Ang-2-VEGF-A CrossMab, a Novel Bispecific Human IgG1 Antibody Blocking VEGF-A and Ang-2 Functions Simultaneously, Mediates Potent Antitumor, Antiangiogenic, and Antimetastatic Efficacy

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Abstract

Purpose: VEGF-A blockade has been clinically validated as a treatment for human cancers. Angiopoietin-2 (Ang-2) expression has been shown to function as a key regulator of tumor angiogenesis and metastasis.

Experimental Design: We have applied the recently developed CrossMab technology for the generation of a bispecific antibody recognizing VEGF-A with one arm based on bevacizumab (Avastin), and the other arm recognizing Ang-2 based on LC06, an Ang-2 selective human IgG1 antibody. The potency of Ang-2-VEGF CrossMab was evaluated alone and in combination with chemotherapy using orthotopic and subcutaneous xenotransplantations, along with metastasis analysis by quantitative real-time Alu-PCR and ex vivo evaluation of vessels, hypoxia, proliferation, and apoptosis. The mechanism of action was further elucidated using Western blotting and ELISA assays.

Results: Ang-2-VEGF-A CrossMab showed potent tumor growth inhibition in a panel of orthotopic and subcutaneous syngeneic mouse tumors and patient or cell line-derived human tumor xenografts, especially at later stages of tumor development. Ang-2-VEGF-A CrossMab treatment led to a strong inhibition of angiogenesis and an enhanced vessel maturation phenotype. Neoadjuvant combination with chemotherapy resulted in complete tumor regression in primary tumor-bearing Ang-2-VEGF-A CrossMab-treated mice. In contrast to Ang-1 inhibition, anti-Ang-2-VEGF-A treatment did not aggravate the adverse effect of anti-VEGF treatment on physiologic vessels. Moreover, treatment with Ang-2-VEGF-A CrossMab resulted in inhibition of hematogenous spread of tumor cells to other organs and reduced micrometastatic growth in the adjuvant setting.

Conclusion: These data establish Ang-2-VEGF-A CrossMab as a promising antitumor, antiangiogenic, and antimetastatic agent for the treatment of cancer.

Introduction

Tumor angiogenesis is a hallmark of cancer and requires the coordinated actions of various signal transduction path-
Translational Relevance

VEGF-A therapy with drugs such as bevacizumab is widely used as a treatment for human cancers. Angiopoietin-2 (Ang-2) expression has been shown to function as a key regulator of tumor angiogenesis and metastasis. In several tumor indications, Ang-2 is upregulated and associated with poor prognosis. Ang-2 inhibitors, both as single agents or in combination with chemotherapeutic agents, mediate antitumor effects. In addition, it has been shown that the Ang/Tie and the VEGF/VEGFR systems act in complementary ways suggesting that dual targeting may be more effective than targeting either pathway alone. Accordingly, we generated Ang-2-VEGF-A CrossMab, a novel bevacizumab-based bispecific human IgG1 antibody, acting as a dual-targeting inhibitor of the two key angiogenic factors VEGF-A and Ang-2. We demonstrate that Ang-2-VEGF-A CrossMab combines good pharmacological properties and potent antitumor, antiangiogenic, and antimetastatic activity. These data support the investigation of the Ang-2-VEGF-A CrossMab in clinical trials (NCT01688206).

are a poor prognostic factor and correlate with disease progression and metastasis (9–11). Accordingly, Ang-2 was identified as a regulator of glioma (12), breast cancer (13), and melanoma cell migration and invasion (14), and has been shown to drive lymphatic metastasis of pancreatic cancer (15). Recent data also demonstrated that Ang-2 inhibitors, both as single agents or in combination with anti-VEGF therapy mediate antitumor effects (16–18) and interfere with metastasis formation (19). Recently, different approaches have been described to target the angiopoietin/Tie axis in clinical trials (20, 21).

Given the cooperative and complementary fashion of Ang-2- and VEGF-induced angiogenesis and metastasis, cotargeting of both ligands in a bispecific manner represents an encouraging approach to improve the outcomes of current antiangiogenic therapies. A number of bispecific antibodies has been described, including the bifunctional CovX-Body CVX-241 targeting Ang-2 and VEGF via peptides covalently linked to a catalytic antibody (22). As most bispecific antibody formats deviate significantly from the natural IgG format, we aimed to develop bispecific antibodies that differ only minimally from natural occurring antibodies. In this way, we have recently described a novel method for the production of heterodimeric bivalent bispecific human IgG1 antibodies (CrossMabs) that display the classical IgG architecture, and exhibit favorable IgG-like properties in terms of pharmacokinetic, diffusion, tumor penetration, production, and stability (23). We have subsequently applied the CrossMab technology to generate a bispecific antibody recognizing VEGF-A with one arm, based on bevacizumab (Avastin) and Ang-2 with the other arm, based on LC06; an Ang-2 selective human IgG1 antibody (24). Ang-2-VEGF-A CrossMab is being developed for the treatment of multiple cancer indications aiming to substantially improve clinical outcomes.

In this study, we evaluated the therapeutic potential of Ang-2-VEGF-A CrossMab. The experiments show that it mediates potent antitumor, antiangiogenic, and antimetastatic efficacy in a panel of cancer models and represents a promising approach to achieve sustained tumor control.

Materials and Methods

Therapeutic antibodies and treatment

A2V CrossMab (anti-human VEGF-A and anti-human/murine Ang-2; Supplementary Fig. S1A) was generated as previously described (23). LC06 (anti-murine/human Ang-2; ref 24), bevacizumab (Avastin, anti-human VEGF-A), or B20-4.1 (anti-murine/human VEGF-A; ref 25) served as monotherapies. Dual Ang-2-VEGF-A targeting was achieved using A2V CrossMab or the combination of LC06 and B20-4.1. Murine/human Ang1/2 targeting was achieved using LC08 (24). Omalizumab (Xolair, anti-human IgE) was used as control IgG. Optimal antibody dosages (10 mg/kg qw, i.p.) were based on pilot experiments. Docetaxel (Taxotere, Sanofi-Aventis) was dissolved in PBS and injected intravenously at 10 mg/kg. A summary of the therapeutic antibodies is supplied in Supplementary Table S1.

Animals

Eight to 10-week-old female SCID/beige or Balb/c mice (Charles River Laboratories) were maintained under specific pathogen-free conditions with daily cycles of 12-hour light/12-hour darkness. All experimental procedures were conducted in accordance with committed guidelines as approved by local government (GV-Solas; Felasa; TierschG).

Statistical analysis

Results are expressed as mean±SEM. Differences between experimental groups were analyzed by Student t test or Wilcoxon signed-rank test, respectively. A value of P < 0.05 was considered statistically significant.

Additional and more detailed experimental procedures are provided in Supplementary Methods.

Results

Ang-2-VEGF-A CrossMab retards tumor growth in orthotopic and subcutaneous cancer models at later stages of tumor development

On the basis of a method for the generic production of bivalent bispecific human IgG1 antibodies (23), we have generated a human IgG1 antibody neutralizing VEGF-A and Ang-2 function simultaneously (Supplementary Fig. S1A and S1B). Bevacizumab was selected as the parental antibody and the light chain was left unaltered, whereas a CH1-Cx crossover was introduced into the Ang-2 binding antibody arm. Heterodimerization of the two heavy chains was achieved by using the “knobs into holes” (KH) methodology (26). Ang-2-VEGF-A CrossMab (hereinafter referred to as A2V CrossMab) can be produced in Chinese
hamster ovary (CHO) cells with productivity volumes in the range of 3 to 4 grams per liter, which is similar to standard IgG processes, shows thermodynamic and long-term stability comparable to conventional IgG antibodies (data not shown), and exhibits identical cross-reactivity and affinity as the respective parental antibodies (Supplementary Fig. S2).

First, we investigated the functional consequences of Ang-2-VEGF-A inhibition in orthotopic slowly growing KPL-4 breast tumors expressing human Ang-2 (Supplementary Fig. S3A). The tumors were treated when they reached a mean tumor size of 70 mm³. Mice treated with 10 mg/kg (qw × 5, i.p.) control antibody (omalizumab; anti-human IgE) showed a mean tumor burden (mtb) of 431.5 with control antibody (Fig. 1B, mtb of 442.0 during the course of the trial in contrast to mice treated (Fig. 1A; mtb of 148.1 with four intraperitoneal injections tumor bearing mice were treated when tumors had reached a mean size of 150 mm³ with four intraperitoneal injections of a mean tumor size of 70 mm³. Mice treated with 10 mg/kg (qw × 5, i.p.) control antibody (omalizumab; anti-human IgE) showed a mean tumor burden (mtb) of 431.5 ± 67.5 mm³ at the end of the experiment (Fig. 1A). Administration of an equivalent dose of A2V CrossMab yielded a potent retardation of tumor growth, with a final mtb of 102.3 ± 21.2 mm³ (Fig. 1A, P < 0.001). Because of the strong antitumor activity of all three therapies resulting in more or less tumor stasis, there was however no statistically significant differentiation to anti-Ang-2 (Fig. 1A; mtb of 149.4 ± 27.9 mm³, P = 0.19) or anti-VEGF-A monotherapy (Fig. 1A; mtb of 148.1 ± 30.7 mm³; P = 0.27). Next, we performed a therapeutic trial at a later stage of tumor development to investigate whether A2V CrossMab also inhibited growth of advanced orthotopic tumors. KPL-4 tumor bearing mice were treated when tumors had reached a mean size of 150 mm³ with four intraperitoneal injections of 10 mg/kg A2V CrossMab. Mice that received A2V CrossMab showed tumor stasis or partial regression (TGI value of 115%, Table 1) with a mtb of 159.0 ± 10.5 mm³ (Fig. 1B) during the course of the trial in contrast to mice treated with control antibody (Fig. 1B, mtb of 442.0 ± 92.3 mm³, P = 0.001), anti-Ang-2 (Fig. 1B, mtb of 259.8 ± 47.7 mm³, P = 0.03) and anti-VEGF-A monotherapy (Fig. 1B; mtb of 219.7 ± 23.6 mm³, P = 0.004). Furthermore, A2V CrossMab therapy also resulted in potent tumor growth inhibition in various other syngeneic, patient- and cell line-derived xenograft tumor models, especially when treatment started at larger tumor sizes (> 200 mm³) for subcutaneous tumors and 150 mm³ for orthotopic KPL-4 tumors (Table 1). VEGF-dependent smaller tumors (≤ 120 mm³) with low Ang-2 expression (e.g., MDA-MB-231, MCF-7, Colo205, Calu-3 and PC-3; Supplementary Fig. S3A; Table 1) were inhibited by anti-VEGF-A therapy at maximum efficacious doses with no statistically significant effect of additional anti-Ang-2 treatment, even though a trend in improved efficacy could be observed in all cases.

The efficacy of A2V CrossMab was further characterized in a dose–response trial (2–36 mg/kg, qw × 8, i.p.) in Colo205 tumors (an established model for anti-Ang-2 treatment (ref. 16; Supplementary Fig. S3B). Further analysis determined a dose-related increase of serum levels across the dosing range of 2 to 36 mg/kg (Supplementary Fig. S3C). A2V CrossMab was well tolerated and no body weight loss (Supplementary Fig. S3D) or other overt adverse effects were observed (data not shown). Moreover, an improved median overall survival was observed after A2V CrossMab treatment (Supplementary Fig. S3E).

The results demonstrate that administration of A2V CrossMab retards tumor growth in various tumor models. Especially in larger tumors, A2V CrossMab therapy showed statistically significant differences in antitumor efficacy compared with the respective monotherapies.

**Ang-2-VEGF-A CrossMab impairs tumor angiogenesis and promotes improved vessel maturation**

A2V CrossMab treatment resulted in a complete shutdown of angiogenesis in the VEGF-induced cornea pocket assay (Supplementary Fig. S4A and SB). To further elucidate the mechanism of action behind the observed tumor growth inhibition previously described (Fig. 1B), we characterized the effects of administration of A2V CrossMab on the phenotype of advanced KPL-4 tumors (150 mm³; Fig. 1B). Tumors from mice treated with A2V CrossMab displayed a more than 50% diminished vascular density compared with tumors from control-treated mice (Fig. 2A; P = 0.04). In addition, blood vessels exhibited an increased pericyte coverage (Fig. 2B; P < 0.04), an indicator of a tumor blood vessel maturation.

Figure 1. Tumor growth inhibition of Ang-2-VEGF-A CrossMab on small and advanced orthotopic KPL-4 xenografts. A, KPL-4 small tumor (mean 70 mm³) and (B) advanced tumor (mean 150 mm³) growth curves in SCID/beige mice receiving A2V CrossMab (10 mg/kg), anti-VEGF-A (bevacizumab, 10 mg/kg), anti-Ang-2 (10 mg/kg), or control antibody (omalizumab, 10 mg/kg) once weekly i.p. [n = 10; (A) *, P < 0.001 vs. control, (B) **, P ≤ 0.03 vs. single and control treatments]. Treatment started at day of randomization (arrow, day 38). Animals were randomized in small (70 mm³) and advanced (150 mm³) tumor groups.
Table 1. Antitumor activity of A2V CrossMab in different tumor models

<table>
<thead>
<tr>
<th>Tumor models (syngeneic, patient-derived and xenograft models)</th>
<th>Mean tumor size at treatment start (mm$^3$)</th>
<th>Indication</th>
<th>A2V CrossMab</th>
<th>TGI (%)</th>
<th>Anti-Ang-2</th>
<th>TGI (%)</th>
<th>Anti-VEGF-A</th>
<th>TGI (%)</th>
<th>A2V superiority over anti-Ang-2</th>
<th>A2V superiority over anti-VEGF-A</th>
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<tbody>
<tr>
<td>KPL-4 Breast cancer</td>
<td>70</td>
<td>90</td>
<td>80</td>
<td>45</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.003</td>
</tr>
<tr>
<td>MDA-MB-231 Breast cancer</td>
<td>80</td>
<td>56</td>
<td>70</td>
<td>44</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.002</td>
</tr>
<tr>
<td>Colo205 Colon cancer</td>
<td>120</td>
<td>77</td>
<td>92</td>
<td>67</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>MC38# Colon cancer</td>
<td>60</td>
<td>40</td>
<td>67</td>
<td>20</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>SUDHL-4 Lymphoma (B)</td>
<td>290</td>
<td>77</td>
<td>67</td>
<td>20</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>H460M2 Lung cancer</td>
<td>120</td>
<td>73</td>
<td>73</td>
<td>20</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>Calu-3 Lung cancer</td>
<td>120</td>
<td>73</td>
<td>73</td>
<td>20</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>Panc-1 Pancreatic cancer</td>
<td>160</td>
<td>120</td>
<td>120</td>
<td>80</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>PC-3 Prostate cancer</td>
<td>85</td>
<td>72</td>
<td>72</td>
<td>20</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>RXF-486 RCC (pol)</td>
<td>115</td>
<td>66</td>
<td>66</td>
<td>40</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>HCC07-0409Av7 Gastric cancer (pol)</td>
<td>250</td>
<td>71</td>
<td>71</td>
<td>20</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>N87 Gastric cancer</td>
<td>120</td>
<td>70</td>
<td>70</td>
<td>20</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>NOTE: Green, statistically significant; gray, statistically not significant; n.s., not tested; pd, patient-derived.</td>
<td></td>
<td></td>
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phenotype. Despite the strong reduction in the number of tumor blood vessels, no significant differences in tumor cell apoptotic, necrotic, and proliferative index were noted (Fig. 2C and D and Supplementary Fig. S5A). Furthermore, in only a small fraction (0.2%–0.8%) of the entire tumor area, we observed slight but insignificant increase in tumor hypoxia as detected by CAIX staining (Supplementary Fig. S5B). Moreover, we did not observe any major changes in the level of tumor hypoxia in different xenografts at endpoint analysis (Supplementary Fig. S6A and S6B). To further analyze early and late hypoxic responses to A2V CrossMab treatment, we analyzed tumor CAIX levels in Colo205 bearing animals (Supplementary Fig. S6C). Concentration levels of CAIX in tumor tissue increased during early treatment (day 10), but decreased at the end of the study (day 97). We confirmed this finding by alternative hypoxia measurements using [18F]-FMISO PET in a patient-derived HCC xenograft (Supplementary Fig. S6D). Thus, despite an early transient induction of tumor hypoxia, prolonged A2V CrossMab treatment prompts tumor vessels to normalize and readjust their shape and phenotype that may help to restore tumor oxygen supply. Collectively, our findings indicate that loss of Ang-2/VEGF-A inhibits angiogenesis and retards advanced tumor growth by promoting tumor vessel regression while at the same time boosting tumor vessel maturation.

Ang-2-VEGF-A CrossMab improves chemotherapeutic efficacy and leads to complete regression of well-established tumors

Vessel normalization mediated by bevacizumab and other antiangiogenic agents has gained interest as a therapeutic option to improve chemotherapeutic drug delivery and anticancer treatment (27). We therefore hypothesized that improved vascular coverage by pericytes mediated by A2V CrossMab therapy could further enhance chemotherapeutic efficacy. Orthotopic KPL-4 breast tumor bearing mice were treated after tumors reached a mean tumor size of 100 mm³ with docetaxel either alone or in combination with A2V CrossMab or anti-Ang-2 or anti-VEGF-A (bevacizumab), respectively (Fig. 3A, first arrow). Interestingly, in anti-Ang-2, and anti-VEGF-A groups that had been combined with docetaxel, therapy resulted in regression of orthotopic KPL-4 breast tumors. However, in these groups and also in the docetaxel monotherapy group, tumor growth resumed upon cessation of therapy (day 50, second arrow, Fig. 3A). In contrast, 100% of the A2V CrossMab-treated mice (n = 10) remained tumor free even after treatment termination (Fig. 3A) and chemotherapy-induced changes in body weight were stabilized (day 50, second arrow, Fig. 3B). The study was terminated after 180 days, at which time the A2V CrossMab treated long-term survivors were necropsied with no visible evidence of residual tumor.

Figure 2. Ex vivo analysis of advanced orthotopic KPL-4 tumors (150 mm³; tumor growth curves shown in Fig. 1B) reveals potent antiangiogenic properties and an enhanced normalization phenotype of tumor vessels mediated by A2V CrossMab. Tumors were collected 3 days after last dosing. A, quantifications and representative pictures of CD34+/vascular density (n = 5; *P < 0.04 vs. control). B, double staining using anti-CD31 (red) and anti-desmin (green) antibodies and quantification of pericyte coverage calculated as the average number of desmin positive pixels (green) near the vascular endothelium (red; n = 5; *P < 0.04 versus control and monotherapies). C, percentage of apoptotic tumor cells (Caspase-3 staining) after treatment. D, necrotic tumor areas (H&E staining). Quantification displayed as percentage of necrotic regions compared to total tumor area. Scale bars: 200 μm (A and B); 500 μm (C); 1000 μm (D).
their metastasizing properties (31), from their subcutaneous properties of lung metastatic H460M2 cells, selected for analyzed treatment effects on hematogenous dissemination anti-Ang-2-VEGF-A combination therapies. First, we analyzed invasion and metastasis of tumor cells under mono- and progression (9, 11, 14). This prompted us to investigate angiogenic factors work collaboratively to regulate angiogenesis (32) and thereby resistance to antiangiogenic single-agent therapy occurs by switching on of compensatory angiogenic rescue programs (3). We therefore conducted a time course study with Colo205 tumors to determine the dynamics of human VEGF-A expression during Ang-2 monotherapy with sacrifice of 4 to 7 tumor-bearing mice each at 11, 12, 28, 34, and 42 days after tumor cell inoculation (Fig. 5A). Mice were treated with five intraperitoneal injections of 10 mg/kg control, A2V CrossMab or monotherapies, respectively, after tumors had reached a mean size of 130 mm³. While control animals showed heterogeneous yet moderate VEGF-A upregulation during the course of the entire study, mice treated with anti-Ang-2 monotherapy exhibited a strong shift towards VEGF-A upregulation beginning at day 28 as compared with A2V CrossMab-treated mice (Fig. 5B, red-dotted boxes; P = 0.03). Anti-VEGF-A monotherapy caused an upregulation of human Ang-2 by Colo205 tumor cells evident at day 42 as compared with A2V CrossMab-treated mice (Fig. 5C, red dotted-boxes; P = 0.02). This compensatory mechanism led to activation of proangiogenic tumor vasculature, exemplified by induction of VEGFR-2 in anti-VEGF monotherapy groups (Fig. 5D, red-dotted boxes; P = 0.02 versus anti-Ang-2 and P < 0.001 vs. anti-VEGF). In contrast, A2V CrossMab therapy resulted in downregulation of VEGFR-2 at the end of the study.

Anti-Ang-2-VEGF-A treatment reduces hematogenous spread of tumor cells and inhibits growth of postsurgical metastases

A possible link between antiangiogenic treatment and increased metastasis has been a matter of debate (28, 29). On the other hand, normalization and pruning of dysfunctional, leaky tumor blood vessels is discussed to contribute to a reduction of tumor cell dissemination (30). Interestingly, Ang-2 overexpression is associated with metastatic progression (9, 11, 14). This prompted us to investigate invasion and metastasis of tumor cells under mono- and anti-Ang-2-VEGF-A combination therapies. First, we analyzed treatment effects on hematogenous dissemination properties of lung metastatic H460M2 cells, selected for their metastasizing properties (31), from their subcutaneous transplantation site (Fig. 4A). Tumor-derived DNA released by circulating H460M2 tumor cells was detected in the peripheral blood of mice (day 17 after tumor cell inoculation) by human-specific Alu repeats (Fig. 4B). We observed a significant reduction in tumor DNA following anti-Ang-2-VEGF-A combination therapy that remained below the detection limit until the end of the study, irrespective of primary tumor size (day 32; Fig. 4B; P = 0.03). In a subsequent experiment, we tested whether a reduction of tumor cell dissemination into the blood stream correlates with a diminished metastatic spread to other organs. Subcutaneous Colo205 tumors were first-line treated with anti-VEGF-A, then after 51 days, randomized to treatment with either anti-VEGF-A, anti-Ang-2 monotherapy or anti-Ang-2-VEGF-A combination therapy. Treatment with either anti-Ang-2 alone or anti-Ang-2-VEGF-A combination therapy resulted in a significant reduction of tumor cell dissemination to the lungs (Fig. 4C; P = 0.02). We next tested the effect of adjuvant anti-Ang-2-VEGF-A combination treatment on distant spontaneous metastasis generated after primary H460M2 tumor removal. Mice were randomized postsurgery based on primary tumor weight to ensure equal tumor burden between treatment groups (data not shown). Mice receiving postsurgical adjuvant anti-Ang-2-VEGF-A therapy showed significantly decreased metastatic tumor burden as measured by histology and Alu-PCR (Fig. 4D, P = 0.01).

Our data suggest that anti-Ang-2-VEGF-A treatment can reduce early metastatic spread and interferes postsurgically with the outgrowth of metastases.

Ang-2 and VEGF-A exhibit angiogenic synergy in a mutually compensatory fashion

Angiogenic factors work collaboratively to regulate angiogenesis (32) and thereby resistance to antiangiogenic single-agent therapy occurs by switching on of compensatory angiogenic rescue programs (3). We therefore conducted a time course study with Colo205 tumors to determine the dynamics of human VEGF-A expression during Ang-2 monotherapy with sacrifice of 4 to 7 tumor-bearing mice each at 11, 12, 28, 34, and 42 days after tumor cell inoculation (Fig. 5A). Mice were treated with five intraperitoneal injections of 10 mg/kg control, A2V CrossMab or monotherapies, respectively, after tumors had reached a mean size of 130 mm³. While control animals showed heterogeneous yet moderate VEGF-A upregulation during the course of the entire study, mice treated with anti-Ang-2 monotherapy exhibited a strong shift towards VEGF-A upregulation beginning at day 28 as compared with A2V CrossMab-treated mice (Fig. 5B, red-dotted boxes; P = 0.03). Anti-VEGF-A monotherapy caused an upregulation of human Ang-2 by Colo205 tumor cells evident at day 42 as compared with A2V CrossMab-treated mice (Fig. 5C, red dotted-boxes; P = 0.02). This compensatory mechanism led to activation of proangiogenic tumor vasculature, exemplified by induction of VEGFR-2 in anti-VEGF monotherapy groups (Fig. 5D, red-dotted boxes; P = 0.02 versus anti-Ang-2 and P < 0.001 vs. anti-VEGF). In contrast, A2V CrossMab therapy resulted in downregulation of VEGFR-2 at the end of the study.

![Image of tumor volume and body weight graphs](https://www.aacrjournals.org/clin-cancer-research/article-pdf/19/24/6734/565820/6734.pdf)
arguing for a quiescent vascular status (Fig. 5D, red-dotted box). In vitro, Ang-2 adenoviral transduction of endothelial cells also resulted in the upregulation of VEGF-R2 (Supplementary Fig. S7). Interestingly, our findings suggest a compensatory function between Ang-2 and VEGF-A, which may mutually substitute each other upon inhibition, thereby antagonizing the monotherapeutic treatment effects. Ang-2-VEGF-A inhibition does not aggravate the adverse effect of anti-VEGF-A treatment on healthy vessels. Treatment with VEGF inhibitors causes microvascular pruning in healthy organs (33). In contrast to unselective Ang-1/2 inhibition, treatment of healthy mice with a selective Ang-2 antibody does not affect healthy vessels (24). We therefore sought to analyze the effect of Ang-2-VEGF-A inhibition on healthy vessels in the mouse trachea. When analyzing the morphology of quiescent vessels, selective Ang-2 inhibition combined with anti-VEGF-A treatment (using the mouse VEGF-A cross-reactive surrogate antibody B20-4.1; ref. 25) did not aggravate the adverse effect of anti-VEGF-A treatment on healthy vessels (Fig. 6A and B). On the contrary, combined unselective anti-Ang-1/Ang-2/VEGF-A treatment further reduced the number of capillary branching points/μm² in the trachea by 28% compared with anti-VEGF-A monotherapy (Fig. 6A and B; P = 0.01). These results imply a key differentiation between selective Ang-2 and unselective Ang-1/Ang-2 inhibition in combination with anti-VEGF-A treatment. Selective anti-Ang-2/VEGF-A treatment does not enhance VEGF-A-mediated vessel pruning, providing an improved safety profile over pan-Ang inhibitors.

Discussion

Ang-2-VEGF-A CrossMab is a novel bevacizumab-based bispecific human IgG1 antibody against the two key
Figure 5. Coordinated action of VEGF-A and Ang-2 during cancer therapy. A, subcutaneous Colo205 tumors (n = 20; *, P < 0.001 versus anti-VEGF-A and anti-Ang-2) were harvested at 11, 12, 28, 34, and 42 days post randomization (red dotted lines), and the levels of (B) human VEGF, (C) human Ang-2, or (D) VEGFR-2 were determined by ELISA and Western blot analysis. Data were normalized to tumor size by determining the total protein content of the tumor homogenate. Red dotted boxes refer to treatment-induced expression level changes with (B) *, P ≤ 0.03 versus anti-Ang-2 (days 28, 34, 42); (C) *, P ≤ 0.02 versus anti-VEGF (days 28, 34, 42); (D) *, P ≤ 0.02 versus anti-Ang-2 (days 28, 24); *, P ≤ 0.001 versus anti-VEGF (days 28, 34, 42).
Angiogenic factors VEGF-A and Ang-2. This study demonstrates that the dual blockade of VEGF-A and Ang-2 shows greater effects over the blockade of either of these factors alone. Combinatorial anti-Ang-2-VEGF-A therapy has additive effects on inhibition of advanced tumor growth, angiogenesis, and metastasis and targets angiogenic escape pathways that can be observed in the clinic under anti-VEGF monotherapy (7, 34).

High levels of both VEGF-A and Ang-2 in breast cancer, non–small cell lung cancer, ovarian cancer, and acute myelogenous leukemia correlate with a worse prognosis than cancer indications expressing high levels of either protein alone (35–37). Ang-2 and VEGF-A co-operatively promote tumor growth in mouse tumor models, for example. Ang-2 overexpression does not stimulate the growth of hepatocellular carcinoma unless VEGF-A is simultaneously upregulated (38). In this study, we show a clear disadvantage of Ang-2 primary tumor monotherapy due to a strong upregulation of VEGF-A that can be responsible for tumor escape mechanisms and poor clinical response (3, 39). Our results suggest a role for Ang-2 as a sensitizing molecule for VEGFR-2 consistent with other studies (40). In this way, upregulation of Ang-2 and consequently VEGFR-2 during anti-VEGF-A monotherapy may open up substitutional signaling pathways that pave the way for restoration of tumor growth and progression.

Dual targeting of Ang-2 and VEGF-A slows down tumor growth in a variety of tumor models (16–18, 22). Interestingly, with regards to the clinical situation, we show that Ang-2-VEGF-A dual targeting exerts better therapeutic effects especially on larger tumors as compared with the monotherapies. In the light of recent findings which support the hypothesis that larger tumors consist of different vessel types that do not all respond equally to anti-VEGF-A therapy (41), such a therapeutic profile of is special interest.

The vessel normalization paradigm is supported by the fact that anti-VEGF/R therapy is most effective when combined with chemotherapy (42). In this study, A2V CrossMab treatment reduced tumor vessel density, stabilized vessel architecture, and abrogated hypoxia. These findings are supportive of tumor vascular normalization that is achieved by reducing the proportion of unstable blood vessels that initiate angiogenesis. A2V CrossMab induced enhanced vessel normalization resulted in improved chemotherapeutic activity and hence complete tumor regression compared to monotherapies, most likely due to improved drug delivery, and unlike the short-lived effects often seen during the normalization window after anti-VEGF-A monotherapy (43). Thus, highlighting the potential for A2V CrossMab to modulate the tumor vasculature more favorably and thereby prevent cancers from becoming more malignant and metastatic, and to increase the responsiveness to chemotherapy.

Recent studies suggest that targeting the VEGF/VEGFR pathway alone, although effective in reducing tumor blood vessel density, only temporarily retards tumor growth, and may even promote tumor aggressiveness and metastasis (28, 29). Interestingly, in addition to its antiangiogenic effects, Ang-2 targeting was reported to have additional beneficial effects on tumor metastasis inhibition (12–15). Moreover, a positive correlation between Ang-2 overexpression and metastasis can be observed in the clinic (9–11). In this study, we show that Ang-2-VEGF-A dual targeting inhibits early tumor cell dissemination to the blood and other distant organs as well as late stage lung metastatic growth. Despite promising preclinical evidence (44, 45), adjuvant bevacizumab therapy (e.g., in colorectal cancer) has been disappointing so far (46). Given the additive effect on tumor growth inhibition, enhanced chemotherapeutic efficacy and impact on distant tumor metastasis, it is tempting to speculate that transient positive effects observed using bevacizumab in the adjuvant setting (47) could be enhanced by Ang-2-VEGF-A dual targeting.

A non-overlapping toxicity profile will be a determining factor for combining antiangiogenics in the clinic. Different approaches have been described to target the angiopoietin/Tie axis in early clinical trials (20, 21). The most common side effects reported in patients with cancer include fatigue,

<table>
<thead>
<tr>
<th>Branching points/µm²</th>
<th>Control</th>
<th>Anti-Ang-1</th>
<th>Anti-Ang-2</th>
<th>Anti-VEGF-A</th>
<th>Anti-Ang-1/2</th>
<th>Anti-VEGF-A/Ang-1/2</th>
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<td></td>
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<td>2</td>
<td>4</td>
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**Figure 6.** Anti-Ang-2-VEGF treatment does not further affect healthy vessels. A, representative immunofluorescent pictures of CD31 stained tracheal whole mount sections of Balb/c mice treated with 25 mg/kg i.p. control (g3), anti-VEGF (B20-4.1), anti-Ang-2 (LC06), anti-Ang1/2 (LC08) and the combinations of anti-Ang-2-VEGF (LC06 and B20-4.1) and anti-Ang1/2-VEGF (LC08 and B20-4.1) once weekly × 10. Scale bars: 100 µm. B, quantification of capillary branching points in random regions (n = 5) of 230 × 520 µm in each mouse whole mount tracheas (n = 5; *, P ≤ 0.01 vs. anti-VEGF-A and anti-Ang-2-VEGF-A).
and safety of Ang-2-VEGF-A CrossMab suggest that it represents a novel and effective therapeutic opportunity for patients with cancer with the potential to replace bevacizumab as a pan-tumor agent.

Disclosure of Potential Conflicts of Interest

C. Klein, F. Hering, and M. Thomas have ownership interest (including patents) in Roche. No potential conflicts of interest were disclosed by the other authors.

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Ang-2-VEGF-A CrossMab, a Novel Bispecific Human IgG1 Antibody Blocking VEGF-A and Ang-2 Functions Simultaneously, Mediates Potent Antitumor, Antiangiogenic, and Antimetastatic Efficacy

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