Combined Therapy with Direct and Indirect Angiogenesis Inhibition Results in Enhanced Antiangiogenic and Antitumor Effects

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ABSTRACT

The multifaceted nature of the angiogenic process in malignant neoplasms suggests that protocols that combine antiangiogenic agents may be more effective than single-agent therapies. However, it is unclear which combination of agents would be most efficacious and will have the highest degree of synergistic activity while maintaining low overall toxicity. Here we investigate the concept of combining a “direct” angiogenesis inhibitor (endostatin) with an “indirect” antiangiogenic compound [SU5416, a vascular endothelial growth factor receptor 2 (VEGFR2) receptor tyrosine kinase (RTK) inhibitor]. These angiogenic agents were more effective in combination than when used alone in vitro (endothelial cell proliferation, survival, migration/invasion, and tube formation tests) and in vivo. The combination of SU5416 and low-dose endostatin further reduced tumor growth versus monotherapy in human prostate (PC3), lung (A459), and glioma (U87) xenograft models, and reduced functional microvessel density, tumor microcirculation, and blood perfusion as detected by intravital microscopy and contrast-enhanced Doppler ultrasound. One plausible explanation for the efficacious combination could be that, whereas SU5416 specifically inhibits vascular endothelial growth factor signaling, low-dose endostatin is able to inhibit a broader spectrum of diverse angiogenic pathways directly in the endothelium. The direct antiangiogenic agent might be able to suppress alternative angiogenic pathways up-regulated by the tumor in response to the indirect, specific pathway inhibition. For future clinical evaluation of the concept, a variety of agents with similar mechanistic properties could be tested.

INTRODUCTION

Without vascularization, solid tumors grow only to 1–2 mm (1). Thus, tumor growth and metastasis are angiogenesis dependent, and microvascular endothelial cells (ECs) recruited by a tumor have become an important second target in cancer therapy (2). As a result, angiogenesis inhibitors have been developed to target vascular ECs and block tumor angiogenesis. Targeting ECs that support tumor growth is particularly promising because these cells are genetically stable and, therefore, less likely to accumulate mutations that would allow them to develop drug resistance (3). A paramount challenge for antiangiogenic therapy is to design combination protocols that can counteract the diverse angiogenic stimuli produced by the tumor and its microenvironment. Studies suggest that the addition of antiangiogenic agents to conventional therapeutic strategies, e.g., chemotherapy (4–7), radiation (8–10), or other tumor-targeting agents (11), will increase clinical efficacy (12–15). For relapsed multiple myeloma, the antiangiogenic agent thalidomide has become an important treatment option (16), and its combination with dexamethasone is now being advocated for newly diagnosed multiple myeloma (17).

Angiogenesis inhibitors have been divided into two classes, namely, “direct” and “indirect” (18). Direct angiogenesis inhibitors such as endostatin target the microvascular ECs and prevent them from responding to various proangiogenic stimuli. In contrast, indirect angiogenesis inhibitors interfere with the proangiogenic communication between the tumor–cell and endothelial–cell compartments. This indirect inhibitory effect can be achieved by (a) inhibiting the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) in the tumor; (b) blocking the activity or neutralizing the proangiogenic proteins in circulation [e.g., using solubilized VEGF receptor 2 (VEGFR2)-Fc fragments]; or (c) blocking the expression or activation of their receptor on ECs (e.g., VEGFR2). Because of the nature of indirect angiogenesis inhibition, tumors are thought to be able to circumvent the inhibitory effect by switching the production of proangiogenic factors (e.g., from VEGF to bFGF). Indeed, recent studies have shown that tumors produce different angiogenic factors over the course of their growth (19). Because there is the potential of tumor “escape” when specific, indirect antiangiogenic agents e.g., anti-VEGF are delivered individually, appropriate combination protocols involving these agents are needed to achieve the greatest benefit (20). We considered that endostatin plus a VEGF receptor antagonist would be a good combination for further study because of the pervasive role of VEGF in tumor growth and the direct action of endostatin. Another rationale for investigating the combination of endostatin and VEGF inhibition is their closely balanced relationship under physiological conditions, which may become imbalanced as the cancer or inflammatory disease progresses (21, 22). Circulating endostatin levels have been shown to be elevated in patients with renal cell carcinoma (23) and hepatocellular carcinoma (24), and to be correlated with circulating VEGF levels. Additionally, effects of exogenous endostatin have been suggested to be mediated, at least in part, by their ability to down-regulate VEGF expression within the tumor (25). Endostatin decreases VEGF-induced vascular leakage and suppresses VEGF-induced retinal neovascularization and retinal detachment (26). Elevated VEGF levels with an imbalance between proangiogenic cytokines and endostatin are associated with human metastatic liver tumors (27). The extent of the imbalance between VEGF and endostatin was a predictive marker for the outcome of vitreous surgery in patients with proliferative diabetic retinopathy (28).

In the present report, we evaluate the concept of combining a direct (endostatin) and an indirect (SU5416, an inhibitor of VEGFR2 signaling) angiogenesis inhibitor on human ECs in vitro and on human xenograft tumors in vivo. We show that the therapeutic efficacy of SU5416 can be enhanced by the addition of a low dose of the endogenous angiogenesis inhibitor endostatin. The combined therapy with SU5416 and endostatin enhanced the antiangiogenic effects in human ECs in vitro and enhanced tumor growth delay of human xenografts (prostate adenocarcinoma PC3, non-small cell lung cancer (NSCLC) A549, and glioblastoma U87). Additionally, tumor angiogenesis was quantified and visualized using a new cranial window model (A549), and blood perfusion was detected by contrast-
enhanced Doppler ultrasound. The data confirm that combined treatment with direct and indirect inhibitors of angiogenesis may result in synergistic antiangiogenic activity, improving the overall antitumor efficacy of these agents.

MATERIALS AND METHODS

Reagents and Cell Culture. Primary isolated human umbilical vein ECs (HUVECs; Promocell, Heidelberg, Germany) and human dermal microvascular ECs (HDMECs; Promocell) were cultured up to passage 9. Cells were maintained in culture at 37°C with 5% CO₂ and 95% humidity in serum-reduced (5% FCS) modified Promocell medium, supplemented with 2 ng/ml VEGF and 4 ng/ml bFGF (Promocell). This combination of growth factors (VEGF and bFGF) optimized growth kinetics. Human adenocarcinoma prostate tumor cells (PC3) and human glioblastoma multiforme cells (U87; Tumorbank DKFZ, Heidelberg, Germany), were cultured in DMEM (10% FCS). Human recombinant VEGF and bFGF proteins were purchased from Promocell.

The angiogenesis inhibitor SU5416 was synthesized at SUGEN Inc., as described previously (29). SU5416 is an ATP-competitive inhibitor of the VEGFR2 receptor tyrosine kinase, with a Ki value of ~160 nM. It also inhibits platelet-derived growth factor receptor β tyrosine kinase, with a Ki value of 320 nM (30, 31). Endostatin treatment was performed with soluble recombinant human (in vitro) and mouse (in vivo) endostatin (Pichia pastoris; Calbiochem, Schwalbach, Germany).

Endothelial and Tumor Cell Proliferation Assay. Proliferation assay was performed as described previously by O’Reilly et al. (32) with minor modifications. Briefly HUVEC and HDMEC passages 6–9 were grown to confluence in modified Promocell medium supplemented with 5% FCS, containing 2 ng/ml VEGF and 4 ng/ml bFGF (endothelial standard condition). PC3 and U87 cells were cultured in DMEM supplemented with 10% FCS (tumor cell standard condition). Cells were harvested by trypsinization at 37°C and neutralized with trypsin-neutralizing solution (TNS). A suspension of 50,000 cells in modified Promocell medium/DMEM was added to 25-cm² flasks (Becton Dickinson, Heidelberg, Germany). The cells were incubated for 24 h under standard conditions, incubated with angiogenesis inhibitors for 1 h in medium with no additional growth factor supplementation and thereafter were incubated for another 72 h in standard condition. Cells were then dispersed in trypsin, were resuspended, and were counted in a Coulter counter.

Clonogenic Assay. ECs (HUVECs, HDMECs) and tumor cells (PC3, U87) were grown under standard conditions. To investigate the effects of endostatin and SU5416 on clonogenic survival, increasing numbers of cells (10² to 5 x 10⁶) were plated in 25 cm² flasks. Cells were incubated with compounds and cultures were returned to the incubator for 14–17 days, after which they were stained with Diff-Quik II solution (Dade Behring, Marburg, Germany), colonies were counted, and the surviving percentage was determined for plating efficiency and clonogenic survival.

Matrigel Invasion Assay. To assess the migration/invasion ability of ECs after treatment with endostatin and SU5416, a Matrigel invasion assay was used. Transwell inserts with an 8-µm pore size were coated with Matrigel (0.78 mg/ml; Becton Dickinson, Heidelberg, Germany). HUVECs and HDMECs were trypsinized, and 200 µl of cell suspension (3 x 10⁶ cells/ml) containing different concentrations of endostatin and SU5416 were added in triplicate transwells. Chemoattractant media (500 µl) was added to the lower wells.

After 12 h of incubation, ECs that had invaded the underside of the membrane were fixed, were stained in thiazine and eosin solution using Diff-Quick II solution (Dade Behring, Marburg, Germany), and were sealed on slides. Migrating cells were counted by microscopy.

EC Morphogenesis Assay: Tube Formation. To examine the ability of HUVECs and HDMECs to produce tubular structures in vitro, 24-well plates were coated with 300 µl of Matrigel (Becton Dickinson, Heidelberg, Germany). This extract of the Engelbreth-Holm-Swarm (EHS) murine sarcoma, which contains basement membrane components, is liquid at 4°C and forms a gel when warmed to 37°C. When plated on Matrigel, HUVECs (48,000 cells/well) undergo differentiation into capillary-like tube structures in modified Promocell medium (10% FCS) supplemented with VEGF (2 ng/ml) and bFGF (4 ng/ml). Angiogenesis inhibitors were added, and, after a 6-h incubation on the Matrigel at 37°C/5% CO₂, the medium was aspirated, and the cells were fixed and stained with Dif-FQuick II reagents (Dade Behring AG). The ECs were assessed in pictures taken with a microscope.

Apoptosis. At various times, up to 72 h after therapy, fluorescence-activated cell sorting analysis (FACS; Becton Dickinson, San Jose, CA) was performed. Cells were fixed in Hank’s solution and 70% ethanol. After centrifugation of the cells by centrifugation and removing the supernatant, the cells were washed in PBS. Cells were again pelleted and the supernatant was discarded. Next, the cells were resuspended in the staining solution of PBS, RNase, and propidium iodide; and fluorescence-activated cell sorting measurement for apoptotic cells was performed.

Animal Studies. The in-house and governmental animal protection committees approved all of the experiments, and the animals were cared for according to the guidelines for laboratory animals established by the German government. For tumor growth experiments with s.c. growing human xenografts, female athymic mice (BALB/c, nu/nu, 8 weeks of age; weight: 20–20 g) were obtained from Charles River Laboratories (Sulzfeld, Germany). The animals were maintained under clean room conditions in sterile rodent microisolator cages (Ventimack, Heidelberg, Germany). Animals received sterile rodent chow and water ad libitum. Human prostate carcinoma cells (PC3) and glioblastoma (U87) cells were injected s.c. into the right hind limb (5 x 10⁶ cells in 100 µl of PBS). Tumors grew for 7–10 days (U87) or 15–20 days (PC3) until established. Animals were randomized for therapy when tumor volume reached 50–75 mm³. Tumor volume was determined three times weekly by direct measurement with calipers and was calculated by the formula Volume (V) = length x width x depth x 0.5. Animals were treated with 7.5 µg (in 100 µl of PBS) recombinant mouse endostatin twice daily (every 12 h) by s.c. injections (0.75 mg/kg/day). In animals with U87 tumors, SU5416 was administered i.p. dissolved in 50 µl of DMSO (25 mg/kg, two times weekly). In animals with PC3 tumors, SU5416 was administered s.c. dissolved in 100 µl of carboxymethylcellulose (CMC)-based vehicle (100 mg/kg, twice weekly). The observations were ended for ethical reasons, when the tumor volumes became large compared with the animal size.

Histology and Immunohistochemistry. Tumors for histological analysis were harvested from three animals at day 10 after the start of antiangiogenic therapy and at the end of observation, were fixed in buffered formalin, and were embedded in paraffin. Tissue slices (5 ± µm) were stained with H&E. General tissue morphology was visualized by H&E. To assess the tumor cellular proliferation (%Ki-67 positivity) immunohistochemical staining was performed using the MIB-1 monoclonal mouse antihuman Ki-67 antigen (Dako, Hamburg, Germany). Sections were counterstained with H&E. Ki-67 staining was quantified by counting the number of positively stained cells of 200–250 nuclei in 10 randomly chosen fields at ×200.

Blood Perfusion Using Intermittent Power Doppler Ultrasound. Intermittent Doppler sonography is a useful method to visualize and quantify functional parameters of tumor vascularization in vivo. It is based on the i.v. bolus injection of an ultrasound contrast agent and the destruction of the contrast microbubbles by the intermittent power Doppler ultrasound. The dynamics of the refilling of these bubbles is a measure for blood perfusion (33). Mice carrying the s.c. growing U87 glioblastoma were mock treated or treated with endostatin, SU5416, or their combination as described above (n = 3, each). Ten days after the start of therapy, mice were examined after the i.v. injection of 100 µl of Levovist (300 mg/ml) using intermittent power Doppler-sonography (Siemens-Acuson, Sequoia 512, fixed 7 MHz linear transducer, Mechanical Index 1.9) as described earlier (34).

Intravenous Microscopy of A549 NSCLC in a Cranial Window Preparation. For intravital microscopy, adult male severe combined immunodeficient (SCID) mice (age, 5–6 weeks; weight, 20–25 g) were obtained from Charles River Laboratories.Chunks (volume 0.5–1 mm³) of solid tumors (A549 NSCLC; Tumorbank DKFZ) from donor mice were implanted into a cranial window preparation in which the calvaria serves as the site for implantation (35). To produce the chunks, 10⁵–10⁷ cells of the A549 cell line were s.c. injected into the hind limbs of severe combined immunodeficient donor mice. For cranial window preparation, 20 mice were anesthetized, and the scalp was shaved, depilated, and disinfected. An ~1-cm² oval surface area of the scalp and the perisutural area below were removed and a hole 2 mm x 1 mm x 0.5 mm was drilled into the calvaria. After implantation of a tumor chunk into this hole, the preparation was sealed with a glass coverslip and bone cement. Within the 1st week after implantation, tumors were investigated every 24 h to determine
the first appearance of newly formed vessels and the onset of perfusion in these vessels. On days 7, 14, 21, and 28 after tumor implantation, the tumor growth i.e., the two-dimensional tumor surface was documented with standardized microscopic digital photography of the cranial window using bright field microscopy at $\times 10$ and was measured with a computer-based analysis program (AnalySIS V3.0; Soft Imaging System, Münster, Germany). Additionally, the functional vessel density ([FVD], the length of perfused microvessels per tumor surface) as a measure for angiogenesis was assessed by intravital fluorescence video microscopy. FITC-labeled dextran (Sigma; $M_r$, 2,000,000; 0.1 ml of a 5% solution in 0.9% NaCl) as a plasma marker was injected into a tail vein. For off-line analysis, a custom-made computer program was used (CapImage; Engineer Office Dr. Zeintl, Heidelberg, Germany). Animals were treated from day 15 on, after randomization into four groups: endostatin ($n = 5$; 0.75 mg/kg/day in twice daily injections s.c. in 100 μl of PBS), SU5416 ($n = 5$; 100 mg/kg/week s.c. in 100 μl of carboxymethylcellulose every other day), combined endostatin with SU5416, and control ($n = 5$; 100 μl of PBS daily s.c.). On day 28 after implantation, experiments were terminated for ethical reasons.

**Statistical Analysis.** Tumor growth experiments were designed to assess the response to treatment by using the reduction in tumor growth. For this purpose, the tumor volume $V$ was normalized to $V_0$ at the onset of treatment (day 0) to compare tumor growth. Statistical evaluation of tumor growth was undertaken by comparing the standardized volumes for each day. In addition, the general response to treatment was assessed on the basis of the time, undertaken by comparing the standardized volumes for each day. In addition, the general response to treatment was assessed on the basis of the time, $T_A$, required to reach four times the initial tumor volume. For multiple comparisons in tumor growth experiments, histological quantitation, and in vitro experiments, we used the Kruskall-Wallis ANOVA for nonparametric variables. For parametric variables, ANOVA was used along with Fisher’s least-significant-difference (LSD) method using the software program Statistica 5.0 (StatSoft, Hamburg, Germany). All of the analyses were two-tailed. Data of intravitreal microscopy were analyzed statistically using the software program SigmaStat for Windows (Version 2.03; SPSS AG, Zurich, Switzerland). Depending on parametric or nonparametric distribution of data, one-way repeated measures ANOVA or Friedman repeated measures ANOVA on ranks was used with Turkey test for all pairwise multiple comparison procedures. A P of 0.05 was considered significant.

**RESULTS**

Endostatin and VEGF Inhibition Have Synergistic Antiproliferative Effects on ECs. EC proliferation is an important step in tumor angiogenesis. We first investigated the effects of endostatin in combination with SU5416 on endothelial and tumor cell proliferation in cultures. Fig. 1, A and B, show dose-response curves, demonstrating that each compound alone significantly inhibited EC proliferation. To determine whether combinations exhibited increased antiproliferative activity, isobolographic analysis was performed. Different combinations of concentrations were added to HUVECs. From the dose-response curves, the concentration at which HUVEC proliferation was inhibited to 60% of control ($IC_{60}$) was calculated. Fig. 1C shows the isobologram generated from these data (36). When compared with the theoretical (diagonal) line representing (zero interaction) additive effectivity, all of the values from the combination treatment fall below the line indicating synergistic activity for this end point.

For additional in vitro combination experiments, an endostatin dose of 100 ng/ml ($IC_{60}$) and a SU5416 dose of 1 μM ($IC_{70}$) were chosen. Using these drug concentrations, the combination of endostatin and SU5416 appeared to have synergistic antiproliferative effects in HUVEC and also in a second human (HDMEC) EC (HUVEC: expected proliferation, 80% × 70% = 56%; measured proliferation, 38%; $P < 0.01$; HDMEC: expected combined inhibition from single effects 72% × 62% = 44%; measured combined inhibition, 28%; $P < 0.01$; Fig. 1D).

The combination using these drug concentration (100 ng/ml endostatin, 1 μM SU5416) also exhibited some weak antiproliferative activity in U87 glioma and PC3 tumor cells ($P < 0.05$), but it was modest and appeared to be only additive (Fig. 1E).

Combined Endostatin and VEGF Inhibition Enhances Reduced Clonogenic Survival in ECs. Clonogenic survival is an important criterion of cell survival in response to antitumor agents because the final cell death may occur only after additional cell divisions. A single application of endostatin or of SU5416 reduced the clonogenic survival of ECs significantly ($P < 0.05$; Fig. 2). The combination of endostatin and SU5416 induced a synergistic inhibition of clonogenic
survival in HUVECs (expected survival fraction, 0.75; measured, 0.32) and HDMECs (expected, 0.68; measured, 0.29). There was also a modest reduction of clonogenic survival in U87 and PC3 tumor cells after treatment with the angiogenesis inhibitors alone, but this was statistically significant only for SU5416. As expected, the combinations were more effective than single agents but, in contrast to the effect on ECs, appeared to be only additive.

Endostatin and SU5416 Enhance Apoptosis in ECs. To investigate potential mechanisms of the antiproliferative effect of endostatin and SU5416, we analyzed the apoptosis fraction of HUVECs and HDMECs. Fig. 3A demonstrates that both endostatin and SU5416 used alone induced apoptosis. The combination of endostatin and SU5416 appeared to be additive for the induction of apoptosis. Qualitatively similar results were obtained with Hoechst staining of HUVEC cytopsins and counting the apoptotic cells under the microscope (data not shown). Fig. 3B demonstrates that neither endostatin nor SU5416 alone induced significant apoptosis in PC3 and U87 cells in vitro. The combination of endostatin and SU5416 weakly induced apoptosis in the tumor cells.

These observations suggest that EC apoptosis can contribute to the potent suppression of EC proliferation and clonogenic survival observed on treatment with endostatin and SU5416.

Endostatin and SU5416 Reduce EC Migration and Invasion. EC migration and invasion are critical for tumor angiogenesis. We examined the invasion of HUVECs and HDMECs through Matrigel-coated transwell inserts (Fig. 4). Endostatin (100 ng/ml) or SU5416 (1 μM) significantly reduced HUVEC and HDMEC migration/invasion. Interestingly, a combination of endostatin and SU5416 showed a more-than-additive reduction of EC invasion (HUVEC: expected, 47% migrated cells, observed, 23% migrated cells; HDMEC: expected, 40%, observed, 21%).

Combined Endostatin and SU5416 Reduce EC Tube Formation. The production of tubular structures is another important step in angiogenesis. We, therefore, investigated the effects of endostatin and SU5416 on HUVEC and HDMEC EC tube formation. As shown in Fig. 5, control ECs, plated on Matrigel and incubated with control medium, aligned to form lumen-like structures and anastomosing tubes with multicentric junctions. EC cultures, treated with either endostatin or SU5416 alone, formed fewer tubes as well as fewer and weaker anastomoses. Endostatin not only quantitatively reduced the number of tubes but also altered the morphology of the cells. Cells treated with endostatin appeared to be contracted and condensed. When both treatments, endostatin and SU5416, were combined, the effects were greater than after each treatment alone, suggesting at least an additive inhibition of tube formation. Few single cells remained...
and were morphologically highly altered, and no capillary-like network could be detected.

**Tumor Growth in Human Prostate and Glioblastoma Tumor Models in Mice.** Next we investigated whether the findings of antiangiogenic activity in vitro translated to in vivo efficacy in s.c. xenografts in nude mice. For tumor growth quantification, we used the human PC3 prostate adenocarcinoma and the human glioblastoma U87 cell lines. After tumor cell injection, tumors were allowed to grow until established (7 days for U87 and 21 days for PC3). Then mice were randomized and were divided into therapy groups. Treatment was designated as day 0. Low-dose endostatin (0.75 mg/kg/day) was administered twice daily (every 12 h) from day 0 to day 14 for both PC3 prostate adenocarcinoma (A) and U87 glioblastoma (B). SU5416 was administered at 25 mg/kg two times weekly during the entire observation period (C) or up to day 15 (U87). Each group contained 8–12 mice, data points, means of tumor volume $V$ normalized to the initial volume $V_0$, SD.

**Materials and Methods.** The day treatment started and were allowed to establish, as described in "Materials and Methods." The day treatment started was designated as day 0. Low-dose endostatin (0.75 mg/kg/day) was administered twice daily (every 12 h) from day 0 to day 14 for both PC3 prostate adenocarcinoma (A) and U87 glioblastoma (B). SU5416 was administered at 25 mg/kg two times weekly during the entire observation period (C) or up to day 15 (U87). Each group contained 8–12 mice, data points, means of tumor volume $V$ normalized to the initial volume $V_0$, SD.

**Histological Examination.** Histological examination of PC3 xenografts at day 10 after treatment started showed marked differences in tumors from animals that received endostatin, SU5416, or the combination therapy versus controls (Fig. 7). Extensive tumor necrosis with less mitotic figures were observed in H&E sections after endostatin, SU5416, and the combination therapy. Tumor cellular proliferation (% Ki-67 positivity) was markedly reduced after endostatin, SU5416, and the combination therapy. Quantitative analysis of the tumor cell proliferation (% Ki-67 positivity) showed a significant 1.8-fold reduction after SU5416 ($P < 0.01$) versus endostatin and SU5416, or the combination therapy versus controls (Fig. 7). Extensive tumor necrosis with less mitotic figures were observed in H&E sections after endostatin, SU5416, and the combination therapy. Tumor cellular proliferation (% Ki-67 positivity) was markedly reduced after endostatin, SU5416, and the combination therapy.

**Table 1 Response of PC3 and U87 xenograft tumors growing s.c. after treatment with endostatin, SU5416, and the combination endostatin + SU5416**

<table>
<thead>
<tr>
<th>Tumor/Treatment</th>
<th>Days required for tumors to grow four times the original volume ($T_4$) ± SE</th>
<th>Growth delay (days)</th>
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<tr>
<td>PC3 control</td>
<td>15.5 ± 1.5</td>
<td>4.6</td>
</tr>
<tr>
<td>PC3 SU5416</td>
<td>20.1 ± 1.8$^a$</td>
<td>7.8</td>
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<tr>
<td>PC3 Endostatin</td>
<td>23.3 ± 2$^a$</td>
<td></td>
</tr>
<tr>
<td>PC3 SU5416 + Endostatin</td>
<td>37.6 ± 2$^{a,b}$</td>
<td>22.1</td>
</tr>
<tr>
<td>U87 control</td>
<td>3.2 ± 0.8</td>
<td>3.5</td>
</tr>
<tr>
<td>U87 SU5416</td>
<td>6.7 ± 1.1$^a$</td>
<td>7.1</td>
</tr>
<tr>
<td>U87 Endostatin</td>
<td>10.3 ± 1.3$^a$</td>
<td></td>
</tr>
<tr>
<td>U87 SU5416 + Endostatin</td>
<td>14.8 ± 1.3$^{a,b}$</td>
<td>11.6</td>
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$^a P < 0.01$ versus control.

$^b P < 0.05$ versus each monotherapy.
significantly higher than that induced by each therapy alone ($P < 0.05$ for each comparison against a single therapy). Qualitatively similar results were observed for U87 tumors.

Thus, for both PC3 and U87, the combination therapy endostatin and SU5416 was significantly more effective than each therapy alone, which suggested at least an additive tumor growth-inhibitory capacity for the combination.

**Tumor Blood Perfusion.** To analyze tumor blood perfusion, we used intermittent-power Doppler ultrasound as a noninvasive method to determine blood flow in the s.c. growing U87 tumors. Ten days after the start of antiangiogenic therapy, a total of 12 animals were examined immediately after i.v. injection of $100 \mu l$ of Levovist 300 ultrasound contrast agent. An example of the ultrasonographic visualization of tumor blood flow is demonstrated in Fig. 8. A reduction of blood perfusion was observed after treatment with either endostatin or SU5416. The combination treatment of endostatin and SU5416 resulted in a further reduction in tumor blood perfusion.

**Intravital Microscopy of A549 Lung Carcinoma Cranial Window Model.** Intravital microscopy in conjunction with the cranial window system can be used as an experimental in vivo model to

<table>
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<tr>
<th></th>
<th>control</th>
<th>SU5416</th>
<th>Endostatin</th>
<th>End.+SU5416</th>
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<tr>
<td>H&amp;E ×400</td>
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<td>Ki-67 ×400</td>
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*Fig. 7. Histological examination of PC3 tumor xenografts (day 10 after therapy started with endostatin, SU5416, or combined treatment) growing s.c. in balb c nu/nu mice. Marked tumor necrosis in H&E staining after SU5416 and endostatin treatment and in the combination treatment group (End.+SU5416; ×400). Immunostaining of Ki-67 antigen (dark brown reaction product) at ×200 and ×400 shows a reduction of proliferating tumor cells after SU5416 and endostatin. The combination treatment further reduces the tumor cell proliferation versus each therapy alone.*

*Fig. 8. Visualization of tumor blood perfusion by noninvasive intermittent-power Doppler-sonography in U87 human glioblastoma growing s.c. at the hind limbs of balb c nu/nu mice. Representative Doppler ultrasound pictures are taken at maximal signal intensity after i.v. bolus injection of $100 \mu l$ of ultrasound contrast agent (12 s after injection). Animals were examined 10 days after therapy started, as in Fig. 6. A, control; B, SU5416; C, endostatin; D, SU5416+endostatin.*
characterize tumor angiogenesis, microcirculation, and tumor growth with high temporal and spatial resolution. This model was initiated by implanting chunks of A549 NSCLC into holes in the calvaria (Fig. 9). In the first week after implantation, tumors were examined every 24 h by bright field microscopy to observe the newly formed vessels and the onset of perfusion in these vessels. On days 7, 14, 21, and 28 after tumor implantation, functional microvessel density was additionally analyzed by intravital fluorescence using FITC-labeled dextran. The onset of perfusion in newly formed vessels occurred within 24 h after the first appearance of the new vessels. After 7 days, all of the tumors were established and had functional microcirculation. Animals were then randomized into four groups: control, endostatin, SU5416, and the combination of endostatin and SU5416. Tumor growth analysis showed a typical growth for solid tumors in the two-dimensional tumor diameter up to day 14, the day that treatment started. From that time on, all three of the treatment groups showed inhibited tumor growth reaching ~50% of the control group. Starting at 1.0 ± 0.2 mm² on the day of implantation, the tumors reached a final two-dimensional tumor surface by day 28 of 4.3 ± 2.8 mm² (control), 2.1 ± 0.9 mm² (SU5416), 2.5 ± 0.7 mm² (endostatin), and 2.4 ± 1.4 mm² (SU5416 and endostatin). The differences between the treatment groups and the control did not reach statistical significance (P > 0.05 for each comparison). Only if the three treatment groups were pooled and considered as one treatment group was the tumor growth reduction statistically significant (P < 0.05). In contrast, the analysis of FVD as a measure of angiogenesis revealed highly statistically significant differences (P < 0.001) over the time of investigation within all of the groups, and between groups at the same time point of investigation. The data of FVD are presented in detail in Table 2. Intravital fluorescence microscopy showed that, in all of the treatment groups, FVD decreased significantly with the beginning of treatment, compared with the controls. This effect was most distinct in the group treated with the combination of SU5416 and endostatin. Endostatin and SU5416, as well as the combination treatment, were able to significantly decrease FVD versus control and versus days 7 within the same experimental group. The strongest antiangiogenic response was observed in the group treated with the combination of SU5416 and endostatin: Here on days 21 and 28 after tumor implantation, the decrease in FVD was significant not only versus control but also versus SU5416 and endostatin alone. Thus, the combination of endostatin and SU5416 demonstrated strong antiangiogenic activity and reduced microcirculation and tumor growth in the cranial tumor window model.

DISCUSSION

For antiangiogenic therapy to be successful, it must inhibit diverse angiogenic stimuli produced by the tumor and its microenvironment while limiting overall toxicity. Thus, it has been suggested that combination antiangiogenesis protocols may be more effective than monotherapies. To date, it is unclear which agent combinations would be most efficacious and have the highest degree of synergistic activity.

Here we demonstrate that this bimodal therapy of direct and indirect angiogenesis inhibition resulted in marked enhancement of anti-

characterize tumor angiogenesis, microcirculation, and tumor growth with high temporal and spatial resolution. This model was initiated by implanting chunks of A549 NSCLC into holes in the calvaria (Fig. 9). In the first week after implantation, tumors were examined every 24 h by bright field microscopy to observe the newly formed vessels and the onset of perfusion in these vessels. On days 7, 14, 21, and 28 after tumor implantation, functional microvessel density was additionally analyzed by intravital fluorescence using FITC-labeled dextran. The onset of perfusion in newly formed vessels occurred within 24 h after the first appearance of the new vessels. After 7 days, all of the tumors were established and had functional microcirculation. Animals were then randomized into four groups: control, endostatin, SU5416, and the combination of endostatin and SU5416. Tumor growth analysis showed a typical growth for solid tumors in the two-dimensional tumor diameter up to day 14, the day that treatment started. From that time on, all three of the treatment groups showed inhibited tumor growth reaching ~50% of the control group. Starting at 1.0 ± 0.2 mm² on the day of implantation, the tumors reached a final two-dimensional tumor surface by day 28 of 4.3 ± 2.8 mm² (control), 2.1 ± 0.9 mm² (SU5416), 2.5 ± 0.7 mm² (endostatin), and 2.4 ± 1.4 mm² (SU5416 and endostatin). The differences between the treatment groups and the control did not reach statistical significance (P > 0.05 for each comparison). Only if the three treatment groups were pooled and considered as one treatment group was the tumor growth reduction statistically significant (P < 0.05). In contrast, the analysis of FVD as a measure of angiogenesis revealed highly statistically significant differences (P < 0.001) over the time of investigation within all of the groups, and between groups at the same time point of investigation. The data of FVD are presented in detail in Table 2. Intravital fluorescence microscopy showed that, in all of the treatment groups, FVD decreased significantly with the beginning of treatment, compared with the controls. This effect was most distinct in the group treated with the combination of SU5416 and endostatin. Endostatin and SU5416, as well as the combination treatment, were able to significantly decrease FVD versus control and versus days 7 within the same experimental group. The strongest antiangiogenic response was observed in the group treated with the combination of SU5416 and endostatin: Here on days 21 and 28 after tumor implantation, the decrease in FVD was significant not only versus control but also versus SU5416 and endostatin alone. Thus, the combination of endostatin and SU5416 demonstrated strong antiangiogenic activity and reduced microcirculation and tumor growth in the cranial tumor window model.

DISCUSSION

For antiangiogenic therapy to be successful, it must inhibit diverse angiogenic stimuli produced by the tumor and its microenvironment while limiting overall toxicity. Thus, it has been suggested that combination antiangiogenesis protocols may be more effective than monotherapies. To date, it is unclear which agent combinations would be most efficacious and have the highest degree of synergistic activity.

Here we demonstrate that this bimodal therapy of direct and indirect angiogenesis inhibition resulted in marked enhancement of anti-

Table 2. Analysis of functional vessel density (FVD) in A549 lung carcinoma in the intracranial window preparation (n = 5 animals in each group. Mean ± SD)

<table>
<thead>
<tr>
<th>FVD (mm²/mm²)</th>
<th>FVD (mm²/mm²)</th>
<th>FVD (mm²/mm²)</th>
<th>FVD (mm²/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1</strong>day</td>
<td>Control</td>
<td>SU5416</td>
<td>Endostatin</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>7</td>
<td>49 ± 4</td>
<td>53 ± 5</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>14</td>
<td>110 ± 10*</td>
<td>117 ± 7*</td>
<td>110 ± 8*</td>
</tr>
<tr>
<td>21</td>
<td>115 ± 9§</td>
<td>86 ± 5±§</td>
<td>97 ± 9±§</td>
</tr>
<tr>
<td>28</td>
<td>116 ± 4*$</td>
<td>84 ± 6±$</td>
<td>93 ± 9±$</td>
</tr>
</tbody>
</table>

| 1 | day after tumor implantation  
| 2 | $P < 0.05$ vs. control within the same time of investigation  
| 3 | $P < 0.05$ vs. SU5416 within the same time of investigation  
| 4 | $P < 0.05$ vs. endostatin within the same time of investigation  
| 5 | $P < 0.05$ vs. day 7 after implantation within the same experimental group  
| 6 | $P < 0.05$ vs. day 14 after implantation within the same experimental group  

*P < 0.05 vs. control within the same time of investigation  
$P < 0.05$ vs. SU5416 within the same time of investigation  
§P < 0.05 vs. endostatin within the same time of investigation  
$P < 0.05$ vs. day 7 after implantation within the same experimental group  
$P < 0.05$ vs. day 14 after implantation within the same experimental group  

Fig. 9. Intravital microscopy as an in vivo model to characterize tumor angiogenesis, microcirculation, and tumor growth with high temporal and spatial resolution (see Table 2). A549 human NSCLC xenograft tumors were grown in a cranial window of severe combined immunodeficient mice until day 15, when tumors and microvessels were established. Bright field microscopy shows tumor diameter, and intravital fluorescence microscopy using FITC-labeled dextran revealed functional microvessel density and tumor microcirculation. Therapies with endostatin, SU5416, and their combination started on day 15. In the control group, microvessel density and tumor diameter increased significantly on days 14, 21, and 28 versus day 7. Both endostatin- and SU5416-treated animals have decreased vessel density and tumor growth versus control. The combination of endostatin and SU5416 completely abrogates microvessels and further reduces tumor growth compared with either therapy alone. Top panels, pictures of the cranial window preparation were taken on day 28 after tumor implantation at ×10. Bottom panels, images were processed with PaintShop Pro (version 7.0) by cutting out the tumor field in the perfusion (FITC-dextran) images to elucidate the perfusion in the tumor area. Dotted lines, two-dimensional tumor surface. A, control; B, SU5416; C, endostatin; D, SU5416 + endostatin. Scale bars, 1 mm.
angiogenic effects in vitro and in vivo. In vitro, the combination of endostatin and SU5416, a synthetic, small-molecule, ATP-competitive inhibitor of the VEGFR2 receptor tyrosine kinase, showed higher activity than each therapy alone with respect to EC proliferation, clonogenic survival, migration, tube formation, apoptosis induction, and cell cycle blockade (data not shown). In vivo, in human prostate cancer (PC3), NSCLC (A549), and glioblastoma (U87) xenograft tumor models, the combination therapy resulted in enhanced tumor cellular proliferation reduction, enhanced tumor growth delay, and reductions in tumor cell proliferation, microvasculature, and blood flow.

In the PC3 prostate cancer model, tumors treated with SU5416 in combination with low-dose endostatin [compared with doses applied, e.g., in the study by O’Reilly et al. (32)] were found to be only 4.5-fold of the size of control tumors at 40 and 60 days. A regression was induced in some tumors, which was not achieved with either therapy alone. The U87 glioblastoma model was used as an example of an aggressive tumor that is associated with poor prognosis in the clinic (1-year survival, <10%) and that exhibits high resistance to conventional therapies. The combined therapy of U87 xenografts with SU5416 and endostatin not only resulted in significant tumor growth delay but also in reduced tumor blood perfusion, as shown by contrast-enhanced Doppler ultrasound. In vivo angiogenesis was further investigated through the application of a novel cranial window model in which the microvascular development in NSCLC (A549) could be assessed using intravital microscopy. One aim of this model was to mimic the bone microenvironment as a common site of NSCLC metastasis. In contrast to the traditional cranial window models in which the tumor is directly implanted in the cranium, we implanted the tumor into a hole in the calvaria allowing it to grow in the bone microenvironment. Using this intrasossear tumor window, we observed results similar to those seen in the s.c. tumor xenografts of PC3 and U87. Tumor growth, angiogenesis, and microcirculation were strongly impaired after treatment with SU5416, endostatin, or the combination of the two. The antiangiogenic effects in the combination group were significantly greater than after each monotherapy, as seen by the strong reduction of FVD in the cranial window model of A549 lung carcinoma. Our results in tumor growth inhibition can be explained in part by the assumption that tumor growth inhibition occurs after the impairment of angiogenesis, as described in Jain et al. (37).

Because of the ability of endostatin to suppress angiogenesis under a variety of circumstances [high ambient concentrations of bFGF, VEGF, interleukin-8, or platelet-derived growth factor (20)] and the strength of SU5416 against the important VEGF receptor target in particular, we show that the combination of endostatin and VEGFR inhibition increased the antiproliferative activity in ECs and tumors in vivo. Endostatin as a single agent has shown measurable antiangiogenic activity in its first two Phase I studies (38). With increasing doses of endostatin, generally decreased tumor blood flow and metabolism was measured by positron emission tomography (PET) scans. Further tumor biopsy analysis revealed a significant increase in tumor cell apoptosis and EC apoptosis after 8 weeks of therapy (39). It is thought that in conjunction with other antiangiogenics or chemotherapeutics, the efficacy of endostatin will be improved (40, 41) using a versatile repertoire of activities for endostatin that contribute to its antiangiogenic function (42).

Using genome-wide expression profiling, we recently demonstrated that many key regulators of proangiogenic pathways, including several genes related to VEGF signaling (such as, e.g., Ap1 transcription factors, Thrombin receptors, Stats, and HIF-1α), are directly down-regulated after endostatin treatment in human microvascular ECs in vitro. Thus, endostatin may act cooperatively with the VEGF receptor inhibitor, because of the ability of endostatin to down-regulate VEGF upstream and downstream signaling components on the phosphorylation and transcriptional levels.8

Perhaps more importantly, endostatin may additionally abrogate the angiogenic escape mechanisms in activated endothelium, which could circumvent the blockade of VEGF signaling. Thus, the notable therapeutic enhancement observed may occur for two reasons: (a) the endostatin-induced down-regulation of angiogenic pathways that are turned on by the high-intratumoral- or developmental-stage-specific angiogenic heterogeneity (44); and (b) the ability of endostatin to suppress the alternative angiogenic mechanisms up-regulated by the blockade of VEGF signaling.

Taken together, our results suggest that angiogenic inhibitors that possess presumed distinct modes of action may, in fact, show increased therapeutic effectiveness when these agents are combined. Enhanced antiangiogenic effectiveness has been reported for the combination of endostatin with angiostatin (44, 45) and for endostatin with chemotherapeutic agents (46). In contrast, the dual blockade of VEGFR and epidermal growth factor receptor (EGFR) using small molecules that block receptor tyrosine kinases of the same family did not result in enhanced antitumor activity (11). In fact, only when VEGFR blockade or epidermal growth factor receptor blockade was combined with gemcitabine, were supra-additive therapeutic effects observed.

Our chosen models, lung cancer, prostate cancer, and glioblastoma, are common malignant diseases. Patients suffering from lung cancer or glioblastoma, in general, share a poor prognosis, with the exception of the rare cases of early-stage lung cancer, despite continuing efforts to improve survival rates using combinations of surgery, radiotherapy, and chemotherapy (47, 48). In the case of prostate cancer, effective therapies exist, but the severe side effects of local and systemic tumor therapy rationalize improved therapies.

In summary, the strategy of combining direct and indirect angiogenesis inhibition to achieve a synergistic effect warrants clinical investigation. A variety of agents with mechanistic properties that are similar to the properties of the specific drugs used here could be evaluated in humans.

ACKNOWLEDGMENTS

We thank Drs. P. Peschke and F. Kiessling for suggestions and support with the animal work and Thuy Trinh for excellent in vitro work, as well as Professor M. Wannemacher for support of the entire study.

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Combined Therapy with Direct and Indirect Angiogenesis Inhibition Results in Enhanced Antiangiogenic and Antitumor Effects


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