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Chemotherapy enhances metastasis formation via VEGFR-1-expressing endothelial cells

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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 vascular endothelial growth factor 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.
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Introduction

Not all cancer patients treated with chemotherapy show response to treatment. Moreover, a small subset of patients experiences early progression during systemic anti-cancer 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 contain increased levels of several pro-tumorigenic 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 post-chemotherapy, an experimental mouse metastasis model was designed in which the direct, cytotoxic anti-tumor 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 two widely used chemotherapeutic agents enhanced lung metastasis formation. This phenomenon was observed in several mouse strains, injected intravenously (iv) 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
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upregulated by endothelial cells in response to chemotherapy. Finally, systemic administration of antibodies targeting VEGFR-1 reversed the chemotherapy-induced tumor cell retention in the lungs, reducing the number of lung colonies.
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Materials & Methods

Cell Culture
C26 colon carcinoma cells and COS-7 were maintained in DMEM (Lonza, Verviers, Belgium) with 5% FCS, 2 mM 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, Grand Island, NY) 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 (Chicago, IL, USA), 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≥6/group (Charles River (Sulzfeld, Germany) were injected ip with chemotherapy at MTD levels: cisplatin 6 mg/kg, paclitaxel 20 mg/kg (Pharmachemie BV, Haarlem, The Netherlands), or vehicle controls. Four days later, 4x10^4 C26(-mCh) cells were iv injected into the tail veins of BALB/c mice, or 6x10^3 C26 cells into Rag2<sup>−/−</sup>;IL2Rγ<sup>−/−</sup> BALB/c mice (7), or 1x10^5 B16F10 mouse melanoma cells into C57Bl/6 mice. Anti-mouse VEGFR-1 antibody MF1 and anti-mouse VEGFR-2 were kindly provided by ImClone Systems Inc., New York. One day before injection of tumor cells, MF1 (40 mg/kg), DC101 (800 μg/mouse) or a vehicle control was administered ip.

Bioluminescence Imaging
Mice were anesthetized with isoflurane (IsoFlo, Abbott Laboratories Inc., UK) and ip injected with n-luciferin (potassium salt; Biosynth AG, Staad, Switzerland) 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, Paris, France). 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.
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**Lentiviral Transduction**

For C26-mCh, lentiviral particles were produced by seeding $1.2 \times 10^6$ Cos-7 cells onto a 10cm dish and transient transfection using fuGENE-6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany) 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 40µg/ml polybrene. Transduction 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 iv injected into mice that had been pretreated with chemotherapy or vehicle controls. 24 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 DAPI and per lung 10 random three-dimensional fields were evaluated for mCherry-expressing cells on a Zeiss LSM 510 META (40x). 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, Heidelberg, Germany), followed by 488-/647-conjugated secondary antibodies (AlexaFluor, Molecular Probes Inc, Oregon, USA) and DAPI. CLSM evaluation was performed.

**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 (Abidgon, UK), CD31-APC and isotype controls from eBioscience (Vienna, Austria), CD11b-FITC from Miltenyi Biotec (Bergisch Gladbach Germany). Remaining FACS antibodies were obtained from BD Biosciences Pharmingen (Breda, The Netherlands): CXCR4-FITC.
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VEGFR-2-PE, CD45-PerCP, CD117-APC and VCAM-1-FITC. After RBC lysis, analysis was performed on a FACSCalibur II. For in vitro FACS experiments, bEND.3 cells were plated out in 6-well plates. After 24 hrs, 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 3-(4,5dimethylthiazolyl-2)-2,5-diphenyltetrazoleumbromide (MTT) assays (Roche Diagnostics) were performed 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 µM calcein-AM (Molecular Probes, Leiden, The Netherlands) in HBSS. VCAM-1 and ICAM-1 antibodies (BD Pharmingen) were added 1:200. 5 x 10^4 calcein-labeled C26 were added to triplicate wells. After 50 minutes at 37°C, non-adhered 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±SEM. Statistical significance was assessed by Student’s two-tailed t-test. A p-value<0.05 was considered significant and represents significance compared to untreated controls, unless indicated otherwise.
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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 (Figure 1A). In this experimental lung metastasis model, mice were pretreated with chemotherapy, followed by iv tumor cell injection four 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 two commonly used chemotherapeutic agents, paclitaxel or cisplatin, followed by iv injection of C26 mouse colon carcinoma cells, a significantly enhanced number of lung colonies was present after 13 days (Figure 1B). Paclitaxel augmented lung colony formation more than 3-fold, whereas cisplatin gave rise to a 6-fold increase compared to untreated mice. This corresponded to a significant increase in lung weight of these mice for cisplatin-treated animals (Figure 1C). We next performed bioluminescence imaging 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 to 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 iv injected with B16F10 mouse melanoma cells. Consistent with our findings in BALB/c mice, chemotherapy pretreatment resulted in enhanced pulmonary metastasis (Figure 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 performed an identical experiment in Rag2\(^{−/−}\);IL2R\(γ\)c\(^{−/−}\) 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 (Figure 1F). These results indicate that suppression of any of these three components of the adaptive immune system by chemotherapy does not account for the observed effect.
Furthermore, dextran perfusion studies were performed to study the integrity of the lung vasculature. Cisplatin pretreatment did not increase vascular leakage at the time of tumor cell injection (Figure 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 iv injection. To this end, mCherry-expressing C26 clones (C26-mCh) were generated by lentiviral transduction. Puromycin selection yielded a clone that was highly fluorescent (Figure S2A) with a proliferation rate comparable to the original cell line both in vitro (Figure S2B) and in vivo, as determined by the number of lung colonies two weeks after iv injection (Figure S2C). Furthermore, mCherry expression was maintained in lung colonies harvested two weeks after iv injection of C26-mCh cells (Figure 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 iv injection of C26-mCh tumor cells 4 days later. 24 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 (Figure 2A,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

Since 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 (ECs). To test this hypothesis, in vitro adhesion assays were performed. 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 three 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 Mn^{2+}. Neither of these agents had any effect (data
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not shown). Next, we primed the EC monolayer with TNFα or IL-1β, two 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 (Figure 2C). Pretreatment of bEND3 monolayers with paclitaxel showed enhanced tumor cell adhesion as well (Figure 2D). Furthermore, similar results were found when mouse primary lung ECs were pretreated with cisplatin followed by adherence of C26 tumor cells (Figure 2E).

TNFα or IL-1β are commonly used in static adhesion assays since they enhance expression of adhesion proteins VCAM-1 and ICAM-1 on ECs, two 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 (Figure 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 (Figure S3B). Similar results were found when blocking ICAM-1 or VCAM-1 on the endothelium (Figure 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 four 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<sup>hi</sup>CD45<sup>ne</sup> cells. The number of activated vascular cell adhesion molecule 1 (VCAM-1)-expressing ECs was similar in both groups (Figure 3A). However, when studying expression of vascular endothelial growth factor (VEGF)-receptors, a significant increase in VEGFR-1 expression on lung ECs was found, whereas the percentage of VEGFR-2-expressing ECs remained unchanged (Figure 3A). VEGFR-1 expression was only increased in activated, VCAM-1-expressing ECs (Figure 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 performing co-immunofluorescence of CD31 and VEGFR-1. Indeed, a larger percentage of CD31+ ECs co-expressed VEGFR-1 in lungs obtained from mice that had been pretreated with chemotherapy (Figure 3B and 3C).

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 performed using pan-hematopoietic cell marker CD45. Pulmonary levels of VEGFR-1-expressing hematopoietic cells remained unchanged 4 days after chemotherapy (Figure 3D). In addition, no increases of VEGFR-1-expressing myeloid cells (VEGFR-1+CD11b+; Figure S4A), VEGFR-1–expressing hematopoietic progenitor cells (VEGFR-1+CD45+CD117+; Figure S4B), and VEGFR-1-expressing hemangiocytes (15) (VEGFR-1+CD45+CXCR4+; Figure 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 (Figure S4D) nor hemangiocytes (Figure S4E). Together, these experiments confirm that VEGFR-1 upregulation indeed takes place in non-hematopoietic CD31\textsuperscript{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 \( \mu \)M cisplatin for 4 hours. After washing-out of the chemotherapeutic drug, ECs were maintained in culture medium for four 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 (Figure 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 other analyzed organs. In the lungs, on average 12.3\% of ECs was CD31\textsuperscript{high}, comprising over 2.2\% of all cells in the lungs. In liver and spleen, only 0.11\% of all cells is a CD31\textsuperscript{high} EC, whereas in brain this percentage was as low as
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0.05%. These numbers were too low to allow meaningful statistical comparison between controls and chemotherapy pre-treated 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.). Since 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 (Figure 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 (Figure 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 iv injection (16).

In order 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 three days later. One day after MF1 injection, C26-mCh tumor cells were injected iv. Cisplatin therapy was again found to significantly enhance the number of C26-mCh cells in the lungs after 24 hours (Figure 4B,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 (Figure 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, co-treatment 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 (Figure 4D). This was successfully reproduced in C57Bl/6 mice injected iv 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 iv with B16F10 cells (Figure S5A), whereas pulmonary metastasis were in fact blocked by in a second model of BALB/c mice injected iv with C26 tumor cells (Figure 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 ECs. Indeed, DC101 therapy by itself - in contrast to MF1 therapy - diminished the number of lung metastases in BALB/C mice injected iv with C26 tumor cells (Figure S5B), and C26 proliferation was diminished by DC101 in vitro as determined by MTT (Figure S5C). Additionally, in contrast to VEGFR-1, we did not find an increase in VEGFR-2 expression following chemotherapy exposure of ECs in vivo (Figure 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.
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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 iv 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 as well as the formation of chemotherapy-induced pulmonary metastases.

In the early 1970s, irradiation of mouse lungs before iv 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, two papers by Hanna and coworkers demonstrated 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, since \( \text{Rag2}^{-/-};\text{IL2Ryc}^{-/-} \) mice display a similar metastatic load as compared to 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).
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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 ten-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 anti-tumor effects in C26 and B16 tumor cells, in our hands (unpublished results LD and EV) as well as in literature (31-33). Furthermore, pre-treatment with RTKIs has been reported to enhance iv 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 set-up, 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 iv 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
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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 (CTCs) 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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References


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Figure Legends

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 iv. Lung colonies were analyzed 13 days later by (B) counting surface metastases and (C) determining lung weight. (D) Similar experiments were performed with C26-luc cells. After 13 days mice were injected with n-luciferin and BLI was performed. (E) C57Bl/6 mice (n=10 per group) were pretreated with chemotherapy followed by 1x10^5 B16F10 tumor cells iv 4 days later. Pulmonary surface metastases were counted after 13 days. (F) Immune deficient Rag2^-/-;IL2R^-/- 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.

Figure 2. Chemotherapy pretreatment enhances early retention of tumor cells in the lungs.
(A,B) Mice were pretreated with cisplatin or vehicle control. Four days later, C26 cells were administered iv; 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 TNFα (left panel) or IL-1β (right panel). After 4 hrs, calcein-labeled C26 were added. After 50 minutes, non-adherent C26 cells were taken off and wells were washed three times with HBSS containing EGTA and Mg^{2+}. Adherent tumor cells were quantified by a fluorescence plat reader and plotted normalized to the adhesion of tumor cells to vehicle-pretreated endothelium; (D) Similar experiments were performed after bEND.3 pretreatment with paclitaxel and stimulation with TNFα; (E) Cisplatin pretreatment of primary mouse lung ECs enhanced initial binding of tumor cells after stimulation with TNFα; Cis: cisplatin, Tax: paclitaxel; ns, not significant,* p<0.05, ** p<0.01, ***p<0.001.

Figure 3. Chemotherapy enhances VEGFR-1 expression on ECs in vivo and in vitro
(A) Mouse lungs were harvested four days after treatment with cisplatin or vehicle control. Single cell samples were prepared and stained for flow cytometry with antibodies to VCAM-1 (upper left panel), VEGFR-2 (upper middle panel), VEGFR-1 (upper right panel) in all CD45^-negCD31^high ECs.
VEGFR-1 in VCAM-1+ ECs (lower left panel) and in VCAM-1− ECs (lower middle panel). (B,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.

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 μg/ml. MTT assays were performed on 3 following days to determine the proliferation rate compared to vehicle control. (B,C,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 iv. For (B,C) mouse lungs were perfused, fixed and sectioned at day 1. After DAPI staining (blue), 300 μm 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.
Figure 1.

A. 

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<thead>
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<th>0</th>
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Chemotherapy i.p.  
Tumor cells i.v.  
Analysis lung metastases

B. 

![Graph showing surface metastases and statistical significance](image)

C. 

![Graph showing lung weight and statistical significance](image)

D. 

![Image showing Vehicle and Cisplatin treatment](image)

E. 

![Graph showing surface metastases and statistical significance](image)

F. 

![Graph showing surface metastases and statistical significance](image)
Figure 2.
Figure 4.

A. Graph showing MTT intensity over days after start therapy with Vehicle and MF1 50 µg/ml.

B. Bar graph showing cells per field for NaCl, Cis, MF1, Cis + MF1 groups. P-values indicated: **0.0025, 0.0089. ns for Cis + MF1.

C. Images comparing Vehicle, Cis, MF1, and Cis + MF1 groups. Arrows indicate areas of interest.

D. Bar graph showing surface metastases for Vehicle, Cis, MF1, MF1 + Cis groups. P-values indicated: ***p<0.0001, p<0.0001 for Cis + MF1.
Chemotherapy enhances metastasis formation via VEGFR-1-expressing endothelial cells


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