Activin Receptor-like Kinase 1 Ligand Trap Reduces Microvascular Density and Improves Chemotherapy Efficiency to Various Solid Tumors

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Abstract

Purpose: Antiangiogenic therapy, mostly targeting VEGF, has been applied in cancer patients for the last decade. However, resistance to anti-VEGF therapy and/or no significant benefit as monotherapy agent is often observed. Therefore, new antiangiogenic strategies are needed. In the current study, we investigated the therapeutic effect of interfering with the bone morphogenetic protein (BMP)9/activin receptor–like kinase (ALK)1 signaling pathway by using an ALK1-Fc ligand trap.

Experimental Design: We analyzed the potential antiangiogenic and antitumor effects of ALK1-Fc protein as monotherapy and in combination with chemotherapy in vivo in mouse models of melanoma, head and neck cancer, and invasive lobular breast carcinomas. ALK1-Fc sequesters BMP9 and 10 and prevents binding of these ligands to endothelial ALK1, which regulates angiogenesis.

Results: Treatment of mice with ALK1-Fc strongly decreased the tumors’ microvascular density in the three different mouse cancer models. However, this effect was not accompanied by a reduction in tumor volume. An immunohistochemical analysis of the tumor samples revealed that ALK1-Fc treatment increased the pericyte coverage of the remaining tumor vessels and decreased the hypoxia within the tumor. Next, we observed that combining ALK1-Fc with cisplatin inhibited tumor growth in the breast and head and neck cancer models more efficiently than chemotherapy alone.

Conclusions: The addition of ALK1-Fc to the cisplatin treatment was able to enhance the cytotoxic effect of the chemotherapy. Our results provide strong rationale to explore combined targeting of ALK1 with chemotherapy in a clinical setting, especially in the ongoing phase II clinical trials with ALK1-Fc.

Introduction

Angiogenesis, the process leading to the development of new blood vessels, is essential for the outgrowth of solid tumors, providing nutrients and a mechanism for dissemination of cancer cells (1, 2). Notably, the newly formed tumor vasculature is structurally and functionally abnormal leading to an aberrant tumor microenvironment characterized by interstitial hypertension, hypoxia, and acidosis. Collectively, these abnormalities contribute to impaired delivery and reduced efficacy of therapeutics to solid tumors.

Antiangiogenesis therapies aim to prevent the formation of new blood vessels and ultimately starve the cancer cells and inhibit tumor progression. A broad number of molecules have been identified and shown to play key roles in this process (3). Among them, VEGF has been used as the prime antiangiogenic target in the clinic for the last decade (4). Unfortunately, in contrast to the promising results from preclinical studies, the use of these antiangiogenic agents as monotherapy has yielded only modest therapeutic benefit in some tumor types, whereas it has failed in others (5–7). Moreover, after an initial response, resistance to this therapy is often observed. In addition, long-term suppression of VEGF signaling gives rise to compensatory mechanisms by other angiogenic pathways (8). Therefore, alternative antiangiogenic targets should be explored (9).

When combined with chemotherapy, synergistic effects have been reported (10, 11). However, clinical trials of breast cancer patients with the anti-VEGF antibody bevacizumab, also in combination with chemotherapy have shown variable results and no clear clinical improvement yet (12). A major challenge remains...
ALK1-Fc Improves Chemotherapy Efficiency

Translational Relevance

ALK1-Fc (dalantercept) is a ligand trap targeting the activin receptor-like kinase-1 and is currently being tested in clinical trials for antiangiogenic therapy. ALK1-Fc prevents binding of selective bone morphogenetic proteins to endothelial ALK1, which controls (tumor) angiogenesis. In the current study, we show strongly enhanced efficacy of chemotherapy, when combined with ALK1-Fc treatment in various mouse models for solid tumors. These results provide a strong rationale to explore combined targeting of ALK1 with chemotherapy in a clinical setting, especially in the ongoing phase II clinical trials with ALK1-Fc.

that a large number of cancer patients do not respond at all, or only minimally, to antiangiogenic therapies. Activin receptor-like kinase (ALK)-1 represents a promising target for antiangiogenic therapy in solid tumors. ALK1 forms part of a TGFβ receptor signaling complex on endothelial cells and plays an important role in regulating angiogenesis (13, 14). Bone morphogenetic protein (BMP)9 and BMP10 bind directly to ALK1 (15, 16). ALK1-Fc, a chimeric protein consisting of the ALK1 extracellular domain fused to the Fc-part of an antibody, functions as a ligand trap and sequesters BMP9 and -10 and prevents binding of these ligands to the endothelial ALK1 receptor, thereby decreasing angiogenic responses in vitro (17). However, the role of BMP9 in angiogenesis remains controversial and seems to be strongly context and concentration dependent (18).

A previous study has shown that administration of a BMP9-neutralizing antibody or ALK1-Fc causes increased retinal vascularization due to elevated BMP10 levels (19). Conversely, ALK1-Fc treatment was shown to inhibit MCF-7 breast cancer growth in vivo (20). Interestingly, ALK1-Fc was recently reported to have therapeutic effects in multiple patients with advanced cancer (21). Therefore, identifying what types of tumors might be sensitive to this therapy could yield long-term survival benefits to these patients.

In this study, we analyze the effects of targeting ALK1 ligands with the ALK1 ligand trap ALK1-Fc in vivo using three different mouse cancer models of melanoma, head and neck cancer, and invasive lobular breast carcinomas. Mortality rates of patients with melanoma, head and neck cancer and breast cancer have not significantly decreased in past years. Currently, these tumors account for over 20% of the estimated new cases of cancer in 2014 and approximately 10% of cancer-related mortality (data from American Cancer Society), indicating a clinical need for new therapies. Angiogenesis has a well-recognized role in these types of tumors and several studies have shown a correlation between disease progression and vascular density. However, to date, few clinical trials have shown promising results in these types of cancers using antiangiogenic agents. Therefore, we sought to determine the antitumor effects of interfering with the ALK1 signaling pathway by using an ALK1-Fc ligand trap.

Materials and Methods

Antibodies and recombinant proteins

CD31 (PECAM) antibodies were purchased from Santa Cruz Biotechnology and NG2 antibodies from Millipore. Anti-human FC antibodies were purchased from R&D systems and rat anti F4/80 antibodies from ebiosciences. To detect hypoxic areas, hypoxia probe was purchased (Burlington). ALK1-Fc is a fusion protein comprised of the extracellular domain of human ALK1 fused to the Fc region of IgG and was generously provided by Acceleron Pharma. ALK1-Fc was dissolved in 20 mmol/L Tris-HCl and diluted in 0.9% NaCl. The Fc domain of IgG1 was used as a control (MOPC-21; Bio Express).

Lentiviral vectors were generated by cloning a sequence containing the ALK1, ALK2, or Flt extracellular domain fused to an Fc expression sequence in pLV puromycin resistance-containing plasmids.

Cell lines

KEP1-11 mouse breast cancer cells (22) were isolated from spontaneous breast tumors in E-cadtherin+/−, p53+/− mice and cells were transduced with luciferase. Cells were kept in DMEM/F12 supplemented with 10% FCS and penicillin/streptomycin. KEP1-11 cells with stable expression of Fc, ALK1-Fc, ALK2-Fc, and Flt-Fc were generated by infecting cells with lentiviruses and selection with 5 µg/mL puromycin (Sigma-Aldrich). Luciferase-expressing human OSC19 head and neck cancer cells were described before (23) and maintained in DMEM-containing 10% FCS, penicillin/streptomycin, MEM-NEAA (1%), MEM vitamin mixture (1%), Glutamax (all Sigma-Aldrich), 1 mmol/L pyruvate (Thermo Scientific).

Animal experiments

All animal experiments using breast and head and neck cancer cells were approved by the animal ethics committee of the Leiden University Medical Center (the Netherlands). All animal studies using melanoma cells were approved by the animal welfare committee of Erasmus MC (the Netherlands).

Treatments consisted of 10 mg/kg bodyweight ALK1-Fc (RAP-041, an ALK1 extracellular domain/Fc fusion protein), Fc control protein or DC101 antibody (10 mg/kg) administered by intraperitoneal (i.p.) injection twice per week. Cisplatin treatment consisted of 3 rounds, 5 mg/kg i.p. injections at indicated time points. Doxorubicin was administered by intravenous injection. Hypoxia probe was injected intraperitoneally 45 minutes before sacrifice. For perfusion studies, 50 µg FITC-lectin (Vector labs) was injected intravenously and after 5 minutes the mouse was anesthetized and perfused with PBS.

For the melanoma model, C57Bl6 mice (Harlan) were used at a weight of 25 grams. Mice were brought under anesthesia and received subcutaneous Temgesic (Schering-Plough) injection as pain medication. Mice were housed under standard conditions or at 30°C and 60% humidity for window chamber experiments.

Window mice for intravital microscopy were generated as previously described (24). Briefly, dorsal skin-fold window chambers were installed on the back of mice (n = 5) and B16BL6 tumors were generated by inserting a small tumor fragment in the exposed fascia and allowed to grow. Eight days after installment of the windows and transplantation of the tumors, treatment with ALK1-Fc was started with an interval of 2 days between injections. Treatment was continued for the remainder of the experiment. Tumor growth and tumor vessel formation was followed over time. For this purpose, endothelial nitric oxide synthase (eNOS)-Tag GFP mice were used constitutively expressing GFP in endothelial cells allowing visualization of vascular bed formation as...
described elsewhere (25). Images were made with a Zeiss 510 Meta confocal microscope (Carl Zeiss). GFP in vessels was visualized by an argon laser (488 nm) with fluorescent band pass filters set to 505 to 550. Mice were positioned on a heated stage and the windows fixed to prevent motion. Acquired images were analyzed using ImageJ (Wyne Rasban, NIH, Bethesda, MD).

For the efficacy study, tumor fragments were implanted subcutaneously in the flank of mice (n = 10–13) as previously described and allowed to grow until palpable (average tumor diameter 4 mm). Treatment was started with ALK1-Fc at that time point and continued every 3 days. Doxorubicin (Pharmachemie B.V. Haarlem, the Netherlands) was added to the treatment starting on the third ALK1-Fc administration with the same interval. Treatment was continued for the remainder of the experiment. For the head and neck cancer models, 6- to 8-week-old female Balb/c mice (Charles River, strain CBBy/Cg-doc1–nu/J) of 20 grams were used. Mice were brought under anesthesia and received subcutaneous Temgesic injection as pain medication. A total of 10 × 10^3 luciferase-expressing OSC19 cells dissolved in 10 µL PBS were injected in the tip of the tongue of the mice (26). Three days after injection, mice were imaged as described above. Treatment with ALK1-Fc or Fc was given twice per week (10 mg/kg, i.p.) and cisplatin was administrated at days 8, 13, and 18. At day 20, mice were sacrificed and tumors and lymph nodes were removed for further analysis. Blood was collected and serum and plasma samples were stored at −80°C until use.

For the breast cancer model mice (6/group, total 12 mice/group) were brought under anesthesia by intraperitoneal injection of a ketamine/sedanum/atropine mixture. Next, an incision was made and the 4th mammary gland was exposed. A total of 30 mls of 1:1 medium/Matrigel (BD Biosciences) solution were injected in the 4th mammary gland. The wound was closed and mice received subcutaneous Temgesic injection as pain medication. After 2 weeks, mice were injected with d-Luciferin (Synchem; 0.2 mg/g bodyweight), brought under anesthesia by isoflurane inhalation (Pharmachemie), and imaged using the Xenogen, IVIS Lumina (Perkin-Elmer). Furthermore, tumor volume was estimated using caliper measurements and calculated using the formula: Tumor volume = (length^2 × breadth)/2. After 2 weeks, when tumors were palpable, mice were treated twice per week intraperitoneally with 10 mg/kg ALK1-Fc, or Fc control protein. For cisplatin studies, mice were treated once per week intraperitoneally for 3 weeks with 5 mg/kg cisplatin (Tocris). Weight was monitored several times per week. After 6.5 weeks, mice were sacrificed and tumors and metastases were collected and processed for standard immunohistochemistry. Blood was collected and serum and plasma samples were stored at −80°C until use.

Immunohistochemistry

Tumors were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Sequential sections (4µm) were cut and mounted on superfract glass slides (ThermoScientific). Immunohistochemistry was performed as described before (27). In short, slides were incubated overnight with primary antibodies, followed by biotinylated secondary antibodies (all obtained from Dako). Staining was visualized by the vectastain system (Vector Laboratories) and diaminobezidine staining (Sigma-Aldrich). Counterstain was performed with hematoxylin and slides were mounted using entellan (Merck). For microvessel density analysis, binary images were generated and staining was quantified using ImageJ software. Four fields per tumor were analyzed to calculate the average microvessel density per mouse.

Immunofluorescence

Immunofluorescence staining was performed as described previously (28). In brief, immunofluorescence staining of tumors samples were performed on 4-µm paraffin-embedded sections. After deparafinization and rehydration, antigen retrieval was performed by boiling in 0.01 mol/L sodium citrate, pH 6.0, for 10 minutes followed by overnight incubation at 4°C with NG2 and CD31 antibodies. Next, sections were incubated with secondary antibodies labeled with Alexa Fluor 488, 555, or 647 (Invitrogen/Molecular Probes) at 1:250. Nuclei were visualized by DAPI, which was included in the mounting medium, Prolong G (Invitrogen/Molecular Probes).

TGFβ1 and BMP9 ELISAs

Plasma BMP9 levels were measured by ELISA as described before (29). TGFβ1 levels were measured using a commercially available mouse TGFβ1 Duoset ELISA (DY1086, R&D Systems) as described before (30, 31).

Western blot analysis

To evaluate expression of the Fc constructs in the stable cell lines, cells were lysed and 10 µg protein was separated by 10% SDS-PAGE. Western blotting with anti-Fc antibodies (1:1,000, R&D Systems) was performed as described before (27).

Statistical analysis

Differences between groups were calculated using the Student t test or Mann–Whitney U test when appropriate.

Results

ALK1-Fc reduces tumor vascular density without inhibiting tumor size in melanomas, head and neck cancer, and breast cancer

The proangiogenic ALK1 receptor has been shown to bind to BMP9, which is present in circulation (32). Before testing the effect of sequestering these proteins in vivo by injecting ALK1-Fc, we investigated the expression of BMP9 in human tumor samples. Immunohistochemical analysis revealed that BMP9 was clearly expressed in the squamous cell tongue carcinoma and invasive lobular breast carcinoma samples tested (Supplementary Fig. S1). To determine the effect of ALK1-Fc protein on vascularization and tumor development, OSC19 human oral squamous carcinoma cells were injected in the tip of the tongue of Balb/c nude mice. These cells carry a luciferase construct, allowing us to follow tumor growth and development in time by bio luminescence imaging (BLI; Fig. 1A). Size of these fast developing tumors was determined by quantifying the luciferase signal after the administration of luciferin (Fig. 1B). Initial imaging 4 days after injection was performed to randomize the mice in groups with equivalent signal and thereafter mice were treated systemically with the antiangiogenic agent. The impact of ALK1-Fc was compared with Fc control and DC101, a well-known anti-mouse VEGF receptor (VEGFR) antibody (33) Treatment with ALK1-Fc did not affect
tumor growth compared with the control group (Fc). As expected, tumor growth inhibition was observed in mice treated with DC101, resulting in a significantly decreased tumor size at day 20 (Fig. 1A and B). At this point, tumors were retrieved and immunohistochemical analysis was performed. Staining with CD31-specific antibodies, a marker for endothelial cells, revealed that treatment with either ALK1-Fc or DC101 led to significantly reduced vascular density in the tumors (Fig. 1C, representative images are shown in supplementary Fig. S2A). These results suggest that interference with ALK1 or VEGF signaling inhibits tumor angiogenesis, but only the latter affects growth of oral squamous cell tumors.

Figure 1. Effect of ALK1-Fc treatment on tumor growth and vascularity in head and neck cancer and melanoma mouse models. A, bioluminescence images of Balb/C nude mice injected with OSC-19 luciferase-expressing cells in the tongue (day 20). Mice were treated with Fc, ALK1-Fc or DC101 (n = 8/group), and pictures from 3 representative mice per group are shown. B, tumor growth was assessed by quantification of the luciferase signal on days 4, 7, 10, 13, 17, and 20. C, the antiangiogenic effect of the treatments was verified by immunohistochemical analysis of the tumor samples and quantification of the CD31-positive area. D, dorsal skinfold window chambers were installed on the back of C57/Bl6 mice and a small tumor piece from subcutaneous B16 tumors was inserted in the exposed fascia. After 8 days, treatment with either ALK1-Fc or control Fc started and tumor development was assessed by measuring the tumor surface on days 0, 3, 5, and 7 (n = 2). E, to visualize the vascularization of the tumors, eNOS-GFP mice were used, which constitutively express GFP in endothelial cells. Representative images from the window chambers show the vascularization of the tumors upon treatment with Fc or ALK1-Fc (n = 5 per group). F, quantification of vascular density in Fc and ALK1-Fc–treated melanomas (day 13).
To assess the therapeutic effects of ALK1-Fc in melanoma, a syngeneic B16 melanoma model was used and the development of the tumor and vasculature was followed using intravital microscopy in a dorsal skin-fold window chamber. Administration of ALK1-Fc did not affect tumor growth (Fig. 1D), but had an effect on vascular density of the melanomas (Fig. 1E and F). In the Fc-treated mice, 34% GFP-positive pixels (reflecting vessel quantity) were observed, whereas in the ALK1-Fc–treated mice this was reduced to 19%. This corresponds to a reduction of about 45% of the vascular density (Fig. 1E and F).

Next, the potential therapeutic effect of ALK1-Fc treatment on the growth and vascularization of tumors was tested on a model for invasive lobular breast carcinomas (22). Mice were injected orthotopically with the luciferase-expressing KEP1-11 breast cancer cells. After 2 weeks, when tumors were palpable, mice were treated with PBS, Fc, ALK1-Fc, or DC101. Our data show that tumor size, as monitored by BLI (Fig. 2A) and weight of the tumors ex vivo was not affected by treatment with ALK1-Fc, whereas it was reduced after treatment with DC101 (Fig. 2B). Immunohistochemical detection of endothelial cells using a CD31-specific antibody revealed strongly reduced vascular density in both the ALK1-Fc–treated and the DC101–treated tumors compared with the Fc control tumors (Fig. C and D). As tumor-infiltrating macrophages have been shown to have important roles in the regulation of tumor angiogenesis, we stained for F4/80 to determine macrophage accumulation in the tumors. Our data show no significant difference in macrophage accumulation in ALK1-Fc–treated mice versus Fc control mice (Supplementary Fig. S2B).

To analyze whether ALK1-Fc could interfere with tumor initiation, we generated KEP1-11 breast cancer cell lines expressing ALK1-Fc, ALK2-Fc (ALK2 is another BMP type-I receptor), Fc (negative control), and Flt-Fc (soluble VEGF-receptor, positive control). Expression of the constructs was verified using western blot analysis on tumor cell lysates (Supplementary Fig. S3A). After orthotopic transplantation of the cells, tumor development was

Figure 2. Effects of ALK1-Fc on tumor growth and vascular density in breast cancer mouse models. A, bioluminescence images of nude mice orthotopically injected with KEP1-11 luciferase-expressing cells in the fourth mammary gland. Mice were treated with PBS, Fc, ALK1-Fc, or DC101 (n = 8/group) and pictures from 3 representative mice per group are shown (week 7). B, mice were sacrificed and tumors were retrieved and weighted. Data, mean ± SEM. C, representative pictures of CD31 staining on the tumors from each group are shown (Original magnification 100×). D, quantification of the CD31-positive area. E, breast cancer KEP1-11 luciferase-expressing cells with stable expression of Fc, ALK1-Fc, ALK2-Fc, and Flt-Fc were orthotopically injected in nude mice and tumor burden and growth was followed by BLI. Representative pictures from 3 mice per group are shown. F, tumor growth was assessed by caliper measurement and quantification of the tumor volume at end stage is shown. Graph represents mean ± SEM. G, representative pictures of immunohistochemical analysis of vascularization in tumors using CD31-specific antibody (original magnification 100×). H, quantification of the CD31-positive area.
followed by BLI and tumor growth was assessed by quantification of the luciferase signal and caliper measurements. ALK1-Fc expression reduced circulating BMP9 levels in mice to undetectable levels (Supplementary Fig. S3B), whereas tumors injected with ALK2-Fc showed increased BMP9 levels (Fig. 2E and F). Only Flt-Fc expression by these cells led to a significant reduction in tumor size (P = 0.03). Immunohistochemical analysis of the tumors showed decreased CD31 expression in ALK1-Fc and Flt-Fc–expressing tumors compared with Fc control tumors (Fig. 2G and H).

Together, these data show that although treatment with ALK1-Fc did not result in decreased tumor size, it lead to a strong reduction of the vascular density in vivo in three different mouse models for melanomas, head and neck cancer, and invasive lobular breast carcinoma.

ALK1-Fc treatment leads to increased coverage of tumor vasculature by NG2-expressing cells

To investigate why a strong reduction in vascular density does not result in tumor growth inhibition, KEP1-11 mouse breast tumor samples were stained with NG2-specific antibodies, a marker for pericytes. The immunohistochemical analysis revealed increased NG2 staining in ALK1-Fc–treated mice (Fig. 3A and B) or mice bearing ALK1-Fc–expressing tumors (Supplementary Fig. S4A and S4B). Interestingly, compared with controls, the number of pericytes was not increased in tumors from DC101–treated mice (Fig. 3A and B) or tumors formed after inoculation of Flt-Fc–expressing cells (Supplementary Fig. S4A and S4B).

We performed immunofluorescent stainings to visualize the localization of the NG2-positive pericytes within the tumors. Mice treated with ALK1-Fc contained CD31-positive cells in the tumor vessels tightly covered by NG2-expressing cells. Endothelial cells forming vessel-like structures in the other treatment groups (PBS, Fc, and DC101) were found poorly covered by NG2-positive cells (Fig. 3C).

To functionally address the effect of the aforementioned changes on the tumor vasculature, we injected mice with a probe that binds specifically to hypoxic areas. ALK1–Fc–treated tumors showed a reduction in the intratumoral hypoxia compared with tumors from control groups (Fig. 3D and E). To analyse perfusion in response to ALK1-Fc, mice were injected with FITC-lectin prior to sacrifice, and FITC signal relative to CD31 immunofluorescent signal was quantified and calculated for KEP1-11 tumor samples. Our result appears to support an increased perfusion in ALK1-Fc treated mice (n = 3) versus Fc treated controls (n = 2) (Fig. 3F).

Taken together, these results show that ALK1-Fc treatment leads to remodeling of the tumor vasculature with increased pericyte coverage. In addition, we observed increased perfusion and reduced intratumoral hypoxia in mice treated with ALK1-Fc.

ALK1-Fc increases chemotherapy efficiency in melanoma, head and neck cancer, and breast tumors

Considering our previous results, we hypothesized that pretreatment with ALK1-Fc could lead to a normalized phenotype of the tumor vasculature with a temporary improvement of the tumor perfusion that could result in a better delivery and efficacy of chemotherapy in tumors. Therefore, we tested whether the coadministration of ALK1-Fc together with a chemotherapeutic drug could increase its cytotoxic effect.

First, the combinatorial effects were evaluated in the KEP1-11 breast cancer model. KEP1-11 cells showed a dose-dependent cisplatin-induced growth arrest (Supplementary Fig. S5A and S5B). Next, KEP1-11 cells expressing the different Fc constructs were injected in the mammary gland of Balb/c nude mice. After 4 weeks, mice were treated with 3 rounds of cisplatin. In all mice treated with cisplatin, tumor growth was inhibited when compared with the untreated control group. In tumors originating from ALK2-Fc and Flt-Fc–expressing cells cisplatin caused a growth inhibition that was comparable with the control Fc tumors, whereas ALK1-Fc–expressing tumors showed a further reduction of tumor growth. Notably, complete stasis of ALK1-Fc–expressing tumors was observed from the start of cisplatin treatment until the end of the experiment (Fig. 4A and B).

To obtain further confirmation of the increased cytotoxic effect of cisplatin in combination with ALK1 targeting in a clinically more relevant setting, mice were injected with the KEP1-11 breast cancer cells. Once palpable tumors were formed (2 weeks after injection), treatment with PBS, Fc, or ALK1-Fc was started. After 2 additional weeks, the therapy was combined with three rounds of cisplatin. Consistently, with our previous results, administration of ALK1-Fc significantly impaired tumor growth when combined with cisplatin treatment. Furthermore, sustained tumor regression was observed in the last phase of the experiment, during the 2-week period after the last round of chemotherapy. At the end of the experiment, tumor volume in ALK1–Fc–treated groups was significantly smaller, compared with the other groups (PBS and Fc), and similar to the size at week 5, just after initiation of cisplatin treatment. Taken together, these data suggest that pretreatment with ALK1–Fc renders KEP1-11 tumors more responsive to chemotherapeutic agents, and represses further tumor development when combined with cisplatin (Fig. 4C and D).

We next analyzed the response of B16 melanomas, implanted in the back of C57BL/6 mice, to Fc or ALK1-Fc alone or in combination with doxorubicin (DXR). Mice received two rounds of either ALK1-Fc or Fc prior to the initiation of the chemotherapy. Starting from day 8, 3 cycles of DXR (2 mg/kg) were administered together with Fc or ALK1-Fc in the designated groups. ALK1-Fc alone had no effect on the tumor growth (Fig. 4E). DXR exhibited a mild effect on these fast-growing tumors, which was enhanced by coadministration of ALK1-Fc (Fig. 4E). In the combination treatment with ALK1-Fc, inhibition of melanoma growth was observed a few days after the first administration of the doxorubicin. This growth inhibition continued until the end of the experiment, suggesting that pretreatment with ALK1-Fc leads to increased delivery and/or efficacy of chemotherapy in melanomas.

Finally, ALK1-Fc combination therapy was tested in the human OSC19 head and neck cancer model. OSC19 cells were injected and mice were treated with ALK1-Fc alone or combined with cisplatin. Bioluminescence (BLI) revealed a slight effect of cisplatin alone, which was significantly improved by ALK1-Fc (Fig. 5A and B). Immunohistochemical analysis showed that only in the ALK1-Fc groups CD31 staining and hypoxia were decreased (Fig. 5C and D). While ALK1-Fc did not affect apoptosis when given as a single agent, it did increase the cytotoxic effect of cisplatin (Fig. 5E).

Taken together, our data show that combined treatment of ALK1-Fc together with either doxorubicin or cisplatin enhances the efficiency of the chemotherapeutics in B16 melanomas, OSC19 head and neck cancers, and the KEP1-11 mammary tumors.
Figure 3.
Effect of ALK1-Fc treatment on vessel normalization. A, changes in the number of pericytes in breast tumors after PBS, Fc, ALK1-Fc, or DC101 treatment (n = 10–12 per group). Immunohistochemical analysis was performed using NG2-specific antibody (Original magnification 100×). B, quantification of the NG2-positive area. Data, mean ± SEM. C, representative images of CD31 (red), NG2 (green), Dapi (blue), immunofluorescence staining in KEP1-T1 breast tumors treated with PBS, Fc, ALK1-Fc, or DC101. Lower row shows merged images. D, representative images of hypoxia immunofluorescent staining (red) in breast tumors originated after orthotopic injection of KEP1-T1 cells. Mice were treated with PBS, Fc, ALK1-Fc, or DC101. At end stage (week 7), hypoxia probe was injected prior to sacrifice and was detected by staining the tumor samples with a specific fluorescent antibody. E, quantification of the hypoxic positive area in the tumor samples. F, quantification of FITC-lectin perfusion in KEP1-T1 tumors treated with Fc or ALK1-Fc. Data, mean ± SD, n = 2 (Fc) or 3 (ALK1-Fc) mice, P = 0.01.
Discussion

In this study, we found that administration of ALK1-Fc can strongly decrease tumor angiogenesis in vivo. Treatment with ALK1-Fc significantly reduced the number of vessels in mouse models for melanoma, breast cancer and head and neck cancer. However, despite this effect, tumor size remained unaltered. Further analysis showed that the remaining vessels present in the ALK1-Fc-treated tumors exhibited increased number of pericytes, while they were absent or loosely associated with the endothelial cells in the control groups. This change in the tumor vasculature was accompanied by a reduction in hypoxia and an enhancement...
of tumor perfusion. Combination of ALK1-Fc with chemotherapy further increased the antitumoral effect of the cytotoxic agent leading to a reduction in tumor growth in B16, KEPI-11, and OSC-19 cancer models. ALK1-Fc was able to increase the cisplatin-induced apoptosis on OSC-19 tumor cells. ALK1-Fc treatment was assessed by quantification of the luciferase signal. Data represents mean ± SEM. C, tongue tumor samples were embedded in paraffin and stained with CD31 specific antibody for endothelial cells. Vascular density (CD31-positive area) from all the tumors was quantified from the images taken from these samples. Data represented are mean ± SEM. D, at day 20, hypoxia probe was injected to the mice prior to sacrifice and was detected by staining the tumor samples with a specific fluorescent antibody. Quantification of the hypoxic positive area in the tumor samples is shown. E, quantification of cleaved caspase-3-positive nuclei (apoptotic cells) in OSC19 tumor samples.

**Figure 5.** Combination of ALK1-Fc with cisplatin in a head and neck cancer mouse model leads to decreased tumor size by enhancing the cytotoxic effect of chemotherapy. A, growth of tumors originated from OSC19 luciferase-expressing cells injected in the tip of the tongue of nude mice was followed by BLI. Representative bioluminescence images from 3 mice per group are shown at end stage (day 20). Mice received Fc or ALK1-Fc alone, or in combination with cisplatin. B, tumor size was assessed by quantification of the luciferase signal. Data represents mean ± SEM. C, tongue tumor samples were embedded in paraffin and stained with CD31 specific antibody for endothelial cells. Vascular density (CD31-positive area) from all the tumors was quantified from the images taken from these samples. Data represented are mean ± SEM. D, at day 20, hypoxia probe was injected to the mice prior to sacrifice and was detected by staining the tumor samples with a specific fluorescent antibody. Quantification of the hypoxic positive area in the tumor samples is shown. E, quantification of cleaved caspase-3-positive nuclei (apoptotic cells) in OSC19 tumor samples.
promoted apoptosis of tumor cells and tumor shrinkage, a phenomenon that we have not observed in our current study. Surprisingly, in all three models used in the current study, strong effects on the tumor vasculature, but not on tumor growth were observed. Treatment of mice with ALK1-Fc alone did not increase apoptosis of cancer cells. Only when combined with chemotherapy, a further enhancement of the cytotoxic effect of cisplatin was seen. This effect could indeed be mediated by an enhanced delivery of the drug to the tumor and/or increased cisplatin sensitivity due to reduced hypoxia.

Noteworthy, our data contradicted the results from the first clinical trial using ALK1-Fc that yielded positive effects in head and neck cancer patients (21). The discrepancy between our results obtained in this experimental mouse model and the human clinical trial might be due to the rapid growth of the tumors and/or the lack of an intact immune system in this xenograft model, which could be contributing to the beneficial effect of ALK1-Fc observed in these patients. The temporary enhancement of perfusion could not only lead to increased delivery and efficiency of chemotherapeutic agents, but also to improve the access of the host immune system to the tumors.

Previous studies have shown a correlation between an increase in hypoxia in tumors and the development of metastasis in various models. As ALK1-Fc seems to decrease hypoxia in these tumors, this could be an additional benefit of the treatment. Unfortunately, the tumor models that we have used do not allow us to look at metastasis formation, as spontaneous metastases do not occur at high frequency. A recently published study on ALK1-Fc in breast cancer however indeed showed an effect on metastasis formation in a breast cancer model (37).

For breast cancer, many subtypes are known and also the angiogenic factors playing a role in the induction of tumor angiogenesis seem to be dependent on the histologic subtype. The differences in tumor subtype were also highlighted in a recently published article where the authors could show a reduction in tumor volume in breast cancer after treatment with ALK1-Fc in a different type of breast cancer (pyMT genetically engineered mouse breast cancer model; ref. 37). Another study showed that invasive lobular carcinomas have a similar microvascular density compared with invasive ductal breast carcinomas. In the latter, expression of VEGF mRNA and protein levels were found to be higher than in the lobular types and correlated significantly with the microvacular density of these tumors (38). This observation suggests that in invasive lobular breast carcinomas, other angiogenic factors besides VEGF might play a significant role. Indeed, we could show strongly decreased angiogenesis in the model for invasive lobular carcinomas by targeting BMP9.

Other approaches to inhibit ALK1 signaling are via small molecules or neutralizing antibodies (39, 40). One of these antibodies, PF-03446962, currently under phase II clinical development, inhibits binding of BMP9 to ALK1 and prevents BMP9-induced recruitment of the coreceptor endoglin into the signaling complex (41, 42). This antibody has been shown to inhibit tumor growth and could overcome resistance to anti-VEGF therapy (36). Although we could previously show that TGFβ can bind to ALK1 in endothelial cells, it can only do so in the presence of TGFβ type II receptor (43). TGFβ does not bind to ALK1 alone when expressed on cells (15) or in vitro using purified TGFβ and ALK1-Fc proteins in a Bicore analysis. Therefore, we do not expect that ALK1-Fc has an effect on TGFβ signaling. Consistent with this notion, we found that ALK1-Fc does not affect TGFβ-induced Smad1 or TGFβ-induced Smad2 phosphorylation, whereas ALK1-Fc does block BMP9-induced Smad1 phosphorylation (17).

Currently, multiple phase II clinical trials are ongoing for the treatment of solid tumors with ALK1-Fc (dalantarecept; NCT01720173, NCT01642082), also in combination with sorafenib (NCT02024087) or axitinib (NCT01727336; ref. 44). Interestingly, a phase II clinical study on the effect of ALK1-Fc on head and neck squamous cell carcinomas has been initiated (NCT01458392). Our current findings indicate that the combination of ALK1-Fc with chemotherapy might be very effective, especially as positive effects of ALK1-Fc treatment (as monotherapy) in head and neck cancer patients have been reported (21). Furthermore, it would be very interesting to test the effect of ALK1-Fc combination treatment in breast cancer patients, where current antiangiogenic therapies do not show strong therapeutic effects in terms of survival benefit.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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