Contribution of Granulocyte Colony-Stimulating Factor to the Acute Mobilization of Endothelial Precursor Cells by Vascular Disrupting Agents

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Abstract

Vascular disrupting agents (VDA) cause acute shutdown of abnormal established tumor vasculature, followed by massive intratumoral hypoxia and necrosis. However, a viable rim of tumor tissue invariably remains from which tumor regrowth rapidly resumes. We have recently shown that an acute systemic mobilization and homing of bone marrow–derived circulating endothelial precursor (CEP) cells could promote tumor regrowth following treatment with either a VDA or certain chemotherapy drugs. The molecular mediators of this systemic reactive host process are unknown. Here, we show that following treatment of mice with OXi-4503, a second-generation potent prodrug derivative of combretastatin-A4 phosphate, rapid increases in circulating plasma vascular endothelial growth factor, stromal derived factor-1 (SDF-1), and granulocyte colony-stimulating factor (G-CSF) levels are detected. With the aim of investigating whether G-CSF is involved in VDA-induced CEP mobilization, mutant G-CSF−/− mice were treated with OXi-4503. We found that as opposed to wild-type controls, G-CSF−/− mice failed to mobilize CEPs or show induction of SDF-1 plasma levels. Furthermore, Lewis lung carcinomas grown in such mice treated with OXi-4503 showed greater levels of necrosis compared with tumors treated in wild-type mice. Evidence for rapid elevations in circulating plasma G-CSF, vascular endothelial growth factor, and SDF-1 were also observed in patients with VDA (combretastatin-A4 phosphate)-treated cancer. These results highlight the possible effect of drug-induced G-CSF on tumor regrowth following certain cytotoxic drug therapies, in this case using a VDA, and hence G-CSF as a possible therapeutic target. [Cancer Res 2009;69(19):7524–8]

Introduction

The growth of new blood vessel capillaries in tumors—therapeutic inhibition of which is now a clinically validated strategy—seems to be mediated not only by the local division of pre-existing differentiated vascular endothelial cells ("sprouting angiogenesis"; ref. 1) but also by a systemic process involving the mobilization and homing of a variety of bone marrow–derived cell (BMDC) populations (2). These include circulating endothelial precursor cells (CEP), which can incorporate into the lumens of growing blood vessels and differentiate into endothelial cells (2, 3).

We recently reported that exposure of tumor-bearing mice to cytotoxic-like vascular disrupting agents (VDAs) can cause a rapid (i.e., within hours) mobilization of BMDCs, some of which are CEPs, followed by their homing to the viable rim of tumor tissue (4), which characteristically remains after treatment with VDAs, and surrounds a sometimes massive necrotic tumor center (4, 5). This acute reactive host response contributes to the rapid regrowth of the drug-treated tumors and thus compromises the durability of the initial antitumor effect induced by VDA treatment. Furthermore, we recently reported that certain cytotoxic chemotherapy drugs (administered at maximum tolerated doses) such as taxanes or 5-flourouracil, can also cause acute mobilization of CEPs (6). In both cases, combination treatment with an antiangiogenic drug, e.g., DC101, a VEGFR-2 neutralizing antibody (4, 6, 7), blocks CEP mobilization and thus enhances the antitumor activity of the cytotoxic therapy used (4, 6). Given the remarkably acute and robust nature of the BMDC response induced by VDAs, and its consequences for tumor response and effects on therapeutic benefit, we decided to investigate the possible molecular mediators of this therapy-induced mobilization of proangiogenic BMDCs, including CEPs. To do so, we used a potent VDA, i.e., the microtubule-inhibiting agent, OXi-4503, a second-generation prodrug derivative of the VDA combretastatin-A4 phosphate (CA4P; ref. 5).

Materials and Methods

Human blood samples. Patients with advanced solid tumors who had given consent to participate in a phase I study evaluating escalating doses of CA4P given in combination with bevacizumab (8) participated in this study (n = 15). Patients entered as one of three cohorts and
received 45, 54, or 63 mg/m² of CA4P i.v. in the first treatment cycle without bevacizumab. For the purpose of this study, blood was collected at baseline (pretreatment), and 4 h after CA4P treatment. The protocol was approved by the ethics committee.

**Tumor and animal models.** All animal studies were conducted in accordance with the animal care guidelines at Sunnybrook Health Sciences Centre (Toronto, Canada), and at Washington University (St. Louis, MO), and described in detail in the Supplementary Online Material.

**Evaluation of circulating endothelial progenitor cells by flow cytometry.** Blood was obtained from anaesthetized mice by retro-orbital sinus bleeding. Viable CEPs were quantitated using flow cytometry, as described previously (3), and in the Supplementary Online Material.

**Tissue processing and imaging.** Tissue processing and immunohistochemistry were performed as described previously (4, 9), and in the Supplementary Online Material.

**Evaluation of circulating granulocyte colony-stimulating factor, vascular endothelial growth factor, and stromal derived factor-1 plasma levels by ELISAs.** Tumor-free or 500 mm³ MeWo human melanoma–bearing nude mice, as well as tumor-free granulocyte colony-stimulating factor receptor (G-CSF-R) and wild-type control mice were treated with an i.p. injection of 100 mg/kg of OXi-4503, or PBS as a control. Four hours later, mice were sacrificed and blood was drawn by cardiac puncture. In patients, blood samples were obtained by intravenous puncture. Plasma was separated using heparin and subsequently stored at −70 °C until assayed. Levels of mouse or human vascular endothelial growth factor (VEGF), stromal derived factor-1 (SDF-1), and G-CSF were assessed by using commercially available sandwich ELISAs (R&D Systems).

**Statistical analysis.** Data are expressed as mean ± SD and the statistical significance of differences in mean values was assessed by two-tailed Student’s t test. Differences between means of experimental/treated groups compared with control-untreated or baseline (pretreatment) groups (unless indicated otherwise) were considered significant at values of $P < 0.01$ (**) or $0.05 < P < 0.01$ (*).

**Results**

Rapidly elevated plasma levels of predominantly host-derived G-CSF, VEGF, and SDF-1 are induced by OXi-4503. To investigate whether induction of certain growth factors may be involved in the acute mobilization of CEPs by VDA treatment, we analyzed plasma from non-tumor–bearing or 500 mm³ MeWo human melanoma–bearing nude mice obtained 4 hours after treatment with OXi-4503 for human and mouse G-CSF, VEGF, and SDF-1 using specific ELISAs. Emphasis was placed on these particular factors because all are known to induce CEP mobilization (10–12). The results in Fig. 1A to C show acute increases in both normal and tumor-bearing mice of mouse G-CSF, VEGF, and SDF-1 (10– to 20-fold, 4– to 5-fold, and 2-fold increases, respectively). Interestingly, levels of human G-CSF were significantly lower 4 hours after OXi-4503 treatment. Human VEGF levels were not detectable in untreated tumor-bearing mice, but a relatively small amount (15–18.32 pg/mL) was detected in two out of five mice, 4 hours after OXi-4503 treatment. Of note, we recently reported marked elevations of plasma SDF-1 in non–tumor-bearing mice treated with maximum tolerated dose paclitaxel, which contributed to the rapid CEP spike observed similar in nature to the VDA-induced effect (6). Overall, these results suggest that several factors known to mediate the mobilization of CEPs are acutely increased by OXi-4503 treatment and are, in the main, derived from the host.
Elevated G-CSF contributes to the rapid CEP mobilization induced by VDA treatment. G-CSF is well known as a mobilizing factor for hematopoietic stem cells, as well as CEPs (12, 13). Given the pronounced elevation of G-CSF induced by OXi-4503, we asked whether it has a direct effect on CEP mobilization following VDA treatment. To do so, tumor-free BALB/c mice were infused with 5 μg/kg of human recombinant G-CSF, and blood was drawn 4 and 24 hours later for the evaluation of CEPs. The results in Supplementary Fig. S1 show that G-CSF induced a 3-fold to 4-fold spike in CEPs levels, similar to what we observed using OXi-4503. Similar results were obtained using C57Bl/6 mice (data not shown). Next, we evaluated the levels of CEPs and relative tumor response as measured by necrosis in mutant G-CSF receptor (G-CSF-R−/−) mice (14) after OXi-4503 treatment. Figure 2A and B and Supplementary Fig. S2 show that levels of CEPs in G-CSF-R−/− mice do not significantly change within the first 24 hours, in contrast to their wild-type controls. In addition, transplanted LLC tumors grown in G-CSF−/− mice or wild-type control mice were allowed to grow until the tumors reached 500 mm3, at which point, the mice were treated with OXi-4503. Three days later, tumors were removed and evaluated for tumor response by measuring necrosis. The results in Fig. 2C and D show that whereas tumors grown in wild-type mice, which exhibited a prominent necrotic center exceeding ~15% of the total tumor area, tumors treated in G-CSF-R−/− mice had a significantly greater degree of tumor necrosis (~35%). These results suggest a direct effect of G-CSF on promoting CEP mobilization, which contributes, at least in part, to the rapid regrowth of tumors from the viable tumor rim, after VDA treatment.

To further assess the contribution of G-CSF to CEP mobilization, plasma levels of SDF-1, VEGF, and G-CSF were evaluated in G-CSF-R−/− mice, 4 hours after they were treated with OXi-4503. The results in Fig. 3 show that both VEGF and G-CSF plasma levels were substantially elevated, although the baseline levels of G-CSF in G-CSF-R−/− mice were remarkably higher in comparison to their levels in wild-type control mice. Interestingly, SDF-1 levels were down-regulated in G-CSF-R−/− mice, which suggests that SDF-1 may also be secondarily involved in G-CSF–induced CEP mobilization following OXi-4503 treatment.

Increases in circulating G-CSF, VEGF, and SDF-1 plasma levels in cancers treated with a VDA. To evaluate whether some of our preclinical results could be reproduced in the clinical setting, blood was collected from patients with advanced solid tumors enrolled in a phase I clinical study (8) at baseline (pretreatment) and 4 hours after treatment with CA4P. Plasma was separated for the evaluation of G-CSF, VEGF, and SDF-1 levels using specific ELISAs. The results in Fig. 4 show that the levels of all three cytokines were substantially increased within 4 hours of CA4P treatment, similar to the results we observed in mice using G-CSF-R−/− mice following treatment with OXi-4503. CEP levels (A) and WBC counts (B) were evaluated at baseline, 4, and 24 h after OXi-4503 treatment in non–tumor-bearing G-CSF-R−/− mice (filled columns) and their respective wild-type controls (open columns). C, relative necrosis in tumor sections from both G-CSF-R−/− and wild-type control mice following treatment with OXi-4503 (bar, 100 μm). D, quantitative data on the percentage of necrosis area from all tumor areas.

**Cancer Research** 2009; 69: (19). October 1, 2009 7526 www.aacrjournals.org

Published OnlineFirst September 8, 2009; DOI: 10.1158/0008-5472.CAN-09-0381
Overall, these preliminary clinical results are remarkably consistent with the respective preclinical findings.

**Discussion**

Cytotoxic anticancer drugs, or treatments, including chemotherapy, radiation, and VDAs can often cause major tumor responses manifested by tumor cell death subsequently leading to various degrees of tumor shrinkage. However, the benefit of such responses, at least in terms of increased survival times, is often limited because of rapid tumor repopulation (15). Consequently, developing and optimizing strategies to slow down such rapid tumor repopulation are of considerable therapeutic interest (15). Some possible strategies include closer spacing of cytotoxic therapies such as “dose-dense” chemotherapy, or hyperfractioned radiation (15), or the combined use of biological therapies that target cell proliferative mechanisms, including antiangiogenic drugs, during the treatment-free intervals in between successive cycles of cytotoxic therapy (4, 6, 15–17).

Tumor cell repopulation/regrowth has generally been viewed as an intrinsic tumor cell-driven property (15). However, our previous studies implicated that a number of cytotoxic drugs can rapidly activate various host processes which can contribute significantly to tumor repopulation, one of which involves acute mobilization and tumor homing of proangiogenic BMDCs, including CEPs (4, 6). In our recent study, we reported that SDF-1 is one of the molecular mediators of CEP mobilization following paclitaxel treatment, as neutralizing anti-SDF-1 antibodies that were administered in combination with chemotherapy resulted in the failure of induction of CEP spikes (6). Here, using a VDA as the cytotoxic-(like) agent, we have implicated another molecular mediator responsible for CEP mobilization. The increase in circulating G-CSF was remarkable, being in the range of one order of magnitude, and moreover, it occurred within 4 hours of VDA treatment. However, G-CSF-R−/− mice failed to induce elevated levels of both CEPs and SDF-1. Eash and colleagues reported that G-CSF mobilizes neutrophils within hours, probably by directly down-regulating CXCR4 expression (18). Moreover, AMD3100, a CXCR4 antagonist, was also found to mobilize CEPs and other hematopoietic stem cells within 4 hours (19). Whether G-CSF acts in a similar fashion to AMD-3100 by down-regulating CXCR4 expression on CEPs remains to be determined in future studies. In addition, we also observed a significant and rapid reduction in levels of WBC counts, following treatment with OXi-4503, in G-CSF-R−/− mice but not in wild-type controls, which is consistent with recent findings by Petit and colleagues (20), implicating the direct effect of VDAs on hematopoietic cells. However, how G-CSF acts to maintain normal...
WBC counts in wild-type mice following VDA treatment remains unknown.

Also noteworthy with respect to our results was the observation of rapid elevation of G-CSF, VEGF, and SDF-1 plasma levels observed in the peripheral blood of cancer patients treated with CA4P, results which are obviously similar in some respects to the rapid elevations of the three cytokines in our preclinical study. Although we did not evaluate the levels of CEPs in clinical samples, recent studies have shown that cancer patients treated with either maximum tolerated dose paclitaxel-based chemotherapy or a VDA (known as AVE8062) combined with cisplatin, exhibited a rapid elevation in CEP levels (6, 21).

One implication of our results relates to the outcome of cytotoxic dose-dense chemotherapy regimens which are made possible by the use of recombinant G-CSF growth factor support to accelerate host recovery from myelosuppression. Although there is no clinical evidence at present to suggest that administration of recombinant G-CSF worsens clinical outcomes, it is conceivable that the antitumor benefits obtained by using such dose-dense (and hence more intensive) chemotherapy regimens are less than would otherwise be the case. Moreover, regarding the possibility of G-CSF–induced mobilization of BMDCs possibly facilitating tumor angiogenesis, we also note the recent findings by Shojaei and colleagues who reported that mobilization of proangiogenic Gr1+CD11b+ myeloid suppressor cells can be induced indirectly by G-CSF through up-regulation of Bv8/prokineticin (22).

In summary, our results suggest a host-reactive molecular mechanism contributing to rapid tumor regrowth following cytotoxic anticancer drug therapy using VDAs, and suggest that G-CSF could conceivably be exploited transiently as a therapeutic target under selected circumstances, e.g., as a means of enhancing the efficacy of VDA therapy. Such targeting may be particularly suitable in situations in which the cytotoxic drug treatment is not associated with myelosuppression. In this regard, VDAs are not associated with high-grade myelosuppression (5, 8, 21).

Disclosure of Potential Conflicts of Interest

D.J. Chaplin is an employee of OXiGENE Inc. G. Rustin is a member of the clinical advisory board of OXiGENE Inc. R.S. Kerbel is a member of the scientific advisory board of OXiGENE Inc. The other authors disclosed no potential conflicts of interest.

Acknowledgments

Received 2/2/09; revised 7/21/09; accepted 7/29/09; published OnlineFirst 9/8/09.

Grant support: NIH, the National Cancer Institute of Canada, and Canadian Institutes of Health Research (R.S. Kerbel); NIH R01 HL073762 (D.C. Link); National Cancer Institute NIC B01 CA 90722 (J.M. Arbeit); the Associazione Italiana per la Ricerca sul Cancro, Istituto Superiore di Sanità, and the sixth EU Framework Programme (Integrated Project "Angiostargeting" contract no. 504743) in the area of "Life sciences, genomics and biotechnology for health" (F. Bertolini); and sponsored research agreements with ImClone Systems New York and OXiGENE, Inc., Boston (R.S. Kerbel); Cancer Research UK and the Oxford NIHBI Biomedical Research Centre (A. Harris); and Dr. Saal van Zwansenberg Stichting fellowship (L. Daenen). The human study was sponsored and fully funded by OXiGENE, Inc.

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We thank Cassandra Cheng for her excellent secretarial assistance.

References

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