

Chemotherapy Enhances Metastasis Formation via VEGFR-1-Expressing Endothelial Cells

Laura G.M. Daenen¹, Jeanine M.L. Roodhart¹, Miranda van Amersfoort², Mantre Dehnad¹, Wijnand Roessingh¹, Laurien H. Ulfman³, Patrick W.B. Derkx², and Emile E. Voest¹

Abstract

Recent studies suggest that chemotherapy, in addition to its cytotoxic effects on tumor cells, can induce a cascade of host events to support tumor growth and spread. Here, we used an experimental pulmonary metastasis model to investigate the role of this host response in metastasis formation. Mice were pretreated with chemotherapy and after clearance of the drugs from circulation, tumor cells were administered intravenously to study potential "protumorigenic" host effects of chemotherapy. Pretreatment with the commonly used chemotherapeutic agents cisplatin and paclitaxel significantly enhanced lung metastasis in this model. This corresponded to enhanced adhesion of tumor cells to an endothelial cell monolayer that had been pretreated with chemotherapy *in vitro*. Interestingly, chemotherapy exposure enhanced the expression of VEGF receptor 1 (VEGFR-1) on endothelial cells both *in vitro* and *in vivo*. Administration of antibodies targeting VEGFR-1 reversed the early retention of tumor cells in the lungs, thereby preventing the formation of chemotherapy-induced pulmonary metastases. The data indicate that chemotherapy-induced expression of VEGFR-1 on endothelial cells can create an environment favorable to tumor cell homing. Inhibition of VEGFR-1 function may therefore be used to counteract chemotherapy-induced retention of tumor cells within the metastatic niche, providing a novel level of tumor control in chemotherapy. *Cancer Res*; 71(22); 1–10. ©2011 AACR.

Introduction

Not all cancer patients treated with chemotherapy show response to treatment. Moreover, a small subset of patients experiences early progression during systemic anticancer therapy. Prime examples include accelerated growth of non–small cell lung cancers in patients after induction chemotherapy (1), and rapid tumor cell proliferation in oropharyngeal cancer patients who responded poorly to chemotherapy (2).

This early progression during therapy is generally thought to be part of the natural course of disease, meaning that the tumor would have progressed in a similar fashion if it had not been treated. However, accumulating evidence suggests that chemotherapy may also induce tumor-promoting changes in the microenvironment as part of a systemic host response. Blood of cancer patients treated with chemotherapy contains increased levels of several protumorigenic growth factors and

mobilized bone marrow-derived progenitor cells, that can home to the tumor and subsequently contribute to angiogenesis (3, 4). High levels of these cells correspond to primary tumor progression in mice and decreased survival in patients (3, 4). Thus, in addition to the direct effects of the therapy on the cancer, chemotherapy elicits a host response that can be tumor growth promoting (5). Benefits of anticancer treatment must therefore be weighed with respect to potential protumorigenic effects.

We hypothesized that when tumors can overcome the potent, cytotoxic effects of chemotherapy through resistance, the signals to the microenvironment will obfuscate the benefits of treatment and may actually facilitate disease progression and metastatic spread.

To study the host effects occurring postchemotherapy, an experimental mouse metastasis model was designed in which the direct, cytotoxic antitumor effects were absent. Mice were pretreated with chemotherapy and after clearance of the drug from circulation, tumor cells were administered intravenously. Using this model, we here show that pretreatment with 2 widely used chemotherapeutic agents enhanced lung metastasis formation. This phenomenon was observed in several mouse strains, injected intravenously with different tumor cell lines. Chemotherapy pretreatment resulted in an early accumulation of tumor cells in mouse lungs, which corresponded to enhanced adhesion of tumor cells to endothelial cells exposed to cytotoxic agents *in vitro*. Moreover, our data indicate that membrane expression of VEGFR-1 was upregulated by endothelial cells in response to chemotherapy. Finally, systemic

Authors' Affiliations: Departments of ¹Medical Oncology, ²Pathology, and ³Respiratory Medicine, University Medical Center Utrecht, Utrecht, the Netherlands

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Corresponding Author: Emile E. Voest, Department of Medical Oncology (F02.126), University Medical Center Utrecht, P.O. Box 85500, 3508 GA Utrecht, the Netherlands. Phone: 31-88-7556265; Fax: 31-30-2523741; E-mail: e.e.voest@umcutrecht.nl

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administration of antibodies targeting VEGFR-1 reversed the chemotherapy-induced tumor cell retention in the lungs, reducing the number of lung colonies.

Materials and Methods

Cell culture

C26 colon carcinoma cells and COS-7 were maintained in Dulbecco's modified Eagle's medium (DMEM; Lonza) with 5% fetal calf serum (FCS), 2 mmol/L glutamine, 0.1 mg/mL streptomycin, and 100 U/mL penicillin. C26 cells expressing the firefly luciferase gene (C26-luc) were described previously (6). B16F10 cells were maintained in RPMI (Invitrogen) with 5% FCS and antibiotics. bEND.3 immortalized microvascular ECs were a gift of M. Verhaar, UMC Utrecht, and were maintained in DMEM (1 g/L glucose) with 10% FCS and antibiotics. 6011L primary C57Bl/6 lung ECs were purchased from Cell Biologics, maintained in accompanying M1166 growth medium and grown on gelatin-coated plates. All cells were kept at 37°C in a humidified atmosphere with 5% CO₂.

Tumor and mouse models

All animal procedures were approved by the UMC Utrecht Animal Care Ethics Committee. BALB/c and C57Bl/6 mice, $n \geq 6$ /group (Charles River) were injected intraperitoneally (i.p.) with chemotherapy at MTD levels: cisplatin 6 mg/kg, paclitaxel 20 mg/kg (Pharmachemie BV), or vehicle controls. Four days later, 4×10^4 C26 (-mCh) cells were i.v. injected into the tail veins of BALB/c mice, or 6×10^3 C26 cells into *Rag2*^{-/-}; *IL2Rγc*^{-/-}BALB/c mice (7), or 1×10^5 B16F10 mouse melanoma cells into C57Bl/6 mice. Anti-mouse VEGFR-1 antibody MF1 and anti-mouse VEGFR-2 antibody DC101 were kindly provided by ImClone Systems Inc. One day before injection of tumor cells, MF1 (40 mg/kg), DC101 (800 µg/mouse) or a vehicle control was administered i.p.

Bioluminescence imaging

Mice were anesthetized with isoflurane (IsoFlo, Abbott Laboratories Inc.) and i.p. injected with *n*-luciferin (potassium salt; Biosynth AG) at 225 mg/kg, 13 days after tumor cell injection. C26-luc lung metastases were assessed by *in vivo* bioluminescence imaging (BLI) using a Biospace Φ imager and M3 vision software (Biospace Lab). The integrated light intensity as measured by single photon counting of a 10-minute exposure was used to quantify the amount of light emitted by C26-luc cells. A low intensity visible light image was made for overlay images.

Lentiviral transduction

For C26-mCh, lentiviral particles were produced by seeding 1.2×10^6 Cos-7 cells onto a 10-cm dish and transient transfection using fuGENE-6 Transfection Reagent (Roche Diagnostics) with third-generation packaging constructs (8) and a CMV-mCherry transfer vector containing a puromycin selection cassette (a gift from C. Löwik, Leiden UMC, Leiden, the Netherlands). After 48 hours, supernatant was harvested, filtered, and used to transduce 10^5 C26 cells with 40 µg/mL polybrene. Trans-

duction was repeated after 24 hours and 24 hours later puromycin selection was initiated.

Identification of C26-mCh cells in mouse lungs

C26-mCh cells were i.v. injected into mice that had been pretreated with chemotherapy or vehicle controls. Twenty-four hours after injection, lungs were perfused via the left ventricle with PBS-EDTA followed by 1% PFA. Subsequently, 3% agarose/0.5% PFA was administered via the trachea into the lungs. Lungs were harvested and kept on ice, before fixation in 4% PFA. Vibrotome sections (300 µm) of lungs were stained with 4',6-diamidino-2-phenylindole (DAPI) and per lung 10 random 3-dimensional fields were evaluated for mCherry-expressing cells on a Zeiss LSM 510 META (40×). Zeiss LSM Image Brower software Version 4.2.0.121 was used.

Immunofluorescence

Vibrotome sections were prepared, blocked with goat serum, stained with rat-anti-mouse CD31 (BD Biosciences Pharmingen) and rabbit-anti-mouse VEGFR-1 (Santa Cruz), followed by 488-/647-conjugated secondary antibodies (AlexaFluor, Molecular Probes Inc.) and DAPI. CLSM evaluation was done.

Flow cytometry

Mice were pretreated with chemotherapy or vehicle. Organs were harvested 4 days later and single cell suspensions were prepared by cutting and DNase/collagenase treatment. Cells were stained for 30 minutes in PBS-BSA-EDTA with antibodies or isotypes. VEGFR-1-PE antibodies were obtained from R&D Systems, CD31-APC and isotype controls from eBioscience, CD11b-FITC from Miltenyi Biotec. Remaining fluorescence-activated cell sorting (FACS) antibodies were obtained from BD Biosciences Pharmingen: CXCR4-FITC, VEGFR-2-PE, CD45-PerCP, CD117-APC, and VCAM-1-FITC. After RBC lysis, analysis was done on a FACSCalibur II. For *in vitro* FACS experiments, bEND.3 cells were plated out in 6-well plates. After 24 hours, cisplatin was added for 4 hours. Cells were washed twice with PBS and put on DMEM. Four days later, cells were harvested, stained for VEGFR-1 and analyzed.

MTT assay

Cells were plated in 96-well plates and cultured for 24 hours. MF1 or DC101 were added (50 µg/mL) and MTT assays (Roche Diagnostics) were done every 24 hours as per manufacturer's instructions.

EC adhesion assays

bEND3 or 6011L monolayers were grown in 96-wells plates, treated with cisplatin, paclitaxel or vehicle for 4 hours, washed twice with PBS and maintained on medium for 3 days. After washing with PBS, the plate was blocked with 2.5% BSA (Sigma-Aldrich), and incubated for 4 hours with PMA, Mn²⁺, TNFα, IL-1β, or PBS. Integrin β1 or β3 (BD Pharmingen) antibodies were added 1:200. C26 tumor cells were loaded with 4 µmol/L calcein-AM (Molecular Probes) in HBSS. VCAM-1 and ICAM-1 antibodies (BD Pharmingen) were added 1:200. A total of 5×10^4 calcein-labeled C26 were added to triplicate wells. After 50 minutes at 37°C, nonadhered cells were removed and

the plate was washed 3 times with HBSS buffer containing EGTA and Mg²⁺. After the third wash adherent tumor cells were quantified using a FLUOstar Optima (BMG Labtech).

Statistical analysis

Data are expressed as mean \pm SEM. Statistical significance was assessed by Student 2-tailed *t* test. A value of $P < 0.05$ was considered significant and represents significance compared with untreated controls, unless indicated otherwise.

Results

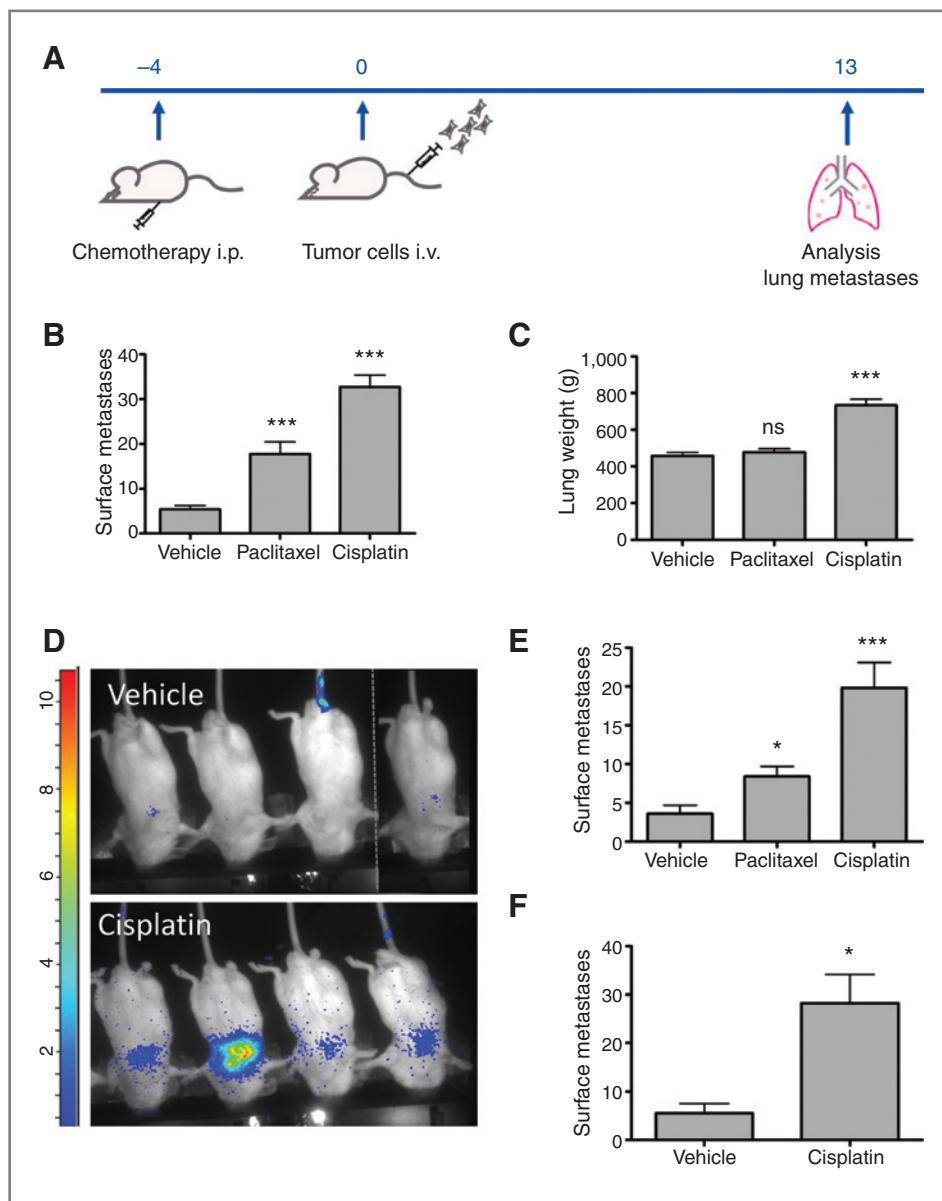
Chemotherapy pretreatment enhances experimental lung metastasis in mouse models

We designed a mouse model to specifically study the host effects that take place after exposure to chemotherapy, and their

potential effects on metastatic spread (Fig. 1A). In this experimental lung metastasis model, mice were pretreated with chemotherapy, followed by intravenous tumor cell injection 4 days later, after the chemotherapeutic agents had been cleared from circulation. Administration of tumor cells after drug clearance prevents direct cytotoxic effects on the tumor cells, but allows investigation of drug-induced host effects. Thirteen days after tumor cell injection, lung colonies were analyzed.

When BALB/c mice were pretreated with either of 2 commonly used chemotherapeutic agents, paclitaxel or cisplatin, followed by intravenous injection of C26 mouse colon carcinoma cells, a significantly enhanced number of lung colonies was present after 13 days (Fig. 1B). Paclitaxel augmented lung colony formation more than 3-fold, whereas cisplatin gave rise to a 6-fold increase compared with untreated mice. This corresponded to a significant increase in lung weight of these

Figure 1. Chemotherapy pretreatment enhances experimental lung metastasis. A, BALB/c mice ($n = 10$ per group) were treated with cisplatin, paclitaxel, or vehicle control. After 4 days, C26 tumor cells were injected intravenously. Lung colonies were analyzed 13 days later by (B) counting surface metastases and (C) determining lung weight. D, similar experiments were done with C26-luc cells. After 13 days mice were injected with *n*-luciferin and BLI was done. E, C57Bl/6 mice ($n = 10$ per group) were pretreated with chemotherapy followed by 1×10^5 B16F10 tumor cells i.v. 4 days later. Pulmonary surface metastases were counted after 13 days. F, immune deficient *Rag2*^{-/-}; *IL2Rγc*^{-/-} BALB/c mice were pretreated with cisplatin, followed by C26 tumor cells iv 4 days later. Pulmonary surface metastases were counted after 13 days; ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



mice for cisplatin-treated animals (Fig. 1C). We next conducted BLI 13 days after injection of luciferase-expressing C26 tumor cells (C26-luc). Figure 1D shows an increase in lung colonization upon pretreatment with cisplatin compared with the untreated mice.

To confirm that these effects can be attributed to a general phenomenon, we repeated the experiments in C57Bl/6 mice that were intravenously injected with B16F10 mouse melanoma cells. Consistent with our findings in BALB/c mice, chemotherapy pretreatment resulted in enhanced pulmonary metastasis (Fig. 1E).

To determine whether suppression of the immune system by chemotherapy played a role in the enhanced number of lung metastases observed in these experiments, we carried out an identical experiment in *Rag2*^{-/-}; *IL2Rγc*^{-/-} BALB/c female mice (7). In these immune-deficient animals—that lack B-lymphocytes, T-lymphocytes, and NK-cells—similar results of chemotherapy pretreatment were found (Fig. 1F). These results indicate that suppression of any of these 3 components of the adaptive immune system by chemotherapy does not account for the observed effect.

Furthermore, dextran perfusion studies were done to study the integrity of the lung vasculature. Cisplatin pretreatment did not increase vascular leakage at the time of tumor cell injection (Supplementary Fig. S1).

Chemotherapy enhances early retention of tumor cells in the lungs

To determine whether the increase in lung colonization after chemotherapy treatment was due to an early event, we scored the number of tumor cells in the lungs of mice 24 hours after intravenous injection. To this end, mCherry-expressing C26 clones (C26-mCh) were generated by lentiviral transduction. Puromycin selection yielded a clone that was highly fluorescent (Supplementary Fig. S2A) with a proliferation rate comparable with the original cell line both *in vitro* (Supplementary Fig. S2B) and *in vivo*, as determined by the number of lung colonies 2 weeks after intravenous injection (Supplementary Fig. S2C). Furthermore, mCherry expression was maintained in lung colonies harvested 2 weeks after intravenous injection of C26-mCh cells (Supplementary Fig. S2D).

To determine differences in the presence of mCherry-expressing tumor cells in the lungs 24 hours after tumor cell injection, mice were pretreated with chemotherapy or vehicle control, followed by intravenous injection of C26-mCh tumor cells 4 days later. Twenty-four hours after tumor cell administration, mice were sacrificed and lungs were perfused, harvested, and sectioned. Interestingly, we observed that the number of fluorescent tumor cells in the lungs of chemotherapy-pretreated mice was significantly enhanced as early as one day after tumor cell injection (Fig. 2A and B). This implies that chemotherapy pretreatment promotes early retention of tumor cells in the lungs.

Chemotherapy enhances tumor cell adhesion to endothelial cells *in vitro*

Because chemotherapy effects were observed at very early stages of metastasis formation, we hypothesized that this was

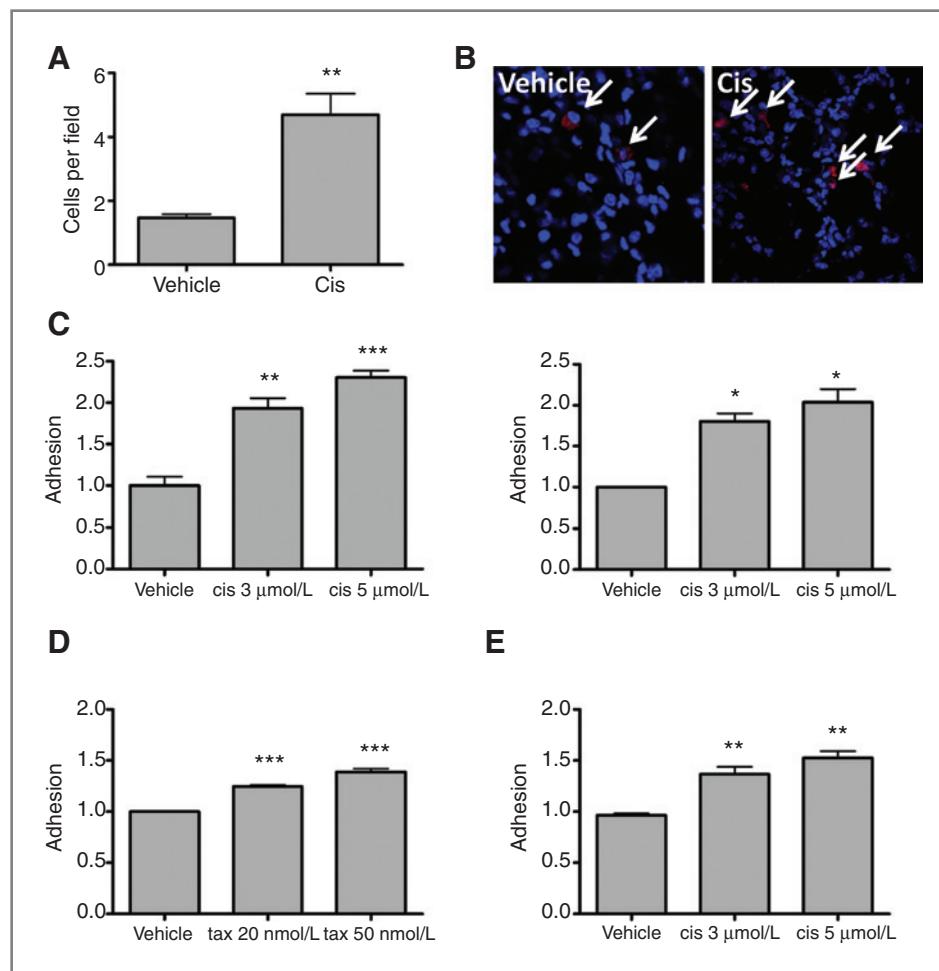
most likely due to enhanced tumor cell adhesion to endothelial cells (EC). To test this hypothesis, *in vitro* adhesion assays were done. Mouse bEND.3 EC monolayers were pretreated with cisplatin and 4 days later, calcein-labeled tumor cells were added and allowed to adhere. After 50 minutes, tumor cells were taken off and the wells were washed 3 times, followed by fluorescent quantification of adherent tumor cells. Remarkably, when ECs were not stimulated *in vitro*, tumor cells rapidly detached in all conditions (data not shown). To exclude the possibility that this was dependent on tumor cells requiring integrins for adhesion, ECs were stimulated with PMA and Mn²⁺. Neither of these agents had any effect (data not shown). Next, we primed the EC monolayer with TNFα or IL-1β, 2 cytokines that are known to circulate in response to cisplatin therapy (9–11). Upon stimulation with either of these cytokines, a significantly higher number of tumor cells remained attached to the cisplatin-pretreated EC monolayer than to the untreated EC monolayer (Fig. 2C). Pretreatment of bEND3 monolayers with paclitaxel showed enhanced tumor cell adhesion as well (Fig. 2D). Furthermore, similar results were found when mouse primary lung ECs were pretreated with cisplatin followed by adherence of C26 tumor cells (Fig. 2E).

TNFα or IL-1β is commonly used in static adhesion assays because they enhance expression of adhesion proteins VCAM-1 and ICAM-1 on ECs, 2 integrin ligands. To further investigate the binding between tumor cells and ECs, we determined whether CAM/integrin-mediated binding played a role in our system. Addition of integrin-stimulating agent PMA to TNFα-stimulated endothelium did not further enhance adhesion of tumor cells (Supplementary Fig. S3A). Similar effects were found for Mn²⁺ addition to TNFα (data not shown). Furthermore, when blocking integrin β1 or β3 on tumor cells before addition onto TNFα-stimulated endothelium, adhesion of tumor cells to vehicle-treated endothelium was decreased, whereas adhesion to cisplatin-stimulated endothelium was unchanged, indicating that these integrins are not important for chemotherapy-induced adhesion (Supplementary Fig. S3B). Similar results were found when blocking ICAM-1 or VCAM-1 on the endothelium (Supplementary Fig. S3C). Together, these experiments show that the chemotherapy-induced adhesion is independent of integrins β1 and β3, and VCAM-1 and ICAM-1, and a different binding mechanism plays a key role here.

Chemotherapy enhances VEGFR-1 expression on ECs *in vivo*

To clarify the chemotherapy-induced changes in ECs, we characterized several cell surface receptors on lung ECs after chemotherapy treatment. Mouse lungs were harvested 4 days after cisplatin administration (when we would usually inject tumor cells) and single cell fractions were prepared for analysis of various EC markers by FACS. ECs were characterized as CD31^{high}CD45^{neg} cells. The number of activated vascular cell adhesion molecule 1 (VCAM-1)-expressing ECs was similar in both groups (Fig. 3A). However, when studying expression of VEGF-receptors, a significant increase in VEGFR-1 expression on lung ECs was found, whereas the percentage of VEGFR-2-expressing ECs remained unchanged (Fig. 3A). VEGFR-1 expression was only increased in activated, VCAM-1-

Figure 2. Chemotherapy pretreatment enhances early retention of tumor cells in the lungs. A and B, mice were pretreated with cisplatin or vehicle control. Four days later, C26 cells were administered intravenously; 24 hours later, mouse lungs were perfused, filled with agarose, fixed, sectioned, and stained with DAPI (blue). The number of mCherry+ tumor cells (red) in the lungs was analyzed by CLSM. C, for adhesion assays, bEND.3 EC monolayers that had been pretreated with cisplatin or vehicle were stimulated with TNF α (left) or IL-1 β (right). After 4 hours, calcein-labeled C26 were added. After 50 minutes, nonadherent C26 cells were taken off and wells were washed 3 times with HBSS containing EGTA and Mg $^{2+}$. Adherent tumor cells were quantified by a fluorescence plate reader and plotted normalized to the adhesion of tumor cells to vehicle-pretreated endothelium. D, similar experiments were done after bEND.3 pretreatment with paclitaxel and stimulation with TNF α . E, cisplatin pretreatment of primary mouse lung ECs enhanced initial binding of tumor cells after stimulation with TNF α ; Cis, cisplatin; Tax, paclitaxel; ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.



expressing ECs (Fig. 3A), suggesting that VEGFR-1 is specifically upregulated in activated endothelial cells upon chemotherapy exposure. We confirmed the enhanced VEGFR-1 in pulmonary ECs of mice following exposure to chemotherapy by conducting coimmunofluorescence of CD31 and VEGFR-1. Indeed, a larger percentage of CD31+ ECs coexpressed VEGFR-1 in lungs obtained from mice that had been pretreated with chemotherapy (Fig. 3B and C).

A different population of VEGFR-1-expressing cells, circulating VEGFR-1-expressing hematopoietic cells, has recently been implicated in tumor growth and progression (12–15). To exclude the possibility that we are in fact studying VEGFR-1-expressing hematopoietic cells, control experiments were done using pan-hematopoietic cell marker CD45. Pulmonary levels of VEGFR-1-expressing hematopoietic cells remained unchanged 4 days after chemotherapy (Fig. 3D). In addition, no increases of VEGFR-1-expressing myeloid cells (VEGFR-1+CD11b+) (Supplementary Fig. S4A), VEGFR-1-expressing hematopoietic progenitor cells (VEGFR-1+CD45+CD117+) (Supplementary Fig. S4B), and VEGFR-1-expressing hemangiocytes (15; VEGFR-1+CD45+CXCR4+) (Supplementary Fig. S4C) were observed in the lungs. Analysis of peripheral blood of mice 4 days after treatment did not show cisplatin-induced

mobilization of VEGFR-1-expressing hematopoietic cells (Supplementary Fig. S4D) nor hemangiocytes (Supplementary Fig. S4E). Together, these experiments confirm that VEGFR-1 upregulation indeed takes place in nonhematopoietic CD31^{high} VCAM-1+ ECs.

To determine whether VEGFR-1 upregulation on ECs is a direct effect of cytotoxic agents, we incubated ECs with chemotherapy *in vitro*. To mimic the acute peak in drug concentration that mice experience *in vivo*, ECs were exposed to 3 or 5 μ mol/L cisplatin for 4 hours. After washing-out of the chemotherapeutic drug, ECs were maintained in culture medium for 4 days, corresponding to the time point at which tumor cells were injected in our *in vivo* experiments. Interestingly, a significant upregulation of VEGFR-1 on ECs was found by flow cytometry, which increased with ascending doses of cisplatin (Fig. 3E). In contrast, VEGFR-2 expression on ECs was not increased (data not shown).

Furthermore, we investigated whether the VEGFR-1 increase was found solely on pulmonary ECs by performing flow cytometry studies on single cell isolates from different organs. In the lungs, we found that the VEGFR-1+ increase was most profound on ECs expressing high levels of CD31. Surprisingly, this population was much less abundant in the

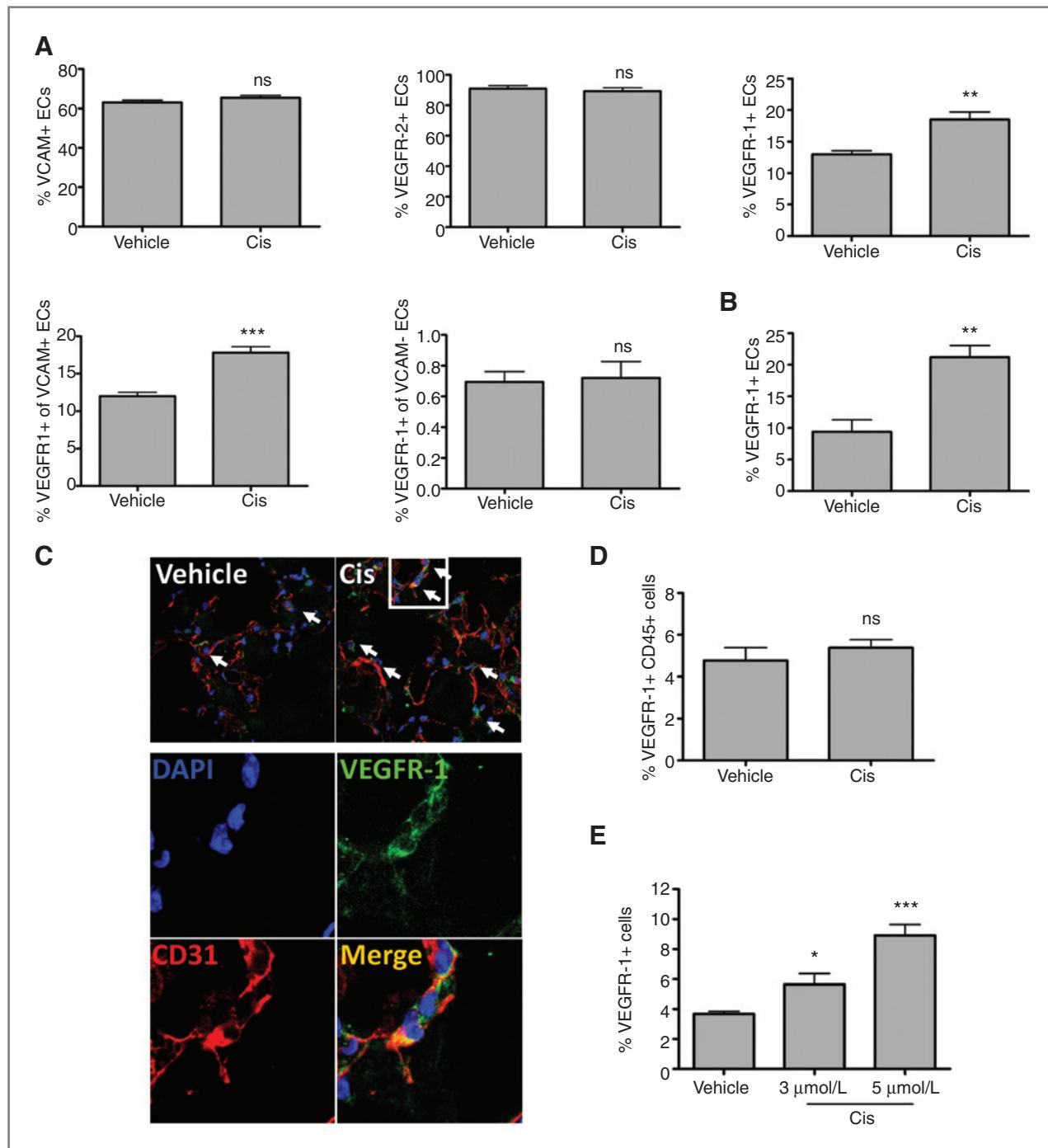


Figure 3. Chemotherapy enhances VEGFR-1 expression on ECs *in vivo* and *in vitro*. **A**, mouse lungs were harvested 4 days after treatment with cisplatin or vehicle control. Single cell samples were prepared and stained for flow cytometry with antibodies to VCAM-1 (top left), VEGFR-2 (top middle), VEGFR-1 (top right) in all CD45^{neg}CD31^{high} ECs, VEGFR-1 in VCAM-1⁺ ECs (bottom left) and in VCAM-1⁺ ECs (bottom middle). **B** and **C**, 4 days after cisplatin or vehicle, 300 μm slides were prepared and stained for CD31 and VEGFR-1. Expression of CD31 (red) and VEGFR-1 (green) was analyzed by CLSM. Cells that expressed both markers were quantified. **D**, expression of CD45 was determined on VEGFR-1⁺ cells harvested from mouse lungs 4 days after chemotherapy. **E**, ECs were incubated with accumulating doses of cisplatin for 4 hours. At day 4 after incubation, cells were harvested, stained with antibodies against VEGFR-1, and analyzed using FACS; Cis, cisplatin; ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

other analyzed organs. In the lungs, on average 12.3% of ECs was CD31^{high}, comprising more than 2.2% of all cells in the lungs. In liver and spleen, only 0.11% of all cells is a CD31^{high}

EC, whereas in brain this percentage was as low as 0.05%. These numbers were too low to allow meaningful statistical comparison between controls and chemotherapy pretreated

animals. Of note, no metastases were found in other organs in our model.

Blocking VEGFR-1 prevents early retention of tumor cells in the lungs and chemotherapy-induced metastases

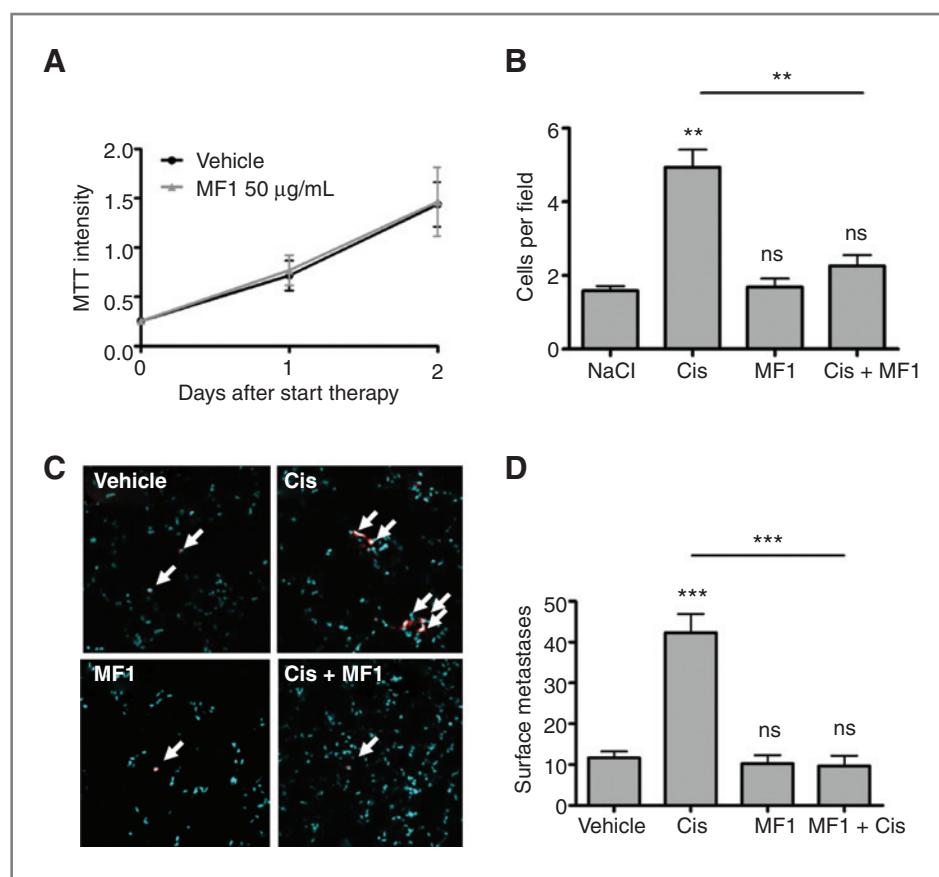
To determine whether tumor cell retention in mouse lungs following chemotherapy exposure could be attributed to VEGFR-1 upregulation on ECs, we obtained neutralizing antibodies targeting mouse VEGFR-1 (Clone MF1, ImClone Systems Inc.). Because we specifically aimed to block host VEGFR-1, we excluded direct effects of these antibodies on the tumor cells. C26 proliferation *in vitro* was not influenced by the addition of MF1 (Fig. 4A) and a single administration of MF1 one day before tumor cell injection in the absence of cisplatin did not diminish the number of lung colonies after 13 days (Fig. 4D, left and third bar). These findings correspond to a recently published study in which continuous MF1 treatment did not change the number of C26 lung colonies after intravenous injection (16).

To test whether MF1 would block early retention of tumor cells in mouse lungs after chemotherapy exposure, we administered chemotherapy to BALB/c mice, followed by MF1 3 days later. One day after MF1 injection, C26-mCh tumor cells were injected intravenously. Cisplatin therapy was again found to significantly enhance the number of C26-mCh cells in the lungs after 24 hours (Fig. 4B and C), while MF1 by itself did not change the number of tumor cells present in the lungs.

However, addition of MF1 to cisplatin completely reversed the chemotherapy-induced tumor cell retention (Fig. 4B,C). To study whether the reduction of pulmonary tumor cell retention at early time points corresponded to an inhibition of the number of surface metastases at later time points, mice were sacrificed 13 days after tumor cell injection. We found that MF1 by itself again did not reduce the number of surface metastases. However, cotreatment of MF1 and chemotherapy was sufficient to prevent the chemotherapy-induced metastases. Mice treated with the combination therapy had as few surface metastases as the untreated control mice (Fig. 4D). This was successfully reproduced in C57Bl/6 mice injected intravenously with B16F10 tumor cells (data not shown), strengthening our finding that VEGFR-1 blockade can specifically reduce the chemotherapy-induced lung colonization by tumor cells.

Furthermore, we tested the specificity of VEGFR-1 in this process. VEGFR-2 blocking antibodies (clone DC101, ImClone Systems Inc.) were administered to mice and their effects on chemotherapy-induced metastasis were determined. We found that antibodies targeting VEGFR-2 did not block cisplatin-induced pulmonary metastasis in C57Bl/6 mice injected intravenously with B16F10 cells (Supplementary Fig. S5A), whereas pulmonary metastasis were in fact blocked by in a second model of BALB/c mice injected intravenously with C26 tumor cells (Supplementary Fig. S5B). The variability of VEGFR-2 effects across models suggests a direct effect of VEGFR-2 on C26 tumor cells rather than a broad effect on

Figure 4. Blocking VEGFR-1 prevents early retention of tumor cells in the lungs and chemotherapy-induced metastases (A) C26 cells were plated and MF1 was added in a concentration of 50 μ g/mL. MTT assays were done on 3 following days to determine the proliferation rate compared with vehicle control. B–D, mice were pretreated with cisplatin or vehicle control at day –4. At day –1, MF1 or vehicle was administered. At day 0, C26 cells were administered i.v. For B and C, mouse lungs were perfused, fixed, and sectioned at day 1. After DAPI staining (blue), 300 μ m slides were analyzed for presence of mCh⁺ tumor cells (red) by CLSM. For D, lung colonies were analyzed by counting surface metastases at day 13. Cis, cisplatin; ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.



ECs. Indeed, DC101 therapy by itself—in contrast to MF1 therapy—diminished the number of lung metastases in BALB/C mice injected intravenously with C26 tumor cells (Supplementary Fig. S5B), and C26 proliferation was diminished by DC101 *in vitro* as determined by MTT (Supplementary Fig. S5C). In addition, in contrast to VEGFR-1, we did not find an increase in VEGFR-2 expression following chemotherapy exposure of ECs *in vivo* (Fig. 3A) nor *in vitro* (data not shown). Overall, we conclude that the endothelial cell response to chemotherapy resulting in enhanced pulmonary metastases is specific for VEGFR-1.

Discussion

In addition to the direct effects of chemotherapy on the tumor, a host response is evoked that may interfere with therapy benefits (1–5). Our study stresses the relevance of this host response. We here show that chemotherapy stimulated tumor cell homing after intravenous injection, resulting in an increased number of lung metastases. Chemotherapy exposure elevated VEGFR-1 expression on endothelial cells. Administration of antibodies targeting VEGFR-1 prevented both arrest of tumor cells in lungs and the formation of chemotherapy-induced pulmonary metastases.

In the early 1970s, irradiation of mouse lungs before intravenous injection of tumor cells was shown to enhance lung colony formation (17). A few years later, similar effects were observed after treatment with cyclophosphamide (18, 19). The rise in the number of experimental pulmonary metastases was shown to be dose-dependent and unrelated to blood clotting after therapy. Subsequently, some preliminary mechanisms have been suggested to participate in this phenomenon (20). In 1981, 2 articles by Hanna and colleagues showed a central mechanistic role for NK cells in cyclophosphamide-induced lung metastases (21, 22). However, our study shows that the observed increase in C26 metastases after cisplatin pretreatment does not depend on components of the adaptive immune system, because *Rag2*^{−/−}; *IL2Rγc*^{−/−} mice display a similar metastatic load as compared with immune competent mice. Thus, it is plausible that distinct cytotoxic agents will induce differential host effects.

Using the commonly prescribed chemotherapeutic drugs cisplatin and paclitaxel, our studies now provide insight in enhanced metastasis formation mediated by changes in vascular ECs, more specifically by upregulation of VEGFR-1. Nowadays, the endothelium is recognized as not simply being an inert lining to vessels, but a highly specialized, metabolically active interface between blood and underlying tissues (23). Even though ECs are not very sensitive to cytotoxic agents because they do not avidly divide, patients frequently develop vascular complications, which may result from damage to ECs (24). In this light, previous studies have shown that VEGFR-1 upregulation in ECs occurred upon mechanical denudation (25). Furthermore, when tumor cells were exposed to cisplatin (or related compound oxaliplatin) enhanced VEGFR-1 expression on their membranes was observed, which was mediated by Akt, Src, or MAP kinase signaling (26, 27). Further research will need to clarify

whether the processes occurring in both cell types after chemotherapy exposure are similar.

VEGFR-1 is a receptor tyrosine kinase that can bind VEGF-A, VEGF-B, and PlGF. VEGFR-1 has a 10-fold higher affinity for VEGF than VEGFR-2, but it has a weak kinase activity (28, 29). Even though much remains unknown regarding its functions, VEGFR-1 has been implicated in metastasis formation. In VEGFR-1TK^{−/−} mice, less metastases were observed than in their wild-type littermates, whereas primary tumor growth was not significantly different (30). Moreover, VEGFR-1-expressing hematopoietic progenitor cells have been shown to initiate a premetastatic niche in mouse lungs, providing a permissive environment for tumor cell colonization (12). In our models, we neither detected mobilization of circulating VEGFR-1-expressing hematopoietic (progenitor) cells into the circulation upon cisplatin administration, nor homing of these cells in the lungs. Yet, chemotherapy created a distinctive niche in the pulmonary endothelium, which is characterized by upregulation of VEGFR-1 on ECs and—similar to the premetastatic niche—can be inhibited by blocking VEGFR-1. It will be very interesting to determine whether the observed VEGFR-1 effects are due to inhibition of VEGFR-1 kinase signaling. Therefore, combining chemotherapy with a specific VEGFR-1 receptor tyrosine kinase inhibitor (RTKI) would be an alternative approach. However, VEGFR-1-specific RTKIs are not presently available, and multi-targeting RTKIs such as sunitinib have antitumor effects in C26 and B16 tumor cells, in our hands (unpublished results LD and EV) and in literature (31–33). Furthermore, pretreatment with RTKIs has been reported to enhance intravenous lung metastases (34), which would further complicate these experiments.

Although the functional role of VEGFR-1 in the "chemotherapy-induced niche" remains to be determined, a direct adhesive role for VEGFR-1 seems unlikely. Indeed, we could not block adhesion *in vitro* with antibodies directed at VEGFR-1 (data not shown). This could be due to limitations of the *in vitro* setup, which obviously does not reflect the complexity of our *in vivo* models. However, an indirect role for VEGFR-1 in adhesion is feasible. Alternatively, and perhaps more importantly, VEGFR-1 could also function in tumor cell survival, invasion, or migration after chemotherapy. Previously, it was shown that primary tumors can facilitate lung colony formation after intravenous injection of tumor cells via upregulation of MMP9 in lung ECs, among other cells (30). The enhanced expression of MMP9 was dependent on VEGFR-1 tyrosine kinase activity, since it was not observed in VEGFR-1TK^{−/−} mice. Together, MMP-9 and VEGFR-1 mediated tumor cell invasion into lung tissues (30). Given the very potent *in vivo* effects of MF1 in prevention of metastasis after chemotherapy, this mechanism could contribute to the early retention of tumor cells observed after chemotherapy exposure.

Our model highlights the specific host events that are evoked by chemotherapy, regardless of the presence of a tumor. There are several situations in which this mechanism may play a clinically relevant role. First of all, all patients will experience chemotherapy-mediated host effects. However, those patients with tumors that are refractory to chemotherapy will most likely suffer most from these effects which may lead to early

progression. Second, circulating tumor cells (CTC) can be found in most patients. Chemotherapy-induced adaptation of the microenvironment may facilitate retention of these cells in distant organs, thereby diminishing the treatment outcome. Similarly, during surgery the number of CTCs increases due to manipulation of the tumor. Hence, neoadjuvant chemotherapy may prime the microenvironment in such a way that these circulating tumor cells have a higher chance of forming metastatic foci. In these, and perhaps other relevant clinical situations, VEGFR-1 blockade could potentially lead to an improved treatment outcome for patients.

In summary, we show that endothelial cell changes occurring upon chemotherapy exposure in mice can create an environment favorable for metastasis formation through expression of VEGFR-1. This study provides a novel rationale for the addition of VEGFR-1 targeting agents to current chemotherapy regimens.

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Laura G.M. Daenen, Jeanine M.L. Roodhart, Miranda van Amersfoort, et al.

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