Targeting Activin Receptor-Like Kinase 1 Inhibits Angiogenesis and Tumorigenesis through a Mechanism of Action Complementary to Anti-VEGF Therapies

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).


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doi: 10.1158/0008-5472.CAN-10-1451

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Abstract

Genetic and molecular studies suggest that activin receptor-like kinase 1 (ALK1) plays an important role in vascular development, remodeling, and pathologic angiogenesis. Here we investigated the role of ALK1 in angiogenesis in the context of common proangiogenic factors [PAF; VEGF-A and basic fibroblast growth factor (bFGF)]. We observed that PAFs stimulated ALK1-mediated signaling, including Smad1/5/8 phosphorylation, nuclear translocation and Id-1 expression, cell spreading, and tubulogenesis of endothelial cells (EC). An antibody specifically targeting ALK1 (anti-ALK1) markedly inhibited these events. In mice, anti-ALK1 suppressed Matrigel angiogenesis stimulated by PAFs and inhibited xenograft tumor growth by attenuating both blood and lymphatic vessel angiogenesis. In a human melanoma model with acquired resistance to a VEGF receptor kinase inhibitor, anti-ALK1 also delayed tumor growth and disturbed vascular normalization associated with VEGF receptor inhibition. In a human/mouse chimera tumor model, targeting human ALK1 decreased human vessel density and improved antitumor efficacy when combined with bevacizumab (anti-VEGF). Antiangiogenesis and antitumor efficacy were associated with disrupted co-localization of ECs with desmin + perivascular cells, and reduction of blood flow primarily in large/mature vessels as assessed by contrast-enhanced ultrasonography. Thus, ALK1 may play a role in stabilizing angiogenic vessels and contribute to resistance to anti-VEGF therapies. Given our observation of its expression in the vasculature of many human tumor types and in circulating ECs from patients with advanced cancers, ALK1 blockade may represent an effective therapeutic opportunity complementary to the current antiangiogenic modalities in the clinic.

Cancer Res; 71(4); 122011 American Association for Cancer Research.

Introduction

Activin receptor-like kinase 1 (ALK1) is a type 1 TGFβ receptor subclass with distinct expression, signaling, and functional properties from other family receptors (1, 2). It is preferentially expressed on proliferating vascular endothelial cells (EC). Genetic studies of ALK1 +/- mice and zebrafish harboring a loss-of-function mutation demonstrated that ALK1 plays a key role in vasculogenesis, particularly in vessel maturation involving recruitment and differentiation of perivascular cells (PC), and in the organization and patency of neo-angiogenic vessels (3–6). In humans, type 2 hereditary hemorrhagic telangiectasia (HHT2), an autosomal dominant vascular dysplasia syndrome, is linked to the loss-of-function mutations of ALK1 (7, 8).

ALK1 is phosphorylated upon forming a membrane complex with TGFβ and its type II receptor, which then phosphorylates the receptor-regulated Smad proteins (Smad1/5/8). Phosphorylated Smad1/5/8 (pSmads) dimerize with Smad4, and the complex translocates to the nucleus triggering
transcriptional regulation of target genes that regulate EC function and angiogenesis (9–12).

Early studies showed that ALK1 signaling is context-dependent and can be either pro- or anti-angiogenic (11–16). Recent reports revealed that ALK1 signaling promotes angiogenesis through a synergistic action of TGFβ and bone morphogenesis protein 9 (17); furthermore, a soluble ALK1/extracellular domain (ECD) Fc-fusion protein (RAP-041) reduced xenograft tumor burden in mice through antiangiogenesis (18). These studies suggest that ALK1 is proangiogenic.

During angiogenesis, many proangiogenic factors (PAF), including VEGF and basic fibroblast growth factors (bFGF), are coordinately overexpressed by tumor, stromal, and infiltrating myeloid cells (19–25). An association between VEGF expression/activity and ALK1 dysregulation in HHT syndrome has been suggested (7, 8, 15, 26, 27), although the molecular and cellular mechanisms of this relationship remain unclear.

This study aimed to verify the proangiogenic role of ALK1 and elucidate its relationship with VEGF in tumor angiogenesis via pharmacologic approaches utilizing ALK1-specific monoclonal antibodies (mAb).

Materials and Methods

Cells and tissues

Human ECs were obtained from Clonetics and cultured in EGM-2 containing serum and a cocktail of growth factors (Lonza). Human lung fibroblast cells (MRC-5) were from Sigma. Human melanoma M24met cells were described previously (28). Human breast cancer MBA-MD-231/Luc cells were from Xenogen Corp. Human peripheral blood samples were collected with informed consent and local Institutional Review Board approval. ALK1 expression in circulating ECs (CEC) was assessed according to a modified protocol using Alexa Fluor-labeled anti-human ALK1 (29). See Supplementary Materials for human tumor and normal tissue specimens.

Reagents and animals

Anti-human ALK1 mAb [Anti-huALK1 (PF-03446962)] was generated by immunizing human immunoglobulin G (IgG) 2-transgenic Xenomouse (30). The antibody potently and selectively binds to human ALK1 with an affinity (Kd) of 7 ± 2 nmol/L (Supplementary Fig. 1A). The murine anti-ALK1 mAb (Anti-muALK1) was generated from mouse hybridomas. Amu-VEGF [mAb against human (hu) and murine (mu) VEGF-A], sunitinib [inhibitor of VEGF receptor tyrosine kinasas (RTK) and several other RTKs], and PF-00337210 (selective inhibitor of VEGF RTKs) were generated at Pfizer. Bevacizumab (anti-huVEGF-A) was from Henry Shein. ALK1/ECD Fc-fusion protein and growth factors were from R&D Systems. Supplementary Table A describes these agents in detail. All mAbs were dosed subcutaneously once weekly (QW), and RTK inhibitors were dosed orally (PO) once daily (QD).

In vitro tubulogenesis assays

ECs and MRC-5 cells were seeded in PAF-reduced Matrigel (BD Biosciences) and treated with testing agents and stimuli diluted in endothelial basal medium (EBM)-2 containing 5% FBS. The supernatant was changed every 3 days. At day 9, cells were fixed with 4% paraformaldehyde and stained with anti-huCD31 mAb (Santa Cruz) and Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen). Images were captured using Cellomics (Thermo Scientific) and quantified using Image-Pro Plus (Media Cybernetics). See Supplementary Methods for other in vitro assays.

In vivo models

See Supplementary Materials for mice used in in vivo studies and general information on conventional xenograft models. For the chimera tumor model, 50 μL of 2 × 10^6 M24met cells mixed with collagen IV and human fibronectin (both BD Biosciences) were intradermally injected into human neonatal foreskin engrafted in severe combined immunodeficiency (SCID) mice (31). Tumor volumes (TV) were calculated according to 0.5 × [length × width]^2. Antitumor efficacy was calculated according to [1 – ΔTreat/ΔControl] × 100, where ΔTreat and ΔControl were average tumor volume changes during the treatment period for the treated and control groups, respectively.

Immunofluorescence staining

Frozen chimera tumor sections (20 μm) were blocked with 5% rabbit serum/0.2% bovine serum albumin (BSA)/0.3% Triton X-100/PBS, huALK1 was stained with a goat anti-ALK1 antibody (Santa Cruz); CD31 was stained with an Alexa Fluor 488-labeled anti-huCD31 antibody (BioLegend) or a rat anti-muCD31 clone (MEC 13.3; BD Biosciences). Murine lymphatic vessels were stained with antibody against LYVE-1 (Abcam). The corresponding Alexa Fluor-labeled secondary antibodies were from Invitrogen. Slides were counter-stained with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories) and images were captured using a Zeiss Axiosvert 200M microscope and an AxioCam HR color digital camera. For quantification, 3 to 5 ‘hotspot’ areas were captured for each slide by at least 2 individuals. Images were analyzed and quantified using Image-Pro Plus.

For perivascular marker staining, tumor sections were coincubated with sheep anti-huCD31 (R&D Systems) and rabbit anti-desmin (Millipore) antibodies, followed by Alexa Fluor-labeled secondary antibodies (Invitrogen). Three-dimensional Z-stack images were captured with a Zeiss Axioplan 2 microscope using AxioVision 4.8 (Carl Zeiss Microimaging). Image reconstruction and surface rendering were done using the 3D Constructor 5.1 plug-in for Image-Pro Plus.

Ultrasound measurements

Contrast-enhanced ultrasound (CE-US) images were acquired using an Acuson Sequoia 512 system (Siemens Medical Solutions), with a 15L8 linear array transducer (32). Time required for each pixel of the tumor to return to 20% of the original amplitude (T20%) was assessed and values were binned in 1.5-second increments.
Statistical analysis

Unless otherwise noted, the statistical significance in mean values was determined by one-way ANOVA (analysis of variance) with a Dunnett’s multiple-comparison posttest (GraphPad Prism). A value of \( P < 0.05 \) is considered significant.

Results

Smad1/5/8 signaling pathway can be inhibited by Anti-huALK1

Human umbilical vein endothelial cells (HUVEC) were used to investigate ALK1 signaling and functional activity due to their relatively high-level surface ALK1 expression compared with other EC lines (Supplementary Fig. 1B and C). Western blotting and quantitative real-time PCR (RT-PCR) assays showed that Anti-huALK1 dose-dependently inhibited serum-stimulated pSmads and Id-1 expression in HUVECs, respectively (Fig. 1A).

To understand the role of ALK1 in tumor-associated angiogenesis, we examined whether PAFs could regulate ALK1 signaling. VEGF and bFGF rapidly induced pSmads and Id-1 in HUVECs as assessed by Western blotting, and these signals were blocked by Anti-huALK1 (Fig. 1B). Anti-huALK1 also attenuated VEGF and bFGF-stimulated pSmads translocation to the nucleus (Fig. 1C). Thus, in addition to TGFB (a known ALK1 ligand), VEGF and bFGF

Figure 1. Modulation of ALK1 signaling PAFs and Anti-huALK1 in HUVECs. A, top, cells were starved overnight and treated with Anti-huALK1 for 2 to 4 hours followed by a 45-minute stimulation with 2.5% FBS. pSmads were detected by Western blotting. \( \beta \)-Actin was used as loading control (\( n = 2 \)). Bottom, cells were treated as above and the Id-1 transcript in the presence of 0.3\( \mu \)C2 of EGM-2 BulletKit (Lonza; 30-minute stimulation) was measured by quantitative RT-PCR. Shown are fitted curves of dose-dependent inhibition of Id-1 by Anti-huALK1 from 3 experiments. B, cells were starved for 2 to 4 hours and incubated with Anti-huALK1 (200 nmol/L) for an additional 1 to 2 hours before a 45-minute stimulation with VEGF (10 ng/mL), bFGF (30 mg/mL), or TGFB (1 ng/mL). pSmads and Id-1 were detected by Western blotting. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was probed as loading control (\( n = 3 \)). C, cells were treated as in B, permeabilized, and stained for pSmads (green). Nuclei were counterstained with DAPI (blue) to indicate the presence of cells after Anti-huALK1 (\( n = 2 \)). D, cells and the experiment details were the same as in B, except that VEGF and TGFB were used as stimuli, and sunitinib (50 nmol/L) and ALK1/ECD (200 nmol/L) were also used as inhibitors in the assay (\( n = 3 \)).
may also activate Smads and Id-1 in an ALK1-dependent manner.

To address whether VEGF canonical signaling was involved in modulating ALK1 downstream components, we applied sunitinib in the signaling assay and observed that sunitinib significantly suppressed pSmads and Id-1 stimulated by VEGF or TGF

These downstream molecules were also inhibited by an ALK1/ECD fusion protein in the same assay (Fig. 1D). These results imply a close interplay between the VEGF/VEGFR and TGF

Anti-huALK1 inhibits PAF-mediated EC proliferation and tube formation

We next investigated whether PAFs contribute to ALK1-mediated EC function. First, using an electronic cell sensor assay (RT-CES, Roche), in which cellular phenotypical changes were measured in real time (see Supplementary Methods), we observed that Anti-huALK1 rapidly inhibited PAF-stimulated HUVEC attachment and spreading (Fig. 2A; Supplementary Fig. 2A). Longer incubation (up to 15 hours) had a similar result (data not shown). Second, in a coculture assay, we observed that Anti-huALK1 significantly inhibited VEGF-stimulated EC tubulogenesis (Fig. 2B and C); tubulogenesis under bFGF and TGFβ1 was moderately inhibited by Anti-huALK1. Anti-huALK1 also inhibited VEGF- and bFGF-mediated tubulogenesis by human pulmonary artery endothelial cells (HPAEC) in the coculture assay (Supplementary Fig. 2B). Thus, VEGF and bFGF can promote EC phenotypical function in an ALK1-dependent manner, consistent with their ability to stimulate ALK1 signaling.

ALK1 promotes tumor growth via angiogenesis

In the AngioReactor model containing PAFs, a mAb against murine ALK1 (Anti-muALK1) significantly inhibited murine
angiogenesis (Fig. 3A; Supplementary Fig. 3A). Anti-muALK1 produced significant tumor growth inhibition (TGI) of MDA-MB-231 human breast cancer xenograft tumors, in which muALK1 was found to be expressed in the vasculature and co-localized with mUCD31 (Fig. 3B; Supplementary Fig. 3B). The TGI was associated with substantial reduction of microvessel density (mUCD31 staining) and moderate inhibition of murine lymphatic vessel density (LYVE-1 staining; Fig. 3B). This is consistent with the observation that human lymphatic ECs express ALK1 (Supplementary Fig. 1C) and that ALK1 signaling is involved in multiple stages of lymphatic development in mice (6). Thus, ALK1 promoted murine blood and lymphatic angiogenesis and xenograft tumor growth.

To investigate whether huALK1 can promote human vessel function during tumor growth, we used a human/mouse chimera tumor model, in which local human angiogenesis was attained in the presence of human melanoma tumors grown in human foreskin engrafted in SCID mice (31). Immunofluorescent staining showed that tumor vessels were positive for huCD31 and huALK1 (Supplementary Fig. 3C). Systemic administration of Anti-huALK1 significantly and dose-dependently reduced huCD31−/microvessel density (huMVD) compared with a nonspecific control IgG2 (Fig. 3C). Anti-huALK1 generated a similar huMVD reduction (40%–73%) compared with sunitinib (33%–58%) and bevacizumab (40%–46%) across several studies (Fig. 3D), suggesting that, similar to VEGF/VEGFRs, ALK1 may be a factor promoting robust human angiogenesis.

**ALK1 may contribute to resistance to anti-VEGF treatments through dysregulation of vessel normalization**

Given the observed involvement of VEGF in ALK1 signaling, we investigated whether tumors secreting a higher level of VEGF are more sensitive to anti-ALK1 treatment. We chose M24met/R xenograft tumors that had acquired resistance to a VEGF RTK inhibitor (PF-00337210), because these tumors express significantly higher huVEGF-A compared with PF-00337210 treatment, compared with untreated tumors (Fig. 4A, i). The vasculature of the M24met/R tumors was found to be ALK1+/CD31+/desmin+ (Fig. 4A, ii–iv; desmin is a marker for PCs and appeared smooth, reminiscent of the so-called “normalized vessels” (33–35). Anti-muALK1 plus PF-00337210 generated a statistically significant TGI (57%) compared with PF-00337210 alone (Fig. 4A, v). In a separate study, Anti-muALK1 alone did not generate any antitumor efficacy (data not shown). Thus, VEGF RTK inhibitor treatment resulted in increased VEGF that may be required for ALK1-mediated angiogenesis. Enhanced efficacy of Anti-muALK1 combined with PF-00337210 was associated with further reduction of CD31− vessels in the tumor than PF-00337210 alone (Supplementary Fig. 4A); furthermore, the remaining vessels in this group showed diminished CD31+/desmin− costaining and increased sprouting (Fig. 4A, vi and vii).

Next we investigated whether huALK1 inhibition can also enhance the TGI of bevacizumab, using Anti-huALK1 in the chimera tumor model. Bevacizumab produced moderate (42%) TGI (Fig. 4B), despite its ability to significantly inhibit huMVD and muMVD (Fig. 4C). Serum samples from the chimera tumor mice contained significantly higher huVEGF-A than muVEGF-A (Supplementary Fig. 4B); thus, the lack of robust TGI by bevacizumab in this model could not be fully explained by its lack of cross-reactivity to muVEGF-A. An alternative explanation may be that tumors in this model have intrinsic resistance to bevacizumab. We questioned whether ALK1 played a role in this resistance. As expected, Anti-huALK1 alone showed little TGI (11%), due to its lack of cross-reactivity to muALK1 (and murine angiogenesis played a significant role in tumor growth). When Anti-huALK1 was combined with bevacizumab, significant TGI was observed (58% compared with control, P < 0.05; Fig. 4B), implying that huALK1 in human vessels was at least partially responsible for bevacizumab resistance in this model.

The above combination treatment did not further reduce huMVD (Fig. 4C), yet markedly improved efficacy. To understand why, we conducted in-depth immunohistochemistry (IHC) analysis of the vessels. Tumors treated with Anti-huALK1 were often left with large, open structures consisting of desmin− PCs but lacked huCD31+, as if Anti-huALK1 "pulled" human ECs out of the vasculature that was once covered with PCs prior to Anti-huALK1 treatment (Fig. 4D, i–iv, arrowheads). In the same tumor, the remaining smaller vessels were huCD31+/desmin− (indicated by §), suggesting that Anti-huALK1 prevented the recruitment of PCs to these vessels. The above observation/interpretation is consistent with the vascular characteristics of ALK1−/− mice (3–5). Conversely, bevacizumab treatment resulted in large, open vessels positive for both huCD31 and desmin (Fig. 4D, v–viii), mimicking a stable/normalized vascular phenotype. Anti-huALK1 plus bevacizumab resulted in vascular structures that were largely either huCD31+ or desmin+ (Fig. 4D, ix–xii). Overall, combination treatment reduced huMVD, vessel sprouting, and tortuosity compared with the control group (Fig. 4D, xiii–xvi). These data suggest that anti-ALK1 quantitatively disrupted the vascular normalization phenotype induced by bevacizumab, which may have contributed to the observed combinatorial antitumor efficacy.

**ALK1 supports functional blood flow of large vessels**

Because vascular normalization may result in better blood flow (BF; refs. 36, 37), we investigated whether Anti-huALK1 could modulate BF in the chimera tumor model using CE-US that can differentiate flow rates within the microvasculature (32). Sixty hours after Anti-huALK1 administration, the development of vessels with fast flow rates was significantly inhibited compared with control tumors (Fig. 5A, arrowheads). Quantification of CE-US imaging parameters further revealed that the prevalence of fast BF (generally associated with large/arterial and functional vessels), but not slow BF (associated with small, leaky vessels), was suppressed in Anti-huALK1–treated tumors compared with control tumors (Fig. 5B and C). A similar conclusion could be drawn from a study using the same model and power Doppler ultrasonography (Supplementary Fig. 5). These results suggest that huALK1 may be involved in promoting efficient BF in functional and established vessels for human angiogenesis.
A

Murine CD31 fluorescence intensity (arbitrary unit)

<table>
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<th>Treatment</th>
<th>CD31 Fluorescence Intensity</th>
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<tr>
<td>Control (no treatment)</td>
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</tr>
<tr>
<td>AID16 (10 mg/kg)</td>
<td>4,000</td>
</tr>
<tr>
<td>Amu-VEGF (5 mg/kg)</td>
<td>6,000</td>
</tr>
<tr>
<td>Anti-muALK1 (10 mg/kg)</td>
<td>8,000</td>
</tr>
<tr>
<td>Amu-VEGF (pre-mix)</td>
<td>*</td>
</tr>
<tr>
<td>Anti-muALK1 (pre-mix)</td>
<td>*</td>
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</table>

B

Green = CD31  Red = ALK1  20x

Weeks of treatment

Tumor volume (mm³)

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<tr>
<td>Anti-muALK1 (10 mg/kg BIW)</td>
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</tr>
<tr>
<td>Amu-VEGF (6 mg/kg Q5D)</td>
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C

Human CD31+ area (arbitrary unit)

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<tr>
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</tr>
<tr>
<td>Anti-huALK1 10 mg/kg (i.v.)</td>
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D

Human CD31+ area (arbitrary unit)

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<th>Treatment</th>
<th>Human CD31+ Area (arbitrary unit)</th>
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<tbody>
<tr>
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<tr>
<td>JBS5: 10 mg/kg QW</td>
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<tr>
<td>Anti-huALK1: 10 mg/kg QW</td>
<td>15,000</td>
</tr>
<tr>
<td>Sunitinib: 40 mg/kg PO QD</td>
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</tr>
<tr>
<td>Bevacizumab: 10 mg/kg QSD</td>
<td>25,000</td>
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</tbody>
</table>

CONTROL: No dose

JBS5: 10 mg/kg QW

Anti-huALK1: 10 mg/kg QW

Sunitinib: 40 mg/kg PO QD

Bevacizumab: 10 mg/kg QSD
ALK1 expression in primary ECs and human tumor specimens

To begin assessing the relevance of ALK1 in human cancer, we investigated huALK1 expression by IHC in 3 tissue microarrays (TMA) containing more than 3,000 human tumor specimens representing more than 100 tumor types. The results showed that huALK1 was expressed primarily in the vasculature of most human tumors with varying frequency and intensity. The expression in tumor cells and normal tissues was generally weak or absent. The top 20 most frequently occurring tumor types were rank ordered on the basis of ALK1 expression score (Fig. 6A; Supplementary Table B). Figure 6B shows representative photomicrographs from 3 tumor types and the corresponding normal tissues. These data imply that ALK1 may be a potential therapeutic target in certain tumor types.

ALK1 expression in CECs from human peripheral blood (HPB) samples was also assessed. Greater levels of total ALK1+/CECs were detected in the HPB of patients with cancer than that of healthy volunteers (Supplementary Fig. 6; Supplementary Table C). The number of viable ALK1+/CECs was even more markedly increased in patients with advanced colon cancer, melanoma, and non–small cell lung cancer than in healthy volunteers (Fig. 6C). Implications of ALK1+/CECs are being investigated in an ongoing phase I study.

Discussion

Here we report that ALK1 signaling and function in ECs may depend on multiple PAFs including VEGF and bFGF, and that ALK1 may significantly contribute to blood and lymphatic angiogenesis, tumor growth, and resistance to therapies involving anti-VEGF or VEGF RTK inhibitors.

We showed that ALK1-mediated signaling, EC phenotypical changes, and differentiation were not only dependent on its natural ligand TGFβ, but also on VEGF and bFGF. TGFβ-stimulated Smads phosphorylation and Id-1 expression, until now thought to be specific to ALK1, were also inhibited by sunitinib, an inhibitor of VEGFRs and several other RTKs (38). Because VEGF and bFGF are not known to bind to ALK1, nor is TGFβ direct binding to VEGFRs expected, one possible explanation for our observations may be that there is intracellular cross-talk between the VEGF/VEGFR, bFGF/FGF receptor, and TGFβ/ALK1 pathways at the βSmads level. Consistent with in vitro observations, in vivo we observed that in mice bearing PF-00337210-treated M24met/R tumors, which produced higher serum VEGF than untreated M24met/R tumors, addition of Anti-muALK1 generated statistically significant antitumor efficacy (Fig. 4A, v). TGFβ signaling has been shown to interact closely with several other pathways in a context-dependent fashion in models of development and pathologic diseases (reviewed in ref. 39). The molecular mechanism of the cross-talk between TGFβ/ALK1 and VEGF/bFGF pathways in angiogenesis is being investigated.

Through antitumor efficacy, functional imaging, and IHC studies, we reveal that the mode of action of ALK1 in angiogenesis may be different from, yet complementary to, that of VEGF/VEGFRs. VEGF/VEGFRs are well known to promote neovascularization with weak, leaky vessels deficient in tight junction and EC–EC interaction. Anti-VEGF therapy can result in vascular normalization (35, 36, 40). In supporting this, we reported that a VEGFR inhibitor selectively decreased total tumor perfusion, but not BF, in mature vessels (41). Although vascular normalization may temporarily improve the delivery of an anticancer agent, it has been associated with relapse and resistance to anti-VEGF therapies in the clinic (40). ALK1 may be essential in supporting the function of “normalized” vessels, as we show that anti-ALK1 disrupted ALK1+ EC–EC co-localization, reduced BF primarily in the large and fast-flow vessels, and improved efficacy in models otherwise resistant to anti-VEGF therapies. Collectively, our data support the hypothesis that ALK1 expression and function following anti-VEGF/VEGFR inhibitor therapy may contribute to vascular adaptation from the VEGF-driven neovascularization to the ALK1-driven productive angiogenesis involving mature vessels. The proposed mode of action of ALK1 may also explain the controversy regarding the role of ALK1 in angiogenesis (11–16). Taken together, our findings suggest a key differentiation between, and yet complementary role of, ALK1 and VEGF/VEGFRs in promoting tumor angiogenesis.
Translation to the clinic

Here we show that the ALK1 protein is broadly but heterogeneously expressed in human tumor vasculature, implying a need for patient selection in the clinic to achieve meaningful benefit from anti-ALK1 therapy. Regarding CECs, both the total and viable ALK1⁺/CEC counts were significantly increased in patients with cancer compared with healthy volunteers. ALK1 expression in tumors on the basis of TMAs was not always consistent with the percentage of viable ALK1⁺/CECs; the latter may be a surrogate marker for the fraction of vessel turnover and may not directly correlate with ALK1 in the vasculature of solid tumors. The discrepancy may also reflect differences in disease stage and treatment history of patients associated with TMAs (mostly treatment-naïve, early-stage cancers) and CEC samples (all from advanced cancers). The significance of ALK1 positivity in CECs is being investigated. Because Id-1, an ALK1 target gene, has been implicated in the mobilization and function of endothelial progenitor cells (42), we hypothesized that ALK1 may play a key role in circulating EC mobilization and associated angiogenesis. Preliminary data from a phase I study showed that

Figure 4. ALK1 inhibition disrupted interaction between ECs and PCs in the M24met/R model (A) and the chimera tumor model (B–D). A, i, ELISA showed increased human VEGF expression in PF-00337210-treated compared with untreated M24met/R tumors; vascular staining (immunofluorescent) was performed and showed co-localization of ALK1 and CD31 (ii) and muCD31 and desmin (iii, iv). v, M24met/R tumors lacked response to PF-00337210 (~0% compared with untreated tumors (*); addition of Anti-muALK1 (10 mg/kg, QW) to PF-00337210 (!) delayed tumor growth by 57% compared with PF-00337210 alone. *, P < 0.01; 10 animals per group. Vessels in the combination group showed increased sprouting (arrows) and tortuosity, and reduced CD31⁺/desmin⁺ co-staining (vi, vii). Rx, treatment. B, chimera tumors were treated with Anti-huALK1, bevacizumab, or a combination of the two agents (treatment started when average TV was 50 mm³ and lasted for 10 days). Data are group average tumor weight ± SEM (n = 5–7 per group). C, quantification of huCD31 and muCD31 in tumors from B. Anti-huALK1 had no effect on muMVD due to lack of cross-reactivity with muALK1. Data are group average ± SEM (n = 5–7 per group; 3–5 hotspots per tumor). *, P < 0.01 compared with control; †, P < 0.01 compared with bevacizumab. D, representative images of huCD31 (green) and desmin (red) double staining of chimera tumors from B. i, iv, vii, xii, and xvi, 3D images. Arrowheads, desmin⁺/huCD31⁻/vessels; §, small vessels were mostly huCD31⁺/desmin⁻; arrows, huCD31⁺ vessels devoid of desmin staining. Details are discussed in the Results section.

Figure 5. Effect of Anti-huALK1 on vascular BF and perfusion. A, CE-US images of chimera tumors at baseline and 60 hours after treatment. The color bar represents time required for T20% (0–10 seconds). The formation of fast-flow vessels (T20% <1.5 seconds; yellow) is evident in the control but not the Anti-huALK1–treated tumor (arrowheads). Fast flow in the kidney cortex is shown below the tumor (arrow), B and C, quantitation of fast-flow (B) and slow-flow blood vessels (C; T20% in 3–10.5 seconds) in the control and Anti-huALK1 groups on days 1 and 4 of treatment. *, P = 0.011 (n = 4–5 per group).
Anti-huALK1 (PF-03446962) reduced total ALK1+/CECs following the first treatment cycle in several patients (ref. 43; Pfizer Inc. Data on file). Additional studies are needed to correlate changes in ALK1+/CECs with clinical activity in order to ascertain their potential utility as predictive biomarkers.

In conclusion, our data suggest that VEGF/bFGF can regulate ALK1 signaling and angiogenesis in vivo, and ALK1 plays an important and compensatory role in vascular angiogenesis. Targeting ALK1 may represent a novel and effective therapeutic opportunity, particularly for the treatment of patients resistant to anti-VEGF therapies.
Disclosure of Potential Conflicts of Interest

D.D. Hu-Lowe, E. Chen, L. Zhang, P. Lappin, T.A. Swanson, B.H. Simmons, J. Wang, J.H. Chen, Z. Feng, S. Berggqvist, G.F. Casperson, W.J. Levin, C.G. Stampino, and D.R. Shalinsky are full-time Pfizer Inc. employees and own Pfizer Inc. stock. P. Mancuso, K.D. Watson, R. Simon, and A. Erbersdobler declare no competing interests. G. Wickman, K. Amundson, X. Jiang, and J. Lippincott disclose work done as employees of Pfizer Inc. K.W. Ferrara has received research funding from Pfizer Inc. W. Fiedler has received research funding and fees for advisory board meetings and invited speeches from Pfizer Inc. F. Bertolini has received compensation from Pfizer Inc. for a consultant/advisory board role.

Acknowledgments

We thank Shile Liang, Paola Marighetti, Ines Martin Padura, Jasmin Otten, Joseph Zachwieja, Tina Lu, and Comparative Medicine, Pfizer for technical support, and Steve Bender, Farbod Shojaei, Jamie Christensen, and Neil Gilson for support and discussion of the manuscript. We thank all of the participating patients and their families, and the global network of investigators, research nurses, study coordinators, and operations staff. Editorial assistance was provided by Jessica Stevens of ACUMED (Tytherington, UK) and was funded by Pfizer Inc.

Grant Support

This study was sponsored by Pfizer Inc. K.W. Ferrara received funding from NIH R01CA103828 and R01CA134639. W. Fiedler received funding from Eppendorfer Krebs-und Lukaamäriteile e.V.

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Received May 4, 2010. revised December 17, 2010; accepted December 22, 2010; published OnlineFirst January 6, 2011.

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Cancer Res  Published OnlineFirst January 6, 2011.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-1451

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