The Highly Metastatic Nature of Uterine Cervical/Endometrial Cancer Displaying Tumor-Related Leukocytosis: Clinical and Preclinical Investigations

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Abstract

Purpose: The aim of this study was to investigate the metastatic potential of uterine cervical and endometrial cancer displaying tumor-related leukocytosis (TRL).

Experimental Design: Clinical data on uterine cervical (N = 732) and endometrial cancer (N = 900) were collected, and the metastatic potential of TRL-positive cancer was evaluated in univariate and multivariate analyses. Tumor and blood samples obtained from patients with cervical cancer, cervical cancer cell lines, and a mouse model of cervical cancer were used to examine the mechanisms underlying the highly metastatic nature of TRL-positive cancer, focusing on tumor-derived G-CSF and the myeloid-derived suppressor cell (MDSC)-mediated premetastatic niche.

Results: Pretreatment TRL was significantly associated with visceral organ metastasis in patients with uterine cervical or endometrial cancer. The patients with TRL-positive cervical cancer displayed upregulated tumor G-CSF expression, elevated G-CSF levels, and increased MDSC frequencies in the peripheral blood compared with the TRL-negative patients. In vitro and in vivo investigations revealed that MDSCs produced in response to tumor-derived G-CSF are involved in premetastatic niche formation, which promotes visceral organ metastasis of TRL-positive cancer. The depletion of MDSCs attenuated this premetastatic niche formation and effectively inhibited the visceral organ metastasis of TRL-positive cancer.

Conclusions: Uterine cervical/endometrial cancer displaying TRL is a distinct clinical entity with high metastatic potential. Tumor-derived G-CSF and the MDSC-mediated premetastatic niche are responsible for the highly metastatic nature of this type of cancer. MDSC-targeting therapy might represent a potential strategy for combating metastasis derived from TRL-positive uterine cancer. Clin Cancer Res; 24(16): 4018–29. ©2018 AACR.

Introduction

Uterine cervical and endometrial cancer are the two most common gynecologic malignancies. In the United States, 12,820 and 61,380 new cases of cervical and endometrial cancer, respectively, and 4,210 and 10,920 deaths due to these diseases were reported in 2016 (1). Metastasis is the primary cause of death in patients with cancer, including those with gynecologic cancer. Patients with visceral organ metastasis are diagnosed with stage IVB disease and are known to have a dismal prognosis, with 5-year survival rates of only 10% to 20% (2–4). Metastasis is a multistep process that involves local tumor cell invasion, the intravasation of the tumor cells into blood vessels, the passage of the cells through the vascular system, their accumulation in capillary beds, and their subsequent extravasation into the organ parenchyma. Moreover, in hostile distant organs, they must escape from host immune surveillance to survive and grow (5).

Many tumors show a predisposition to metastasize to particular organs. In 1889, Stephen Paget published the seminal "seed and soil" hypothesis to explain the nonrandom pattern of metastasis (6): metastasis depends on cross-talk between selected cancer cells (the "seeds") and specific organ microenvironments (the "soil"). In addition to the "seed and soil" hypothesis, recent studies have suggested that "premetastatic niches" might also regulate the process of organ-specific tumor spread. The term "premetastatic niche" refers to a series of changes that occur in metastatic target organs prior to the arrival of metastatic cancer cells. These changes facilitate the invasion, survival, and proliferation of metastatic tumor cells (7).

It has recently been reported that host bone marrow–derived cells (BMDC) that are produced in response to stimulation with tumor-derived chemokines or cytokines play an important role in the metastatic process by forming "premetastatic niches" (8). BMDCs are recruited to metastatic sites before the arrival of tumor cells and create an environment that facilitates the survival of
Tumor-related leukocytosis (TRL) is a paraneoplastic syndrome that is occasionally observed in patients with cancer. Although TRL was found to be associated with worse clinical outcomes, the association between the presence of TRL and the metastatic potential of uterine cervical or endometrial cancer remains unknown. Our results show that TRL is an indicator of visceral organ metastasis in patients with uterine cervical and endometrial cancer. This finding indicates that by performing a simple peripheral blood examination, it might be possible to identify patients who are at greater risk of developing visceral organ metastasis. We also found that tumor-derived G-CSF is responsible for TRL and that MDSC-mediated premetastatic niche formation is involved in the highly metastatic nature of TRL-positive cervical cancer. Moreover, we demonstrated that MDSC inhibition attenuated the formation of premetastatic niche, and prevented cervical cancer metastasis. MDSC-targeting therapy may represent a novel treatment in patients with TRL-positive uterine cancer.

**Materials and Methods**

**Patients and clinical samples**

Permission to proceed with the data acquisition and analysis was obtained from the institutional review board of Osaka University Hospital (Osaka, Japan). A list of patients with uterine cervical cancer (N = 732, April 1996–March 2011) and endometrial cancer (N = 900, April 1996–March 2015) who were treated at Osaka University Hospital was generated from our institutional tumor registry. Patients with coexisting hematologic malignancies, administrations of corticosteroids or recombinant G-CSF, or acute or chronic infection were excluded. No HIV-infected patient was included. Appropriate informed consent was obtained from each patient in accordance with the Declaration of Helsinki, and their clinical data and biopsy tissue specimens and blood samples were examined.

**Definitions of leukocytosis and neutrophilia**

During the period between the initial presentation of the disease and the first day of treatment, all patients underwent at least two blood tests, including complete blood counts, and the lowest neutrophil counts obtained during these tests were used in the current analyses. Pretreatment leukocytosis was defined as the detection of a leukocyte count of $\geq$9,000/μL. Pretreatment neutrophilia was defined as the detection of persistent neutrophil counts of $\geq$7,200/μL on at least two separate occasions, as reported previously (14, 17).

**ELISA**

The G-CSF concentrations of the serum samples were analyzed via an ELISA using the Quantikine assay system for human G-CSF (R&D Systems; #DCS50), according to the manufacturer’s protocol.

**Reagents/antibodies**

The following labeled mAbs were used for the staining experiments: anti-human antibodies: V450-conjugated anti-CD33 (BD Biosciences; #561157) and allophycocyanin-conjugated anti-human leukocyte antigen (HLA)-DR (BioLegend; #307609); mouse antibodies: phycoerythrin-conjugated anti-Gr-1 (R&D Systems; #NCL-L-CD33). The surrounding nonneoplastic stroma served as an internal negative control for each slide, as reported previously (15, 16). Optical images were captured using PROVIS AX80 (Olympus).

**Flow cytometry**

Single-cell suspensions were prepared from mouse blood or lung tissue. Red blood cells were removed using ammonium chloride potassium (ACK) lysis buffer. To prepare human samples, peripheral blood mononuclear cells were separated by gradient centrifugation using Lymphoprep (Axis-Shield; #1114544). Then, the cells were filtered through 40-μm nylon strainers, incubated with antibodies, and analyzed by flow cytometry. Flow cytometric data were acquired on a FACSCanto II flow cytometer and analyzed using the FACSDiva software (BD Biosciences). Cells that had been incubated with irrelevant
isotype-matched antibodies and unstained cells served as controls.

Cell culture
A human cervical tumor cell line, ME180, was purchased from the ATCC. The cells were passaged in our laboratory soon after they were received from the cell bank, before being divided and stocked in liquid nitrogen vessels. Cells were passaged for less than 3 months after resurrection, and were regularly authenticated by examination of morphology and growth characteristics. Cells were routinely screened for mycoplasma species (EZ-PCR Mycoplasma Test Kit; Biological Industries; #20-700-20).

Each experiment was carried out using thawed cells without further authentication. The cells were maintained in DMEM supplemented with 10% FBS and cultured in 5% carbon dioxide at 37°C.

Clone selection
The expression vector for the mouse G-CSF gene (pCAM-G-CSF) and the empty vector (pCAZ) were used in this study, which were described previously (15). The resultant total RNA (1 µg) was used to synthesize cDNA with ReverTra Ace qPCR RT Master Mix (Toyobo; #FSQ-201). PCR was performed using TaqMan PCR master mix (Qiagen; #201443) and specific primers. The PCR primers were purchased from Life Technologies. The sequences of the primers used as follows: mouse glyceraldehyde 3-phosphate dehydrogenase (Gapdh); forward primer, 5′-TTAGCCCCCCTGGGCAA-AGG-3′ and reverse primer, 5′-CTTACCTCTTGAGGGCCATG-3′; mouse S100a8: forward primer, 5′-TCGTCAGATGCGCTGC- AA-3′ and reverse primer, 5′-GACATATCCAGGGACCCACG-3′; mouse S100a9: forward primer, 5′-AGATGGCCCAAACAGGACCTT-3′ and reverse primer, 5′-TAGACCTTTGTTGGGAGCTG-3′; mouse Bv8: forward primer, 5′-AGAGGAAAGGAGGGTTC-3′ and reverse primer, 5′-GCTTCCAGGGCAATGTCTG-3′; human β-actin: forward primer, 5′-CGTACATATTAGGAGAGCTTG-3′ and reverse primer, 5′-GTCCTAGGGAGGACAGTG-3′; human C-X-C chemokine receptor type 2 (CXCR2): forward primer, 5′-ATTCTGGGATCCCTTCAG-3′ and reverse primer, 5′-TCGACATTTGGCGAGGTCT-3′. Amplification was performed using a Takara PCR personal-type thermal cycler (Takara).

Quantitative real-time reverse transcriptase PCR
The qRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems; #4309155) and TaqMan probes on a StepOnePlus sequence detection system (Applied Biosystems). The PCR primers for Gapdh, Cxcl2, S100a8, S100a9, Mmp-9, and Bv8 were designed using Primer3 plus software and were purchased from Eurofins Operon. The sequences of the primers used as follows: mouse Gapdh: forward primer, 5′-TGCGATGGTGATAAAGTGG-3′ and reverse primer, 5′-ATTCTGGGATCCCTTCAG-3′; mouse S100a8: forward primer, 5′-TGGATGTTGTAAAAGGCTGG-3′ and reverse primer, 5′-GGGCAAGAAGGCTGCTGCT-3′; mouse S100a9: forward primer, 5′-ACAGGGAGGAGCTTG-3′ and reverse primer, 5′-AAATGGCCGATAGTCCAGAA-3′. The following TaqMan probes were used: mouse Gapdh (#4352339E), mouse Cxcl2 (#Mm00445553_m1), and mouse Mmp-9 (#Mm00442991_m1). Relative mRNA expression levels were determined using the ΔΔCt relative quantification method.

Isolation of pulmonary MDSCs
The lungs of mice with ME180-GCSF cell-derived or ME180-control cell-derived tumors were perfused with PBS via the ventricle under anesthesia (induced with a mixture of medetomidine, midazolam, and butorphanol; refs. 20, 21). The excised lungs were minced and digested with neutral protease (Roche; #492078001), collagenase (Wako; #034-22563), and DNase 1 (Sigma-Aldrich; #D4527). Single-cell suspensions were prepared via the mechanical dissociation of the lung tissue, which was achieved by passing it through a 40-μm nylon mesh. Red blood cells were lysed with ACK lysis buffer. Pulmonary MDSCs were finally isolated from single-cell preparations of mouse lung cells using an MDSC Isolation Kit (mouse) and a mass spectrometry column (Milltenyi Biorate; #130-042-201), according to the manufacturer's instructions (15). They were subjected to flow cytometry analysis and RNA analysis.

Recurrent transscriptase PCR
RNA was extracted from the cells using TRizol (Life Technologies; #15596018). The resultant total RNA (1 µg) was used to synthesize cDNA with ReverTra Ace qPCR RT Master Mix (Toyobo; #FSQ-201). PCR was performed using TaqMan PCR master mix (Qiagen; #201443) and specific primers. The PCR primers were purchased from Life Technologies. The sequences of the primers used as follows: mouse glyceraldehyde 3-phosphate dehydrogenase (Gapdh); forward primer, 5′-TTAGCCCCCCTGGGCAA-AGG-3′ and reverse primer, 5′-CTTACCTCTTGAGGGCCATG-3′; mouse S100a8: forward primer, 5′-TCGTCAGATGCGCTGC- AA-3′ and reverse primer, 5′-GACATATCCAGGGACCCACG-3′; mouse S100a9: forward primer, 5′-AGATGGCCCAAACAGGACCTT-3′ and reverse primer, 5′-TAGACCTTTGTTGGGAGCTG-3′; mouse Bv8: forward primer, 5′-AGAGGAAAGGAGGGTTC-3′ and reverse primer, 5′-GCTTCCAGGGCAATGTCTG-3′; human β-actin: forward primer, 5′-CGTACATATTAGGAGAGCTTG-3′ and reverse primer, 5′-GTCCTAGGGAGGACAGTG-3′; human C-X-C chemokine receptor type 2 (CXCR2): forward primer, 5′-ATTCTGGGATCCCTTCAG-3′ and reverse primer, 5′-TCGACATTTGGCGAGGTCT-3′. Amplification was performed using a Takara PCR personal-type thermal cycler (Takara).

Histologic sections were cut through each tissue, which was achieved by passing it through a 40-μm nylon mesh. Red blood cells were lysed with ACK lysis buffer. Pulmonary MDSCs were finally isolated from single-cell preparations of mouse lung cells using an MDSC Isolation Kit (mouse) and a mass spectrometry column (Milltenyi Biorate; #130-042-201), according to the manufacturer's instructions (15). They were subjected to flow cytometry analysis and RNA analysis.

Reverse transcriptase PCR
RNA was extracted from the cells using TRizol (Life Technologies; #15596018). The resultant total RNA (1 µg) was used to synthesize cDNA with ReverTra Ace qPCR RT Master Mix (Toyobo; #FSQ-201). PCR was performed using TaqMan PCR master mix (Qiagen; #201443) and specific primers. The PCR primers were purchased from Life Technologies. The sequences of the primers used as follows: mouse glyceraldehyde 3-phosphate dehydrogenase (Gapdh); forward primer, 5′-TTAGCCCCCCTGGGCAA-AGG-3′ and reverse primer, 5′-CTTACCTCTTGAGGGCCATG-3′; mouse S100a8: forward primer, 5′-TCGTCAGATGCGCTGC- AA-3′ and reverse primer, 5′-GACATATCCAGGGACCCACG-3′; mouse S100a9: forward primer, 5′-AGATGGCCCAAACAGGACCTT-3′ and reverse primer, 5′-TAGACCTTTGTTGGGAGCTG-3′; mouse Bv8: forward primer, 5′-AGAGGAAAGGAGGGTTC-3′ and reverse primer, 5′-GCTTCCAGGGCAATGTCTG-3′; human β-actin: forward primer, 5′-CGTACATATTAGGAGAGCTTG-3′ and reverse primer, 5′-GTCCTAGGGAGGACAGTG-3′; human C-X-C chemokine receptor type 2 (CXCR2): forward primer, 5′-ATTCTGGGATCCCTTCAG-3′ and reverse primer, 5′-TCGACATTTGGCGAGGTCT-3′. Amplification was performed using a Takara PCR personal-type thermal cycler (Takara).
Gelatin zymography
Serum-free conditioned medium was collected from the pulmonary MDSC culture. Gelatin zymography was performed as described previously (23).

Chemotaxis assay
Cancer cells (5 × 10^4/well) were seeded in the top chamber of a 24-well plate. The top chamber contained an 8-μm pore membrane, which had been precoated with fibronectin (Corning; #356008). The indicated concentrations of recombinant mouse CXCL2 were placed in the bottom chamber of the 24-well plate as a chemoattractant. After 24 hours' incubation, the number of cells that had migrated was quantitated by counting the mean number of cells in three randomly chosen areas per well (magnification: ×200).

Statistical analysis
Continuous data were compared between groups using the Student t-test or the Welch t-test or the Wilcoxon rank-sum test. Frequency counts and proportions were compared between groups using the χ^2 test or Fisher exact test, as appropriate. Differences were considered statistically significant at P < 0.05. All analyses were conducted using SAS version 12.0 for Windows (SAS Institute Inc.).

Results
Highly metastatic nature of uterine cancer involving pretreatment leukocytosis
A total of 1,632 patients with uterine cancer (732 patients with cervical cancer and 900 patients with endometrial cancer) were included in this study. The clinicopathologic characteristics of these patients are summarized in Supplementary Table S1. Visceral organ metastasis was observed in 31 (4.2%) and 33 (3.7%) patients with cervical and endometrial cancer, respectively. The lungs and liver were the most common and second most common sites of distant metastasis.

Using the patients' clinical data, we next investigated the metastatic potential of TRL-positive uterine cancers. Pretreatment TRL (leukocyte ≥9,000/μL) was observed in 13.5% (99 of 732) and 8.4% (76 of 900) of patients with cervical and endometrial cancer, respectively (Supplementary Table S1). Among the patients with cervical cancer, visceral organ metastasis was detected in 12.1% (12 of 99) of the patients who displayed pretreatment TRL, which was a significantly higher frequency than the 3.0% (19 of 633) seen in the patients without pretreatment TRL (P < 0.0001; Fig. 1A, i). As expected, the patients with visceral organ metastasis exhibited significantly shorter OS than those without it (Fig. 1B, i). In the multivariate analysis (Table 1), pretreatment TRL was found to be an independent predictor of visceral organ metastasis (OR 3.40; 95% CI, 1.35–8.60; P = 0.0097). Collectively, these results suggest that TRL-positive uterine cervical and endometrial cancer exhibit greater propensity to develop metastases than TRL-negative cancers.

Cancer cell–derived G-CSF as a possible stimulator of metastasis in patients with cervical cancer
To investigate the mechanism responsible for the highly metastatic nature of TRL-positive cancer, we next examined the patients' neutrophil counts. Significantly increased neutrophil counts were observed in the patients with TRL-positive cervical cancer and endometrial cancer (Fig. 1C). Moreover, the presence of pretreatment neutrophilia was significantly associated with visceral organ metastasis (Fig. 1A, ii). In the multivariate analysis (Table 1), pretreatment neutrophilia remained an independent predictor of distant metastasis in both cervical and endometrial cancer. On the basis of these clinical findings, we considered that hematopoietic growth factors, such as G-CSF, might be responsible for the highly metastatic nature of TRL-positive uterine cancers. Then, using biopsy samples obtained at the initial diagnosis, we examined G-CSF expression in cervical cancer according to the patient's neutrophil count. As shown in Fig. 1D (i), greater G-CSF immunoreactivity was observed in the cervical tumors from neutrophilia-positive patients than in those from neutrophilia-negative patients. Consistent with this finding, the neutrophilia-positive patients (neutrophils ≥7,200/μL) exhibited significantly greater serum G-CSF concentrations than the neutrophilia-negative patients (P < 0.0001; Fig. 1D, ii). Similar results were obtained from patients with endometrial cancer (Supplementary Fig. S1; P = 0.0051).

Accumulation of MDSCs in premetastatic lungs in a mouse model of G-CSF–expressing cervical cancer
To investigate the role of tumor-derived G-CSF in the metastasis of TRL-positive uterine cancer, we used a mouse model of G-CSF–expressing cervical cancer; that is, nude mice that had been subcutaneously inoculated with ME180 cervical cancer cells, which had been stably transfected with a G-CSF–expressing vector (ME180-GCSF) or a control vector (ME180-control), as reported previously (15, 16). The expression of G-CSF in the ME180-GCSF cells was verified by immunostaining (Fig. 2A, i). We also confirmed that the mouse model of G-CSF–expressing cervical cancer displayed increased peripheral white blood cell (WBC) and granulocyte counts (Fig. 2A, ii).

Using this model, we investigated the effects of tumor-derived G-CSF on premetastatic lung tissue. Before the in vivo study, we first confirmed that the ME180-GCSF cells and ME180-control cells exhibited equivalent in vitro invasiveness using an invasion assay (Supplementary Fig. S2) Then, to investigate the effects of tumor-derived G-CSF on premetastatic lung tissue, we subcutaneously inoculated mice with ME180-GCSF or ME180-control cells. No pulmonary metastases were observed at 3 weeks after the mice had been subcutaneously inoculated with ME180-GCSF or ME180-control cells (Supplementary Fig. S3). However, interestingly, markedly increased numbers of CD11b^+Gr-1^+ cells were detected in the premetastatic lungs of the ME180-GCSF cell–derived tumor-bearing mice than in the lungs of the ME180-control cell–derived tumor-bearing mice (Fig. 2B). Similar results were obtained in the IHC analysis (Fig. 2C, i). Increased numbers of CD11b^Gr-1^+ cells were also seen in the peripheral blood (Fig. 2B) and premetastatic livers (Supplementary Fig. S4) of the
Figure 1.
Metastatic potential of uterine cancers displaying TRL. A, Association with visceral organ metastasis and pretreatment leukocytosis/neutrophilia in patients with uterine cancer. (i) Visceral organ metastasis was significantly more frequently observed in the patients with cervical cancer ($P < 0.0001$) and patients with endometrial cancer ($P = 0.0072$) with leukocytosis than those without leukocytosis. (ii) Visceral organ metastasis was significantly more frequently observed in the patients with cervical cancer ($P < 0.0001$) and patients with endometrial cancer ($P = 0.0002$) with neutrophilia than those without neutrophilia. **, $P < 0.01$, according to the $\chi^2$ tests. B, Kaplan–Meier estimates of survival based on the presence/absence of visceral organ metastasis. (i) The patients with cervical cancer ($P < 0.0001$) and (ii) patients with endometrial cancer ($P < 0.0001$) with visceral organ metastasis exhibited significantly shorter survival than those without visceral organ metastasis, according to the log-rank test. C, Association between the patients’ neutrophil count and WBC count in cervical/endometrial cancer. Histograms are shown. Scale bars, mean ± SD; **, $P < 0.01$, according to the Wilcoxon rank-sum test. D, (i) G-CSF expression in cervical cancer based on the neutrophil count. Cervical cancer biopsy samples, which were obtained from patients with or without neutrophilia, were stained with anti-G-CSF antibody. Representative photographs of tumors are shown. Scale bars, 500 μm (left), 100 μm (right). (ii) The serum G-CSF concentrations of patients with newly diagnosed cervical cancer according to their neutrophil counts (neutrophils: $\geq$7,200/μL, $n = 19$; neutrophils: $<7,200/\mu$L, $n = 38$; **, $P < 0.01$, according to the Wilcoxon rank-sum test).
Table 1. Univariate and multivariate logistic analyses of prognostic factors for visceral organ metastasis of patients with cervical carcinome and endometrial cancer.

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<th>Multivariate analysisb</th>
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<td>OR (95% CI)</td>
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Endometrioid cancer

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Abbreviations: EA, endometrioid adenocarcinoma; Neu, neutrophil; SCC, squamous cell carcinoma.

* A multivariate analysis in which WBC count was not included as a prognostic variable.

* A multivariate analysis in which Neu count was not included as a prognostic variable.

ME180-GCSF cell–derived tumor-bearing mice. When surface marker expression was examined further, a marked enrichment of the Ly6G+ polymorphonuclear (PMN) MDSC population (also known as granulocytic MDSC) was observed in the premetastatic lungs of ME180-GCSF cell–derived tumor-bearing mice (Supplementary Fig. S5), indicating that these PMN-MDSCs were the dominant subset that was expanded by the tumor-derived G-CSF in this experimental model. Consistent with this finding, the H&E staining of the lungs of the ME180-GCSF cell–derived tumor-bearing mice contained markedly higher numbers of neutrophil-like cells than the lungs of the ME180-control cell–derived tumor-bearing mice (Fig. 2C(ii)). To confirm that the CD11b+Gr-1+ cells exhibited immunosuppressive activity, a T-cell suppression assay was conducted. As shown, the Gr-1+ cells, which usually express CD11b, isolated from the lungs of the ME180-G-CSF cell–derived tumor-bearing mice significantly inhibited T-cell proliferation (Supplementary Fig. S6), indicating that the CD11b+Gr-1+ cells were MDSCs.

We next determined whether the findings obtained in mice are representative of the clinical status of patients with cervical/ endometrial cancer. The peripheral blood of the neutrophilia-positive patients contained significantly higher numbers of MDSCs (Fig. 2D). Moreover, as shown in Supplementary Fig. S7, primary tumor samples obtained from patients with neutrophilia-positive cervical/endometrial cancer with visceral metastasis exhibited strong G-CSF immunoreactivity and contained increased CD33-positive cells (indicative of human MDSCs). Collectively, these results from mice and human materials indicated that tumor-derived G-CSF induces the production of MDSCs from the bone marrow, leading to the recruitment of MDSCs into the premetastatic organs of tumor-bearing mice.

MDSC-mediated formation of premetastatic niches in a mouse model of G-CSF–expressing cervical cancer

Given the increased numbers of MDSCs seen in the premetastatic lungs, we established the "premetastatic niche hypothesis" for TRL-positive uterine cancer (Fig. 3A). To test this hypothesis, we first investigated the MDSC-mediated environmental changes in the premetastatic lungs. The lung tissues of the ME180-GCSF cell–derived tumor-bearing mice expressed significantly higher levels of proinflammatory chemokin attractants (Cxc2, S100a8, S100a9, and B8) than those of the ME180-control cell–derived tumor-bearing mice (Fig. 3B). In addition, the lung tissues of the ME180-GCSF cell–derived tumor-bearing mice expressed significantly higher levels of MMP-9 than those of the ME180-control cell–derived tumor-bearing mice (Fig. 3B).

We next determined whether pulmonary MDSCs express these factors. The MDSCs isolated from the lungs of the ME180-GCSF cell–derived tumor-bearing mice expressed high levels of Cxc2, S100a8, S100a9, and B8 mRNA (Fig. 3C(i) and D(i)). The MDSC isolated from the lungs of the ME180-GCSF cell–derived tumor-bearing mice also secreted the matrix-degrading enzyme MMP-9 (Fig. 3C, ii). Importantly, in an in vitro chemotaxis assay, CXC2 attracted ME180 cells that expressed CXCR2 (Fig. 3D(ii) and D (iii)). Collectively, these results strongly indicate that MDSCs contribute to the formation of premetastatic niches by generating an immunosuppressive, proinflammatory, and proangiogenic environment in premetastatic lungs and attract cancer cells from primary tumors, which might lead to increased visceral organ metastasis.

Role of MDSC-mediated premetastatic niche formation in the pulmonary metastases of cervical cancer cells

We next investigated whether MDSC-mediated environmental changes lead to increased pulmonary metastases. As shown in Fig. 4A (i), 2 to 3 weeks after the premetastatic niche formation, we injected ME180-GCSF cells into the tail veins of the mice. At 4 weeks after the tail vein injections, pulmonary metastases were more frequently observed in the ME180-GCSF cell–derived tumor-bearing mice than in the ME180-control cell–derived tumor-bearing mice (Fig. 4A, ii). To demonstrate whether MDSC-mediated premetastatic niche formation facilitates the pulmonary metastases of cervical cancer, we inhibited MDSC formation in the same experimental models. Treating the mice with anti-Gr-1 antibody for a total of 4 weeks significantly reduced the numbers of MDSCs in the premetastatic lungs and peripheral...
Increased MDSC accumulation in the premetastatic lungs of TRL-positive uterine cancer. **A,** Mouse models of uterine cancer displaying TRL and neutrophilia. Mice were subcutaneously inoculated with ME180-GCSF or ME180-control cells (seven mice per group). Two to 3 weeks after the inoculation procedure, their subcutaneous tumors and blood were collected for analysis. (i) G-CSF expression in the subcutaneous tumors. Scale bar, 50 μm. (ii) WBC/granulocyte counts. Scale bars, mean ± SD; **, P < 0.01, according to the Welch t test (three mice per group). **B,** (i) and (ii) CD11b+Gr-1+ cell populations detected in the lungs and blood. Mice were inoculated with ME180-GCSF or ME180-control cells (seven mice per group). Two to 3 weeks after the inoculation procedure, their blood and perfused lungs were collected and assayed by flow cytometry for MDSCs. Bars, mean ± SD; **, P < 0.01, according to the Wilcoxon rank-sum test or the Student t test. **C,** Representative images of lung sections from mice. Pulmonary MDSCs were stained with (i) anti-Gr-1 antibody and (ii) H&E. Scale bars, 500 μm (top), 100 μm (bottom). **D,** Circulating MDSC counts of the patients with cervical cancer according to their neutrophil counts (neutrophils: ≥7,200/μL, n = 12; neutrophils: <7,200/μL, n = 32). Human MDSCs were defined as CD11b+CD33+HLA-DR- cells. (i) Representative flow cytometry plots. The frequency of CD11b+CD33+HLA-DR- cells is indicated. (ii) Quantification of human MDSC (*, P < 0.05, according to the Wilcoxon rank-sum test).
Figure 3.
The role of G-CSF–induced MDSC-mediated premetastatic niche formation in the pulmonary metastases of cervical cancer. A, Proposed premetastatic niche mechanism in TRL-positive uterine cancer. B, The roles of tumor-derived G-CSF in the formation of the premetastatic niche. Three weeks after their subcutaneous inoculation with ME180-GCSF or ME180-control cells, the mice were killed and perfused with PBS. Pulmonary Cxcl2, S100a8, S100a9, Bv8, and Mmp-9 expression was evaluated by qRT-PCR (five mice per group). Data are presented as relative expression levels compared with Gapdh expression. Bars, mean ± SD; **, P < 0.01 and *, P < 0.05, according to the Welch t test or the Wilcoxon rank-sum test. C, The role of pulmonary MDSCs in the expression of proinflammatory factors. Three weeks after their subcutaneous inoculation with ME180-GCSF cells, the mice were perfused with PBS. Then, MDSCs were collected from their lungs. (i) S100a8, S100a9, and Bv8 mRNA expression in pulmonary MDSCs as evaluated by RT-PCR. (ii) MMP-9 activity of pulmonary MDSCs as assessed by gelatin zymography. Medium alone was used as a negative control. D, CXCL2-CXCR2 axis–dependent mobilization of cancer cells toward MDSCs. (i) CXCL2 expression in the pulmonary MDSCs. Three weeks after their subcutaneous inoculation with ME180-GCSF cells, the mice were perfused with PBS. MDSCs were collected from their lungs, and the expression of CXCL2 in the MDSCs was evaluated by qRT-PCR. (ii) CXCR2 expression in the ME180 cells as evaluated by RT-PCR. (iii) The chemotactic migration of ME180-GCSF cells toward CXCL2. Chemotaxis was assessed by counting the number of cells that migrated (n = 4). Scale bars, mean ± SD. **, P < 0.01, according to the Student t test.
blood of the ME180-GCSF cell-derived tumor-bearing mice (Fig. 4B). Moreover, the anti-Gr-1 antibody significantly attenuated the expression of Cxcl2, S100a8, S100a9, Bv8, and Mmp-9 in the premetastatic lungs of the ME180-GCSF cell-derived tumor-bearing mice (Fig. 4C). These results indicate that inhibiting MDSC formation using the anti-Gr-1 antibody effectively prevented premetastatic niche development in the premetastatic lungs of the ME180-GCSF cell-derived tumor-bearing mice. Importantly, when the anti-Gr-1 antibody was administered in combination with the tail vein injection of ME180-GCSF cells [Fig. 4(D)(i)], the pulmonary metastases of ME180-GCSF cells were significantly inhibited (Fig. 4D, ii and iii). Taken together, these results suggest that the MDSC that are produced in response to stimulation with tumor-derived G-CSF are involved in premetastatic niche formation and enhance the metastatic potential of TRL-positive uterine cancer.

Discussion

The predictors of distant metastasis in patients with uterine cervical and endometrial cancer have been investigated previously. In cervical cancer, tumor size, histologic subtype, lymphovascular space involvement (LVSI), deep stromal invasion, and nodal metastasis were reported to be significant predictors of distant metastasis (24). In endometrial cancer, histologic subtype, the grade of the primary tumor, LVSI, myometrial invasion, nodal metastasis, pretreatment thrombocytosis, and the pretreatment serum cancer antigen 125 level were reported to be significant predictors of distant metastasis (25). However, the ability of these conventional risk factors to predict distant metastasis is insufficient.

In this study, we have shown, for the first time, that uterine cancer involving pretreatment TRL or neutrophilia is a distinct entity with a highly metastatic nature. Although the current study focused on visceral metastasis, the presence of pretreatment TRL was also associated with lymph node metastasis in our patients: among the 99 patients with TRL-positive cervical cancer, 56 (57%) had lymph node metastasis, which is significantly higher than 30% observed in patients with TRL-negative cervical cancer ($P < 0.0001$ for Pearson test). Similarly, among the 76 patients with TRL-positive endometrial cancer, 18 (24%) had lymph node metastasis, which is higher than 12% observed in patients with TRL-negative endometrial cancer ($P = 0.0043$ for Pearson test). This clinical finding has important clinical implications, as it indicates that it might be possible to identify patients who are at greater risk of developing metastasis by performing simple and low-cost peripheral blood examinations. Thus, for these patients, a careful pretreatment work-up involving imaging techniques should be performed to detect visceral organ metastasis. Moreover, as subclinical metastasis might be missed during the pretreatment work-up, careful posttreatment follow-up is recommended.

Considering the clinical findings that TRL was associated with increased metastasis in both patients with cervical and endometrial cancer, it is highly possible that there is a common mechanism that promotes metastasis in TRL-positive cervical/ endometrial cancers. In this study, we have shown that tumor-derived G-CSF and MDSC-mediated premetastatic niche formation are responsible for the highly metastatic nature of TRL-positive uterine cancer. MDSCs that infiltrated into premetastatic lung tissue were found to express S100A8, S100A9, Bv8, and Mmp-9. These findings are in line with previous studies in which MDSCs were shown to be involved in premetastatic niche formation (26–29). Mmp-9 produced by MDSCs has been shown to increase the bioavailability of VEGF, promote tumor angiogenesis, and increase tumor cell extravasation and metastatic nodule formation in vivo (28). Moreover, it has been reported that MDSCs secrete S100A8 and S100A9 to enhance the migration and homing of tumor cells (26). As MDSCs express receptors for S100A8/A9, S100A8/9 attracts MDSCs, and the level of S100A8/A9 within the tumor microenvironment is further upregulated via an autocrine loop (26). Bv8 (also known as prokineticin 2 or Prok2) is reported to stimulate the mobilization of MDSCs to the peripheral blood and act as an inducer of tumor angiogenesis (29). In addition to the aforementioned findings, we showed that MDSCs that infiltrated into premetastatic lungs expressed Cxcl2, which attracted ME180 cells that expressed the chemokine receptor CXCR2. Taken together, these results suggest that MDSC increase the metastatic potential of uterine cancer cells by creating a premetastatic niche, that is, a proinflammatory, proangiogenic, and immunosuppressive environment that facilitates tumor cell metastasis.

The findings obtained from our mechanistic investigations could also have important clinical implications. According to our investigations, TRL-positive patients accounted for 14% and 8.4% of patients with newly diagnosed cervical and endometrial cancer, respectively. This clinical finding might play roles in the metastatic process in significant numbers of patients with uterine cervical and endometrial cancer. We have previously shown that MDSCs promote the tumor...
growth or attenuate the therapeutic efficacy of chemotherapy or radiotherapy by suppressing CD8+ T cells or stimulating tumor angiogenesis in TRL-positive cervical cancer (15, 16). Collectively, these results indicate that MDSC play various roles in TRL-positive uterine cancers, and that MDSC-targeting therapy might represent a novel treatment. In this study, we have shown that the inhibition of MDSC formation attenuated premetastatic niche formation and prevented cancer cell metastasis. Thus, we consider that the results of this study provide a scientific rationale for future clinical trials of MDSC-targeting therapy in patients with TRL-positive uterine cancer.

The G-CSF has been widely used clinically during the course of chemotherapy for a variety of cancers to reduce the risk of chemotherapy-induced neutropenia and enable patients to take a maximally effective dose of cytotoxic agents. According to previous investigations, the exogenous G-CSF treatment does not have a negative impact on the survival of patients with colorectal (30), lung (31), or ovarian cancer (32) that are receiving chemotherapy. However, the impact of G-CSF treatment on the survival or the progression of patients with cervical cancer has never been investigated. Moreover, this study indicates that prolonged exposure to a high level of G-CSF may enhance cancer cell metastasis in some circumstances, especially in cases with TRL. Thus, additional clinical studies are needed to evaluate the risk of exogenous G-CSF treatment in patients with TRL-positive or TRL-negative cervical cancer. Until the result comes out, cautious use of G-CSF is recommended.

The limitations of our study need to be addressed. The first is that we used nude mice in this study, as the inoculation of human uterine cancer cells into immunocompetent mice did not result in the development of primary/metastatic tumors. The second is that the mechanism by which the expression of G-CSF is regulated in uterine cancer remains unknown. In a preclinical study of breast cancer, it has been shown that carbonic anhydrase 9 (CAIX)-mediated NF-κB activity is indispensable for G-CSF production in breast cancer cells and that CAIX-mediated G-CSF production is required for the mobilization of granulocyte-like MDSCs from the bone marrow to a pulmonary premetastatic niche (22, 33). Thus, the mechanisms responsible for G-CSF expression in uterine cancer need to be investigated in the future. The third is that we used an anti-Gr-1 antibody to deplete the MDSCs in this study. However, we cannot rule out the possibility that the anti-Gr-1 antibody also affects other cells, such as neutrophils. In addition, due to the absence of a Gr-1 homolog in humans, anti-Gr-1 antibody cannot be used in the clinical setting. Although the specific inhibitor of human MDSCs has not been developed, a variety of clinically feasible strategies to inhibit MDSCs have been identified, and some of the agents [e.g., phosphodiesterase 5 (PDE-5) inhibitors and antagonists of the CXCR2 receptor] are currently under clinical investigations in patients with cancer (34). Hopefully, the next couple of years will bring exciting positive clinical data regarding MDSC-targeting therapies. The fourth is that although we linked the neutrophils to MDSCs in patients with cervical endometrial cancer in this study, we have to recognize that we cannot attribute entire population of increased neutrophils to MDSCs. Thus, the role of other subpopulation of neutrophils in TRL-positive or G-CSF–expressing uterine cancers needs to be investigated in the future. The last is the monotreatment and retrospective nature of our clinical study. A collaborative multi-institutional investigation, preferably in a prospective setting, needs to be conducted to validate our clinical findings.

In conclusion, we have demonstrated that uterine cancer involving TRL or neutrophilia is a distinct clinical entity with a highly metastatic nature. Tumor-derived G-CSF and MDSC-mediated premetastatic niche formation are responsible for the highly metastatic nature of this type of cancer. Novel treatments that selectively target MDSCs represent a potential strategy for combating metastasis from TRL-positive uterine cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Metastatic Nature of Uterine Cancers with Leukocytosis

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