Antiangiogenic and Antitumor Efficacy of EphA2 Receptor Antagonist

Pawel Dobrzanski, Kathryn Hunter, Susan Jones-Bolin, Hong Chang, Candy Robinson, Sonya Pritchard, Hugh Zhao, and Bruce Ruggeri

Division of Oncology, Cephalon, Inc., West Chester, Pennsylvania

ABSTRACT

Tumor-associated angiogenesis is critical for tumor growth and metastasis and is controlled by various pro- and antiangiogenic factors. The Eph family of receptor tyrosine kinases has emerged as one of the pivotal regulators of angiogenesis. Here we report that interfering with EphA signaling resulted in a pronounced inhibition of angiogenesis in ex vivo and in vivo model systems. Administration of EphA2/Fc soluble receptors inhibited, in a dose-dependent manner, microvessel formation in rat aortic ring assay, with inhibition reaching 76% at the highest dose of 5000 ng/ml. These results were further confirmed in vivo in a porcine aortic endothelial cell-vascular endothelial growth factor (VEGF)/basic fibroblast growth factor Matrigel plug assay, in which administration of EphA2/Fc soluble receptors resulted in 81% inhibition of neovascularization. The additive effects of simultaneous inhibition of VEGF receptor 2 and EphA signaling pathways in aortic ring assay and antiangiogenic efficacy of EphA2/Fc soluble receptors against VEGF/basic fibroblast growth factor-mediated neovascularization in vivo indicated a critical and nonredundant role for EphA signaling in angiogenesis. Furthermore, in two independent experiments, we demonstrated that EphA2/Fc soluble receptors strongly (by ~50% versus controls) suppressed growth of ASPC-1 human pancreatic tumor s.c. xenografts. Inhibition of tumor growth was due to decreased proliferation of tumor cells. In an orthotopic pancreatic ductal adenocarcinoma model in mice, suppression of EphA signaling by i.p. administration of EphA2/Fc (30 µg/dose, three times a week for 56 days) profoundly inhibited the growth of primary tumors and the development of peritoneal, lymphatic, and hepatic metastases. These data demonstrate a critical role of EphA signaling in tumor growth and metastasis and provide a strong rationale for targeting EphA2 receptors for anticancer therapies.

INTRODUCTION

Angiogenesis is a process of remodeling of a primitive vascular network into mature vasculature through sprouting, branching, and differential growth of blood vessels. It is a complex process involving endothelial cell proliferation, chemotactic migration, and functional maturation, as well as differential recruitment of supporting cells. Tumor-associated angiogenesis is critical for tumor growth and is controlled by a balance between pro- and antiangiogenic factors. The growing evidence demonstrates the heterogeneity of tumor angiogenesis, probably arising from vastly different microenvironments of individual tumors (1–3). Because of the heterogeneous nature of tumor angiogenesis, effective antiangiogenic therapy should be optimized and might require interference with multiple angiogenic pathways. Indeed, our own observations (4) and other published data (5) strongly indicate that various inhibitors of angiogenesis exhibit type- and stage-specific antitumor effects. Development of functional vascular networks is mediated by several receptor tyrosine kinases acting at distinct phases of this process. In addition to vascular endothelial growth factor (VEGF) and Tie2 receptors, which have long been recognized as key angiogenic receptor tyrosine kinases, Eph receptors have been identified as critical regulators of angiogenesis (6, 7).

Eph receptors represent the largest family of receptor tyrosine kinases, currently consisting of 14 members. Eight ligands for Eph receptors, called ephrins, have been identified to date. The Eph receptors and ephrins are divided into two classes, A and B, based on structural homologies and binding specificities. EphrinA ligands bind preferentially to EphA receptors, whereas ephrinB ligands bind to Eph B receptors; however, within the class, interactions, with some exceptions, are promiscuous (8, 9). Unlike the majority of ligands for receptor tyrosine kinases, which function as soluble molecules, ephrins are anchored on plasma membranes, thus restricting ephrin-Eph interactions to sites of direct cell-cell contact. The vast body of biochemical and genetic evidence demonstrates an active signaling role for ephrins (10–12). Thus, Eph receptors and ephrins are capable of inducing reciprocal, bidirectional signaling between interacting cells. Initial studies indicated that ephrins and Eph receptors play an important role in development of the neuronal systems (9, 12). However, rapidly accumulating evidence clearly demonstrates the critical role of Eph-ephrin signaling in angiogenesis. Eph receptors and their ligands, ephrins, have emerged as essential regulators of angiogenesis in vivo, equal in genetic importance to other ligand-receptor systems such as VEGF and angiopoietins (6). Although early in vitro studies indicated the potential role of ephrins and their receptors in the biology of the vascular system (13, 14), the expression and knockout studies of ephrinB2 and EphB2, EphB3, and EphB4 receptors firmly established them as key players in the formation of the vasculature (15–17). In addition, a number of in vivo and in vitro studies have clearly demonstrated involvement of the EphA2 receptor in angiogenesis (13, 14, 18).

The initial report on ephrinB2 knockout mice provided the first compelling genetic evidence of the key role of ephrin/Eph axis in vascular development (15). Knockout animals died around embryonic day 11 from multiple cardiovascular abnormalities. Interestingly, vasculogenesis occurred normally, but angiogenesis was disrupted. Detailed analysis of the vascular system in targeted animals revealed reciprocal expression of ephrinB2 and EphB4 receptor in arterial and venal endothelial cells, respectively. Not surprisingly, targeted mutation in EphB4 phenocopied mutation in ephrinB2 (16). In addition, Adams et al. (17) have demonstrated expression of EphB2 and EphB3 as well as ephrinB1 in and around the developing vasculature. Double knockouts lacking EphB2 and EphB3 displayed vascular defects reminiscent of ephrinB2-targeted animals. This genetic evidence has been corroborated by in vitro and in vivo studies, which demonstrated the ability of ephrinB1 and ephrinB2 to directly induce sprouting angiogenesis (17) and EphB1-induced neovascularization in mouse corneal micropocket assay (19). Furthermore, ephrinA1, the first ligand for an Eph receptor to be identified, was cloned from human umbilical vein endothelial cells as a gene, which was up-regulated during in vitro capillary tube formation (20). Subsequent studies in mice revealed that ephrinA1 is highly expressed in embryonic (18) but not adult vasculature, suggesting a role in vascular morphogenesis. Although ephrinA1 had no discernible mitogenic effects on cultured endothelial cells, it promoted their chemotaxis and induced angiogenesis in vivo in rat cornea pocket assay (14). EphrinA1 and EphA2 were
also shown to mediate assembly of human umbilical vein endothelial cells into capillary-like structures. This process could be blocked by the dominant negative form of EphA2 (21). Interestingly, expression of EphA2 has not been reported in normal adult vasculature but is widely up-regulated during tumor-associated angiogenesis (21, 22). Based on the existing data, it can be reasonably anticipated that inhibition of Eph signaling would impair neoangiogenesis. We decided to test this notion and to analyze effects of inhibition of Eph signaling on angiogenesis and tumor growth. Five Eph receptors, EphA2 and EphB1–4, have been implicated, thus far, in angiogenesis (23). We chose to focus on EphA2 because of the growing evidence that it is overexpressed in a wide spectrum of human tumors, including breast, lung, colon, prostate, and kidney carcinomas (21, 24, 25). The increased expression was detected in both endothelial and tumor cells (21, 22). In a recent report, Brantley et al. (22) demonstrated that antagonizing EphA signaling resulted in inhibition of tumor neoangiogenesis and tumor growth, thus providing the first functional evidence for the role of Eph A class receptors in tumor angiogenesis. In transgenic mouse models of mammary carcinogenesis, EphA2 (and EphB4) were detected in undifferentiated and invasive primary tumors of mice expressing the H-Ras oncogene, but not in the well-differentiated and nonmetastatic mammary tumors of c-Myc-expressing mice (26). In addition, forced expression of EphA2 in the breast epithelial cell line MCF-10A resulted in a cellular transformation and marked increase in tumorigenicity (24). Recent studies indicated that Eph receptors have the ability to regulate integrin activity and thus affect cell-extracellular matrix interactions and cell motility, key events in tumor invasiveness and metastasis (27, 28). Despite a wealth of information linking increased expression of the EphA2 and other Eph receptors to cancer, the molecular mechanisms underlying the specific role of Eph signaling in tumor-associated angiogenesis and in tumor formation, progression, and metastasis remain poorly understood. In this report, we analyzed the effects of inhibition of EphA signaling on angiogenesis and tumor growth.

**MATERIALS AND METHODS**

**Cell Lines and Reagents.** ASPC-1 human pancreatic adenocarcinoma cells were obtained from American Type Culture Collection and maintained as recommended. Recombinant EphA2/Fc and other soluble receptors were obtained from R&D Systems. CEP-5214 was synthesized in the department of Chemistry at Cephalon, Inc. as described previously (4), solubilized in DMSO, and stored in amber glass vials.

**Animals.** Female athymic nu/nu mice (6–8 weeks old; Charles River, Wilmington, MA) were maintained 5 mouse/cage in microisolator units on a standard sterilizable laboratory diet (Teklad Labchow; Harlan Teklad, Madison, WI). Animals were housed under humidity- and temperature-controlled conditions, and the light/dark cycle was set at 12 h intervals. Male Sprague Dawley rats (250–300 g) were obtained from Charles River and housed 5 animals/cage in a conventional vivarium facility. All mice and rats were quarantined 1 week before experimental manipulation. All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committees of Cephalon, Inc.

**Expression Studies.** EphrinA1 and EphA2 receptor expression and activation in various tumors were analyzed by Western blot and immunoprecipitation. Tumors were homogenized in lysis buffer (Cell Signaling) supplemented with a mixture of protease inhibitors (Complete; Roche). Lysates were cleared by a 20-min centrifugation, and protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories). Total levels of expression were analyzed by Western blot using EphA2-specific (Upstate Biotechnology) or ephrinA1-specific (Santa Cruz Biotechnology) antibodies. Tyrosine phosphorylation of EphA2 receptors was analyzed by immunoprecipitation of EphA2 receptors with anti-EphA2 antibody (Upstate Biotechnology), followed by Western blot with phosphotyrosine-specific 4G10 antibody (Upstate Biotechnology). Blots were developed using Pierce Supersignal System. To analyze effects of EphA2/Fc administration on EphA2 and focal adhesion kinase (FAK) activity, ASPC-1 cells were plated at 60% confluence to assure formation of cell-cell contacts. EphA2/Fc or IgG was added to cell cultures, and 24 h later, cell extracts were prepared. EphA2 receptor expression and phosphorylation were detected as described above. FAK activation was analyzed by Western blotting using anti-FAK [pY925] phosphospecific antibody (BioSource International).

**Ex Vivo Aortic Ring Assay.** Rat aortic ring explant cultures were prepared by a modification of established protocols (29, 30) as described previously (4). Briefly, aortic rings were isolated from male Sprague Dawley rats were incubated in freshly prepared rat tail collagen as detailed previously (4) and transferred to 16-mm wells (4-well NUNC dishes), each containing 0.5 ml of serum-free embryonic basal media (EBM). EphA2/Fc and IgG were mixed with serum-free EBM at desired concentrations before the addition or replacement of media to collagen-embedded aortic ring explant cultures. Cultures were incubated at 37°C in a humidified CO2 atmosphere, and the media were replaced every second day. Microvessel outgrowths from replicate explant cultures (n = 4) were counted under bright-field microscopy following an established protocol (29, 30). Experiments were done at least twice, and microvessel counts in treated and control cultures were analyzed by one-way ANOVA and the Student-Newman-Keuls multiple comparison test, with P < 0.05 deemed significant.

**Matrigel Plug Assay.** The Matrigel plug implantation assay used in these studies was a modified version of an established protocol (31, 32) as described previously (4). Briefly, porcine aortic endothelial cell (PAECs) were grown to confluence in Ham’s F-12 medium supplemented with 10% fetal bovine serum. Cells were used between passages 5 and 10. Nude mice received bilateral s.c. injection with 0.5 ml of Matrigel synthetic basement membrane (Collaborative Research, Waltham, MA) containing 1 × 106 PAECs/plug and recombinant murine VEGF and basic fibroblast growth factor [bFGF (St. Louis Biotechnology Systems, Minneapolis, MN)] at 20 and 250 ng/ml, respectively (final concentrations per plug). Mice bearing PAEC-VEGF/bFGF-Matrigel implants were randomized into control (n = 10) and EphA2/Fc-treated (n = 10) groups. Control animals received s.c. injection around the plug with 100 μl of saline, and treated animals received injection of 100 μl of EphA2/Fc in saline, 25 μg/dose, every second day. After 10 days, animals were euthanized by asphyxiation, and the Matrigel plugs were removed. The extent of neoangiogenesis was quantified by analyzing hemoglobin content in each plug, as described previously (32). The hemoglobin content of the PAEC-VEGF/bFGF-Matrigel plugs has been reported to be directly proportional to the degree of neoangiogenesis in each plug (30). Results from in vivo experiments are expressed as mean g/dl of hemoglobin ± SE. Statistical analyses of the data were done using the paired student t test, with P < 0.05 deemed significant.

**Subcutaneous Tumor Xenograft Model.** Human pancreatic ductal carcinoma xenografts were established by inoculating 10 6 ASPC-1 cells into the right flank of female athymic nude mice in a serum-free media mixed with an equal volume of Matrigel (Collaborative Research), as described previously (4). Tumors were allowed to reach about 200 mm3 before animals were randomized into control (n = 7) and EphA2/Fc-treated (n = 7) groups. Control animals received peritumoral injections of 125 μl of IgG in saline (30 μg/dose), and the treated group received peritumoral injection with 125 μl of EphA2/Fc in saline (30 μg/dose). Reagents were administered 3×/week for 26 days. Tumor volumes were determined with vernier calipers every 3–4 days, as described previously (4). Tumor measurements were expressed as absolute volumes, as well as normalized to individual tumor volumes at day 1, the initiation of dosing (relative tumor volumes), to assess changes in the rate of tumor growth relative to treatment. Statistical analyses of tumor data were done using the Mann-Whitney rank sum test, when analyzing data for the data set, by one-way ANOVA and the Dunnet’s multiple comparison test, with P < 0.05 deemed significant. Animal body weights were determined and analyzed using a similar time course.

**Immunohistochemistry.** ASPC-1 human tumor xenografts from control and EphA2/Fc-treated animals were excised and fixed in neutral buffered formalin for 24–48 h. The paraffin-embedded sections were deparaffinized and rehydrated, and endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Proliferating cell nuclear antigen (PCNA) immunostaining was done with Dako EPOS anti-PCNA/horseradish peroxidase antibody (DAKO Corp.) as recommended by the manufacturer. Sections were developed with diaminobenzidine and counterstained with methyl green.

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Orthotopic Pancreatic Ductal Adenocarcinoma (PDAC) Tumor Model.

Human Colo357 pancreatic carcinoma xenograft tissue that had been thrice serially passaged in nude mice to select for a phenotype distinguished by peritoneal, lymphatic, and hepatic metastases of pancreatic origin was surgically implanted onto the pancreas of nude mice as described previously (33). Briefly, ~8 mm³ tumor xenograft tissue fragments were trimmed free of necrotic tissue and placed in sterile, undiluted Matrigel synthetic basement membrane (Collaborative Research) on ice for 60 min before surgical implantation in the nude mouse host. At approximately 8 weeks of age, female nu/nu mice were anesthetized with a mixture of ketamine/xylazine given by i.m. injection. After establishment of satisfactory anesthesia, a left lateral laparotomy was performed using aseptic technique, and the spleen and pancreas were exteriorized by gentle traction. Two of the ~8-mm³ tumor xenograft fragments prepared as described above were anchored to the posterior surface of the splenic portion of the pancreas of each mouse with a 6-0 Prolene suture. The abdominal incision was closed with 6-0 Vicryl, and the skin was closed with skin staples. Seven days after surgical implantation, mice (n = 5 mice/group) were randomized into two treatment groups and received either a physiological saline vehicle i.p. or treatment with Eph A2/Fc (30 µg/mouse, i.p., 3×/week for 56 days). Mice were monitored for palpable tumor burden, ascites production, severe morbidity, and body weight loss, as well as histological assessment of their primary and metastatic lesions. On necropsy (day 56 of treatment), examination of both the abdominal and thoracic cavities was performed to determine the extent of gross metastatic spread of the orthotopically implanted PDAC tumor. A metastatic score was assigned as follows: a score of I was given if the mouse had a primary mass with 0–10 mesenteric lymph nodes and no other visible organ or peritoneal or thoracic cavity spread. A score of II was given if the mouse had a primary mass with 10–100 mesenteric lymph nodes and no other visible organ or peritoneal or thoracic cavity spread. A score of III was given if the mouse had a primary mass with too numerous to count mesenteric lymph nodes along with plaques visible on the diaphragm, the presence or absence of gross liver nodules, minimal to moderate degree of ascites, and no thoracic cavity spread. Finally, a score of IV was assigned if the mouse had a primary mass with too numerous to count mesenteric lymph nodes, the presence of gross liver nodules, and a moderate to severe degree of ascites. Weights of the primary pancreatic tumor with attached spleen, liver, and lungs for each mouse were obtained.

RESULTS

Effects of Eph Receptor Antagonists on Angiogenesis in the Aortic Ring Assay. To evaluate the role of Eph receptors in angiogenesis, we performed experiments in the ex vivo rat aortic ring explant model. This system allows for quantitative assessment of effects on microvessel growth, vessel maturation, and remodeling, including interactions with periendothelial cells (34). The role of Eph signaling was tested by using Eph/Fc chimeric molecules consisting of the extracellular domain of the receptor fused to the Fc part of IgG. Such chimeric molecules, by binding to their cognate ephrins, would prevent productive interactions between endogenous receptors and ligands. Because binding of Eph receptors to ephrins is generally promiscuous within a class, EphA/Fc chimeras should block all ephrin A present and thus inhibit signaling from all EphA receptors, but not from EphB receptors. The opposite applies to EphB/Fc fusion molecules. Such fusion molecules have been shown to inhibit tyrosine phosphorylation of EphB1 and EphB2 receptors and to block capillary tube formation of human renal microvascular endothelial cells (35). It should be noted that Eph/Fc fusion molecules might increase signaling downstream of ephrins. Initially, effects of EphA2/Fc fusion molecules were evaluated. In two independent experiments, a strong, dose-related inhibition of microvessel growth by EphA2/Fc was observed. In the representative experiment shown in Fig. 1, EphA2/Fc demonstrated a 41%, 62%, 71%, and 76% inhibition of microvessel growth at 500, 1000, 2500, and 5000 ng/ml, respectively, relative to controls. Inhibition was apparent from the early stages of microvessel formation and persisted throughout the experiment (Fig. 1, right panel). No cytotoxicity was observed with EphA2/Fc treatment. These results strongly implicate EphA2 signaling in microvessel formation. We also evaluated the effects of EphB/Fc chimeric molecules in this assay. In these experiments, we used an equimolar mix of EphB1/Fc and EphB3/Fc. We tested effects of various concentrations (200–5000 ng/ml) of EphB/Fc molecules on vascular sprouting and compared them with untreated or IgG-treated aortic rings. We found (Fig. 2) that exposure of aortic rings to high concentrations (~2500 ng/ml) of EphB/Fc fusion molecules significantly induced microvessel sprouting, both in terms of increased number and length of sprouts. Morphologically, sprouts were very long and heavily branched and clearly different from controls. The EphB/Fc fusion protein-induced angiogenesis is likely to be mediated by increased ephrinB signaling. Genetic studies have demonstrated that ephrin B2-mediated signaling is necessary for the formation of the vascular system (35).

The differential effects of EphA2 and EphB chimeric molecules
indicated specificity of the antiangiogenic response and suggested that signaling from EphA and EphB receptors might have different functions in angiogenesis. We further extended these observations by directly comparing effects of EphA2, EphA4, EphA1, and EphB1/B3 soluble receptors on angiogenesis. As shown in Fig. 3, administration of EphA2/Fc and EphA4/Fc exhibited similar inhibitory activity on microvessel growth; in contrast, EphA1/Fc did not affect angiogenesis in this assay. It is important to note that EphA2 and EphA4 have similar binding specificities for ephrins A, whereas EphA1 was shown to preferentially bind to ephrinA1 (9). The equimolar mix of EphB1/Fc and EphB3/Fc again induced vessel sprouting.

**Effects of Simultaneous Inhibition of EphA2 and VEGF Receptor (VEGFR) 2 Receptors.** A growing body of evidence demonstrates the heterogeneity of tumor angiogenesis, which might depend on a variety of angiogenic factors (2, 3, 37). Recent reports also indicated that tumors have the ability to respond to inhibitors of angiogenesis by compensatory up-regulation of other angiogenic pathways (37–39). In addition, phenotypes of Eph and ephrin knockout animals suggest that Eph receptors are involved in later stages of angiogenesis and in vessel maturation and thus might function at different phases of tumor neovascularization than VEGFRs (6, 23). Consequently, targeting Eph receptors in combination with VEGFRs might provide additive antiangiogenic efficacy. We tested this notion in the aortic ring assay. Microvessel growth was evaluated in aortic rings treated with suboptimal concentrations of a VEGFR2 inhibitor (32), CEP-5214 (10 nM and 20 nM), EphA2/Fc (500 nM/g), and combinations of both. Fig. 4 demonstrates that CEP-5214 alone inhibited vessel growth by 52% and 55% at 10 and 20 nM, respectively, whereas EphA2/Fc showed 30% inhibition. Simultaneous inhibition of both signaling pathways resulted in 90% (for CEP-5214 at 10 nM) and 95% (for CEP-5214 at 20 nM) inhibition, indicating at least additive antiangiogenic effects. These data suggest that VEGFR2 and EphA signaling pathways play nonredundant roles in angiogenesis in the rat aortic ring model and point to potential benefits of a combinatorial administration of various inhibitors of angiogenesis.

**EphA2/Fc Inhibits Neovascularization in Matrigel Plug Assay.** The robust and reproducible inhibition of angiogenesis by EphA2/Fc in the aortic ring assay prompted us to evaluate the effects of inhibiting EphA signaling on neovascularization in vivo in a Matrigel plug assay. This model allows quantitative analysis of antiangiogenic efficacy in vivo. Briefly, athymic mice were injected with Matrigel containing VEGF, bFGF, and PAECs. One day after implantation, mice received s.c. administration of either vehicle (control group) or 25 μg/dose of EphA2/Fc every second day. Animals were sacrificed on day 10, plugs were excised, and angiogenesis was quantified by measuring hemoglobin content of each plug. Gross evaluation of the plugs revealed that numerous large vessels easily identifiable in control plugs were entirely missing in EphA2/Fc-treated animals. The measurements of hemoglobin further confirmed these observations and demonstrated that EphA2/Fc administration resulted in 81% reduction of neovascularization relative to vehicle controls (Fig. 5). Thus, in an in vivo model, inhibition of EphA signaling resulted in a strong suppression of angiogenesis.

**Effects of Administration of EphA2/Fc on Tumor Growth.** Having shown antiangiogenic activity of EphA2/Fc in ex vivo and in vivo models, we next decided to test effects of inhibition of EphA2 signaling on the growth of tumor xenografts.

To select the appropriate tumor model, we initially evaluated expression of Eph receptors and ephrins, which had been implicated in angiogenesis, in various tumor xenograft models used routinely in our laboratory. Specific primers were designed, and expression of ephrinA1, ephrinB1, and ephrinB2 and EphA2, EphB2, EphB3, and EphB4 receptors was analyzed by reverse transcription-PCR in xenografts derived from A375 (melanoma) cells, Colo320 (colon carcinoma) cells, and U87 (glioblastoma) cells. We found that all analyzed genes were expressed in all human xenografts tested (data not shown). To further confirm and extend these data, we analyzed expression of EphA2 receptor and ephrinA1 at the protein level by Western blot in various xenograft models. Fig. 6 demonstrates that all tumors expressed readily detectable levels of ephrinA1. We next evaluated expression of EphA2. As shown in Fig. 6, middle panel, all tumors tested expressed EphA2, with ASPC-1 (human pancreatic carcinoma), A375 (human melanoma), and Calu6 (human lung adenocarcinoma) expressing the highest levels. These data confirm and expand results obtained by reverse transcription-PCR analysis. Overlapping expression of EphA2 receptor and its ligand, ephrinA1, should facilitate extensive interactions resulting in the activation of the receptor. This notion was tested by analyzing tyrosine phosphorylation of EphA2 receptors. Fig. 6, bottom panel, shows that EphA2 receptors were tyrosine phosphorylated in all tested tumors; however, levels of phosphorylation varied widely. We found the highest levels of phospho-
Fig. 4. Effects of simultaneous administration of vascular endothelial growth factor receptor inhibitor and EphA2/Fc on microvessel growth in aortic ring assay. Aortic ring explants were treated with a synthetic vascular endothelial growth factor receptor 2 inhibitor, CEP-5214 (10 and 20 nM), and EphA2 (500 ng/ml), either alone or in combination, as indicated. Values are the mean number of microvessels in treated group (n = 4) compared with the mean number of microvessels (n = 4) in IgG-treated controls. Results (left panel) are presented as percentage of inhibition of microvessel growth relative to controls. Microvessels were counted at day 6, the peak of angiogenesis. Inhibition was apparent from early stages of microvessel growth and persisted throughout the experiment (right panel). Data were analyzed by one-way ANOVA and Student-Newman-Keuls test. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 5. Effects of EphA2/Fc soluble receptors on neovascularization in vivo. Effects of EphA2/Fc administration on neovascularization in vivo were evaluated in the porcine aortic endothelial cell (PAEC)-vascular endothelial growth factor/basic fibroblast growth factor-Matrigel implant model. Nude mice received injection with Matrigel containing PAECs, vascular endothelial growth factor, and basic fibroblast growth factor. One day after implantation, mice were given either saline (controls) or EphA2/Fc soluble receptor (25 μg/dose) s.c., every second day, over a 10-day dosing regimen. Matrigel implants were excised, and the neovascularization was quantified by measuring the hemoglobin relative to controls. Microvessels were counted at day 6, the peak of angiogenesis. Inhibition was apparent from early stages of microvessel growth and persisted throughout the experiment (right panel). Data were analyzed by one-way ANOVA and Student-Newman-Keuls test. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 6. EphrinA1 and EphA2 receptor expression in various tumor xenograft models. EphrinA1 and EphA2 receptor expression and activation were analyzed by Western blot and immunoprecipitation. Extracts were prepared from A375, Colo320, Calu-6, ASPC-1, U87MG, and SVR tumor xenografts. In addition, extracts from murine lungs and NIH3T3 cells were tested. EphrinA1 (top panel) and EphA2 receptor (middle panel) expression was evaluated by Western blot using specific antibodies. EphA2 receptor activation was analyzed by immunoprecipitation with EphA2-specific antibody followed by Western blot with phosphotyrosine-specific 4G10 antibody (bottom panel). For ASPC-1, signal is shown from a shorter exposure time than that used for other samples. N/D, not determined.
Statistical analyses of the data were done using the Mann-Whitney rank-sum test.

Volumes were normalized to individual tumor volumes at the beginning of the dosing. Relative tumor volumes were injected peritumorally at 30 μg/dose with IgG or EphA2/Fc, respectively, three times a week for 26 days. Tumor volumes were measured every 3–4 days. Relative tumor volumes were normalized to individual tumor volumes at the beginning of the dosing. Statistical analyses of the data were done using the Mann-Whitney rank-sum test.

Fig. 7. EphA2/Fc soluble receptors inhibit growth of ASPC-1 human pancreatic carcinoma xenografts. ASPC-1 human pancreatic carcinoma cells were injected s.c. into female athymic mice. After tumors reached a volume of ~200 mm³, animals were randomized into control (n = 7) and treated (n = 7) groups. Control and treated groups were injected peritumorally at 30 μg/dose with IgG or EphA2/Fc, respectively, three times a week for 26 days. Tumor volumes were measured every 3–4 days. Relative tumor volumes were normalized to individual tumor volumes at the beginning of the dosing. Statistical analyses of the data were done using the Mann-Whitney rank-sum test.

showed more widespread expression of PCNA, often extending toward the center of a tumor. We do not know whether the observed suppression of cell proliferation is due to decreased angiogenesis or other mechanisms.

EphA2/Fc Administration Inhibits Primary Tumor Growth and Metastatic Burden in an Orthotopic Model of Human PDAC.

To evaluate antitumor efficacy of inhibition of EphA2 signaling on local and distant metastatic tumor growth, a more clinically relevant orthotopic tumor model was selected. We decided to use a pancreatic model to confirm and extend results obtained in the s.c. ASPC-1 pancreatic xenograft model (Fig. 7). Using Western blot analysis, we demonstrated high expression and phosphorylation levels of EphA2 receptors in the PDAC model, both in primary tumors and in liver and lymph node metastases (data not shown). Human PDAC xenograft tissue, which had been serially passaged in nude mice to select for an aggressive phenotype characterized by peritoneal, lymphatic, and hepatic metastases, was surgically implanted onto pancreas of nude mice. Seven days after implantation, animals randomized into control group (n = 5) and EphA2/Fc-treated group (n = 5) received i.p. injection, 3 times a week, with IgG or EphA2/Fc, respectively. As shown in Table 1, administration of EphA2/Fc significantly reduced growth of primary tumors and metastatic burden of animals. The median weight of primary tumors was reduced by about 30% after EphA2/Fc administration. Furthermore, the incidence of mesenteric lymph nodes metastases was significantly reduced with EphA2/Fc treatment: only two animals developed metastases to lymph nodes, including one that had <10 mesenteric nodules. This was in strong contrast to the control group, in which all animals developed a high number (too numerous to count) of metastatic mesenteric lymph nodes. The presence of liver and lymphatic metastases correlated with occurrence of ascites within the peritoneal cavity and was strongly reduced in EphA2/Fc-treated animals. Administration of EphA2/Fc resulted in pronounced reduction of metastatic burden, compared with control animals, which was reflected in four of five animals having the lowest metastatic score I.

As a group, these animals exhibited no or minimal metastatic spread. One animal in the EphA2/Fc-treated group developed widespread metastases and obtained the highest score (score IV), similar to animals in the control group (four of five animals obtained score IV, and one obtained score III). These data indicate that suppression of EphA2 signaling had a strong inhibitory effect on tumor growth and metastasis.

Effects of EphA2/Fc Soluble Receptors on ASPC-1 Cells in Culture.

We have shown previously that inhibiting EphA signaling by EphA2/Fc soluble receptors resulted in a strong, dose-dependent inhibition of angiogenesis in aortic ring assays and in vivo in a Matrigel plug assay. Furthermore, administration of EphA2/Fc soluble receptors suppressed growth of tumors in the ASPC-1 tumor xenograft model and in the orthotopic PDAC model. It has been anticipated that soluble receptors repressed EphA signaling by blocking interactions between endogenous ephrins and EphA receptors. To test whether EphA2/Fc indeed inhibited EphA2 activation, tyrosine phosphorylation of EphA2 receptors was analyzed after incubation of ASPC-1 cells with increasing concentrations of the soluble receptor. ASPC-1 cells were plated at about 60% confluence to allow direct cell-cell contacts, a prerequisite for receptor activation. After attach-
Table 1 EphA2/Fc soluble receptors inhibit growth of primary tumors and metastatic burden in orthotopic model of human ductal pancreatic adenocarcinoma

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* Metastatic score: I, primary mass, 0–10 mesenteric lymph nodes, no other visible spread; II, primary mass, 10–100 mesenteric lymph nodes, no other visible spread; III, primary mass, too numerous to count mesenteric lymph nodes, plaques on diaphragm, ± liver nodules, minimal to moderate ascites; IV, primary mass, too numerous to count mesenteric lymph nodes, + liver spread, moderate to severe ascites.

ment of cells, increasing concentrations of EphA2/Fc (1 and 3 μg/ml) were added. After a 24-h incubation, cell extracts were prepared, and EphA2 activation was assessed. Fig. 9, top panel, demonstrates that exposure to the soluble receptors resulted in a dose-dependent inhibition of EphA2 receptor phosphorylation and, presumably, of its activity.

It has been reported previously (28) that EphA2 receptors can interact directly with the FAK, one of the major regulators of integrin activity. Activation of EphA2 receptors led to a rapid inactivation of FAK and, consequently, to an inhibition of integrin activity and increased cell motility (28). Thus, interfering with EphA2 activity should have opposite effects. To test this notion, we evaluated FAK phosphorylation in ASPC-1 cells treated with EphA2/Fc chimeric molecules. Fig. 9, middle panel, demonstrates that repressing EphA2 activation resulted in a dose-dependent increase of FAK phosphorylation. Increased FAK activity can lead to decreased cell motility and invasiveness, key events in tumor growth and metastasis. These data indicate that antitumor efficacy of EphA2 antagonists can be mediated by angiogenesis-dependent and -independent mechanisms.

The widespread overexpression of EphA2 receptors in tumors suggests that EphA2 signaling might represent a survival pathway or confer a growth advantage to tumor cells. To test this notion, ASPC-1 cells were exposed to increasing concentrations (0.3, 1, and 3 μg/ml) of EphA2/Fc. After 48 h, cells were counted and compared with untreated controls. No significant reduction in cell growth rate was observed (data not shown). It should be noted that incubation of the cells with EphA2 soluble receptors, in addition to repressing EphA signaling, should induce ephrin-mediated reverse signaling. Effects of the prolonged stimulation of ephrin signaling on the cell growth rate remain to be investigated.

**DISCUSSION**

Development of functional vascular networks requires high a degree of coordination between various cell types involved and is mediated by several receptor tyrosine kinases acting at different stages of angiogenesis. The VEGFR2 and Tie2 receptors have long been recognized as key players in this process. However, a vast biochemical and genetic evidence has implicated Eph receptors as another critical regulator of angiogenesis. Although there is ample evidence demonstrating the critical role of Eph-ephrin signaling in vascular morphogenesis, there is only limited information concerning its role in tumor neovascularization, despite the fact that various Eph receptors have been found to be overexpressed in vasculature as well as in tumor cells in a wide spectrum of malignancies. Based on the existing data, it could be reasonably anticipated that inhibition of Eph signaling would impair angiogenesis and possibly tumor growth. Indeed, the recent article by Brantley et al. (22) provided the first functional evidence of the critical role of EphA signaling in tumor neovascularization. In this report, we have shown that interfering with EphA2 receptor signaling resulted in a pronounced inhibition of angiogenesis in ex vivo and in vivo model systems and strongly inhibited growth of ASPC-1 pancreatic tumor xenografts as well as growth of primary tumors and metastatic burden in the PDAC orthotopic model. To antagonize EphA signaling, we used a soluble EphA2/Fc receptor consisting of an extracellular domain of EphA2 hooked to a Fc region of IgG. Such chimeric molecules should inhibit EphA2 signaling by binding to endogenous ephrins class A and blocking productive interactions between endogenous receptors and ligands. Similar chimeric molecules have previously been used to inhibit Eph signaling (22, 35). It should be emphasized that EphA2/Fc could inhibit signaling from other EphA receptors. Furthermore, by binding to ephrins, EphA2/Fc is expected to activate ephrin-initiated reverse signaling. We selected EphA2 receptor as a primary target because its widespread expression in tumors, in both clinical specimens and tumor xenografts (21, 24–26, 40, 41), suggested a role in tumor progression. The effects of inhibition of EphA signaling were first evaluated in an aortic ring model. Incubation of aortic rings with increasing concentrations of EphA2/Fc resulted in a strong, dose-dependent inhibition of microvessel growth. These data strongly implicated EphA2 signaling in microvessel formation. The specificity of inhibition was confirmed by testing effects of other EphA soluble receptors on microvessel growth (Fig. 3). The specific responses to various EphA soluble receptors indicated that EphA2/Fc-mediated inhibition of angiogenesis is indeed due to interfering with Eph signaling rather than due to nontpecific toxicity. This notion was further supported by our find-
ings that exposure of aortic rings to EphB soluble receptors induced angiogenesis in a rapid and profound manner. The strong induction of microvessel growth by EphB1/Fc and EphB3/Fc chimeras was probably mediated by reverse signaling induced by ephrins class B. The reverse signaling by ephrins B has been implicated repeatedly in angiogenesis. Adams et al. (36) have demonstrated that deletion of the cytoplasmic tail of ephrinB2 caused severe phenotype in mice with major defects in angiogenic remodeling, indicating that signaling through ephrinB2 is required for vascular morphogenesis. Furthermore, these authors (36) reported that a direct activation of ephrinB ligands by EphB3/Fc or EphB4/Fc soluble receptors induced angiogenic sprouting in adrenal-cortex-derived microvascular endothelial cells. Interestingly, EphB1/Fc and EphB2/Fc fusion proteins exhibited no stimulatory effects, suggesting some functional specificity of the response. In contrast, EphB1/Fc induced migration of human renal microvascular endothelial cells and promoted neovascularization in vivo in a mouse corneal micropocket assay (19). The differential effects of EphA and EphB soluble receptors suggested that signaling pathways activated by different classes of Eph receptors might exert different functions in the aortic ring model. The strong and reproducible antiangiogenic effects induced by EphA2/Fc were further confirmed in in vivo experiments in a Matrigel plug assay. This approach has been widely used to quantitatively assess induction or inhibition of neovascularization into biocompatible polymer matrices implanted s.c. (31). Subcutaneous administration of EphA2/Fc around the implant resulted in a strong inhibition of angiogenesis, as evidenced by an 81% reduction of hemoglobin content in plugs of EphA2/Fc-treated animals. These results were comparable with data obtained with VEGFR2 (4) and Tie2 inhibitors in this system, indicating that EphA signaling is an equally critical factor in vascular morphogenesis in vivo. It is noteworthy that in our assay, angiogenesis was driven by VEGF and bFGF, two key angiogenic factors, which were present in Matrigel implants. Thus, the strong antiangiogenic efficacy of EphA2/Fc molecules indicated a critical and nonredundant role for EphA signaling in neovascularization, which could not be compensated by other angiogenic factors. In accordance with our data, Brantley et al. (22) demonstrated that VEGF-induced endothelial cell migration could be blocked by EphA2/Fc soluble receptors and proposed a cooperative model of EphA-mediated tumor angiogenesis in the context of VEGFR2 signaling. This conclusion was further supported by our results in aortic ring experiments, in which simultaneous inhibition of VEGFR2 and EphA signaling pathways resulted in much stronger, at least additive, antiangiogenic efficacy, than achieved by a single agent. These data point to potential clinical benefits of combinatorial treatment aiming at simultaneous inhibition of various angiogenic targets.

Having demonstrated antiangiogenic activity of EphA2/Fc in ex vivo and in vivo models, we next tested the effects of inhibition of EphA2 signaling on tumor growth in a xenograft model. To select the most appropriate model, we analyzed expression of EphA2, ephrinA1, and other Eph receptors and ephrins implicated in angiogenesis in various xenografts. In accordance with previously published reports (21, 22), we found expression of EphA2 receptors and ephrinA1 in all tumors tested, albeit at different levels. The overlapping expression of EphA2 and ephrinA1 resulted in phosphorylation and, presumably, activation of the receptor in all tumor models analyzed; however, levels of phosphorylation varied widely in different tumor types. We noted that levels of phosphorylation of EphA2 did not correlate with expression levels of ephrinA1. Because of the promiscuity of Eph-ephrin interactions, EphA2 receptors could be activated by other class A ephrins, whose expression was not analyzed in xenograft models. Based on the highest expression and phosphorylation levels, ASPC-1 pancreatic carcinoma was selected for tumor studies. We reasoned that high levels of EphA2 activity were indicative of active involvement in tumor growth. Peritumoral injections of EphA2/Fc resulted in a strong inhibition of tumor growth in two independent studies, which reached 47% and 54% at the end of experiments. It should be noted that the dosing of EphA2/Fc chimeric molecules was not optimized. Inhibition of tumor growth was due to strongly reduced numbers of actively proliferating tumor cells in EphA2/Fc-treated animals, as demonstrated by reduced staining with antibody against PCNA. We do not know whether inhibition of tumor growth and suppression of cell proliferation resulted from inhibition of tumor neovascularization or through some other mechanisms. Based on our data demonstrating strong antiangiogenic properties of EphA2/Fc in the aortic ring assay and in vivo in the Matrigel plug assay, we would expect that inhibition of EphA signaling strongly affected tumor angiogenesis and contributed, at least in part, to the suppression of growth of ASPC-1 xenografts. The dose, schedule, and route of administration of EphA2/Fc in xenograft experiments were based on the Matrigel studies, in which EphA2 exhibited strong antiangiogenic activity. This interpretation is in accordance with the recently published data, which demonstrated that EphA2 soluble receptors inhibited tumor angiogenesis in cutaneous window assays and tumor growth in vivo (22). Inhibition of tumor growth correlated with decreased vascular density and tumor cell proliferation (22). However, we cannot rule out that direct antitumor efficacy also contributed to inhibition of tumor growth. Indeed, immunocytochemistry of ASPC-1 xenografts demonstrated that the majority of tumor cells expressed EphA2 receptors. Tumor cell-derived expression of EphA2 has been readily detected in other xenografts and clinical tumors (21, 22, 24).

The antitumor efficacy of EphA2/Fc was not limited to s.c. tumor xenograft models but was also evident in a very aggressive orthotopic model of human PDAC. In this model, suppression of EphA signaling not only inhibited growth of primary tumors but also profoundly reduced the metastatic burden of animals. EphA2/Fc administration strongly inhibited development of peritoneal, lymphatic, and hepatic metastases, typical for this model, with four of five EphA2/Fc-treated animals exhibiting no or minimal metastatic spread. The significant inhibition of metastasis in a clinically relevant model further underscores the antitumor efficacy resulting from inhibition of EphA2 signaling.

Despite the wealth of information linking increased expression of EphA2 and other Eph receptors to cancer, molecular mechanisms underlying the specific role of Eph signaling in tumor formation, progression, and metastasis remain poorly understood. Zelinski et al. (24) have demonstrated that forced expression of EphA2 receptors was sufficient to transform MCF-10 mammary epithelial cells. The transformed cells acquired tumorigenic potential, giving rise to invasive tumors. This study provided direct evidence that EphA2 overexpression can promote tumor formation independently of its angiogenic activity. Recent studies (27, 28) have shown that Eph receptors have the ability to down-regulate integrin activity and thus to promote de-adhesion and cell motility, key events in tumor invasiveness and metastasis. Activation of EphA2 receptors in PC3 prostate cancer cells resulted in inhibition of FAK activity and cell rounding (28). We demonstrated that EphA2/Fc chimeric molecules inhibited EphA2 activation and increased FAK phosphorylation in ASPC-1 cell cultures. Similar analysis was conducted on extracts prepared from ASPC-1 tumors treated with EphA2/Fc chimeric molecules; however, due to the high tumor-to-tumor variability, results were difficult to

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1 B. Ruggeri, unpublished observations.
interact. In addition to regulating cell-extracellular matrix interactions, several studies have linked EphA2 expression to altered cell-cell contacts. Down-regulation of expression of ephrinA1 inhibited growth of HT29 colon carcinoma cells (42), but only when cultured in three-dimensional spheroids, suggesting that EphA2 signaling might play a role in alleviating contact inhibition. Several reports linked expression and functions of EphA2 receptors to E-cadherin (42–44), further implicating this receptor in regulation of adhesive properties of tumor cells. Together, these studies demonstrated that EphA2 signaling in tumor cells could promote tumor progression by increasing cell invasiveness, motility, and metastatic potential. In this context, inhibition of EphA2 signaling in tumor cells by EphA2/Fc soluble receptors could suppress such an aggressive behavior and inhibit tumor growth and metastasis. This kind of angiogenesis-independent mechanism could have contributed to the marked reduction of metastatic spread detected in the PDAC model after administration of EphA2/Fc soluble receptors.

In our studies, we aimed at inhibiting EphA2 signaling because its widespread expression in tumor cells and tumor vasculature suggested a role in tumor progression. However, as discussed previously, EphA2/Fc soluble receptors would inhibit signaling from other Eph class A receptors. Such inhibition could also contribute to angiogenic and antitumor efficacy of EphA2/Fc. It should be noted that EphA2 receptor is the only class A receptor that has been implicated thus far in angiogenesis. The high levels of expression and activation of EphA2 receptors in ASPC-1 xenografts suggest that inhibition of EphA2 signaling by EphA2/Fc was a primary mechanism underlying the angiogenic and antitumor efficacy of the soluble receptors.

In this report, we demonstrated a critical role of EphA2 signaling in angiogenesis and tumor growth. Our proof of concept experiments are in accordance with results of Brantley et al. (22) and provide a strong mechanistic rationale for developing inhibitors of EphA2 signaling for anticancer therapies. Targeting EphA2 and possibly other Eph receptors might provide strong clinical benefits because of their critical role in tumor angiogenesis, tumor invasion, and metastasis.

REFERENCES


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22-24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 3 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = -0.16 and the normal differential is 65 per cent M and 35 per cent L, then

0.65 (+0.27) + 0.35 (-0.16) = +0.12

a figure identical to the observed +0.12 for normal leukocytes.
Antiangiogenic and Antitumor Efficacy of EphA2 Receptor Antagonist

Pawel Dobrzanski, Kathryn Hunter, Susan Jones-Bolin, et al.


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