Abstract

**Purpose:** Targeting the tumor vasculature by vascular disrupting agents (VDAs) has shown therapeutic activity in mouse models. In most cases, however, VDA efficacy is substantially compromised by the inability of these drugs to completely kill tumor cells located at the periphery of the tumor mass. In this study, we investigated anti-tumor effects of L19mTNFα, a fusion protein composed of L19 (scFv), specific for the angiogenesis-associated ED-B containing fibronectin isoform, and murine TNFα, in xenograft models of esophageal cancer.

**Experimental design:** We evaluated ED-B expression in esophageal cancer samples. Subsequently, we generated subcutaneous xenografts from primary tumors, treated them with the L19mTNFα scFv, and determined effects on tumor vasculature, viability and proliferation, and VEGF expression and infiltration by hematopoietic cells. To overcome tumor resistance, L19mTNFα scFv was combined with vandetanib, a tyrosine kinase inhibitor of VEGF receptor, epidermal growth factor receptor, and RET signaling.

**Results:** ED-B was broadly expressed by esophageal cancer cell lines, as well as xenografts and primary surgical samples of esophageal cancer. Administration of L19mTNFα acutely damaged tumor vasculature and increased necrosis, indicating a VDA-like activity of this immunoconjugate. This event was followed, however, by rapid tumor growth recovery associated with increased expression of VEGF and recruitment of CD11b+Gr1+ myeloid cells into tumors. Combination of L19mTNFα with vandetanib severely impaired vascular functions in tumors, leading to a reduction of cell proliferation and increased necrosis, without apparent signs of toxicity.

**Conclusion:** These findings indicate that a combination of vascular damaging agents with antiangiogenic drugs could represent a promising therapeutic strategy for esophageal cancer. Clin Cancer Res; 17(3); 447–58. ©2010 AACR.

Introduction

Esophageal cancer is an aggressive neoplasm. On a global basis, cancer of the esophagus is the sixth leading cause of cancer death worldwide. Although esophageal squamous cell carcinoma cases have steadily declined in Europe and the United States, the incidence of adenocarcinoma has increased more rapidly than any other cancer type and parallels the rise in population trends in obesity and reflux disease (1).

Despite advances in surgical techniques and treatment, the prognosis of esophageal cancer has slowly improved over the past decades. Indeed, the 5-year overall survival rate remains low (14%), due to detection of the disease at advanced stages, high risk of relapse after surgery, and lack of effective drugs in relapsing or nonoperable patients. In most cases, cancer cells disseminate early and innovative strategies are needed to improve treatment of systemic disease.

Tumor growth and metastasis are generally regulated by the recruitment of new blood vessels and there is some evidence in support of activation of angiogenesis in esophageal cancer. Among proangiogenic factors, VEGF is expressed by 30% to 60% of surgical samples and has a prognostic value in patients undergoing curative surgery (2–4). Moreover, many pathological studies have shown that in esophageal carcinoma the microvessel density (MVD) increases in proportion to disease progression, tumor size and stage, and depth of invasion [reviewed in...
(5)]. Recent advances in morphological investigations, such as the Microfil technique and ultra-high magnification endoscopy, have enabled detailed examination of vascular networks, highlighting dramatic vascular changes already in the early phase of esophageal cancer progression (5).

Angiogenesis occurs in parallel with remodeling of the extracellular matrix, involving qualitative changes in its structure. Fibronectin is an extracellular matrix component abundantly expressed in a variety of normal tissues. Different fibronectin isoforms can be generated by alternative splicing of fibronectin pre-mRNA, a process in part modulated by cytokines and extracellular pH (6). The ED-B domain of fibronectin, a sequence of 91 amino acids identical in mice, rats, and humans, which is inserted by alternative splicing into the fibronectin molecule, specifically accumulates around neovascular structures (7–9). The fibronectin isoform containing ED-B (B-FN) is immunohistochemically undetectable in normal adult tissues, and it is a marker of physiological and pathological angiogenesis, representing a scaffold for migration of endothelial cells into tissues (10).

The possibility of selectively targeting the tumor vasculature using L19 (scFv), a human recombinant antibody (Ab) fragment specific for B-FN, has been reported in several experimental animal models and cancer patients [reviewed in (6)]. Conjugation of L19 to certain cytokines allows their selective accumulation within the tumor microenvironment and enhances their anti-cancer properties; in particular, the fusion protein L19mTNFα immunoconjugate acts as a vascular disrupting agent (VDA) (11). Moreover, when injected with melphalan in immunocompetent tumor-bearing mice, L19mTNFα induces complete and long-lasting tumor eradication, triggering the generation of a specific T-cell–dependent immune response to tumor antigens (12, 13). Clearly, L19mTNFα exerts complex therapeutic activities in immunocompetent syngeneic mouse tumor models, involving vascular effects, direct cytotoxic effects, and immune-mediated mechanisms. In the present article, we focused selectively on the toxic effects exerted by murine TNFα on endothelial cells in early-passage esophageal cancer xenografts grown in immunodeficient mice. We show that blockade of the VEGF pathway by vandetanib, an inhibitor of VEGF receptor (VEGFR), epidermal growth factor receptor (EGFR), and RET tyrosine kinases [reviewed in (14)], synergizes with L19mTNFα and potentiates its anti-tumor effects by substantial impairment of vascular function and tumor cell proliferation. These results indicate the potential of this combination therapy for this highly aggressive malignancy.

Materials and Methods

Cell lines and establishment of xenografts

The human esophageal cancer cell lines OE21 and Kyse-30 (both squamous cell carcinoma) and OE19 and OE33 (both adenocarcinoma) were purchased from ECACC and used within 6 months from resuscitation. OE19, OE21, and OE33 cells were grown in RPMI1640 medium (Euroclone) supplemented with 10% FCS and 2 mM glutamine; Kyse-30 cells were grown in RPMI1640+ Ham’s F12 medium (1:1) with 2% FCS and 2 mM glutamine. The cultures were routinely maintained at 37°C in a humidified 5% CO2/95% air atmosphere.

For tumor establishment, 6-week-old severe combined immunodeficient (SCID) mice (Charles River) were either injected subcutaneously (s.c.) into both dorsolateral flanks with 1.0 × 106 Kyse-30 cells in a 200 μL total volume or implanted with 1–3 mm3 tumor pieces of surgical samples. Primary tissues were collected following informed consent from all patients included in the study. EAC#2 and EAC#8 xenografts were derived from esophageal adenocarcinoma and mucinous cell carcinoma, respectively. Following tumor formation, the animals were sacrificed and the tumor mass was cut and implanted into new recipients as described above.

L19mTNFα was administered intravenously (i.v.) at 0.14 μg/g, and the mice were sacrificed 24–50 h later. In long-term experiments, L19mTNFα was administered once a week for 4 weeks. Vandetanib was administered daily by oral gavage for 3 consecutive days at 50 mg/kg/d. In long-term experiments, vandetanib was administered as described above during the first week and every other day during the following 3 weeks. To measure perfusion, mice were injected with Dextran 70 kDa–FITC (Invitrogen; 360 μmol/L, 50 μL/mouse) 3 min before sacrifice. Perfused vessels were visualized by confocal microscopy using a Zeiss LSM 510 microscope (Zeiss).

Tumor volume (mm3) was measured by a caliper and calculated according to the following formula: $V = \frac{4}{3}\pi \times L \times \frac{L}{2} \times 0.5$, where $L$ is the longest diameter, $l$ is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. Procedures involving animals and their care were approved

Translational Relevance

Despite improvement in surgical techniques and treatment, slow progress has been made in long-term survival of patients with esophageal cancer, in part due to the lack of effective systemic therapies. Here, we report that ED-B, an extracellular matrix component, is commonly expressed in the stroma of esophageal cancer but not by the normal mucosa. In xenograft models of esophageal cancer, increased pronecrotic and anti-tumor effects were observed by targeting ED-B by the vasculotoxic L19-TNFα immunoconjugate along with concomitant therapy with vandetanib, a tyrosine kinase inhibitor that inhibits vessel re-growth. These data should provide a valuable resource for future basic and clinical studies addressing the role of integrated vasculotoxic and anti-angiogenic therapy in esophageal cancer.

Reverse transcription PCR and qualitative PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen) according to manufacturer’s instructions. cDNA was synthesized from 0.5 to 1 μg of total RNA using Superscript II first-strand system for RT-PCR (Invitrogen). Primers used for PCR analysis are reported in Supplementary Table 1. Reverse transcription PCR and qualitative PCR were carried out using the avidin-biotin-peroxidase complex technique and 3-3’ dianobenzidine as chromogen (Vector Laboratories, Burlingame), and the sections were then lightly counterstained with Mayer’s hematoxylin. Parallel negative controls, obtained by replacing primary Abs with PBS, were run in all cases. Moreover, in each experiment, sections of previously tested VEGF-positive tissue were processed for use as positive controls. In a set of experiments, cell proliferation in tumor sections was evaluated by staining with rabbit anti-phospho histone H3 Ab (Ser10; Cell Signaling), as reported elsewhere (17). For each sample, 6–9 hfp (×400) were scored using an integrated grid.

Immunofluorescence analysis

To evaluate ED-B expression, 5 μm thick tumor sections were incubated with either anti-human B-FN BC-1 (15) or ED-B L19 IgG1 (16) primary antibodies, washed, and incubated with the appropriate Alexa Fluor 546-conjugated secondary Abs (Invitrogen). Microvessels and pericytes were stained by rat anti-CD31 (clone MEC 13.3; Becton Dickinson, San Jose) and rabbit anti-NG2 (Chemicon) Abs, respectively. To evaluate apoptosis, 5-μm-thick tumor sections were incubated with cleaved caspase-3 (Asp175; Cell Signaling Technology) primary Ab, washed, and incubated with the anti-rabbit Alexa Fluor 546 (Invitrogen) secondary Ab. Nuclei were stained with TOPRO-3 (Invitrogen).

Confocal laser scanning microscopy was carried out with a Zeiss LSM 510 microscope using argon (488 nm) and helium-neon (543–633 nm) laser sources. The number of fields analyzed varied between 5 and 10 per sample, depending on the size of tumor sections; at least 4 samples per group were analyzed. Images were collected at a magnification of ×200 if not otherwise indicated. MVD or percentage of perfusion was quantified by screening for the areas of highest vascularity or perfused vessels; for each sample, 10 representative fields at ×200 magnification were counted.

Histology and immunohistochemistry

Quantification of necrosis was carried out by using the Laser Microdissection Software version 4.3 of a laser-assisted microdissector (AS-LMD Leica Microsystems). Briefly, the sample was first completely scanned and divided into a variable number of identical fields, depending on its total surface. In every field, the total tumor area and the area of necrosis were measured by the software and expressed as μm². Finally, to calculate the percentage of necrotic areas, the sum of the necrotic areas in all fields was divided by the total area of the sample.

Evaluation of VEGF expression was carried out on formalin-fixed, paraffin-embedded tumor sections, rehydrated and processed for immunohistochemistry. Tumor sections were labeled with a mouse anti-VEGF monoclonal antibody (mAb) (clone VG-1; Abcam); immunostaining was performed using the avidin-biotin-peroxidase complex method and 3-3’ dianobenzidine as chromogen (Vector Laboratories, Burlingame), and the sections were then lightly counterstained with Mayer’s hematoxylin. Parallel negative controls, obtained by replacing primary Abs with PBS, were run in all cases. Moreover, in each experiment, sections of previously tested VEGF-positive tissue were processed for use as positive controls. In a set of experiments, cell proliferation in tumor sections was evaluated by staining with rabbit anti-phospho histone H3 Ab (Ser10; Cell Signaling), as reported elsewhere (17). For each sample, 6–9 hfp (×400) were scored using an integrated grid.

Flow cytometric analysis

PE anti-CD11b (clone M1/70; BD Pharmingen) and FITC anti-Ly6G (eBioscience) Abs were used for the detection of CD11b⁺/Gr1⁺ in single-cell suspensions obtained from xenografted tumors. Leukocytes and activated macrophages were detected by staining with biotin anti-CD45.2 (BD Pharmingen) and biotin anti-F4/80 (CALTAG Laboratories) Abs, respectively, followed by staining with streptavidin-PE. To evaluate apoptosis, cells were incubated with Annexin V-FITC in Hepes buffer containing propidium iodide (PI), using the Annexin-V Fluos Staining Kit (Roche Diagnostics). Samples were analyzed on an EPICS-XL cytometer using Expo32 software (Beckman Coulter).

Statistical analysis

Results were expressed as mean value ± SE or, where indicated, ±SD. Statistical analysis was performed using the nonparametric Mann–Whitney Rank Sum Test or the Median Test. Differences were considered statistically significant at a P value of 0.05 or less.

Results

ED-B is commonly expressed in esophageal cancer

ED-B expression was initially evaluated in several esophageal cancer cell lines grown in vitro. By quantitative PCR analysis, ED-B levels were apparently correlated to those of total FN (Supplementary Fig. 1). ED-B was expressed at much higher levels by Kyse-30 and OE-33 cells compared with OE-21 or OE-19 cells (Supplementary Fig. 1). Following generation of s.c. xenografts of Kyse-30 cells, expression of ED-B in tumors was also analyzed. ED-B was broadly expressed by Kyse-30 tumors as well as by EAC#2 and EAC#8 xenografts, which were derived from esophageal adenocarcinoma or mucinous cell carcinoma surgical samples, respectively (Fig. 1A). In Kyse-30 xenografts, ED-B expression was abundant and ED-B⁺ areas were often found around blood vessels, whereas in EAC#2 xenografts, ED-B expression was scattered and not clearly overlapping with microvessels (Supplementary Fig. 2). Histologic analysis of Kyse-30 xenografts showed growth of solid nests of polygonal cells, similar to human squamous carcinoma, whereas EAC#2 xenografts showed glandular structures, compatible with adenocarcinoma; EAC#8 xenografts showed the typical histology of a mucinous adenocarcinoma, with...
tumor cells scattered in abundant extracellular mucin (Supplementary Fig. 3). Finally, ED-B was detected in 5 of 6 squamous cell carcinoma and 5 of 6 adenocarcinoma samples analyzed, although with highly heterogeneous expression levels (Fig. 1B), but not in normal esophageal mucosa (Fig. 1A). Altogether, these results indicate that ED-B is produced by cancer cells and is commonly expressed in the stroma of esophageal cancer.

**Acute anti-tumor effects of L19mTNFα in xenografts of esophageal cancer**

Kyse-30 tumors were chosen for initial evaluation of the therapeutic activity of L19mTNFα, in view of their relatively high ED-B expression levels. Mice bearing established s.c. xenografts were injected with the immunoconjugate and analyzed 24 h later. Marked reduction of tumor size (40%) as well as larger areas of necrosis were found in L19mTNFα-treated Kyse-30 tumors compared with controls (39.3% ± 6.5% vs. 16.3% ± 4.9%, P < 0.05) (Fig. 2A). Similar results were obtained following L19mTNFα administration to mice bearing EAC#2 and EAC#8 xenografts, which were derived from surgical samples and contained far less ED-B as compared with Kyse-30 tumors. Treatment increased the extent of tumor necrosis in both EAC#2 and EAC#8 tumors (Fig. 2C and Supplementary Fig. 4); on the contrary, effects on tumor size were minimal in EAC#2 tumors (Fig. 2C) and more pronounced in EAC#8 tumors (Supplementary Fig. 4). Moreover, alterations in the structure of tumor stroma with apparent loss of extracellular matrix and disruption of glandular-like structures were observed in EAC#2 tumors treated with L19mTNFα (Fig. 2C). A moderate reduction in MVD, strong impairment of vessel perfusion, and increased apoptosis of both endothelial and tumor cells were also noticed in L19mTNFα-treated tumors compared with controls (P < 0.005), thus highlighting the vascular-damaging properties of this drug (Fig. 2B and Supplementary Fig. 5). Similar findings were obtained with EAC#8 xenografts (Supplementary Fig. 4).

Finally, we also investigated whether L19mTNFα could exert direct anti-tumor activity, although this was not likely to occur in view of the relatively species-specific effects of TNFα. In this regard, analysis of Kyse-30 cells treated with...
Figure 2. Acute effects of L19mTNFα on esophageal tumors. A and C, L19mTNFα administration is followed by tumor shrinkage and increased necrosis. SCID mice injected s.c. on both flanks with Kyse-30 cells (1 × 10⁶ cells/injection, n = 4 mice/group) or implanted with EAC#2 tumor fragments (n = 5 mice/group) were treated or not with one injection of L19mTNFα (0.14 μg). Reductions of tumor volumes 24 h after treatment are shown. *P < 0.05, compared with PBS controls (Mann–Whitney Rank Sum Test). At histologic analysis, L19mTNFα-treated tumors show increased amounts of necrosis. Hematoxylin & eosin representative images are shown (magnification: ×100). Columns indicate quantitative analysis of necrotic areas in n = 8 (Kyse-30) or n = 10 (EAC#2) samples per group. *P < 0.05, compared with PBS controls (Mann–Whitney Rank Sum Test). B and D, effects of L19mTNFα on tumor angiogenesis. Vascularization of Kyse-30 (left) or EAC#2 (right) tumors was evaluated by anti-CD31 staining (red), whereas perfused vessels were identified by injection of Dextran-FITC 70 kDa (green). Nuclei were stained by TOPRO-3 (blue). Representative images are shown. Quantitative analysis was performed on n = 9 samples per group. *P < 0.05; **P < 0.005, compared with PBS controls (Median Test).
murine or human TNFα failed to demonstrate increased apoptosis compared with control cells (Supplementary Fig. 6), indicating that Kyse-30 cells are resistant to this cytokine. Due to the lack of adaptation to in vitro culture conditions, we were unable to investigate this with EAC#2 cells.

**Esophageal tumors recover rapidly after L19mTNFα treatment**

To evaluate long-term effects, Kyse-30 or EAC#2 tumors were analyzed 2–4 weeks after administration of the immunonoconjugate. Following initial shrinkage, tumors did not further regress, although their size remained significantly smaller compared with control tumors by the end of the observation period (Fig. 3A and C). Necrosis, however, was less abundant in the treatment group compared with controls for both Kyse-30 and EAC#2 tumors, at variance with findings at early time points. The increase in necrosis in the control group is likely due to the larger volume of these xenografts compared with short-term experiments; on the contrary, low levels of necrosis in L19mTNFα-treated xenografts probably reflect tumor cell repopulation following the initial damage. Intriguingly, weekly administrations of L19mTNFα to mice bearing EAC#2 tumors did not further reduce tumor size nor did they substantially change tumor necrosis, compared with a single administration of the immunonoconjugate (data not shown), indicating that tumors can recover relatively quickly following administration of L19mTNFα.

Interestingly, a trend toward increased MVD values was noticed in Kyse-30 and EAC#2 tumors receiving a single administration of L19mTNFα compared with controls (Fig. 3B and D). Percentage of perfusion was significantly higher in L19mTNFα-treated Kyse-30 tumors as compared with controls (median values 36.4 and 8.5, respectively), while it was comparable in treated versus control EAC#2 samples (Fig. 3B and D). Altogether, these findings show that L19mTNFα initially behaves as a vascular damaging agent in Kyse-30 and EAC#2 xenografts, but treatment does not completely eradicate the tumors that remain stable in size and shift toward revascularization.

**Mechanisms of adaptation of tumors to L19mTNFα**

To investigate possible reasons for the rapid recovery of a functional vasculature following L19mTNFα treatment, VEGF expression in tumors was analyzed. VEGF was expressed in EAC#2 tumors and its expression increased 50 h after treatment with the VDA (Fig. 4A). Quantitative PCR analysis confirmed this finding and indicated that it was essentially the human VEGF transcript to increase after L19mTNFα treatment (Fig. 4A). Intriguingly, most microvessels in L19mTNFα-treated tumors lacked pericyte coverage, as shown by double staining with anti-CD31 and anti-NG2 (Fig. 4B). The presence of large numbers of immature vessels may reflect ongoing angiogenesis due to increased VEGF expression or be due to TNFα-induced regression of the pericyte component of preexisting microvessels. However, as we could not find evidence of increased endothelial cell proliferation at the time point analyzed (not shown), the latter explanation is favored.

Moreover, in view of the growing role of hematopoietic cells in tumor angiogenesis and resistance to anti-angiogenic therapy (18), we also examined the numbers of some leukocyte subpopulations in EAC#2 tumors. Leukocyte infiltration, as evaluated by CD45 staining, was not prominent in these tumors (Fig. 4C), yet it slightly increased following L19mTNFα administration. Notably, CD11b+Gr1+ cells were increased 9-fold 50 h after therapy, whereas F4/80+ cell numbers were relatively low in these tumors compared with other models, and they did not significantly change following L19mTNFα administration (Fig. 4C).

Because CD11b+Gr1+ cells have angiogenic potential (19), they could possibly contribute—along with increased VEGF production by the tumor cells—to promote rapid repair of the damaged tumor vasculature after administration of L19mTNFα.

**Vandetanib inhibits growth of EAC#2 xenografts**

Because VEGF was abundantly expressed in EAC#2 xenografts, we evaluated the possibility that vandetanib, a potent orally available inhibitor of VEGFR2 and, to a lesser degree, EGFR and RET tyrosine kinase activity (14) might improve the therapeutic activity of L19mTNFα. To this end, SCID mice bearing established EAC#2 xenografts were treated daily for 3 days with vandetanib, either following one administration of L19mTNFα or as a monotherapy. Treatment was well tolerated; no apparent signs of toxicity were observed. Vandetanib caused 52.7% reduction in tumor size compared with controls (vehicle alone), accompanied by increased necrotic areas (37.0% ± 6.4% vs. 7.8% ± 1.4%; Fig. 5A). Administration of vandetanib after L19mTNFα caused a dramatic increase in necrosis compared with controls (82.0% ± 4.3% vs. 7.8% ± 1.4%) or either agent alone. Viable tumor areas underwent immunofluorescence analysis, which demonstrated a significant reduction in MVD and percentage of perfusion in vandetanib-treated mice compared with controls and extremely reduced values of these parameters in the group receiving combined treatment (Fig. 5B). Furthermore, we found only minor changes in proliferation or apoptosis in EAC#2 tumors treated with vandetanib, whereas the group receiving L19mTNFα had significantly reduced numbers of proliferating cells and increased numbers of apoptotic cells compared with controls (Fig. 5C). Finally, combined treatment was associated with a further decrease of cell proliferation compared with L19mTNFα-treated tumors (P < 0.05).

**Sustained tumor growth inhibition by chronic L19mTNFα and vandetanib administration**

We finally investigated whether combined L19mTNFα and vandetanib therapy could bring about durable therapeutic effects. SCID mice bearing established EAC#2 xenografts were treated with L19mTNFα, vandetanib, or the combination of the 2 drugs for 4 weeks. Measurements of tumor size disclosed that the strongest effect was...
Figure 3. Esophageal tumors recover rapidly after L19mTNFα treatment. A and C, kinetics of tumor development following L19mTNFα administration. SCID mice were injected s.c. on both flanks with Kyse-30 cells (1 × 10^6 cells/injection, n = 5 mice/group) or implanted with EAC#2 tumor fragments (n = 5 mice/group) and treated or not with L19mTNFα (0.14 μg/g) (arrow). Tumor growth curves were determined. Histological analysis disclosed increased necrotic areas in control tumors compared with those treated with L19mTNFα. H&E representative images are shown (magnification: ×200 and ×100). Quantitative analysis was done on n = 10 samples per group. *P < 0.05; **P < 0.001 (Mann–Whitney Rank Sum Test). B and D, restoration of tumor microvasculature in L19mTNFα-treated tumors. Microvessels were stained by anti-CD31, whereas perfusion was evaluated following injection of Dextran-FITC 70 kDa. Representative images are shown. Quantitative analysis was performed in n = 7–10 samples per group. *P < 0.05, compared with PBS controls (Median Test).
apparently exerted by vandetanib monotherapy, followed by the combination of L19mTNFα and vandetanib (Fig. 6A). At gross inspection, however, tumors treated with both drugs differed from those treated with vandetanib monotherapy, in that they were less solid and contained some hemorrhagic fluid (Fig. 6B). We therefore performed histologic analysis to estimate the amount of remaining viable tumor. Larger necrotic areas were detected in tumors treated by the combination of the two drugs compared with vandetanib-treated tumors (Fig. 6B), in line with results obtained following short-term treatments. Analysis of vascular parameters failed to show marked differences in the MVD of the different samples, whereas the percentage of perfusion was generally lower in tumor samples receiving either treatment, with the highest impairment of perfusion in samples treated with the combination of L19mTNFα and vandetanib (Fig. 6C). Inhibition of tumor growth was not attributable to systemic toxicity because no significant weight loss was seen in any of the treatment groups (not shown). Proliferation was not affected by monotherapy with either drug, whereas combination of vandetanib and L19mTNFα significantly reduced numbers of pH3+ cells (P < 0.05) (Fig. 6D).

In conclusion, these findings show that combined treatment with L19mTNFα and vandetanib exerts increased anti-tumor effects in esophageal cancer xenografts.

Figure 4. VEGF expression, pericyte coverage, and tumor-associated hematopoietic cells 50 h after injection of L19mTNFα. A, VEGF expression is increased in L19mTNFα-treated compared with control EAC#2 tumors. Pictures show representative patterns of VEGF expression by IHC in tumor samples (n = 5 per group) treated with L19mTNFα versus PBS; magnification: ×400. The columns report relative expression data of both human and murine VEGF transcripts in tumors by quantitative PCR analysis (mean ± SEM; n = 3 samples/group). B, measurement of pericyte coverage in EAC#2 tumors by anti-NG2/anti-CD31 staining followed by IFA. Representative fields are shown on the right; columns indicate the percentage of NG2+ microvessels (mean ± SE). Quantitative analysis was performed in n = 4 samples per group; 6–10 microscopic fields/sample were analyzed. *P < 0.05, compared with PBS controls (Mann–Whitney Rank Sum Test). C, determination of CD45+ F4/80+ and CD11b+ Gr1+ in EAC#2 tumors treated or not with L19mTNFα by flow cytometric analysis. Quantitative analysis was done on n = 7–10 samples per group. *P < 0.05; **P < 0.001, compared with PBS samples (Mann–Whitney Rank Sum Test). Right, one representative analysis of CD11b+ Gr1+ cells in EAC#2 tumors.
Discussion

In this study, we analyzed expression of the oncofetal B-FN in esophageal cancer and investigated a new therapeutic approach with the use of 3 different tumor models, 2 of which were derived from patients’ samples without prior adaptation to in vitro growth. B-FN appears to be commonly expressed both in adenocarcinomas and squamous cell carcinomas of the esophagus. Although expression levels are rather heterogeneous in tumors, it is important to note that relatively low-level B-FN expression—as found in EAC#2 xenografts—did not preclude therapeutic efficacy of L19mTNFα.

The L19 antibody, fused to human TNF, is currently investigated in two phase II clinical trials in patients with cancer. In this study, the use of murine TNFα was mandatory, in view of the lower biological activity of human TNF in the mouse setting, and the therapeutic properties of L19-mTNFα have previously been documented (11–13). L19mTNFα is not a canonical VDA (20), but its effects on blood vessels and tumor morphology indicate that it behaves as such in esophageal tumor xenografts, in line with previous observations (11). Indeed, it has been reported that TNFα acts as a VDA in its own right (21), and similar to other vasoactive drugs, modifies the actin cytoskeleton and permeability through the RHP/RAC pathway (22); its conjugation to L19 enables specific targeting of its biological activity to tumors.

Treatment of EAC#2 xenografts with L19TNFα also resulted in loss of ECM and altered stromal structure (Fig. 2C). Because L19TNFα recruits leukocytes into tumors (Fig. 4B), we speculate that proteolytic enzymes released by these cells could contribute to this phenomenon, which was not seen in the case of Kyse-30 xenografts, which show a definitely different histologic pattern. Signals stemming from extracellular matrix components may regulate the behavior of malignant cells (23); therefore, it could be that this peculiar stromal alteration contributes by as yet unknown mechanisms to the therapeutic effects of the immunoconjugate.

Figure 5. Vandetanib monotherapy or combined with L19mTNFα inhibits growth of EAC#2 xenografts. A, kinetics of tumor development. SCID mice were implanted with EAC#2 tumor pieces (n = 5 mice/group) and treated or not with vandetanib (50 mg/kg/d for 3 days) as monotherapy or combined with one administration of L19mTNFα (0.14 μg/g). Histological analysis (left) shows larger necrotic areas in EAC#2 tumors treated with both L19mTNFα and vandetanib. Magnification: 1000. Columns indicate quantitative analysis of necrotic areas in n = 10 samples of each group. ∗P < 0.005, compared with the PBS group (Mann–Whitney Rank Sum Test). B, anti-vascular effects of vandetanib are amplified by the combined regimen. Microvessels were stained by anti-CD31, whereas perfusion was evaluated following injection of Dextran-FITC. Representative images are shown. Quantitative analysis was performed on n = 10 samples per group. ∗P < 0.05; ∗∗P < 0.001, compared with the PBS group (Medlan Test). C, evaluation of proliferation and apoptosis in tumors by pH3 and cleaved caspase-3 staining. The columns indicate mean ± SD values; 3–6 samples per group were analyzed. ∗P < 0.05; ∗∗P < 0.005, compared with the control group (Mann–Whitney Rank Sum Test).
**Figure 6.** Durable therapeutic effects of chronic L19mTNFα/vandetanib treatment in EAC#2 xenografts. A, kinetics of tumor growth. SCID mice were implanted with EAC#2 (n = 5–6 mice/group) and treated or not with L19mTNFα (0.14 μg/g) and/or vandetanib (50 mg/kg/d). Mice were sacrificed 24 days later. *P < 0.05, compared with L19mTNFα/vandetanib-treated tumors (Mann–Whitney Rank Sum Test). B, macroscopic appearance of tumors. Left, representative images of intact tumors just excised from the animals; Right, the internal side of the same tumor samples. Representative histologic images of tumor samples show the necrotic areas. Magnification: ×100. Columns indicate quantitative analysis of necrotic areas in n = 6 samples of each group. *P < 0.05 (Mann–Whitney Rank Sum Test). C, representative images of tumor vascularization and perfusion. Quantitative analysis is performed on n = 6–10 samples per group. *P < 0.05; **P < 0.005 (Mann–Whitney Rank Sum Test). D, evaluation of proliferation in tumors. Columns indicate mean ± SD values of phosphohistone 3 (pH3) positive cells in n = 3–5 samples of each experimental group; *P < 0.05.
One key finding of our study is the marked anti-tumor effects observed following combined treatment of esophageal cancer xenografts with L19mTNFα combined with vandetanib. These results expand the list of therapeutic agents that can be combined successfully with VDA administration in preclinical models (20, 24). Combining VDAs with vehicles targeting angiogenesis is a rational step, because the re-growth from the viable tumor rim that remains after VDA therapy is driven by angiogenesis. Indeed, preclinical studies combining a VDA with a drug targeting the VEGF pathway have shown that the viable rim almost entirely disappears when the anti-angiogenic drug is added, resulting in enhanced anti-tumor effects (25, 26). Moreover, it has recently been shown that VDAs can cause direct upregulation of VEGF (27), and indeed, we also observed this in our tumor model (Fig. 4A). This increased level of VEGF is likely to be functionally neutralized by treatments that specifically block the VEGF pathway.

On the contrary, we also noticed that L19mTNFα administration is followed by a rather rapid increase in the numbers of tumor-associated CD11b+Gr1+ cells, which have been associated with resistance to anti-VEGF therapy, mainly through production of the proangiogenic factor Bv8 (28). Bv8 binds two highly related G-protein–coupled receptors, EG-VEGFR/PKR1 and EG-VEGFR/PKR2 (29). As the proangiogenic effect of Bv8 would not likely be neutralized by vandetanib, this could explain—among other possibilities—the persistence of some blood vessels and residual viable tumors even after treatment by the combination of the two drugs. Importantly, vandetanib did not perturb CD45+ or CD11b+Gr1+ cell numbers in tumors (not shown), marking a difference between VDAs and anti-angiogenic drugs that has been noticed before with DC101, an anti-VEGFR Ab (25).

Intriguing results came from analysis of cell proliferation and apoptosis in tumors. In fact, although VDAs have been reported to increase cell proliferation in the viable rim in certain experimental settings (30), we did not observe this in EAC#2 tumors following short-or long-term treatment with L19mTNFα. Rather, L19mTNFα—but not vandetanib—appears to lower cell proliferation and increase apoptosis in tumors following acute treatment (Fig. 5C). The different effects of the two drugs on tumor cell viability and proliferation—which cannot be attributed to direct effects of either drug on tumor cells or to differences in their antiangiogenic activity—might result from the singular stromal effects of L19mTNFα described above, which were not observed in vandetanib-treated tumors and could contribute to reduce cell proliferation and trigger anoikis.

Importantly, L19mTNFα and vandetanib together had synergistic effects on vascular perfusion (Fig. 6C). Altogether, these findings suggest that starvation of tumors by highly effective anti-vascular therapy is feasible, although we concede that further work—including evaluation of the therapeutic effects in other tumor xenografts—_is_ required to strengthen this hypothesis and prove that this drug combination is indeed able to improve the therapeutic outcome in the long term and investigate whether tumors will relapse following discontinuation of the treatment.

Notwithstanding these promising results, there are some potential concerns about the use of a VDA with antiangiogenic agents. In particular, such combinations may exacerbate vascular-associated toxicities that have been reported with VDAs (31, 32). Although we have not observed this in mice, it is not possible to predict how these agents could behave in combination in patients. Clinical studies indicate mild toxicity of vandetanib (33, 34), while the maximum tolerated dose of L19mTNFα will be calculated at the end of the ongoing phase I trials, thus providing the basis to design appropriate clinical trials using a combination of the two compounds.

Anti-angiogenic therapy has been postulated to induce a state of dormancy in the tumor (35), based on early findings of repeated treatments of experimental tumors with endostatin (36). However, first-generation anti-angiogenic drugs mainly targeting the VEGF pathway have failed to bring about tumor dormancy, leading at best to transitory control of tumor growth and moderate clinical benefits (37). Our results indicate that substantial impairment of vascular function and tumor cell proliferation—that is, by combining L19mTNFα with vandetanib—is a strategy that appears promising to achieve this goal.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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