Published OnlineFirst October 4, 2013; DOI: 10.1158/1078-0432.CCR-13-0081

Clinical Cancer Research

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

Yvonne Kienast[1], Christian Klein[5], Werner Scheuer[1], Romi Raemsch[1], Erica Lorenzon[1], Dirk Bernicke[1], Frank Herting[1], Sidney Yu[6], Huynh Hung The[7], Laurent Martarello[8], Christian Gassner[2], Kay-Gunnar Stubenrauch[2], Kate Munro[9], Hellmut G. Augustin[3,4], and Markus Thomas[1]

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. *Clin Cancer Res; 19(24); 6730–40. ©2013 AACR.*

Introduction

Tumor angiogenesis is a hallmark of cancer and requires the coordinated actions of various signal transduction pathways (1). Among those, VEGF-A plays a major role as a key molecule for tumor progression, angiogenesis, and vascular permeability. The clinical efficacy of angiogenesis inhibitors targeting VEGF marked a milestone in the field of angiogenesis research (2); however overlapping and compensatory alternative angiogenic pathways provide escape mechanisms that likely limit the full potential of VEGF monotherapies (3).

The Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2 (Ang-1 and Ang-2), have been implicated in the remodeling of the tumor vasculature. Ang-1 acts as a regulator of vascular maturation and stabilization. In contrast, Ang-2 promotes angiogenesis and tumor growth by (i) destabilizing Tie2 expressing stalk cells, thereby priming the vasculature to respond to angiogenic stimuli (4, 5), and (ii) induction of sprouting tip cell migration in a Tie2-independent manner via integrins (6). Ang-2 can be responsible for compensatory tumor revascularization and growth during anti-VEGF therapy (7) and has been shown to interfere with anti-VEGFR-2–induced vessel normalization (8). In several tumor indications, upregulated Ang-2 levels

Authors' Affiliations: [1]Discovery Oncology and [2]Biologics Research, Pharmaceutical Research and Early Development (pRED), Roche Diagnostics GmbH, Penzberg; [3]Department of Vascular Biology and Tumor Angiogenesis, Medical Faculty Mannheim (CBTM), Heidelberg University; [4]Division of Vascular Oncology and Metastasis, German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), Heidelberg, Germany; [5]Discovery Oncology, pRED, Roche Glycart AG, Schlieren, Switzerland; [6]Department of Nuclear Medicine, Singapore General Hospital; [7]National Cancer Center, Singapore; [8]Roche Translational Medicine Hub, Singapore; and [9]Roche Products Limited, Welwyn Garden City, United Kingdom

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

Y. Kienast and C. Klein contributed equally to this work.

Corresponding Author: Markus Thomas, Roche Diagnostics GmbH, Nonnenwald 2, 82377 Penzberg, Germany. Phone: 49-8856-60-3602; Fax: 49-8856-60-79-3602; E-mail: markus.thomas@roche.com

doi: 10.1158/1078-0432.CCR-13-0081

Published OnlineFirst October 4, 2013; DOI: 10.1158/1078-0432.CCR-13-0081

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 chemo- or anti-VEGF therapy, 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 pharmaceutical 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-Cκ crossover was introduced into the Ang-2 binding antibody arm. Heterodimerization of the two heavy chains was achieved by using the "knobs into holes" (KiH) 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 mm^3. Mice treated with 10 mg/kg (qw $\times$ 5, i.p.) control antibody (omalizumab; anti-human IgE) showed a mean tumor burden (mtb) of 431.5 $\pm$ 67.5 mm^3 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 $\pm$ 21.2 mm^3 (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 $\pm$ 27.9 mm^3; $P = 0.19$) or anti-VEGF-A monotherapy (Fig. 1A; mtb of 148.1 $\pm$ 30.7 mm^3; $P = 0.27$). 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 mm^3 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 $\pm$ 10.5 mm^3 (Fig. 1B) during the course of the trial in contrast to mice treated with control antibody (Fig. 1B, mtb of 442.0 $\pm$ 92.3 mm^3, $P = 0.001$), anti-Ang-2 (Fig. 1B, mtb of 259.8 $\pm$ 47.7 mm^3, $P = 0.03$) and anti-VEGF-A monotherapy (Fig. 1B; mtb of 219.7 $\pm$ 23.6 mm^3, $P = 0.004$). 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 mm^3 for subcutaneous tumors and 150 mm^3 for orthotopic KPL-4 tumors; Table 1). VEGF-dependent smaller tumors ($\leq$ 120 mm^3) 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 $\times$ 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 mm^3; 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; $P \leq$ 0.04), an indicator of a tumor blood vessel maturation

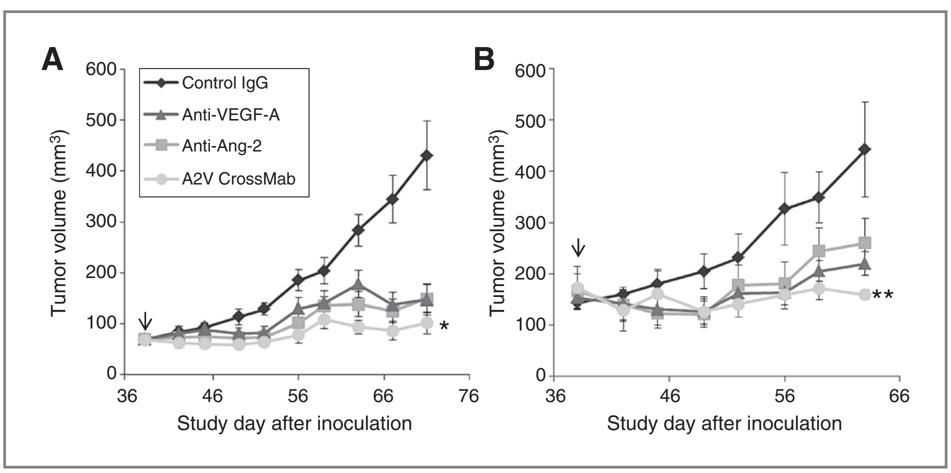

Figure 1. Tumor growth inhibition of Ang-2-VEGF-A CrossMab on small and advanced orthotopic KPL-4 xenografts. A, KPL-4 small tumor (mean 70 mm^3) and (B) advanced tumor (mean 150 mm^3) growth curves in SCID/beige mice receiving A2V CrossMab (10 mg/kg), anti-VEGF-A (bevacizumab, 10 mg/kg), anti-Ang-2 (10 mg/kg), or control antibody (omalizumab, 10 mg/kg) once weekly i.p. [$n = 10$; (A) *, $P <$ 0.001 vs. control, (B) *, $P \leq 0.03$ vs. single and control treatments]. Treatment started at day of randomization (arrow, day 38). Animals were randomized in small (70 mm^3) and advanced (150 mm^3) tumor groups.

Published OnlineFirst October 4, 2013; DOI: 10.1158/1078-0432.CCR-13-0081

Table 1. Antitumor activity of A2V CrossMab in different tumor models

Tumor models (syngeneic, patient-derived and xenograft models)	Mean tumor size at treatment start (mm^3)	Indication	A2V CrossMab TGI (%)	Anti-Ang-2 TGI (%)	Anti-VEGF-A TGI (%)	A2V superiority over anti-Ang-2	A2V superiority over anti-VEGF-A
KPL-4	70	Breast cancer	90	78	78	n.s.	n.s.
KPL-4	150	Breast cancer	115	45	80	$^*P \le 0.003$	$^*P \le 0.03$
MDA-MB-231	80	Breast cancer	56	Not tested	44	Not tested	n.s.
MCF-7	80	Breast cancer	70	Not tested	44	Not tested	n.s.
E0771[#]	50	Breast cancer	75	Not tested	Not tested	Not tested	Not tested
Colo205	120	Colon cancer	77	17	63	n.s.	n.s.
Colo205	420	Colon cancer	92	30	44	$^*P = 0.02$	$^*P = 0.02$
MC38#	60	Colon cancer	67	Not tested	Not tested	Not tested	Not tested
SUDHL-4	290	Lymphoma (B)	77	Not tested	55	Not tested	$^*P \le 0.002$
H460M2	130	Lung cancer	73	20	40	$^*P \le 0.01$	$^*P \le 0.01$
Calu-3	120	Lung cancer	39	29	27	n.s.	n.s.
Panc-1	160	Pancreatic cancer	72	52	39	$^*P = 0.03$	$^*P = 0.02$
PC-3	85	Prostate cancer	55	21	34	n.s.	n.s.
RXF-486	115	RCC (pd)	66	Not tested	49	Not tested	$^*P = 0.02$
HCC07-0409Av7	250	HCC (pd)	71	28	44	$^*P = 0.002$	$^*P = 0.001$
GC23-0909	220	Gastric cancer (pd)	64	32	51	$^*P = 0.01$	$^*P = 0.01$
N87	120	Gastric cancer	79	52	39	$^*P = 0.001$	$P = 0.003$

NOTE: Green, statistically significant; gray, statistically not significant (n.s.); white, not tested; pd, patient-derived.
[#], combination of murine cross-reactive surrogate antibodies LC06 and B20-4.1 (Supplementary Table S1). Tumor models KPL-4 (70 and 150 mm^3) and H460M2 are also described in more detail in the article.

Published OnlineFirst October 4, 2013; DOI: 10.1158/1078-0432.CCR-13-0081

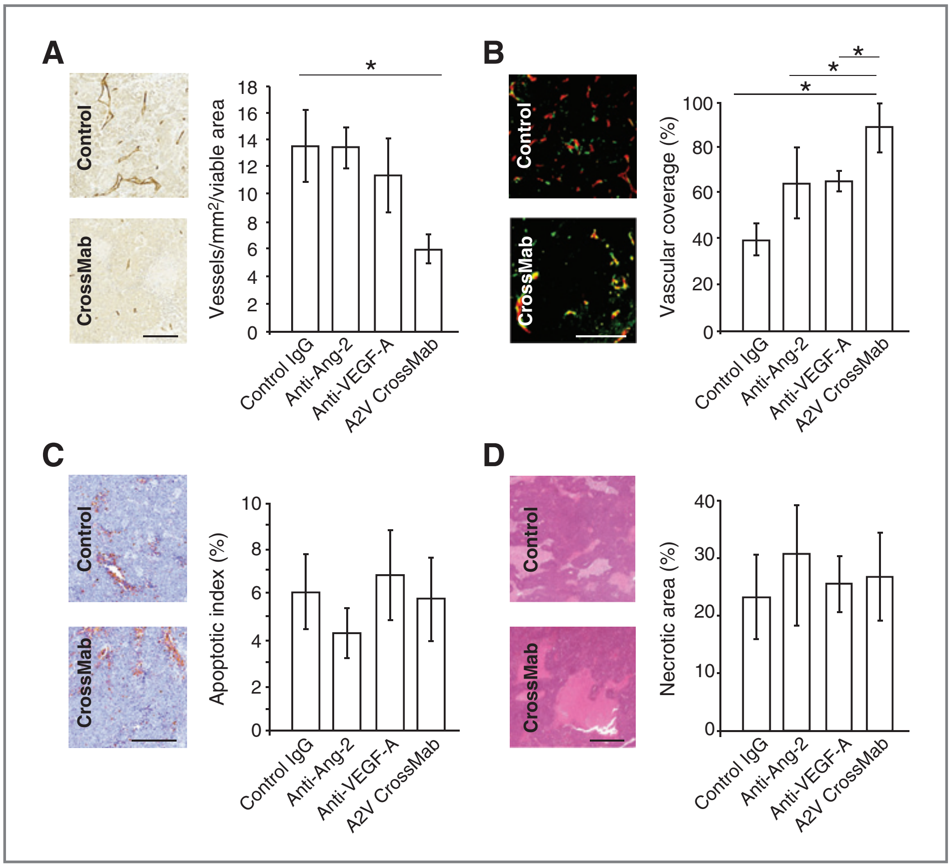

Figure 2. *Ex vivo* analysis of advanced orthotopic KPL-4 tumors (150 mm^3; tumor growth curves shown in Fig. 1B) reveals potent antiangiogenic properties and an enhanced normalization phenotype of tumor vessels mediated by A2V CrossMab. Tumors were collected 3 days after last dosing. A, quantifications and representative pictures of CD34$^+$ vessel 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; $n = 5$; *, $P \leq 0.04$ 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).

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 [^{18}F]-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 mm^3 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.

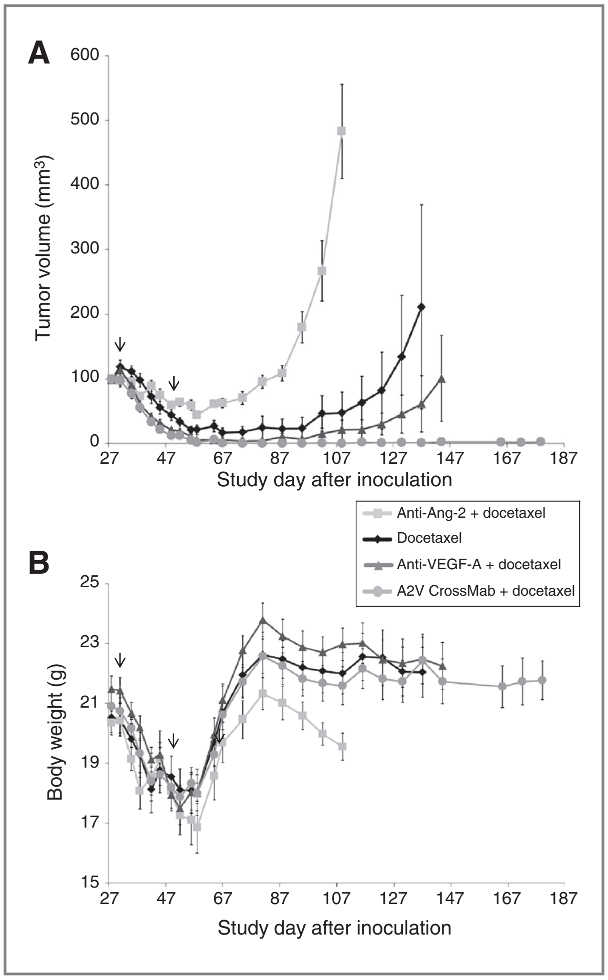

Figure 3. Ang-2-VEGF-A CrossMab enhances chemotherapy and leads to complete tumor regression and long-term cures of mice. A, orthotopic KPL-4 breast tumor bearing SCID/beige mice ($n = 10$) were treated with docetaxel (10 mg/kg) either alone or in combination with A2V CrossMab (5 mg/kg) or monotherapies (anti-Ang-2 or anti-VEGF-A 5 mg/kg each). Arrow at day 28 indicates start, while arrow at day 50 indicates termination of treatment. The study was terminated after 180 days. B, treatment-related changes in body weight are stabilized after treatment termination at day 50 (arrow), except for animals with progressive disease after docetaxel plus anti-Ang-2 therapy.

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 post-surgery 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 mm^3. 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; $P \leq 0.03$). 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; $P \leq 0.02$). 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; $P \leq 0.02$ 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,

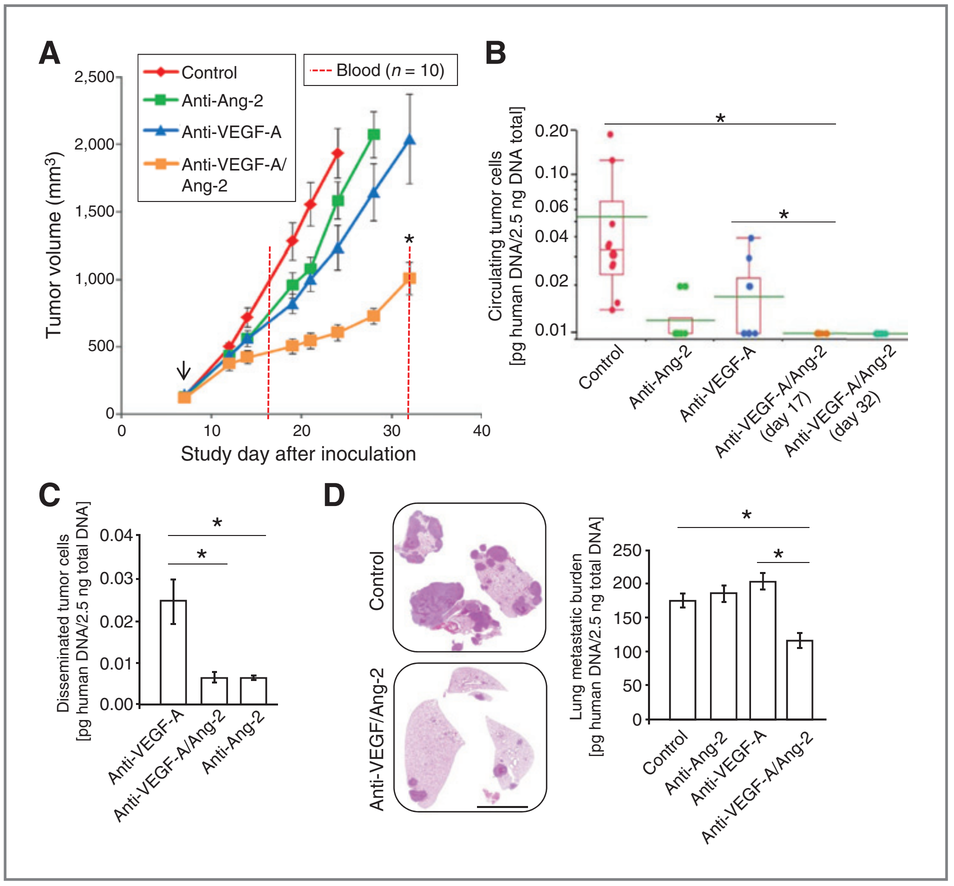

Figure 4. Ang-2-VEGF-A therapy inhibits metastasis in the neoadjuvant and adjuvant setting. A, H460M2 tumor growth curves in SCID/beige mice receiving Ang-2-VEGF-A combination therapy (10 mg/kg LC06 and B20-4.1 each), anti-VEGF (B20-4.1, 10 mg/kg), anti-Ang-2 (10 mg/kg) or control antibody (10 mg/kg) once weekly i.p. ($n = 10$; *, $P \leq 0.01$ versus anti-VEGF-A and anti-Ang-2). Red dotted lines indicate blood sample collection (day 17 and 32, $n = 10$). Mean tumor volumes at day 17 were 1,000 mm^3 (vehicle), 700 mm^3 (anti-Ang-2, anti-VEGF-A), and 500 mm^3 (anti-Ang-2-VEGF-A) or 1,000 mm^3 (day 32, anti-Ang-2-VEGF-A). Arrow indicates start of treatment. B, tumor-derived DNA in blood samples disseminated by subcutaneous H460M2 xenografts was detected by Alu-PCR on day 17 and 32 after tumor cell inoculation ($n = 10$ animals per group; *, $P = 0.03$ versus anti-VEGF-A and control; dots representing equal values are overlapping and data points are thereby partially hidden). C, Colo205 tumor cell dissemination to the lungs in first-line anti-VEGF-A treated SCID/beige mice after anti-Ang-2 treatment in combination with anti-VEGF-A or alone ($n = 5$; *, $P < 0.04$ versus anti-VEGF-A). D, Postsurgically metastasis in a mouse model of spontaneous lung metastasis demonstrated by H&E representative pictures and quantification by Alu-PCR ($n = 10$; *$P \leq 0.02$ vs. anti-VEGF-A and control). Mice received postsurgical adjuvant anti-Ang-2-VEGF-A or monotherapies (10 mg/kg, once weekly i.p. × 3) using the therapeutic antibodies indicated in (A). Scale bars: 500 μm.

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/μm^2 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

Published OnlineFirst October 4, 2013; DOI: 10.1158/1078-0432.CCR-13-0081

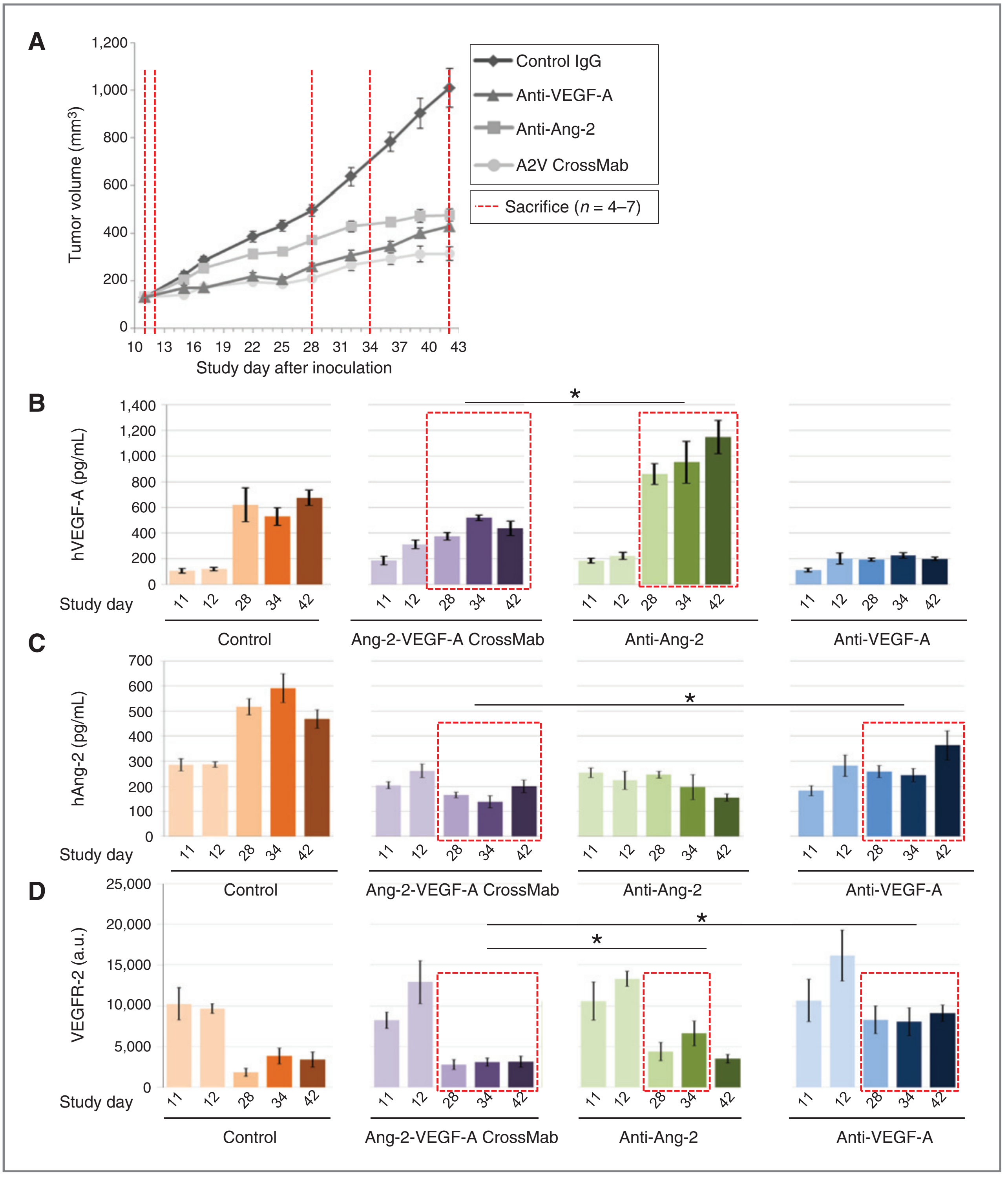

Figure 5. Coordinated action of VEGF-A and Ang-2 during cancer therapy. A, subcutaneous Colo205 tumors ($n = 20$; *, $P < 0.001$ versus anti-VEGF-A and anti-Ang-2) were harvested at 11, 12, 28, 34, and 42 days post randomization (red dotted lines), and the levels of (B) human VEGF, (C) human Ang-2, or (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) *, $P \leq 0.03$ versus anti-Ang-2 (days 28, 34, 42); (C) *, $P \leq 0.02$ versus anti-VEGF (days 28, 34, 42); (D) *, $P \leq 0.02$ versus anti-Ang-2 (days 28, 24); *, $P \leq 0.001$ versus anti-VEGF (days 28, 34, 42).

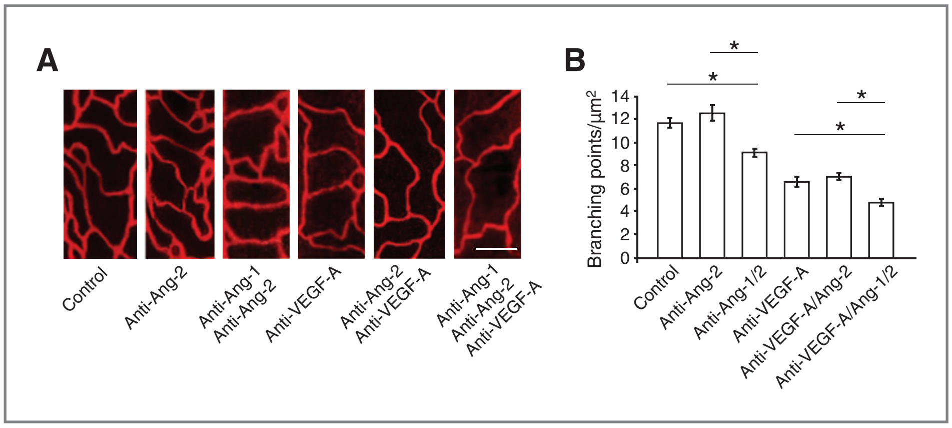

Figure 6. Anti-Ang-2-VEGF treatment does not further affect healthy vessels. A, representative immunofluorescent pictures of CD31 stained tracheal whole mount sections of Balb/c mice treated with 25 mg/kg i.p. control IgG, anti-VEGF (B20-4.1), anti-Ang-2 (LC06), anti-Ang1/2 (LC08) and the combinations 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 ($n = 5$; *, $P \leq 0.01$ vs. anti-VEGF-A and anti-Ang-2-VEGF-A).

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 cel 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 is of 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 angiopoetin/Tie axis in early clinical trials (20, 21). The most common side effects reported in patients with cancer include fatigue, decreased appetite, nausea, upper abdominal pain, back pain, and dyspnea (20). Peripheral edema has only been associated with dual inhibition of Ang-1 and Ang-2 (21). Of note is that the toxicity profile does not appear to overlap with that of VEGF/R inhibitors, in relation to bleeding or thromboembolic events. In fact, results from recently completed clinical trials indicate that dual inhibition of Ang-2 and VEGF-A using anti-Ang-2 peptibody/bevacizumab combination is safe in patients with advanced solid tumors (48, 49). In addition to other side effects, anti-VEGF therapy leads to pruning of quiescent vessels in healthy tissues because they require VEGF survival signals for their maintenance (50). Evaluating the safety profile of A2V CrossMab, we observed that anti-Ang-2-VEGF-A therapy did not aggravate vessel pruning induced by anti-VEGF-A monotherapy, presumably because expression of Ang-2 is negligible in quiescent tissues in baseline conditions (5).

Taken together, we provide supportive data for Ang-2-VEGF-A dual targeting. The proposed mechanism of action suggests substantial beneficial therapeutic impact by targeting tumor angiogenesis and metastasis at the same time. Moreover, synergistic and compensatory roles of Ang-2 and VEGF-A are blocked. Improvement of normalization leads to enhanced chemotherapeutic efficacy and less metastatic spread through leaky vessels. A safety advantage is achieved by no further destruction of physiologic vessels. Expression of Ang-2 and VEGF-A in the same low nanomolar range during cancer progression indicates the potential for a straightforward equimolar and pharmacoeconomic administration scheme achieved by A2V CrossMab in contrast to the combination of monospecific antibodies. The efficacy 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. Herting, and M. Thomas have ownership interest (including patents) in Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: Y. Kienast, C. Klein, E. Lorenzon, L. Martarello, K.-G. Stubenrauch, H.G. Augustin, M. Thomas
Development of methodology: Y. Kienast, C. Klein, W. Scheuer, R. Raemsch, S. Yu, L. Martarello, K.-G. Stubenrauch, M. Thomas
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Scheuer, E. Lorenzon, F. Herting, S. Yu, H.H. The, L. Martarello, C. Gassner, K.-G. Stubenrauch
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Kienast, W. Scheuer, R. Raemsch, E. Lorenzon, D. Bernicke, F. Herting, H.H. The, L. Martarello, C. Gassner, K.-G. Stubenrauch, H.G. Augustin, M. Thomas
Writing, review, and/or revision of the manuscript: Y. Kienast, C. Klein, R. Raemsch, E. Lorenzon, L. Martarello, K. Munro, H.G. Augustin, M. Thomas
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Kienast, W. Scheuer, R. Raemsch, D. Bernicke, M. Thomas
Study supervision: Y. Kienast, F. Herting, L. Martarello, M. Thomas

Acknowledgments

The authors thank Ute Haupt, Melanie Rutz, Franz Osl, Christa Bayer, Stefan Hoert, Gunter Muth, and Stefanie Fischer for their valuable help.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 10, 2013; revised August 27, 2013; accepted September 16, 2013; published OnlineFirst October 4, 2013.

References

1. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. Nature 2005;438:967–74.
2. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature 2011;473:298–307.
3. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer 2008;8:592–603.
4. Thomas M, Felcht M, Kruse K, Kretschmer S, Deppermann C, Biesdorf A, et al. Angiopoietin-2 stimulation of endothelial cells induces alphavbeta3 integrin internalization and degradation. J Biol Chem 2010;285:23842–9.
5. Scharpfenecker M, Fiedler U, Reiss Y, Augustin HG. The Tie-2 ligand angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. J Cell Sci 2005;118:771–80.
6. Felcht M, Luck R, Schering A, Seidel P, Srivastava K, Hu J, et al. Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signaling. J Clin Invest 2012;122:1991–2005.
7. Bullock AJ, Zhang AM, O'Neill AM, Percy V, Sukhatme JW, Mier MB, et al. Plasma angiopoietin-2 (ANG2) as an angiogenic biomarker in renal cell carcinoma (RCC). J Clin Oncol 2010;28:suppl; abstr 4630.
8. Chae SS, Kamoun WS, Farrar CT, Kirkpatrick ND, Niemeyer E, de Graaf AM, et al. Angiopoietin-2 interferes with anti-VEGFR2-induced vessel normalization and survival benefit in mice bearing gliomas. Clin Cancer Res 2010;16:3618–27.
9. Sfiligoi C, de LA, Cascone I, Sorbello V, Fuso L, Ponzone R, et al. Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival. Int J Cancer 2003;103:466–74.
10. Etoh T, Inoue H, Tanaka S, Barnard GF, Kitano S, Mori M. Angiopoietin-2 is related to tumor angiogenesis in gastric carcinoma: possible in vivo regulation via induction of proteases. Cancer Res 2001;61:2145–53.
11. Ochiumi T, Tanaka S, Oka S, Hiyama T, Ito M, Kitadai Y, et al. Clinical significance of angiopoietin-2 expression at the deepest invasive tumor site of advanced colorectal carcinoma. Int J Oncol 2004;24:539–47.
12. Hu B, Jarzynka MJ, Guo P, Imanishi Y, Schlaepfer DD, Cheng SY. Angiopoietin 2 induces glioma cell invasion by stimulating matrix metalloprotease 2 expression through the alphavbeta1 integrin and focal adhesion kinase signaling pathway. Cancer Res 2006;66:775–83.
13. Imanishi Y, Hu B, Jarzynka MJ, Guo P, Elishaev E, Bar-Joseph I, et al. Angiopoietin-2 stimulates breast cancer metastasis through the alpha(5)beta(1) integrin-mediated pathway. Cancer Res 2007;67:4254–63.
14. Helfrich I, Edler L, Sucker A, Thomas M, Christian S, Schadendorf D, et al. Angiopoietin-2 levels are associated with disease progression in metastatic malignant melanoma. Clin Cancer Res 2009;15:1384–92.
15. Schulz P, Fischer C, Detjen KM, Rieke S, Hilfenhaus G, von MZ, et al. Angiopoietin-2 drives lymphatic metastasis of pancreatic cancer. FASEB J 2011;25:3325–35.
16. Hashizume H, Falcon BL, Kuroda T, Baluk P, Coxon A, Yu D, et al. Complementary actions of inhibitors of angiopoietin-2 and VEGF on tumor angiogenesis and growth. Cancer Res 2010;70:2213–23.
17. Koh YJ, Kim HZ, Hwang SI, Lee JE, Oh N, Jung K, et al. Double antiangiogenic protein, DAAP, targeting VEGF-A and angiopoietins in

tumor angiogenesis, metastasis, and vascular leakage. Cancer Cell 2010;18:171–84.

18. Brown JL, Cao ZA, Pinzon-Ortiz M, Kendrew J, Reimer C, Wen S, et al. A human monoclonal anti-ANG2 antibody leads to broad antitumor activity in combination with VEGF inhibitors and chemotherapy agents in preclinical models. Mol Cancer Ther 2010;9:145–56.
19. Mazzieri R, Pucci F, Moi D, Zonari E, Ranghetti A, Berti A, et al. Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. Cancer Cell 2011;19:512–26.
20. Rosen LS, Mendelson DS, Cohen RB. First-in-human dose-escalation safety and PK trial of a novel intravenous humanized monoclonal CovX body inhibiting angiopoietin 2. J Clin Oncol 28:15s, 2010 (suppl; abstr 2524).
21. Herbst RS, Hong D, Chap L, Kurzrock R, Jackson E, Silverman JM, et al. Safety, pharmacokinetics, and antitumor activity of AMG 386, a selective angiopoietin inhibitor, in adult patients with advanced solid tumors. J Clin Oncol 2009;27:3557–65.
22. Doppalapudi VR, Huang J, Liu D, Jin P, Liu B, Li L, et al. Chemical generation of bispecific antibodies. Proc Natl Acad Sci U S A 2010; 107:22611–6.
23. Schaefer W, Regula JT, Bahner M, Schanzer J, Croasdale R, Durr H, et al. Immunoglobulin domain crossover as a generic approach for the production of bispecific IgG antibodies. Proc Natl Acad Sci U S A 2011;108:11187–92.
24. Thomas M, Kienast Y, Scheuer W, Bahner M, Kaluza K, Gassner C, et al. A novel angiopoietin-2 selective fully human antibody with potent anti-tumoral and anti-angiogenic efficacy and superior side effect profile compared to Pan-Angiopoietin-1/-2 inhibitors. PLoS ONE 2013;8:e54923.
25. Liang WC, Wu X, Peale FV, Lee CV, Meng YG, Gutierrez J, et al. Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. J Biol Chem 2006;281:951–61.
26. Ridgway JB, Presta LG, Carter P. 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization. Protein Eng 1996;9:617–21.
27. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 2005;307:58–62.
28. Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell 2009; 15:220–31.
29. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell 2009;15:232–9.
30. Bagri A, Kouros-Mehr H, Leong KG, Plowman GD. Use of anti-VEGF adjuvant therapy in cancer: challenges and rationale. Trends Mol Med 2010;16:122–32.
31. Schneider T, Osl F, Friess T, Stockinger H, Scheuer WV. Quantification of human Alu sequences by real-time PCR–an improved method to measure therapeutic efficacy of anti-metastatic drugs in human xeno-transplants. Clin Exp Metastasis 2002;19:571–82.
32. Visconti RP, Richardson CD, Sato TN. Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). Proc Natl Acad Sci U S A 2002;99: 8219–24.
33. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004;350:2335–42.
34. Goede V, Coutelle O, Neuneier J, Reinacher-Schick A, Schnell R, Koslowsky TC, et al. Identification of serum angiopoietin-2 as a biomarker for clinical outcome of colorectal cancer patients treated with bevacizumab-containing therapy. Br J Cancer 2010;103: 1407–14.
35. Tsutsui S, Inoue H, Yasuda K, Suzuki K, Takeuchi H, Nishizaki T, et al. Angiopoietin 2 expression in invasive ductal carcinoma of the breast: its relationship to the VEGF expression and microvessel density. Breast Cancer Res Treat 2006;98:261–6.
36. Tanaka F, Ishikawa S, Yanagihara K, Miyahara R, Kawano Y, Li M, et al. Expression of angiopoietins and its clinical significance in non-small cell lung cancer. Cancer Res 2002;62:7124–9.
37. Hou HA, Chou WC, Lin LI, Tang JL, Tseng MH, Huang CF, et al. Expression of angiopoietins and vascular endothelial growth factors and their clinical significance in acute myeloid leukemia. Leuk Res 2008;32:904–12.
38. Yoshiji H, Kuriyama S, Noguchi R, Yoshii J, Ikenaka Y, Yanase K, et al. Angiopoietin 2 displays a vascular endothelial growth factor dependent synergistic effect in hepatocellular carcinoma development in mice. Gut 2005;54:1768–75.
39. Zhang D, Hedlund EM, Lim S, Chen F, Zhang Y, Sun B, et al. Anti-angiogenic agents significantly improve survival in tumor-bearing mice by increasing tolerance to chemotherapy-induced toxicity. Proc Natl Acad Sci U S A 2011;108:4117–22.
40. Kunz P, Hoffend J, Altmann A, mitrakopoulou-Strauss A, Koczan D, Eisenhut M, et al. Angiopoietin-2 overexpression in morris hepatoma results in increased tumor perfusion and induction of critical angiogenesis-promoting genes. J Nucl Med 2006;47:1515–24.
41. Nagy JA, Dvorak HF. Heterogeneity of the tumor vasculature: the need for new tumor blood vessel type-specific targets. Clin Exp Metastasis 2012;29:657–62.
42. Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol 2006;3:24–40.
43. Winkler F, Kozin SV, Tong RT, Chae SS, Booth MF, Garkavtsev I, et al. Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. Cancer Cell 2004;6: 553–63.
44. Kienast Y, von Baumgarten L, Fuhrmann M, Klinkert WE, Goldbrunner R, Herms J, et al. Real-time imaging reveals the single steps of brain metastasis formation. Nat Med 2010;16:116–22.
45. Joensson P, Hotz B, Buhr HJ, Hotz HG. A novel antiangiogenic approach for adjuvant therapy of pancreatic carcinoma. Langenbecks Arch Surg 2011;396:535–41.
46. Allegra CJ, Yothers G, O'Connell MJ, Sharif S, Petrelli NJ, Colangelo LH, et al. Phase III trial assessing bevacizumab in stages II and III carcinoma of the colon: results of NSABP protocol C-08. J Clin Oncol 2011;29:11–6.
47. Van CE, Lambrechts D, Prenen H, Jain RK, Carmeliet P. Lessons from the adjuvant bevacizumab trial on colon cancer: what next? J Clin Oncol 2011;29:1–4.
48. Hong D, Gordon M, Appleman L, et al. Interim results from a phase 1b study of safety, pharmacokinetics and tumor response of the angiopoietin1/2-neutralizing peptibody AMG 386 in combination with AMG 706 (motesanib), bevacizumab or sorafenib in advanced solid tumors. ESMO Congress; Stockholm, Sweden; Sept 12–16, 2008. abstr 462PD.
49. Dieras V, Jassem J, Dirix LY. A randomized, placebo-controlled phase II study of AMG 386 plus bevacizumab (Bev) and paclitaxel (P) or AMG 386 plus P as first-line therapy in patients (pts) with HER2-negative, locally recurrent or metastatic breast cancer (LR/MBC). J Clin Oncol 29: 2011 (suppl; abstr 544).
50. Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem 1998;273:13313–6.

Published OnlineFirst October 4, 2013; DOI: 10.1158/1078-0432.CCR-13-0081

American Association for Cancer Research

Clinical Cancer Research

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

Yvonne Kienast, Christian Klein, Werner Scheuer, et al.

Clin Cancer Res 2013;19:6730-6740. Published OnlineFirst October 4, 2013.