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Rapid Chemotherapy-Induced Acute Endothelial Progenitor Cell Mobilization: Implications for Antiangiogenic Drugs as Chemosensitizing Agents

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SUMMARY

Several hypotheses have been proposed to explain how antiangiogenic drugs enhance the treatment efficacy of cytotoxic chemotherapy, including impairing the ability of chemotherapy-responsive tumors to regrow after therapy. With respect to the latter, we show that certain chemotherapy drugs, e.g., paclitaxel, can rapidly induce proangiogenic bone marrow-derived circulating endothelial progenitor (CEP) mobilization and subsequent tumor homing, whereas others, e.g., gemcitabine, do not. Acute CEP mobilization was mediated, at least in part, by systemic induction of SDF-1α and could be prevented by various procedures such as treatment with anti-VEGFR2 blocking antibodies or paclitaxel treatment in CEP-deficient *Id* mutant mice, both of which resulted in enhanced antitumor effects mediated by paclitaxel, but not by gemcitabine.

INTRODUCTION

A number of phase III clinical trials involving bevacizumab, the humanized antibody against VEGF, in combination with chemotherapy administered at the maximum tolerated dose (MTD) have shown median overall survival (OS) or progression-free survival (PFS) benefits in metastatic breast, colorectal, and non-small cell lung cancers (Hurwitz et al., 2004; Sandler et al., 2006; Miller et al., 2007). These trials include the use of 5-fluorouracil (5-FU) and irinotecan in first-line colorectal cancer (Hurwitz et al., 2004), paclitaxel in first-line metastatic breast cancer (Miller et al., 2007), and paclitaxel plus carboplatin in the first-line treatment of non-small cell lung cancer (Sandler et al., 2006). Despite these successes, some other phase III trials utilizing bevacizumab coadministered with conventional chemotherapy have failed to show OS or PFS benefits, e.g., when administered with gemcitabine for the treatment of pancreatic cancer (Burris and Rocha-Lima, 2008). Factors such as type of tumor, stage, prior treatment, bevacizumab drug dose, pharmacogenomic status, or the nature of the chemotherapy drug combined with bevacizumab could all be

SIGNIFICANCE

Chemotherapy remains the most commonly employed form of systemic cancer treatment. Although partial or complete shrinkage of tumor mass is frequently induced in chemotherapy-responsive tumors, the survival benefits of such responses can be compromised by rapid regrowth of the drug-treated tumors. Our results illustrate how rapidly activated systemic host processes involving induction of certain cytokines and mobilization of circulating endothelial progenitors (CEPs) from the bone marrow can contribute to recovery of drug-treated tumors and, moreover, how this can be blunted by combination treatment with a VEGF pathway-targeted antiangiogenic drug. The results also implicate the CXCR4/SDF-1α pathway in therapy-induced CEP responses mediated by certain chemotherapy drugs, and hence as a potential target for improving their antitumor effectiveness.

factors in explaining whether or not, and to what extent, clinical benefit is attained. This serves to emphasize how little is known about the mechanism (or mechanisms) of action of bevacizumab, and possibly other antiangiogenic agents, especially when coadministered with chemotherapy.

Several hypotheses to explain how antiangiogenic drugs act as chemosensitizing agents have been proposed. One of them, the vessel normalization hypothesis, is based on the observation that enhanced tumor vessel leakiness produces elevated interstitial fluid pressures in tumors, which can impede the delivery and diffusion of certain anticancer drugs. In addition, abnormal tumor vasculature is associated with reduced blood flow and perfusion, another function impeding chemotherapy delivery, and also causing tumor hypoxia, which can cause resistance to chemotherapy and radiation. Treatment with certain antiangiogenic drugs can transiently reverse these abnormalities and enhance chemotherapy (or radiation therapy), provided that it is administered during the "normalization window" (Jain, 2005; Winkler et al., 2004). An alternative or additional mechanism is related to the property of rapid tumor cell repopulation that can take place between successive MTD chemotherapy treatments. Addition of an antiangiogenic drug treatment during the chemotherapy drug-free break period should slow down tumor regrowth and thus increase the degree and durability of the tumor response (Kerbel, 2006; Hudis, 2005). A third hypothesis, which essentially provides a mechanistic explanation for the second hypothesis, is based on our prior preclinical observations regarding the induction of circulating endothelial progenitor (CEP) mobilization after treatment with a cytotoxic agent. We have demonstrated that lymphoma-bearing NOD/SCID mice treated with intensive 6 day cycles of MTD cyclophosphamide, separated by 2 week breaks, exhibit substantial increases in viability and mobilization of CEPs posttreatment after showing an initial decline during the cycles of therapy, a phenomenon that in some respect mimics the rebound of neutrophil counts after treatment with myeloablative chemotherapy (Bertolini et al., 2003). We suggested that such a mobilization effect in CEP levels may contribute to and facilitate tumor cell repopulation during the subsequent drug-free break that is necessary to allow recovery from the toxic side effects of such therapy (Bertolini et al., 2003). This could occur by intrinsically promoting tumor vasculogenesis/angiogenesis but also by suppressing the ability of chemotherapy to cause a local antiangiogenic effect in tumors by targeting the endothelial cells of the growing angiogenic neovasculature (Kerbel, 2006; Browder et al., 2000).

Chemotherapy-induced CEP mobilization—i.e., increases in CEP levels observed at the end of the first and second cycles of chemotherapy treatment—is observed in patients treated with anthracycline- and/or taxane-based neoadjuvant chemotherapy (Furstenberger et al., 2006). Furthermore, a surprisingly robust elevation in CEP levels has also been observed within hours of treatment with microtubule-inhibiting cytotoxic-like vascular disrupting agents (VDAs) in mice (Shaked et al., 2006). We also found that CEPs and perhaps other bone marrow (proangiogenic) cells mobilized by VDA treatment home to and colonize the remaining viable tumor rim commonly observed after treatment with a VDA. When an antiangiogenic drug, i.e., DC101, a VEGFR2 blocking antibody, was administered 24 hr prior to the VDA, the VDA-induced CEP surge was largely blocked and the residual viable tumor rim was significantly suppressed, which was followed by increased antitumor efficacy (Shaked et al., 2006). In addition, preliminary evidence for the induction of CEPs after VDA treatment has been reported recently in phase I clinical trials using the VDAs ZD6126 or AVE8062 (Beerepoot et al., 2006; Farace et al., 2007) and CA4P (P. Nathan, personal communication). Overall, these findings suggest that CEPs can contribute to some and perhaps even much of the rapid regrowth of tumors after treatment with a VDA.

VDAs have a unique mechanism of action as a result of targeting the abnormal vasculature of tumors, causing massive tumor hypoxia and inducing tumoral necrosis. Such effects could help trigger the acute CEP mobilization and tumor-homing response. We therefore decided to analyze the impact of conventional chemotherapy drugs, which lack such acute and potent vascular disruptive effects, to determine whether such drugs—still the mainstay of systemic therapy for metastatic disease—nevertheless have similar inductive effects on CEP mobilization and tumor homing, hence assisting the ability of tumors to recover from exposure to such agents. We also decided to assess whether different chemotherapeutic drugs have variable abilities to induce CEP mobilization and whether targeted antiangiogenic drugs or other agents can block chemotherapy-induced CEP responses and hence amplify their effectiveness.

RESULTS

Acute Induction in CEP Levels in Peripheral Blood of Mice Treated with Chemotherapy Drugs Administered near or at the MTD

To study the impact of chemotherapy on tumor growth and angiogenesis mediated by bone marrow-derived CEPs, we asked, similar to our previous observation with VDAs, whether chemotherapy administered at the MTD can induce a rapid induction in levels of viable CEPs. To this end, non-tumor-bearing BALB/c mice were treated with a number of different chemotherapy drugs administered near or at the MTD (in doses indicated in Table S1 available online), and blood was drawn from the retro-orbital sinus 4 and 24 hr later. CEP levels were evaluated using flow cytometry as described previously (Shaked et al., 2005a; Bertolini et al., 2003). The results in Figure 1 show that only certain drugs, most notably paclitaxel, 5-FU, and docetaxel, were found to cause acute elevations in viable CEP levels within 24 hr of a single bolus injection, whereas others failed to do so, e.g., gemcitabine, cisplatinum, and doxorubicin.

Administration of Antiangiogenic Drug prior to Chemotherapy-Induced CEP Spike Blocks Rapid Elevation in CEP Levels

For subsequent studies, we focused on experiments using two distinct chemotherapy drugs given at MTD, i.e., 50 mg/kg paclitaxel, which induced rapid and marked elevations in CEP levels when administered to C57BL/6 mice, and 500 mg/kg gemcitabine, which did not. We first monitored levels of CEPs for up to 96 hr after chemotherapy drug injection in order to rule out delayed alterations in CEP levels. To do this, non-tumor-bearing C57BL/6 mice were treated with either paclitaxel or gemcitabine at the indicated MTD. Blood was drawn by retro-orbital sinus at several time points and processed for evaluation of viable

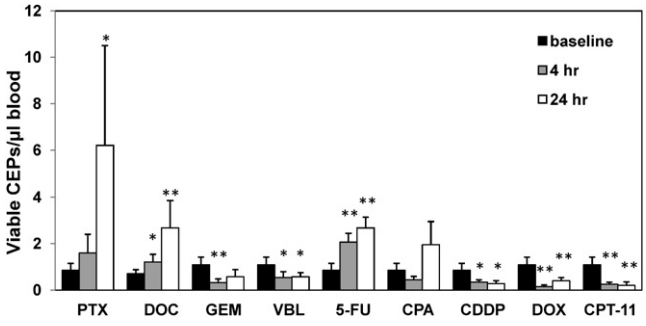

Figure 1. Levels of Viable Circulating Endothelial Progenitors in Non-Tumor-Bearing BALB/c Mice Treated with a Variety of Chemotherapy Drugs near or at the Maximum Tolerated Dose

Eight- to twelve-week-old BALB/c mice (n = 4 or 5 mice per group) were treated with 30 mg/kg paclitaxel (PTX), 40 mg/kg docetaxel (DOC), 120 mg/kg gemcitabine (GEM), 11 mg/kg vinblastine (VBL), 100 mg/kg 5-fluorouracil (5-FU), 250 mg/kg cyclophosphamide (CPA), 6 mg/kg cisplatinum (CDDP), 12 mg/kg doxorubicin (DOX), or 100 mg/kg irinotecan (CPT-11) as indicated in Table S1. Four and twenty-four hours later, mice were bled via retro-orbital sinus for evaluation of viable circulating endothelial progenitors (CEPs) by four-color flow cytometry. Data are expressed as mean ± SD. $0.05 > *p > 0.01$; $**p \leq 0.01$.

CEPs. The results in Figure 2A demonstrate that levels of CEPs in the paclitaxel group increased rapidly within 24 hr and subsequently returned to baseline levels by 48 hr. In contrast, levels of CEPs in the gemcitabine-treated group were maintained at baseline levels for the first 96 hr. Representative flow cytometry plots 4 hr after treatment are presented in Figure S1A. Next we asked whether, similar to VDAs, the administration of DC101, an antiangiogenic anti-mouse VEGFR2 monoclonal antibody (Prewett et al., 1999), 24 hr prior to chemotherapy could block the rapid induction in CEP levels. The results in Figure 2B show that when DC101 was injected 24 hr before chemotherapy, this resulted in a diminished CEP spike in the paclitaxel-treated mice. No significant differences in CEP levels were observed in mice treated with the combination of DC101 and gemcitabine. Similar results were obtained when G6-31, a monoclonal neutralizing antibody against both mouse and human VEGF (Liang et al., 2006), was used in combination with paclitaxel or gemcitabine (Figure S1B).

Rapid Elevation in CEPs after Chemotherapy Treatment Results in Bone Marrow-Derived Cell Colonization of Treated Tumors

A growing body of evidence suggests that a number of different bone marrow-derived cell types promote tumor angiogenesis and growth by various mechanisms. For example, hemangiocytes or recruited bone marrow circulating cells (RBCCs) and Tie2-expressing monocytes (TEMs) have recently been shown to reside at perivascular sites and hence promote angiogenesis in a paracrine manner (Jin et al., 2006; De Palma et al., 2005; Grunewald et al., 2006; Udagawa et al., 2006; Kerbel, 2008). In order to track bone marrow cell homing and retention in treated tumors, experiments were undertaken using GFP^+ bone marrow cells obtained from C57BL/UBI/GFP mice, which were transplanted into lethally irradiated C57BL/6 mice (Shaked et al., 2006). Four weeks later, mice were used as recipients for an injection of Lewis lung carcinoma (LLC) cells. When tumors

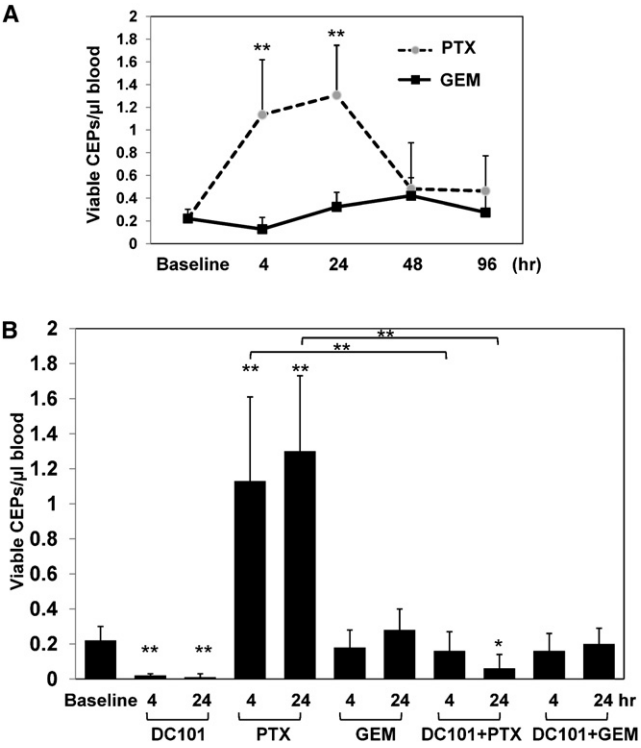

Figure 2. Evaluation of CEPs in Mice Treated with Either Paclitaxel or Gemcitabine in Combination with DC101

(A) Eight- to ten-week-old non-tumor-bearing C57BL/6 mice (n = 4 mice per group) were treated with 50 mg/kg paclitaxel (PTX) or 500 mg/kg gemcitabine (GEM). Blood was drawn from the retro-orbital sinus at time points indicated in the figure and processed for evaluation of viable CEPs using flow cytometry. (B) In a separate experiment, mice were treated with paclitaxel or gemcitabine as described in (A), with or without DC101 administered 24 hr prior to chemotherapy treatment. Blood was drawn via retro-orbital sinus and processed for evaluation of viable CEPs using flow cytometry. $0.05 > *p > 0.01$; $**p \leq 0.01$. Data are expressed as mean ± SD.

reached 500 mm^3, treatment with either bolus-injected MTD paclitaxel or MTD gemcitabine was initiated. Three days later, tumors were removed for evaluation of GFP^+ bone marrow cell colonization and incorporation into the tumor vasculature using both confocal microscopy and flow cytometry techniques, as described in Experimental Procedures. We detected numerous bone marrow-derived GFP^+ cells in tumors that had been treated with paclitaxel, in clear contrast to gemcitabine-treated or untreated control tumors. When DC101 was administered 24 hr prior to chemotherapy, a substantial reduction in the number of GFP^+ bone marrow cells was observed in paclitaxel-treated and untreated tumors. No differences in GFP^+ cell numbers were observed in gemcitabine-treated tumors (Figure 3A; Figure S2A). Of note, the antiangiogenic effect of DC101 on local angiogenesis was insignificant within the first 3 days, as demonstrated previously (Franco et al., 2006).

Next, to further characterize some of the bone marrow cell types colonizing the tumors, tumors from all groups (n = 5 per group) were prepared as single-cell suspensions and subsequently stained for evaluation of bone marrow-derived endothelial cells, TEMs, and hemangiocytes using flow cytometry as described in Experimental Procedures. The results in Figure 3B

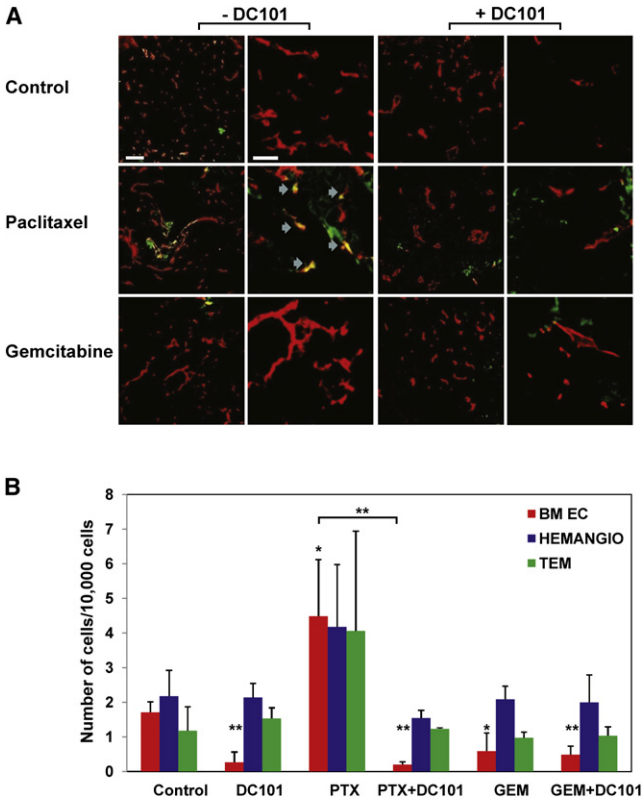

Figure 3. Homing and Colonization of GFP$^+$ Bone Marrow Cells in LLC Tumors after Treatment with Paclitaxel or Gemcitabine in Combination with DC101

C57BL/6 mice (n = 5 mice per group) lethally irradiated and subsequently transplanted with 10^7 GFP$^+$ bone marrow cells obtained from UBI/GFP/C57BL/6 mice were used as recipients for a subcutaneous injection of Lewis lung carcinoma (LLC) cells, which were allowed to grow until they reached 500 mm^3, at which point treatment with paclitaxel (PTX) or gemcitabine (GEM) with or without upfront treatment with DC101 was initiated.

(A) Three days later, tumors were removed and sections were prepared for assessment of GFP$^+$ cell colonization of tumors (green), CD31 staining as an endothelial cell (blood vessel) marker (red), and colocalization of CD31 and GFP$^+$ cells in the paclitaxel-treated group (blue arrows). Left scale bar = 20 μm; right scale bar = 50 μm.

(B) The numbers of bone marrow-derived GFP$^+$ endothelial cells (BM EC), hemangiocytes (HEMANGIO), and Tie2-expressing monocytes (TEM) colonizing tumors were evaluated by flow cytometry using single-cell suspensions prepared from the tumors. Data are expressed as mean ± SD. $0.05 > *p > 0.01$; $**p \leq 0.01$.

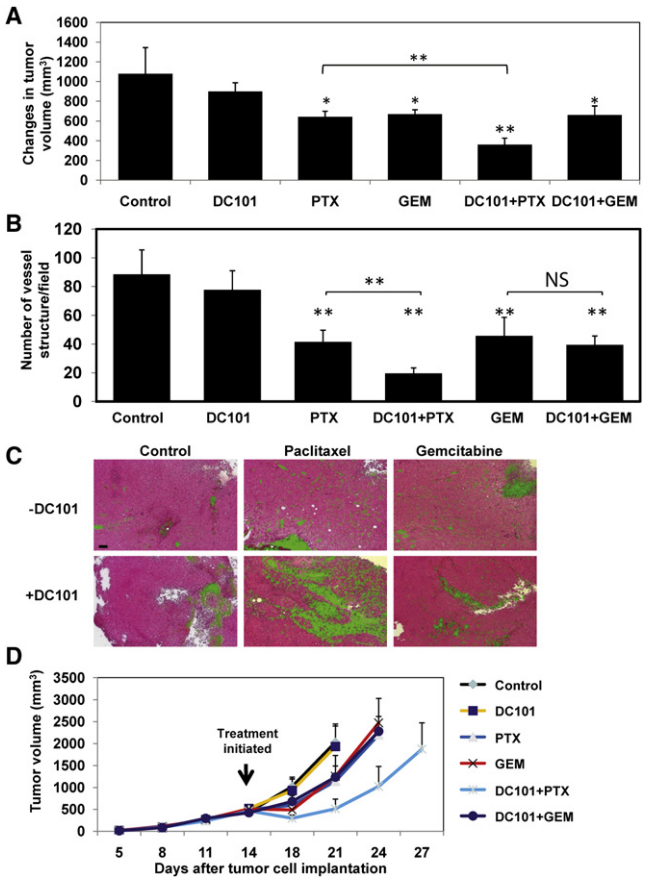

Figure 4. Assessment of LLC Tumor Volume, Microvessel Density, Necrosis, and Long-Term Tumor Growth in Mice Treated with Paclitaxel or Gemcitabine in Combination with DC101

C57BL/6 mice bearing 500 mm^3 LLC tumors (n = 4 or 5 mice per group) were treated with paclitaxel (PTX) or gemcitabine (GEM) with or without DC101 administered 24 hr prior to the chemotherapy drug. Data are expressed as mean ± SD. $0.05 > *p > 0.01$; $**p \leq 0.01$; NS, not significant.

(A) Tumor volumes were assessed before and 3 days after treatment. The change in tumor volume is shown.

(B and C) Three days after treatment, tumors were removed and evaluated for microvessel density after CD31 staining for vessel structure. Data are presented as the number of vessel structures per field (n > 10 fields per tumor) (B) or as necrosis (shown in green) on hematoxylin and eosin staining (C). Scale bar = 100 μm. (See Figure S2B for summary of quantitative data.)

(D) In a separate experiment, LLC tumors implanted in C57BL/6 mice were allowed to reach 500 mm^3, at which point treatment with paclitaxel, gemcitabine (administered at the maximum tolerated dose [MTD]), and/or DC101 was initiated. Tumors were measured regularly using a caliper.

show significant increases in bone marrow-derived endothelial cells as well as increases in hemangiocytes and TEMs (although the latter two did not reach significance) in the paclitaxel-treated group. Administration of DC101 prior to chemotherapy treatment inhibited bone marrow cell colonization of tumors. Overall, these results suggest that paclitaxel treatment induces bone marrow-derived cell mobilization and colonization of tumors and hence may promote tumor cell repopulation and angiogenesis by various mechanisms.

Enhanced Antitumor and Antiangiogenic Activities in Mice Treated with Paclitaxel plus DC101

To further characterize the antitumor and antiangiogenic effects of the combination of paclitaxel plus DC101 in comparison to gemcitabine plus DC101, LLC tumors (n ≥ 5 tumors per group) were evaluated for volume, necrosis, and microvessel density 3 days after either paclitaxel or gemcitabine treatment with or without prior administration of DC101. The results in Figures 4A–4C and Figure S2B demonstrate significant reductions in tumor volume and microvessel density and increases in overall tumor necrosis in tumors treated with the combination of DC101 and paclitaxel in comparison to treatment with paclitaxel alone. In contrast, no significant differences were observed between gemcitabine-treated and DC101 plus gemcitabine-treated tumors. Also noteworthy is the observation that a single dose of

paclitaxel reduced microvessel density, indicating damage to the tumor vasculature, whereas a single injection of DC101 did not cause a drop in microvessel density (Figure 4B).

Next, to explore the long-term antitumor effect of DC101 when administered in combination with paclitaxel or gemcitabine, 5×10^5 LLC cells were implanted subcutaneously in the flanks of C57BL/6 mice. When tumors reached 500 mm^3, a single dose of DC101 was administered, followed by paclitaxel or gemcitabine injection 24 hr later. The results in Figure 4D show that the combination of DC101 and paclitaxel resulted in a substantial antitumor effect manifested by a delayed tumor growth endpoint in comparison to tumors treated with gemcitabine alone, paclitaxel alone, or the combination of DC101 and gemcitabine. Comparable results for long-term enhanced treatment efficacy were obtained for the combination of paclitaxel and DC101 in C57BL/6 mice bearing B16F1 melanomas (Figure S3A). Moreover, we did not observe an enhanced treatment benefit when DC101 was administered prior to doxorubicin (which does not induce a CEP spike; see Figure 1) in C57BL/6 mice bearing LLC (Figure S3B). Overall, these results reinforce our hypothesis that the administration of an antiangiogenic drug immediately prior to a chemotherapy drug that is competent to induce a rapid CEP spike results in enhanced treatment efficacy, whereas little or no enhanced antitumor activity is obtained when it is combined with a chemotherapy drug that does not induce such a CEP spike.

Superior Antitumor and Antiangiogenic Activities in *Id* Mutant Mice Treated with Paclitaxel Compared to Mice Treated with Gemcitabine

To further evaluate whether the treatment efficacy of paclitaxel can be enhanced in the absence of a CEP spike, we tested the antitumor effects of paclitaxel or gemcitabine in $Id1^{+/-}Id3^{-/-}$ (*Id*) mutant mice and compared the treatment effects to those observed in wild-type (WT) controls. *Id* mutant mice cannot mobilize CEPs (Lyden et al., 1999) but are not deficient for other bone marrow-derived proangiogenic cells such as TEMs, tumor-associated macrophages, or tumor-associated neutrophils (Ciarrocchi et al., 2007). Thus, enhanced efficacy of the chemotherapeutic drug can be ascribed directly to the lack of CEP mobilization, as opposed to inhibition of other VEGF-responsive bone marrow-derived cells. For this approach, LLC tumors implanted in mice were allowed to reach 500 mm^3, at which point treatment with either drug was initiated. Of note, a 3 day growth delay in reaching the 500 mm^3 point (20.3 days postimplantation in *Id* mutant mice versus 17.2 days in WT mice) was observed in tumors grown in *Id* mutant mice in comparison to the respective tumors grown in WT mice, in line with previous observations (Lyden et al., 1999; Shaked et al., 2006) (data not shown). Three days after treatment, tumors ($n \geq 5$ tumors per group) were measured and then removed for evaluation of tumor hypoxia, vessel perfusion, microvessel density, cell proliferation, and apoptosis. Consistent with our hypothesis, Id loss in the host animal had no influence on gemcitabine effectiveness, as no significant differences in tumor growth, perfusion, hypoxia, or microvessel density were observed. In contrast, a significant decrease in tumor volume accompanied by an increase in tumor hypoxia and reduction in blood perfusion and microvessel density was observed in tumors grown in *Id* mutant mice treated with paclitaxel compared to WT mice (Figures 5A–5C; Figure S4A–S4C). In addition, we found significant increases in tumor cell apoptosis in paclitaxel-treated tumors grown in *Id* mutant mice in comparison to treated tumors in WT mice. No significant differences in tumor cell apoptosis or proliferation were observed in gemcitabine-treated tumors grown in *Id* mutant versus WT mice (Figure 5D; Figures S4D and S4E). Overall, these results provide further evidence for the tumor growth-enhancing role that acutely mobilized bone marrow-derived cells may play with respect to chemotherapy drugs that induce their mobilization, followed by subsequent homing to tumors. Blocking this chemotherapy-induced host-reactive process resulted in increased treatment efficacy. In previous studies using VDA treatment combined with DC101, these marked short-term tumor-associated differences were found to be predictive of long-term antitumor effects, including prolonged survival (Shaked et al., 2006).

Rapid Induction in SDF-1α Levels May Account for Acute CEP Mobilization after Treatment with Paclitaxel

To further assess the molecular mechanisms responsible for the acute paclitaxel-induced CEP mobilization, plasma samples from non-tumor-bearing C57BL/6 mice (n = 4 mice per group) were obtained 4 hr after treatment with either paclitaxel or gemcitabine administered at the MTD, and circulating VEGF-A, SDF-1α, and G-CSF levels were evaluated, as these are all known to mobilize bone marrow-derived cells including CEPs (Asahara et al., 1999; Jin et al., 2006; Powell et al., 2005). As shown in Figure 6A, both gemcitabine- and paclitaxel-treated mice exhibited significant increases in circulating G-CSF plasma levels and decreases in VEGF-A plasma levels, although not to the same extent. However, levels of SDF-1α were significantly induced only in the paclitaxel-treated mice in comparison to untreated control or gemcitabine-treated mice. No increases in SDF-1α levels were observed in C57BL/6 mice (n = 4 mice per group) 4 hr after they were treated with MTD doxorubicin, which does not induce a CEP spike (Figure S5).

Jin et al. have recently reported that SDF-1α is stored in platelets, and thus hemangiocytes as well as other bone marrow cells expressing CXCR4 may rapidly mobilize from the bone marrow and promote angiogenesis in response to acutely induced SDF-1α secretion from circulating activated platelets (Jin et al., 2006; Avecilla et al., 2004). To test this possibility, platelets isolated from non-tumor-bearing C57BL/6 mice were incubated in vitro for 4 hr with either 5 μM paclitaxel or 50 μM gemcitabine, as previously described (Kroep et al., 1999). Subsequently, platelet cell lysates were generated, and the concentration of SDF-1α content was evaluated by ELISA. No significant differences were observed between any of the groups (data not shown). Next, since Jin et al. (2006) reported that various cytokines may induce release of SDF-1α from platelets, we asked whether paclitaxel might indirectly promote the release of SDF-1α from platelets. To this end, non-tumor-bearing C57BL/6 mice (n = 4 mice per group) were treated with either paclitaxel or gemcitabine administered at the MTD. After 4 hr, mice were bled by cardiac puncture, and platelets were isolated as described in Experimental Procedures. Levels of SDF-1α were evaluated in platelet lysates following normalization of protein content. The results in Figure 6B show that a significant reduction in platelet SDF-1α content was observed only in mice

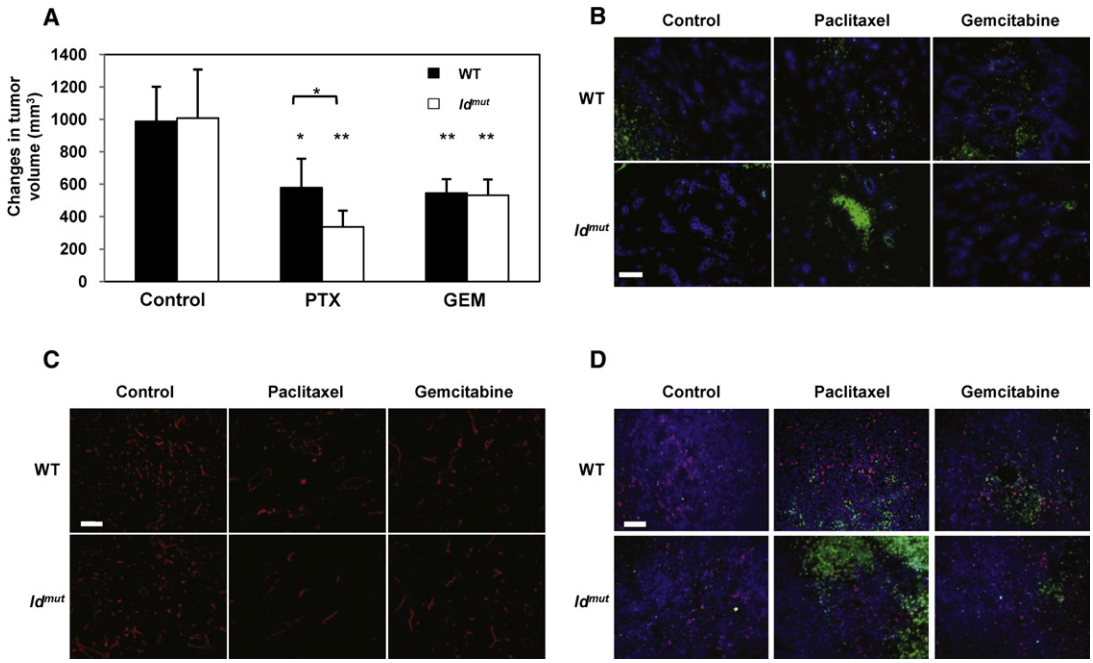

Figure 5. Assessment of LLC Volume, Hypoxia, Perfusion, Microvessel Density, Proliferation, and Apoptosis of Tumors in *Id* Mutant or Wild-Type Mice after Paclitaxel or Gemcitabine Treatment

Id mutant (Id^{mut}) and wild-type (WT) mice bearing 500 mm^3 LLC tumors were treated with paclitaxel (PTX) or gemcitabine (GEM).

(A) Tumor volumes were assessed before and 3 days after treatment. The change in tumor volume is shown. Data are expressed as mean ± SD. $0.05 > *p > 0.01$; $**p \leq 0.01$. (See Figure S4 for summary of quantitative data.)

(B–D) Three days after treatment, tumors were removed and evaluated for vessel perfusion (blue) and hypoxia (green) (B), microvessel density (CD31 staining in red) (C), and proliferation (red) and apoptosis (green) (D). Scale bars = 50 μm.

treated with paclitaxel, as opposed to similar SDF-1α levels in platelets obtained from untreated or gemcitabine-treated mice. Taken together, these results suggest that paclitaxel may induce the CEP spike at least in part by acute release of stored SDF-1α from platelets.

Neutralizing SDF-1α Levels Enhances the Antitumor Activity of Chemotherapy-Induced CEP Spikes

With the aim of assessing whether SDF-1α can account for the rapid CEP mobilization observed after treatment with paclitaxel, non-tumor-bearing C57BL/6 mice were treated with neutralizing anti-SDF-1α antibodies (n = 5 mice per group). Twenty-four hours later, mice were treated with either gemcitabine or paclitaxel, and 4 and 24 hr later, CEP levels were evaluated. The results in Figure 6C show that the SDF-1α neutralizing antibodies substantially blocked induction of CEP levels within 24 hr in paclitaxel-treated mice. No significant differences were observed in mice treated with gemcitabine. To further assess whether blocking SDF-1α might enhance paclitaxel treatment efficacy, mice bearing LLC tumors were treated with polyclonal anti-SDF-1α neutralizing antibodies 24 hr prior to either paclitaxel or gemcitabine treatment. Control mice were treated with nonspecific antisera as described previously (Addison et al., 2000; Phillips et al., 2003). The results in Figure 6D demonstrate that only in mice treated with the combination of SDF-1α neutralizing antibodies and paclitaxel was there evidence of enhanced antitumor efficacy. This enhancement was not observed when the SDF-1α neutralizing antibodies were combined with gemcitabine. Overall, these results suggest that the rapid increase in SDF-1α levels accounts for the acute CEP mobilization after paclitaxel treatment and that, as such, SDF-1α neutralizing antibodies can be used as a de facto antiangiogenic/antivasculogenic-like treatment strategy.

Increases in CEP and SDF-1α Plasma Levels in Patients Treated with Paclitaxel

The preclinical results we obtained previously using VDAs, i.e., rapid elevations in CEP levels within 4 hr after drug administration, have been reproduced in a number of clinical studies (Beerepoot et al., 2006; Farace et al., 2007; P. Nathan, personal communication). Our next aim, therefore, was to evaluate whether there is any indication that our aforementioned preclinical results using chemotherapy can also be observed clinically. To this end, a number of cellular and molecular assays were undertaken using clinical samples from cancer patients treated with chemotherapy at two different centers (the European Institute of Oncology [Milan] and University Medical Center Utrecht [Utrecht, The Netherlands]). Cancer patients were treated with paclitaxel or paclitaxel-based therapy, and the results were compared to patients treated with gemcitabine or with doxorubicin- or cisplatin-based therapies as indicated in detail in Experimental Procedures. The results in Figure 7A show that, similar to our preclinical observations, levels of CEPs significantly and acutely increased from baseline in patients receiving paclitaxel-based therapy. Furthermore, significance was also reached when paclitaxel-based therapy was compared to the

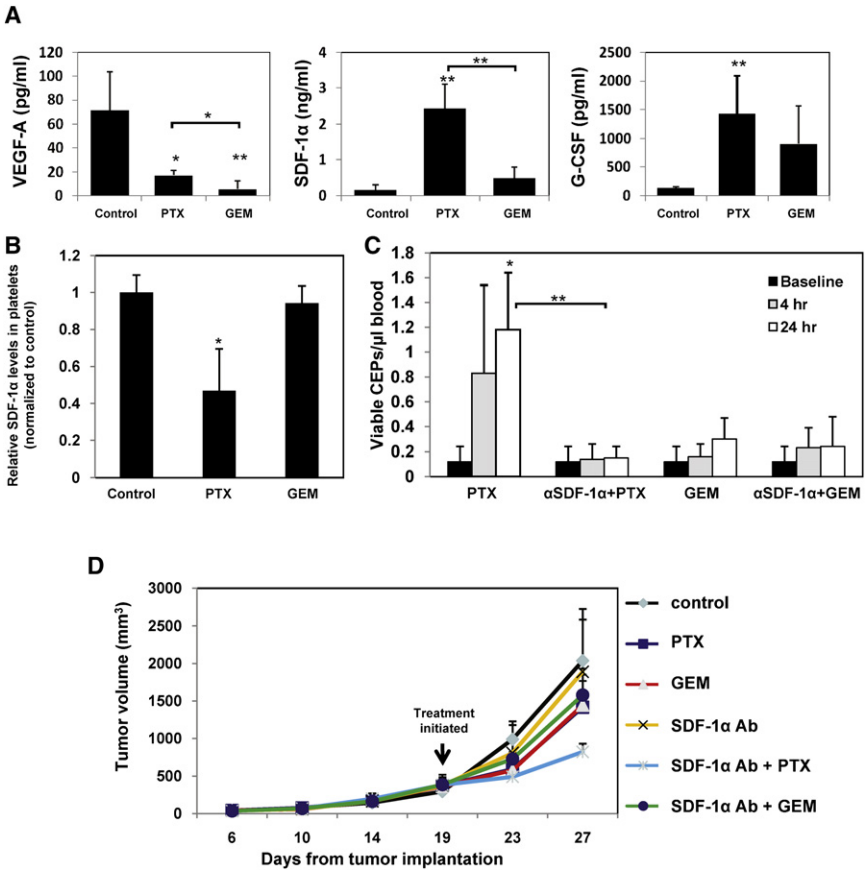

Figure 6. Circulating Levels of Growth Factors after Paclitaxel or Gemcitabine Treatment and Impact of Anti-SDF-1α Antibody on CEPs and Tumor Growth

(A) Non-tumor-bearing C57BL/6 mice (n = 4 mice per group) were treated with paclitaxel (PTX) or gemcitabine (GEM). Four hours later, mice were bled by cardiac puncture and plasma was collected. Levels of murine VEGF-A, SDF-1α, and G-CSF were analyzed by ELISA.

(B) Analysis of SDF-1α content stored in isolated circulating platelets from C57BL/6 mice 4 hr after treatment with paclitaxel or gemcitabine at the MTD.

(C) Non-tumor-bearing C57BL/6 mice (n = 5 mice per group) were treated with SDF-1α neutralizing antibodies. Twenty-four hours later, mice were treated with paclitaxel or gemcitabine. After 4 and 24 hr, mice were bled from the retro-orbital sinus for evaluation of viable CEPs by flow cytometry.

(D) LLC tumors were allowed to grow in C57BL/6 mice until the tumors reached 500 mm^3, at which point the mice were treated with polyclonal SDF-1α neutralizing antibodies in combination with either paclitaxel or gemcitabine. Control mice received nonspecific antiserum treatment. Tumors were measured regularly using a caliper. Data are expressed as mean ± SD. $0.05 > *p > 0.01$; $**p \leq 0.01$.

other treatment groups. In addition, plasma concentrations of SDF-1α, G-CSF, and VEGF were evaluated 4 hr after paclitaxel-based therapy and compared to the plasma concentrations of patients treated with gemcitabine or with doxorubicin- or cisplatinum-based therapies. The results in Figure 7B show that SDF-1α plasma concentrations were rapidly and significantly increased only in patients treated with paclitaxel-based therapy, in contrast to patients treated with the other chemotherapies. A nonsignificant trend was observed in both G-CSF and VEGF plasma concentrations of both groups. Overall, based on the preclinical data and preliminary clinical data obtained, these results indicate that our preclinical mechanistic explanation for the enhanced antitumor activity of bevacizumab when administered in combination with certain chemotherapy drugs may also hold clinically. Future prospective randomized clinical trials will be necessary to confirm this.

DISCUSSION

Our results provide a new perspective regarding the impact that conventional chemotherapy can have on tumor angiogenesis and hence how combination with antiangiogenic drugs may amplify the antitumor effects of chemotherapy. Previously, chemotherapy has been reported to have the potential to cause local tumor antiangiogenic effects by virtue of targeting cycling endothelial cells in sprouting angiogenic blood vessel capillaries within tumors (Browder et al., 2000; Klement et al., 2000; Miller et al., 2001). But at approximately the same time, some chemotherapy drugs administered at MTD can cause a systemic host-mediated counterregulatory response from the bone marrow, comprised at least in part by acute mobilization of CEPs, which subsequently has the potential to stimulate tumor angiogenesis and vasculogenesis. This host response may not only help abrogate the potential local antiangiogenic effect but intrinsically stimulate tumor vasculogenesis/angiogenesis as well, thus acting to limit the duration of tumor responses induced by the cytotoxic chemotherapy drug treatment.

Our results also provide a potential explanation of why not all chemotherapy drugs will necessarily have their efficacy enhanced by the addition of an antiangiogenic agent when the mechanism involves blunting CEP mobilization that is acutely induced by the chemotherapy drug. It should be noted that our experiments were conducted using only a single dose of DC101 prior to chemotherapy, for the purpose of inhibiting CEP mobilization, as we demonstrated previously with VDAs (Shaked et al., 2006). We have not tested the efficacy of repetitive combination treatments, since it has already been demonstrated that DC101 has an antitumor effect due to antiangiogenic mechanisms when administered in such a fashion as a single agent (Prewett et al., 1999). The results may also be pertinent for explaining some of the benefits of other therapeutic approaches that target CEPs. For example, the administration of chemotherapy at close regular intervals using low, nontoxic doses with no prolonged breaks ("metronomic" chemotherapy) (Kerbel and Kamen, 2004) not only avoids acute CEP mobilization but can even target CEPs (Bertolini et al., 2003; Shaked et al., 2005b). It will also be of

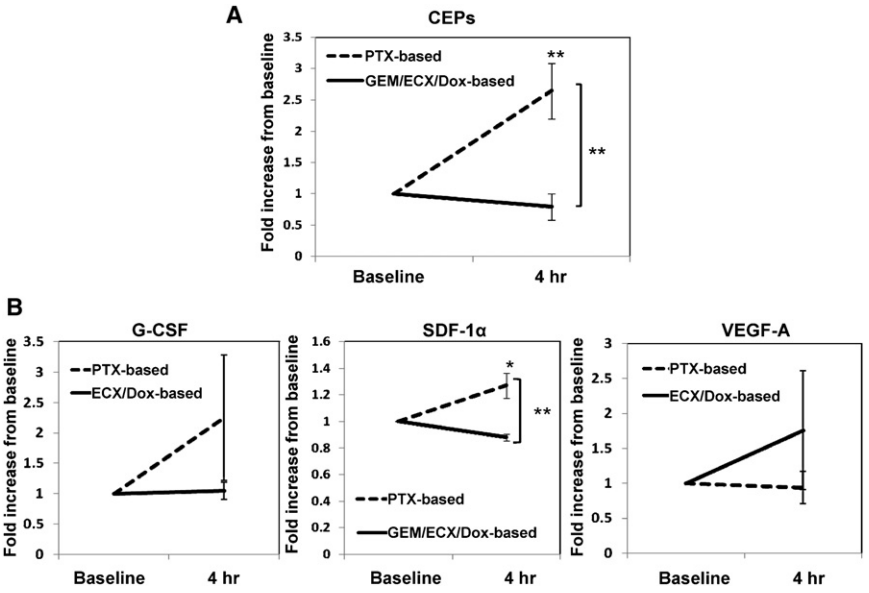

Figure 7. Levels of CEPs and G-CSF, SDF-1α, and VEGF Plasma Concentrations in Cancer Patients Four Hours after Treatment with Various Chemotherapy Drugs Administered at the MTD

Cancer patients (n = 30) were treated with paclitaxel (n = 8) or paclitaxel plus carboplatin (n = 4) (both of which were designated as PTX-based therapy); gemcitabine (GEM) (n = 8); epirubicin, cisplatin, and capecitebin (n = 5) (ECX); or doxorubicin with or without cyclophosphamide (n = 5) (Dox-based therapy). Four hours later, patients were bled intravenously for evaluation of CEPs (n = 12 for PTX-based; n = 18 for GEM/ECX/Dox-based) (A) as well as G-CSF (n = 3 for PTX-based; n = 10 for ECX/Dox-based), SDF-1α (n = 12 for PTX-based; n = 15 for GEM/ECX/Dox-based), and VEGF (n = 3 for PTX-based; n = 10 for ECX/Dox-based) plasma concentrations (B). Results were normalized to the baseline level of each patient to reduce variability due to tumor type, stage, and values obtained from two different centers. Data are expressed as mean ± SEM. $0.05 > *p > 0.01$; $**p \leq 0.01$.

interest to determine whether and to what degree other types of bone-marrow derived proangiogenic cells (Grunewald et al., 2006; De Palma et al., 2005; Udagawa et al., 2006) may be induced (or suppressed) by MTD chemotherapy and thus potentially contribute to tumor recovery after treatment (or response). Notably, some of these populations, e.g., $Gr1^+/CD11b^+$ myeloid cells, may not be suppressed by drugs that target the VEGF-A pathway of angiogenesis (Shojaei et al., 2007). However, our experiments performed in *Id* mutant mice indicate that CEPs play the major role in the systemic response, as these animals are not deficient for other proangiogenic cells (Ciarrocchi et al., 2007).

Our results raise a number of important questions relevant to antiangiogenic drugs and the role of CEPs in tumor angiogenesis. For example, as antiangiogenic small-molecule oral receptor tyrosine kinase inhibitors (RTKIs), which target multiple RTKs including VEGF receptors, have not yet shown an ability to enhance the efficacy of conventional chemotherapy in phase III trials, in contrast to bevacizumab (Kerbel, 2008), could this be due to an inability of such drugs to block CEP mobilization? In this regard, we have recently reported that one such drug, sunitinib, can cause marked elevations in multiple circulating growth factors, cytokines, and chemokines in a dose-dependent and tumor-independent fashion (Ebos et al., 2007). These factors include VEGF, PlGF, G-CSF, SDF-1α, and SCF. Since the receptors for G-CSF and SDF-1α are not affected by sunitinib, and since both G-CSF and SDF-1α are known to mobilize CEPs (Asahara et al., 1999; Jin et al., 2006; Powell et al., 2005), targeting VEGF receptors and c-kit using a drug such as sunitinib may not be sufficient to blunt chemotherapy-induced CEP spikes when they occur. Second, might our results help resolve some of the ongoing controversy regarding the importance of CEPs in tumor angiogenesis? Most studies have shown low (Peters et al., 2005) or even nonexistent (Purhonen et al., 2008) incorporation of CEPs in tumor blood vessels in mouse tumor models (Bertolini et al., 2006); however, as we previously reported for VDAs—which are not yet clinically approved and are being tested only in small numbers of patients—some commonly used chemotherapy drugs such as paclitaxel can also cause a robust mobilization of CEPs that subsequently can home to the drug-treated tumors and incorporate into newly forming vessels. Importantly, such incorporation may be influenced by damage to the tumor (neo)vasculature, thus creating the physiologic need ("signal") for rapid replacement of damaged or destroyed endothelium in the tumor vasculature. Rapid mobilization of CEPs and homing to vessels damaged by adverse cardiovascular events (Urbich and Dimmeler, 2004) could be taken as a model for this host process in the context of cytotoxic drug-induced damage to the tumor vasculature. In this regard, while VDAs are known to cause damage to the tumor vasculature, such a property is less appreciated with respect to chemotherapy. However, there is an expanding literature of chemotherapy-induced damage to endothelial cells in the tumor vasculature (Browder et al., 2000; Klement et al., 2000; Miller et al., 2001), which in some cases can be very rapid (Farace et al., 2007). Indeed our own results, presented here, indicate that MTD chemotherapy can cause rapid drops in tumor microvessel density, even after a single MTD dose as shown in Figure 4B.

Our preclinical results are supported by limited clinical observations testing levels of CEPs and SDF-1α, G-CSF, and VEGF plasma concentrations in cancer patients treated with chemotherapy using paclitaxel. Jin et al. (2006) have recently suggested that elevated levels of SDF-1α induce mobilization of $CXCR4^+$ cells from the bone marrow, perhaps including CEPs, as the majority of them express CXCR4 (Yamaguchi et al., 2003; Athanassakis et al., 2001). Based on our preliminary results, platelets could be one source of the released SDF-1α. The rapid induction of various cytokines may promote platelet activation and hence cause the release of SDF-1α stored in platelets (Jin et al., 2006; Rafii et al., 2008). It has also been suggested that mobilization of activated megakaryocytes from the bone marrow niche can upregulate levels of SDF-1α (Avecilla et al., 2004). Finally, it will be of interest to evaluate the contribution of the mechanism we have proposed here to account for antiangiogenic drug-mediated

enhancement of standard chemotherapy, using drugs such as bevacizumab, relative to other proposed mechanisms. These include transiently induced vessel normalization (Jain, 2005; Winkler et al., 2004) or enhancement of the extent of local damage to the tumor vasculature mediated by chemotherapy, in the clinical setting.

EXPERIMENTAL PROCEDURES

Blood Samples Obtained from Cancer Patients

Blood samples were collected from cancer patients receiving chemotherapy. Sixteen patients with stage IV metastatic breast cancer were treated with either paclitaxel (n = 8) or gemcitabine (n = 8) at the European Institute of Oncology (Milan). The study followed the rules of the European Institute of Oncology ethics committee, and written informed consent was obtained from all patients. In addition, four patients with ovarian cancer were treated with carboplatin and paclitaxel (paclitaxel-based therapy); five patients were treated with either doxorubicin monotherapy or doxorubicin in combination with cyclophosphamide (doxorubicin-based therapy) for malignant sarcoma and breast cancer, respectively; and five patients with esophageal cancer were treated with the combination of epirubicin, cisplatin, and capecitebine (cisplatin-based therapy) at the clinic of the Department of Medical Oncology, University Medical Center Utrecht (Utrecht, The Netherlands). The study was approved by the institutional ethical review board of University Medical Center Utrecht, and written informed consent was obtained from all patients.

Tumors and Animal Models

Eight- to twelve-week-old C57BL/6 or BALB/c mice (obtained from The Jackson Laboratory–West, Sacramento, CA, USA) were treated with chemotherapy drugs. Lewis lung carcinoma (LLC) cells (5×10^5; American Type Culture Collection) were subcutaneously implanted into immunocompetent C57BL/6 mice (The Jackson Laboratory) or C57BL/6 mice previously irradiated and then transplanted with GFP^+ bone marrow cells or were injected into $Id1^{+/-}Id3^{-/-}$ (Id^{mut}) mice and WT C57BL/6 mice. B16F1 melanoma cells (5×10^5; American Type Culture Collection) were implanted into the flanks of immunocompetent C57BL/6 mice. Tumor size was assessed regularly with Vernier calipers using the formula $\text{width}^2 \times \text{length} \times 0.5$. When tumors reached 500 mm^3, treatment was initiated. All mice used for in vivo studies were randomly grouped (n = 4–6 per group). All animal studies were performed according to the Sunnybrook Health Sciences Centre Animal Care Committee (Toronto) and the Canadian Council on Animal Care or the Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center (New York).

Drugs and MTD Drug Concentrations

The following antibodies were used for in vivo therapy: 800 μg/mouse DC101 (ImClone Systems Inc.), a rat monoclonal blocking antibody specific for mouse VEGFR2/Flk-1; 50 μg/mouse monoclonal SDF-1α neutralizing antibodies (R&D Systems); 50 μg/mouse goat polyclonal anti-SDF-1α neutralizing antibodies or goat nonspecific antisera control after Fc fragment digestion; and 5 mg/kg G6-31, a monoclonal anti-mouse/human VEGF neutralizing antibody (Genentech). All doses had been previously determined for optimal activity (Prewett et al., 1999; Schober et al., 2003; Phillips et al., 2003; Liang et al., 2006). Chemotherapy drugs were administered near or at the MTD as indicated in Table S1. All drugs were administered intraperitoneally as a bolus injection. Control mice received the relevant vehicles. DC101, G6-31, and SDF-1α neutralizing antibodies when used with chemotherapy were administered 24 hr prior to the chemotherapy drug injection.

Flow Cytometry

For preclinical evaluation of viable CEPs, blood was obtained from anesthetized mice via retro-orbital sinus bleeding and prepared for CEP labeling as described previously (Bertolini et al., 2003; Shaked et al., 2005a). For clinical samples, CEPs were evaluated in patients as described previously (Bertolini et al., 2006). For detailed information, see Supplemental Experimental Procedures.

For evaluation of GFP^+ bone marrow-derived cells, hemangiocytes, and TEMs resident in tumors, 100–300 mm^3 of tumor tissue (n = 5 samples per group) was prepared as a single-cell suspension as described previously (Baeten et al., 2002). GFP^+ cells expressing CD45, CXCR4, and VEGFR1 were defined as hemangiocytes (Jin et al., 2006). GFP^+ cells expressing CD45, Tie2, and CD11b were defined as TEMs (De Palma et al., 2005). At least 50,000 cells per sample were acquired. All bone marrow cell types were plotted as the absolute cell number in 10,000 cells.

For all flow cytometry experiments, CD133 was purchased from Miltenyi Biotec, Tie2 was produced in-house (Sunnybrook Health Sciences Centre), and VEGFR1 was purchased from R&D Systems. All other antibodies were purchased from BD Biosciences.

Quantitation and Visualization of Tissue Necrosis, Hypoxia, Vessel Perfusion, Tumor Cell Proliferation, and Apoptosis

Tissue processing and immunohistochemistry were performed as described previously (Shaked et al., 2006). For detailed information, see Supplemental Experimental Procedures.

Microscopic Image Acquisition and Analysis

Tumor sections were visualized under a Zeiss Axioplan 2 microscope. Images were captured with a Zeiss AxioCam digital camera connected to the microscope using AxioVision 3.0 software. The number of fields per tumor sample varied from four to ten depending on the tumor size. Analysis of tumor hypoxia, vessel perfusion, and necrosis as well as tumor cell proliferation and apoptosis was carried out by calculating the fraction of the tumor area staining positive for the indicated parameter, using Adobe Photoshop 6.0 software (Adobe Systems). For analysis of microvessel density, the total number of vascular structures ($CD31^+$) per field was counted per tumor sample. At least ten fields per tumor representing all tumor areas were taken ($n \geq 5$ tumors per group).

Analysis of VEGF-A, SDF-1α, and G-CSF Plasma Concentrations

Blood samples obtained by cardiac puncture from mice under anesthesia or intravenously from cancer patients were collected in either Microtainer (Becton Dickinson) plasma separating tubes (for mice) or EDTA tubes (for humans), centrifuged at 4°C, and subsequently stored at −70°C until assayed. Levels of mouse or human VEGF-A, SDF-1α, and G-CSF were assessed using commercially available sandwich ELISAs (R&D Systems).

Isolation of Platelets and Analysis of SDF-1α

Experiments were performed as described previously (Jin et al., 2006). For detailed information, see Supplemental Experimental Procedures.

Bone Marrow Transplantation

Experimental procedures were carried out as described previously (Shaked et al., 2006). For detailed information, see Supplemental Experimental Procedures.

Statistical Analysis

Data are expressed as mean ± SD, and the statistical significance of differences in mean values was assessed by Student's two-tailed t test. Differences between designated groups compared to control untreated group (unless indicated otherwise) were considered significant at values of $0.05 > p > 0.01$ (*) or $p \leq 0.01$ (**). For human samples, data are expressed as mean ± SEM. Comparisons between baseline and 4 hr posttreatment were made by paired t test. Comparisons between groups of patients treated with paclitaxel-based versus other chemotherapy-based therapies were made by unpaired t test. Significance was set as $0.05 > p > 0.01$ (*) or $p \leq 0.01$ (**).

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, one table, and six figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/14/3/263/DC1/.

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