Host response to short-term, single-agent chemotherapy induces matrix metalloproteinase-9 expression and accelerates metastasis in mice

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ABSTRACT

Mounting evidence suggests that bone marrow-derived cells (BMDC) contribute to tumor growth, angiogenesis and metastasis. In acute reactions to cancer therapy, several types of BMDC are rapidly mobilized to home to tumors. While this host reaction to therapy can promote tumor re-growth, its contribution to metastasis has not been explored. To focus only on the effects of chemotherapy on the host, we studied non-tumor bearing mice. Plasma from animals treated with the immunogenic chemotherapy paclitaxel induced angiogenesis, migration and invasion of tumor cells along with host cell colonization. Lesser effects were seen with the non-immunogenic chemotherapy gemcitabine. Conditioned medium from BMDCs and plasma from chemotherapy-treated mice each promoted metastatic properties in tumor cells by inducing MMP9 and EMT. In mice in which Lewis lung carcinoma cells were injected intravenously, treatment with paclitaxel but not gemcitabine or vehicle accelerated metastases in a manner that could be blocked by an MMP9 inhibitor. Moreover, chimeric mice reconstituted with BMDC where MMP9 activity was attenuated did not support accelerated metastasis by carcinoma cells that were pre-treated with chemotherapy before their introduction to host animals. Taken together, our findings illustrate how some chemotherapies can exert pro-metastatic effects that may confound treatment outcomes.
INTRODUCTION

For the last several years, antiangiogenic therapy has emerged as an additional treatment modality in clinical oncology for a number of malignancies that include colorectal (1), non-small cell lung (2), hepatocellular (3), and renal cell (4) cancers. However, the approval of antiangiogenic drugs by the Food and Drug Administration (FDA) has been limited since such drugs sometimes resulted in increased progression-free but not overall survival of patients with certain tumors, e.g., advanced metastatic breast cancer (5). Therefore, doubts have been raised with respect to the overall efficacy of antiangiogenic therapy.

In addition to the local sprouting of tumor microvessels from nearby vessels, endothelial precursor cells (EPCs) were also found to contribute to tumor angiogenesis, by incorporating to the luminal side of the blood vessel wall (6). Although controversies arose with respect to the functional role of EPCs in tumor angiogenesis (7), other types of bone marrow derived cells (BMDC) (8), e.g., hemangiocytes (9, 10), Tie-2 expressing monocytes (11), and myeloid derived suppressor cells (MDSCs) (12), were found to contribute to tumor angiogenesis, by possible paracrine mechanisms.

We recently reported that acute and substantial EPC mobilization occurs in mice that were treated with either vascular disrupting agents (VDAs) or chemotherapy drugs, e.g., paclitaxel (13, 14). These treatments resulted in homing of EPCs to the treated tumors, where they promoted angiogenesis that was accompanied by rapid tumor cell re-population and subsequent tumor re-growth (13, 14). We also demonstrated that the treatment outcome was improved when the mice were treated with an antiangiogenic drug that inhibits EPC mobilization (13, 14). However, an improved outcome was not observed when the antiangiogenic drug was administered prior to chemotherapy such as gemcitabine, which does not affect EPCs mobilization (13). The robust recruitment of EPCs to tumors induced by drugs such as VDAs or paclitaxel may be a consequence, at least in part, of an attempt to repair damage in the tumor vasculature caused by such drugs (15). These results further suggested that antiangiogenic drugs may act as chemosensitizing agents by inhibiting host systemic rebound angiogenesis mediated by bone marrow derived EPCs, in response to cytotoxic drugs treatment (13).

The role of BMDCs in tumors is not only limited to the induction in angiogenesis, but also to metastasis. The metastatic process depends on the interaction between disseminated
tumor cells (termed ‘seeds’) and their specific metastatic microenvironment (termed ‘soil’) (16). Recent evidence suggests that hematopoietic progenitor cells that express the vascular endothelial growth factor receptor-1 (VEGFR1) are crucial for creating the initial microenvironment for metastatic growth in the soil of secondary sites (17). These cells home to distant tumor-specific pre-metastatic sites, where they cluster in order to create ‘pre-metastatic niches’ for tumor cell seeding (17). EPCs have also been reported as being influential for promoting the growth of tumor metastases. Specifically, Gao et al., demonstrated that EPCs are crucial regulators of the ‘angiogenic switch’, which mediates the shift of micrometastases to lethal macrometastases (18). Thus, the formation of metastasis-permissive niches for tumor cell seeding and their growth in other organs depends, at least in part, on several types of BMDCs, and therefore blocking their activity and/or mobilization by targeted drugs may improve treatment outcome.

Numerous molecular factors are also associated with metastasis spread. The initiation of metastases involves the invasion of tumor cells into peripheral tissue, a process which requires the crossing of several physical barriers, such as the endothelial basement membrane. Metaloproteinases (MMP) have been demonstrated to promote metastases in a growing tumor by the proteolysis of the extra cellular matrix (ECM), as well as by activating signaling pathways that are important for tumor cell migration (19). MMP9, like other MMPs, belong to a superfamily of zinc containing proteases, and have been shown to associate with tumorigenesis (19). There is increasing evidence demonstrating that tumor associated macrophages (TAMs), following their invasion into the primary tumor sites, support the motility of metastatic cancer cell extravasation (20). However the role of MMP9 expressed by BMDCs in mice treated with chemotherapy has not been investigated.

In the current study, we hypothesized that chemotherapy may not only increase angiogenesis, but can also accelerate the metastatic process, the latter of which can be modulated by BMDCs, which are acutely mobilized in response to therapy. To test this hypothesis, we used non-tumor bearing mice that were treated with maximum tolerated dose (MTD) of either paclitaxel or gemcitabine chemotherapy, in order to assess the effect of the chemotherapy on the host, and the contribution of the host’s response on tumor and endothelial cell activity. The
results of our study reveal possible new pathways by which acute reaction of the host to some cytotoxic drugs can induce metastasis in mice.

MATERIALS AND METHODS

Animal tumor models and drugs
The use of animals and experimental protocols were approved by the Animal Care and Use Committee of the Technion. Briefly, C57Bl/6 and CB.17 SCID female mice (Harlan, Israel), 8-10 weeks of age, were implanted with Lewis lung carcinoma (LLC) and LM2-4 breast carcinoma cells, respectively. The mice were treated with the following drugs: 50mg/kg paclitaxel, 500mg/kg gemcitabine, or 4mg/kg MMP2-MMP9 inhibitor III. Detailed protocols are available in Supplementary Materials and Methods.

Cells and cell culture
Human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. Neta Ilan (Faculty of Medicine, Technion) and were cultured in gelatin-coated dishes, using a previously described protocol (21). Cells were identified as endothelial cells by the CD31 surface marker. MDA-MB-231, LLC, HT1080, and MCF-7, HEK293-T cell lines were purchased from the American Type Culture Collection (ATCC, USA) and were used within 6 months of resuscitation. LM2-4 tumor cells which were previously established from the MDA-MB-231 cell line, were kindly provided by Prof. Robert Kerbel (Sunnybrook Health Sciences Centre, Toronto, Canada), and were used as previously described (22). All cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS). The SCp-2 murine epithelial cells were kindly provided by Prof. Israel Vlodavsky, Technion, and were cultured according to (23). All cells were passed in culture for no more than 4 months after being thawed from authentic stocks.

Scratch wound assay
The scratch wound assay was performed as previously described (24). Detailed protocols are available in Supplementary Materials and Methods

Modified Boyden chamber assay
The invasion and migration properties of HUVECs and tumor cells were evaluated in either Matrigel- or fibronectin-coated Boyden chambers, using a previously described protocol (25). Detailed protocols are available in Supplementary Materials and Methods.
MMP detection by gelatin zymography
Conditioned medium from BMDCs or MDA-MB-231 cells was analyzed for MMP activity using gelatin zymography (26). Detailed protocols are available in Supplementary Materials and Methods.

Matrigel plug assay
Matrigel (0.5ml) containing 10% plasma from treated C57Bl/6 mice, was injected subcutaneously into each flank of the mice (n=4 mice/group), and the plugs were removed ten days later. Plugs were then prepared for histological and immunohistochemical evaluation, or flow cytometric analysis.

Aortic Ring assay
Aortic ring assay was performed as previously described (10). Detailed protocols are available in Supplementary Materials and Methods.

Single cell suspension
Preparation of a single cell suspension from the Matrigel plugs was performed using a previously described protocol (27).

Flow Cytometry
In order to identify the BMDC types that colonized the Matrigel plugs, single cell suspensions of the cells from the plugs were first prepared, and were then immunostained for flow cytometry analysis as described in Supplementary Materials and Methods.

Immunostaining
The cell types in the Matrigel plugs, the tumor tissues, and the cells from various cultures were identified by immunofluorescence staining as previously described (14, 28). Detailed protocols are available in Supplementary Materials and Methods.

A chimeric mouse model with inhibited MMP9 expression in their BMDCs
Transfection of MMP9 into BMDCs was performed as previously described for Id-1 (18). Detailed protocols are available in Supplementary Materials and Methods.

Statistical analysis
Data are presented as mean ± standard error of the mean (SEM). Statistical significance of differences was assessed by one way ANOVA, followed by Newman-Keuls ad-hoc statistical
test using GraphPad Prism 4 software (La Jolla, CA). Differences between all groups were compared to each other, and were considered significant at values of p<0.05.

RESULTS
The plasma from chemotherapy-treated mice induces endothelial cell invasion, migration and vessel sprouting.
Our previous studies demonstrated that certain types of chemotherapy drugs administered to non-tumor bearing mice can rapidly induce EPC mobilization, in part by the overexpression of numerous factors such as G-CSF and SDF-1α (13, 29). We showed that these effects stimulated angiogenesis in treated tumors and contributed to tumor re-growth. Thus, the host-derived response to chemotherapy, may contribute to tumor angiogenesis and subsequent tumor re-growth (13). Therefore, we further examined the effects of paclitaxel or gemcitabine chemotherapy on the host, and the effect of its response on endothelial cell behavior. To this end, we first treated non-tumor bearing mice with MTDs of either paclitaxel or gemcitabine. After 24 hours, their plasma were collected and then used for assessing endothelial cell migration, invasion, and microvessel sprouting. Plasma from the paclitaxel-treated mice induced a significant increase in migration and invasion of endothelial cells, when compared to that caused by plasma from the vehicle-treated mice. Moreover, plasma from the gemcitabine-treated mice caused a significant increase in endothelial cell migration, but not in endothelial cell migration when compared to plasma from the vehicle-treated mice (Fig.1A-B). Furthermore, we found that plasma from the chemotherapy-treated mice significantly increased microvessel sprouting, when compared to that caused by plasma from vehicle-treated mice as was assessed by aortic ring assay (Fig.1C and Supplementary Fig.1). These results provide evidence that the plasma from paclitaxel- and gemcitabine-treated mice can induce a spectrum of endothelial cell activity following chemotherapy.

The plasma of chemotherapy-treated mice promotes colonization of host cells in Matrigel plugs
In order to further elucidate the effect of chemotherapy on host cells, we implanted Matrigel that contained 10% plasma which has been obtained from chemotherapy-treated mice into the flanks
of C57Bl/6 mice. The plugs were then assessed for the number of host cells that colonized the plugs. The number of host cells, such as the endothelial cells in the Matrigel plugs that contained plasma from the chemotherapy-treated mice was higher than that found in Matrigel plugs that contained plasma from the vehicle-treated mice (Fig.2A-B). We then prepared single cell suspensions from the digested plugs in order to determine the numbers of both hemangiocytes and MDSCs in the implanted plugs, because both are known to contribute to angiogenesis (30, 31). We found that the numbers of both hemangiocytes and MDSCs as well as CD45+BMDCs colonizing the plugs that contained plasma from the chemotherapy-treated mice were significantly greater than those in the plugs that contained plasma of the vehicle-treated mice (Fig.2B). These results indicate that the host reaction to chemotherapy may modulate the tumor microenvironment by recruiting host cells, some of which are hematopoietic cells known to contribute to tumor growth and angiogenesis.

**BMDCs that colonized in chemotherapy-treated tumors express MMP9**

We next asked whether chemotherapy promotes the migration and invasion of BMDCs into tumors. We therefore assessed the migratory properties of BMDCs from chemotherapy-treated mice using the modified Boyden chamber assay. Our results show that the extent of migration of BMDCs from the chemotherapy-treated mice was greater than that of the vehicle-treated mice (Fig.3A).

MMPs have been reported to be important regulators of tumor cell dissemination (19), and promote the invasion of BMDCs into tumors (20, 32). Therefore, we asked whether the host response to chemotherapy may increase secretion and activation of MMPs in BMDCs and/or tumor cells, hence contributing to metastasis. To this end, we determined the levels of MMP9 in conditioned medium of BMDCs from chemotherapy-treated mice, and in conditioned medium of MDA-MB-231 cells pre-exposed to plasma from the chemotherapy-treated mice. The levels of active MMP9 in these two conditioned media were at least twice as high as those in the medium in which BMDCs from the vehicle-treated mice or the MDA-MB-231 cells pre-exposed to plasma from the vehicle-treated mice. The levels of the pro-enzymatic form of MMP9 in conditioned medium of MDA-MB-231 cells pre-exposed to plasma from chemotherapy-treated
mice was more than 10 fold higher than those levels found in MDA-MB-231 cells pre-exposed to plasma from the vehicle-treated mice (Fig.3B-C).

This increase in MMP9 expression in both BMDCs and tumor cells in response to chemotherapy prompted us to investigate whether an increase in MMP9 expression also occurs in the primary tumor and its metastases following chemotherapy. To this end, 500mm³ LLC tumor-bearing mice were treated with either paclitaxel or gemcitabine. Three days later, the mice were sacrificed, and tumors and lungs were removed for determination of the expression of MMP9 in BMDCs that colonized the primary tumor and lungs. A substantial number of MMP9-expressing CD45+BMDCs in the tumor and lungs of the paclitaxel-treated mice, but no in the tumor and lungs of gemcitabine- or vehicle-treated mice was observed (Fig.3D-E and Supplementary Fig.2A-B). Notably, the number of BMDCs that colonized the gemcitabine- or vehicle-treated tumors was lower than the number of BMDCs in paclitaxel-treated tumors, in agreement with our previous publication (13). Collectively, these results suggest that the host response to paclitaxel chemotherapy increases MMP9 secretion by the BMDCs that colonize the primary tumor and/or its metastases.

The plasma from chemotherapy-treated mice induces tumor cell migration and invasion.

Since the plasma from chemotherapy-treated mice induced MMP9 secretion by tumor cells, we posited that chemotherapy can induce tumor cell migration and invasion, and as a result can promote the formation of new metastases. Therefore, we assessed the migratory and invasive properties of MDA-MB-231, LM2-4, LLC, and SCp2 cell lines. Plasma from paclitaxel-treated mice, and to a lesser extent plasma from gemcitabine-treated mice, enhanced the migration and invasion of all tested cells, when compared to that caused by plasma from vehicle-treated mice (Fig.4A-D). An induction in the migration and invasion properties of MDA-MB-231 cells in the presence of plasma obtained from mice 72 hours following chemotherapy was also observed (Supplementary Fig.3). Furthermore, we found that the motility of the MDA-MB-231 cells was greater when these cells were exposed to plasma from paclitaxel-treated mice than that caused by plasma from gemcitabine- or vehicle-treated mice in the scratch wound assay (Fig.4E-F). In order to rule out the possibility that tumor-derived effects following chemotherapy can contribute to the migration and invasion properties of tumor cells in the presence of plasma from
chemotherapy-treated mice, we obtained plasma from 500mm³ LLC-bearing mice 24 hours following paclitaxel or gemcitabine chemotherapy. By using the modified Boyden chamber assay to assess migration and invasion properties of the MDA-MB-231 cells, we found that plasma from chemotherapy-treated LLC-bearing mice induced tumor cell invasion and migration, similarly to plasma obtained from non-tumor bearing mice (Supplementary Fig.4). Collectively, these results indicate that the host reaction to paclitaxel, and to a lesser extent to gemcitabine, can directly promote the migratory and invasive activities of tumor cells, as well as actively causing MMP9 secretion.

**Plasma from paclitaxel-treated mice induces epithelial-to-mesenchymal transition**

The results of recent studies have demonstrated that EMT in tumor cells is essential for cancer metastasis (33, 34). In order to examine this aspect, MCF-7 cells, frequently used for EMT studies (28), were incubated with conditioned medium of BMDCs from chemotherapy-treated mice. Forty-eight hours later, the cells were first fixed and then stained for E-cadherin (an epithelial cell marker) and vimentin (a mesenchymal cell marker) in order to evaluate tumor cells that undergo EMT (28). We found that EMT occurred in MCF-7 cells that were exposed to conditioned medium of BMDCs from the paclitaxel-treated mice, but it did not occur when the cells were exposed to conditioned media of BMDCs from the gemcitabine- and the vehicle-treated mice (Fig.5). Collectively, the results provide additional support for the notion that paclitaxel can induce tumor cells to migrate, invade, and then transiently transform into mesenchymal cells as part of the metastatic process.

**The effect of chemotherapy on metastases in mice**

We next investigated whether chemotherapy can induce metastasis in mice. To test this possibility, C57Bl/6 mice were treated with either paclitaxel or gemcitabine. After 24 hours, mice were intravenously injected with GFP+LLC cells, and 45 days later, the mice were sacrificed and the number of metastatic GFP+LLC cells in the lungs was determined by flow cytometry. In a parallel experiment, the survival of mice in treatment groups was assessed. We found that the number of metastatic lesions and GFP+LLC cells in the lungs of paclitaxel-treated mice were significantly higher than those found in the lungs of gemcitabine- and vehicle-treated
mice (Fig. 6A-C). In addition, the mortality rate of the paclitaxel-treated mice was higher than that of the gemcitabine- and vehicle-treated mice (Fig. 6D). Similar results were obtained in a repeat experiment where we tested whether the host response to chemotherapy was the sole cause of increased metastasis. In this experiment, GFP+LLC cells that had been pre-exposed for 24 hours to plasma from the chemotherapy-treated mice were used (Fig. 6E). Furthermore, in order to investigate whether chemotherapy can accelerate metastases in a more clinically relevant spontaneous metastatic mouse model as well, we used the LM2-4 cells as a model of spontaneous metastases as previously demonstrated (22). Mice bearing metastatic lesions in the lungs were treated with paclitaxel or gemcitabine administered every 21 days for three sequential cycles. The results in Fig. 6F show that as opposed to increased mortality rate found in mice treated with chemotherapy followed by an intravenous injection of LLC cells, mice administered with chemotherapy in multiple cycles in this spontaneous metastasis model revealed increased survival when compared to control vehicle-treated mice. Collectively, the host response to paclitaxel, but not gemcitabine, can accelerate tumor metastasis in short term rapidly administered chemotherapy, but failed to do so in mice bearing spontaneous metastasis following multiple cycles of chemotherapy.

Inhibition of MMP9 can decrease metastasis following paclitaxel treatment.

Our findings that the host response to paclitaxel can increase MMP9 expression in tumor cells and BMDCs, prompted us to further investigate the active involvement of MMPs in metastasis following chemotherapy. We first determined the effect of an MMP inhibitor (MMPi) on the invasion of tumor cells exposed to plasma from chemotherapy-treated mice. We found that the invasion of MDA-MB-231 cells exposed to plasma from the paclitaxel-treated mice was significantly higher than that of MDA-MB-231 cells exposed to plasma from the gemcitabine-treated mice. This invasion was completely abolished by a specific MMP2-MMP9 inhibitor (Fig. 7A). We did not observe changes in the migration properties of MDA-MB-231 cells exposed to plasma from the paclitaxel- or the gemcitabine-treated mice in the presence of the MMPi (data not shown). Next, we investigated the effect of MMPi on metastasis in the paclitaxel-treated mice. First, paclitaxel or vehicle was injected into the mice, and 24 hours later, they were intravenously injected with LLC cells with or without the MMPi. Forty five days later,
we observed that mice from the MMPi monotherapy treatment group succumbed to metastases; however, survival of mice treated with MMPi and paclitaxel was remarkably extended when compared to the mice treated with paclitaxel monotherapy. The remaining mice were sacrificed, and the metastatic lesions in the lungs were counted. The number of metastases in the lungs of the combined paclitaxel and the MMPi treated mice was smaller than that in lungs of the paclitaxel-treated mice (Fig.7B).

An additional experiment to test the effect of inhibiting MMP9 expression in BMDCs on metastasis was performed. MMP9 expression in BMDCs of C57Bl/6 mice was inhibited by 60% by shRNA technique (Supplementary Fig.5). Such BMDCs were transplanted into lethally irradiated mice, and after BMDC reconstitution, mice were injected with LLC cells pre-exposed to plasma from chemotherapy-treated mice. Mouse survival was monitored. The inhibition of MMP9 in BMDCs in mice injected with LLC cells pre-cultured with plasma from paclitaxel-treated mice resulted in extended survival when compared with their control counterparts, which were injected with the same LLC cells (Fig.7C). Notably, the inhibition of MMP9 in BMDCs in mice injected with LLC cells pre-exposed to plasma from vehicle-control mice increased mouse mortality when compared with their control counterparts (Fig.7C). Overall, our results suggest that the inhibition of MMP9 in paclitaxel-treated mice can suppress the metastatic-promoting action of paclitaxel, but reversed therapeutic effects were observed in mice in which MMP9 expression was suppressed in BMDCs after they were injected with tumor cells pre-exposed to plasma from vehicle-treated mice.

**DISCUSSION**

Chemotherapy is one of the most common treatment modalities for cancer, and it has been used in neo-adjuvant, adjuvant and advanced metastatic disease. The results of this study provide insights into the effects of chemotherapy on the host and its reaction on tumor cells. Yamauchi et. al., recently demonstrated that an intravenous injection of HT1080, a human fibrosarcoma cell line which does not usually form metastases, can nevertheless promote them when injected into nude mice which were previously treated with cyclophosphamide chemotherapy (35). We have previously reported that both VDAs and chemotherapy can induce acute mobilization of EPCs in non-tumor bearing mice, therefore suggesting that anti-cancer
drugs can cause host effects which promote angiogenesis and tumor re-growth (13, 14). In this study, we further investigated the effect of plasma from chemotherapy-treated mice on both host and tumor cells. We showed that plasma from chemotherapy-treated mice can induce endothelial cell invasion, migration, and microvessel sprouting. We found that MMP9 is increased in tumors and in BMDCs exposed to plasma from the chemotherapy-treated mice, and demonstrated that conditioned-medium of BMDCs from paclitaxel-treated mice, but not gemcitabine-treated mice induced EMT of MCF-7 cells. All of these tumorigenic effects found shortly after the administration of chemotherapy, markedly increased the mortality rate of mice intravenously injected with tumor cells; however, when chemotherapy was administered in multiple cycles to mice bearing spontaneous metastatic disease, a more clinically relevant tumor model, prolonged survival was actually observed in the chemotherapy-treated groups. Thus, long term chemotherapy may overcome the tumorigenic rapid effects of the host in response to drug administration.

An interesting question which arose from this study deals with the fact that MMP9 inhibition following paclitaxel chemotherapy extended the survival of mice, but the same treatment strategy in vehicle-treated mice resulted in an increased mortality rate. Hiratsuka et.al., demonstrated that MMP9 is induced by VEGFR1, and is expressed in both endothelial cells and macrophages in premetastatic lungs (36). Kaplan et.al., demonstrated that blocking VEGFR1 in pre-metastatic mice can inhibit metastasis growth by disrupting the pre-metastatic niche (17). These studies are not in line with our results in part due to the fact that our experiments were performed on intravenously injected tumor cells, and therefore, pre-metastatic niches were not formed in such mice. Nevertheless, the combination of MMPI and paclitaxel chemotherapy in mice intravenously injected with tumor cells resulted in enhanced survival when compared with mice treated with paclitaxel monotherapy. The results of our study provide additional important evidence that BMDCs can contribute to metastasis, not only in unperturbed tumors, but also following acute cytotoxic drug therapy, despite the myelosuppressive effects commonly found following chemotherapy (37). The suppression of MMP9 expression in BMDCs, using genetic manipulation in a chimeric mouse model, resulted in increased survival of mice following the injection of tumor cells pre-exposed to plasma from chemotherapy-treated mice. Therefore,
blocking or attenuating the activity of BMDCs in the primary tumor and/or its metastases may decrease the potential development of new metastases.

The host-derived factors which can contribute to metastases following chemotherapy were not studied here. It has been demonstrated that tumor cells instigate BMDCs prior to their mobilization to distant micrometastatic sites, and therefore can contribute to metastasis growth (38), in our study we solely tested the effects of chemotherapy which are host-driven with no involvement of tumor cells. Numerous factors have been shown to be induced following therapy, and can therefore promote angiogenesis and metastases (39, 40). We recently demonstrated that a rise in plasma levels of SDF-1α and G-CSF, both of which promote the mobilization of EPCs (41), was observed in certain chemotherapy- and VDA-treated mice (13, 14). Escalating doses of sunitinib, an antiangiogenic receptor tyrosine kinase inhibitor, in non-tumor bearing mice, increased plasma levels of G-CSF, SDF-1α, stem cell factor, and osteopontin (40). Metastases were accelerated in mice which undergo short-term treatment with sunitinib (39). Do all anti-cancer drugs cause tumorigenic effects? It seems that the inhibition of MMP9 expression in paclitaxel-treated mice causes a reduction in metastasis, but this may not be so in combination with other anti-cancer drugs. Collectively, the induction of metastasis may be due, in part, to a rise in numerous host-derived factors following therapy, and therefore necessitates the identification of those metastasis-accelerating factors generated.

In summary, this study was not designed to address the question whether chemotherapy can accelerate metastases. The clinical scenario would suggest that it does not actually do so. However, our results point out on some reasons for the explanation of why anti-cancer drugs may not be as effective as predicted. According to our results it seems that some chemotherapeutic drugs, in addition to their anti-tumor effects, may also induce pro-tumorigenic effects which may apparently result from the host reaction to such a therapy. These negative ‘side effects’ of chemotherapy can promote tumor growth and metastases likely due to an upregulation of tumor-promoting factors. Therefore, adequate combinations of targeted therapies that prevent the occurrence of such responses to chemotherapy in the host may improve treatment outcome.

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Figure Legends

1. Plasma from chemotherapy-treated mice induces angiogenesis. (A) The effect of plasma from chemotherapy-treated mice on the migration and invasion of HUVECs in
the modified Boyden chamber assay, with quantification in (B). (C) Sprouting microvessels in the aortic ring assay (n=3/group), with quantification in Supplementary Fig.1. *, p<0.05, ***, p<0.001.

2. **Host cell colonization of Matrigel plugs containing plasma from chemotherapy-treated mice.** Matrigel that contained 10% plasma from chemotherapy-treated mice, was injected subcutaneously into C57Bl/6 mice (n=4/mice/group). After ten days, plugs were removed, and sections were prepared for (A) hematoxylin and eosin and CD31 immunostaining (per x200 objective-filed), or (B) digested plugs prepared as single cell suspensions for identifying cell types in the plugs using flow cytometry. MDSCs: myeloid-derived suppressor cells. **, p<0.01, ***, p<0.001.

3. **MMP9 expression in BMDC and tumor cells following exposure to chemotherapy.** (A-C) BMDCs from chemotherapy-treated mice (n=5 mice/group) were placed on a modified Boyden chamber to assess invasion (A). The expression levels of pro and active MMP9 were determined by zymography using conditioned medium of either BMDCs obtained from chemotherapy-treated mice (B), or MDA-MB-231 cells pre-exposed to 10% plasma from chemotherapy-treated mice (C). Zymography gels were analyzed by densitometer, and results were plotted as fold increase from the vehicle control. Conditioned medium of HT1080 cells was used as a positive control. Zymography experiments were performed in triplicates. (D-E) The MMP9 expression levels (red) and the CD45+BMDCs (green) that colonized the primary tumor (D) and its lung metastases (E) were determined by immunostaining, per x200 objective-field, with quantification of the positive cells to MMP9 and CD45 in Supplementary Fig.2A-B.

4. **Host-response to chemotherapy accelerates tumor cell invasion and migration.** (A-D) The effect of 10% plasma from the chemotherapy-treated mice on the migration (A) and invasion (C) of MDA-MB-231, LM2-4, LLC, and SCp2 cell lines using the modified Boyden chamber assay and quantification (B and D). (E) The scratch wound
assay was performed using MDA-MB-231 cultured with plasma from the treated mice. (F) The wound area was calculated and plotted. *, p<0.05, **, p<0.01, ***, p<0.001.

5. **Conditioned-medium of BMDCs obtained from treated mice induces epithelial-to-mesenchymal transition of mammary carcinoma cells.** BMDCs from chemotherapy-treated mice were cultured in serum-free medium. After 48 hours the conditioned medium were collected, and was used to evaluate EMT in cultured MCF7 cells. (A) tumor cell morphology (H&E staining, per x200 objective-field), (B) E-cadherin (red), and (C) vimentin (green) expression, per x400 objective-field.

6. **Host-response to chemotherapy accelerates metastasis growth.** (A-D) 8-10 week old C57Bl mice were treated with chemotherapy (n=5 mice/group). After 24 hours, 2.5x10^4 LLC-GFP+ cells were injected intravenously, and 45 days later, the lungs were removed for (A) the evaluation of metastasis lesions in lung sections (tumor cells in green), per x100 objective-field, (B) the percentage of GFP+ cells in lung tissue quantified by flow cytometry, and (C) the number of lesions counted in randomized fields of lung sections (n>15 fields/group). Survival of treated mice (D). (E) The survival of mice intravenously injected with GFP+LLC cells that were exposed for 24 hours to plasma from chemotherapy-treated mice (n=5 mice/group). (F) 6 week old CB.17 SCID mice were implanted in the mammary fad pad with 2x10^6 LM2-4 cells. When tumors reached a size of 250-350mm^3 they were removed, and 3 cycles of MTD chemotherapy was initiated after 48 hours from primary tumor resection (n=5-6 mice/group). The survival of mice from all treated groups is presented. *,p<0.05, **, p<0.01, ***, p<0.001.

7. **MMP inhibitor inhibits paclitaxel-induced metastasis.** (A) The effect of 50µM MMP2-MMP9 inhibitor III (MMPi) on the migration of MDA-MB-231 cells exposed to plasma from chemotherapy-treated mice. (B) The number of LLC lung metastases (per field, n>15 fields/group) in the combined paclitaxel- and MMPi-treated mice after an intravenous injection of 2.5x10^4 GFP+LLC cells, followed by Student t-test statistical analysis. *, p<0.05, **, p<0.01, ***, p<0.001.
Figure 1

A

migration

invasion

Vehicle

Paclitaxel

Gemcitabine

B

Cell migration cells/field

Vehicle Paclitaxel Gemcitabine

Vehicle Paclitaxel Gemcitabine

C

Vehicle Paclitaxel Gemcitabine
Figure 2

A

H&E

CD31

B

Endothelial cells

Hematopoietic (CD45+) cells

Hemangiocytes

MDSCs
Figure 6

A) Vehicle, Paclitaxel, and Gemcitabine images showing cellular distribution.

B) Graph showing % of GFP cells in lungs for Vehicle, Paclitaxel, and Gemcitabine.

C) Graph showing metastasis number/field for Vehicle, Paclitaxel, and Gemcitabine.

D) Survival curves for Vehicle, Paclitaxel, and Gemcitabine.

E) Survival curves for Vehicle, Paclitaxel, and Gemcitabine.

F) Survival curves for Vehicle, Paclitaxel, and Gemcitabine.

Note: Figures D, E, and F are survival curves showing the percentage of survival over days post implantation and days post tumor resection.
Figure 7

A

Cell invasion cells/field

Vehicle Paclitaxel Gemcitabine

- MMPI + MMPI

*** *** ***

B

Number of metastatic lesions/ lung section

Paclitaxel Paclitaxel + MMPI

* *

C

Survival (%)

Vehicle Paclitaxel Vehicle+siMMP9 Paclitaxel+siMMP9

Days post implantation

0 10 20 30 40 50 60 70 80

0 10 20 30 40 50 60 70 80
Host response to short-term, single-agent chemotherapy induces matrix metalloproteinase-9 expression and accelerates metastasis in mice


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