of intratumoral blood vessels were completely occluded just 1 h after injection. Four h after injection, ~80% of the tumor vessels were thrombosed. The number of occluded intratumoral vessels did not change 24 h after the injection, and no additional thrombosis or thrombolysis could be observed.

Fig. 5. Tumor growth retardation studies of three different tumor models [C51 (a and d), F9 (b and e), and FE8 (c and f) tumors] in mice. In experiments a–c, cohorts of mice bearing small tumors were injected with three doses of 14 μg scFv-tTF or saline (arrows). In experiments d–f, mice bearing large tumors (>1 g) were injected with a single dose of 20 μg of scFv-tTF. Tumor sizes were measured with a caliper; standard errors are indicated. □ scFv(D1.3)-tTF; ● saline; ■ scFv(L19)-tTF.
DISCUSSION

The first report by Huang et al. (10) on the targeted induction of intraluminal blood coagulation in tumoral blood vessels using an artificial marker of angiogenesis generated a great interest to learn whether the same strategy would work in tumor models carrying natural markers of angiogenesis. The second report on this strategy, featuring the targeting of the VCAM-1, was less impressive because only a 50% reduction in the tumor growth rate was observed. The authors also postulated that the simultaneous display of target antigen and PS on the luminal surface of blood vessels was an essential requirement for starting the blood coagulation cascade.

The observation of complete remissions of tumor-bearing mice treated with scFv(L19)-tTF described in this article may support an alternative view on the requirements of an effective fusion protein for the complete and selective thrombosis of tumoral vessels. B-FN is an abundant component of the modified extracellular matrix of tumors, which accumulates in the abluminal side of tumor blood vessels. ScFv(L19)-tTF displays an extremely high accumulation on neovasculature a few hours after injection, as measured by quantitative biodistribution analysis using radioiodinated fusion proteins or by \textit{ex vivo} immunofluorescence analysis of tumor sections from mice injected with fluorescently labeled scFv(L19) (data not shown; Ref. 21). It is plausible that the fenestration and leakiness of tumor blood vessels allows 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 in the blood stream with consequent continuation of the blood clotting cascade. Alternatively, fibrin deposition could start in the perivascular space, and would propagate back into the luminal aspects of tumor blood vessels.

The remissions were observed in extremely aggressive tumor models, which are not cured by conventional chemotherapy (data not shown). At the highest dose, complete remissions were observed in 30% of the mice treated. All tumors showed extensive thrombosis of blood vessels, but in some cases residual tumor cells grew back. Typically, these cells would be located in tumor areas in which blood vessel occlusion was not complete. However, the rapid tumor debulking observed with our fusion protein may facilitate removal of residual tumor mass by other means. Furthermore, the clear dose dependence of therapeutic benefit (Fig. 7) obtained with scFv(L19)-tTF suggests that the efficacy of this therapeutic fusion protein would further improve using slightly higher doses or different administration schedules. In principle, a better formulation of the fusion protein, combination with other therapeutic modalities (e.g.,
REFERENCES


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