Interleukin-17A Modulates Circulating Tumor Cells in Tumor Draining Vein of Colorectal Cancers and Affects Metastases

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Abstract

**Purpose:** Metastasis is the major cause of death in patients with colorectal cancer (CRC). Circulating tumor cells (CTC) are believed to cause metastasis and serve as a prognostic marker for mortality in clinical stage IV patients. However, most studies are conducted in late-stage cases when distant metastases have already occurred; thus, such results provide limited clinical use. This study focused on whether CTCs can predict the risk of metastasis after treatment of the primary tumor in early-stage patients with CRC.

**Experimental Design:** CTCs were quantified using EpCAM-positive/CD45-negative immunoselection and flow cytometry in patients with CRC. A mouse model was used to investigate the mechanistic roles of CTCs and interleukin (IL)-17A in metastasis.

**Results:** The number of mesenteric CTCs obtained from stage II patients was higher than that obtained from patients in stages I, III, and IV. In addition, following invasion of orthotopically implanted tumors in our mouse model, we found that CTCs exhibited an increase-then-decrease pattern, accompanied by corresponding changes in serum IL-17A levels and opposing changes in serum granulocyte macrophage colony-stimulating factor (GM-CSF) levels. Ablation of IL-17A and administration of rGM-CSF effectively suppressed the increase in CTCs and prevented metastasis in mice. Moreover, IL-17A promoted cancer cell motility, matrix digestion, and angiogenesis, whereas GM-CSF stimulated the elimination of CTCs by boosting host immunity. Notably, serum levels of IL-17A were also correlated with disease-free survival in patients with CRC.

**Conclusions:** Our results showed that CTCs and IL-17A could serve as prognostic markers and therapeutic targets for CRC metastasis.

Clin Cancer Res; 20(11); 2885–97. ©2014 AACR.

Introduction

Colorectal carcinoma (CRC) is the second most fatal cancer and a major health problem worldwide (1). Although the primary CRC tumor can be readily removed by surgical resection, up to 50% of patients with CRC suffer relapse and eventually die of metastatic disease (2), making cancer metastasis the most frequent cause of CRC-related death. Among all patients with CRC, the clinical courses of stage II patients are the most complex, and the prognoses of these patients are less predictable than those of patients with other stages of CRC. Most patients with stage II CRC, who have neither regional lymph node involvement nor distant metastases, have a 5-year survival rate of approximately 80% (3). However, a subset of stage II patients suffers from very early metastasis and premature mortality (4, 5). Therefore, it is critical to develop an understanding of the mechanisms mediating the rapid disease progression that occurs in these patients.

Metastasis is a multistep process caused by dissemination of malignant cells from the primary tumor (6). When the tumor starts to invade, cancer cells undergo a process called intravasation; the resulting circulating tumor cells (CTC) travel through the bloodstream or lymphatic system and form a metastatic nodule at a distant site (7, 8). The probability of developing distant metastases is thought to be correlated with the number of CTCs in the blood. However, existing reports in the literature are varied and some are conflicting (9–12). Notably, most studies addressing the roles of CTCs in metastasis were conducted in stage IV patients who had already developed distant metastases.
Translational Relevance

Metastasis, the major cause of cancer-related deaths, is promoted by the hematogenous spread of circulating tumor cells (CTCs). CTCs play a prognostic role in patients with stage IV colorectal cancer (CRC); however, the prognostic value of CTCs in early-stage patients remains uncertain. Here, we reported that the number of mesenteric CTCs obtained from stage II patients was higher than those obtained for patients with stages I, III, or IV CRC. In addition, a mouse model demonstrated that CTCs exhibited an increase-then-decrease profile during tumor metastasis. This pattern of CTC dynamics was associated with a corresponding change in serum interleukin (IL)-17A and an opposing change in serum granulocyte macrophage colony-stimulating factor (GM-CSF). Ablation of IL-17A and administration of rGM-CSF effectively diminished the increase in CTCs and prevented metastasis in mice. Thus, IL-17A could serve as a prognostic marker for disease-free survival in patients with CRC and may be a valuable target for the development of novel therapies.

Materials and Methods

Study patients

This study enrolled 172 patients with CRC who underwent potentially curative surgical resection at Taipei Veterans General Hospital between July 2007 and June 2010. Detection and measurement of CTCs were performed during surgery. The enrollment procedures followed the protocols approved by the Internal Review Board of Taipei Veterans General Hospital. All patients provided written informed consent. The follow-up end point was December 31, 2012. The diagnosis of CRC was confirmed in all patients by endoscopic biopsy, and primary tumor staging was confirmed by histologic examination of the resected primary tumor. Adjuvant therapy was administered according to NCCN guidelines. The doctors participating in this study did not know the results of circulating tumor cell measurements when treating the patients. Prognostic assessments were performed retrospectively by analyzing a set of ~5-year clinical follow-up data.

All patients were followed up in the outpatient department every 3 months for the first 2 years, 6 months for the third and fourth years, and annually thereafter. Each follow-up visit included the following examinations: serum CEA, CA-199 levels, chest X-ray, and abdominal sonography. Abdominal/pelvis or chest computed tomography scanning was scheduled yearly or performed whenever a recurrence was suspected.

Patient blood sampling

Sampling of blood from the antecubital veins of patients with CRC and lung cancer was conducted before surgery. During surgery, mesenteric venous blood samples were drawn from the main drainage vein of the diseased segment of the colon, for example, the inferior mesenteric vein for cancer of the sigmoid colon or rectum or the ileocolic vein if the tumor was located on the right side of the colon. To minimize the possibility of releasing CTCs by mechanical manipulation, colonoscopy was scheduled at least 1 day before the surgery. The surgical approach sought vascular control first, that is, ligation of the feeding artery at the beginning, followed by mesenteric vein cannulation and blood drawing. The tumor was left untouched until late in the surgery.

Patient blood analyses

Ten-milliliter blood samples were collected into 10-mL EDTA-containing vacationer tubes and subjected to buffy coat isolation by Ficoll-Paque PLUS gradient (Amersham Biosciences) according to the manufacturer’s protocol (25). Mononucleated cells were stained with phycoerythrin-cyanine7 (PE-Cy7)-conjugated anti-CD45 antibodies (Abs) and fluorescein isothiocyanate (FITC)-conjugated anti-CD326 [epithelium-specific antigen (ESA)] Abs (BD Biosciences) before flow cytometry analysis. The highly conserved hematopoietic marker CD45 was used as an exclusionary marker to eliminate leukocytes from subsequent analysis (26). ESA was used as a positive selection marker for CTCs (27). ESA-positive and CD45-

(13–17). These studies provide no direct evidence regarding whether having higher numbers of CTCs predisposes patients to metastasis.

In addition, in most previous studies, the number of CTCs was calculated from blood drawn by venipuncture of the forearm. These measurements typically ranged from 0 to 8.1 cells/mL of blood (10, 18), which is very close to the limit of detection of current technologies. Although animal experiments have demonstrated the ability of a few stem cells or tumor-initiating cells to give rise to a metastatic lesion (19, 20), there is still no evidence that this event occurs in human colon carcinogenesis/metastasis. Furthermore, the very low number of CTCs found in the peripheral blood poses a technical challenge to the acquisition of accurate measurements, which is crucial for assays used in clinical practice and for interlaboratory comparisons of CTC data.

To quantify CTCs arising from gastrointestinal cancers, several previous studies have demonstrated that collection from the mesenteric veins yields higher detection rates and actual CTC counts than that from the peripheral (forearm vein) blood or central venous blood (21–24). We reason that because the mesenteric vein is the direct venous drainage of the primary CRC tumor, CTCs quantified from the blood of the mesenteric vein rather than the forearm vein would more precisely reflect the level of cancer cell intra-vasation to the blood circulation and could therefore better serve as a risk factor for predicting CRC patients’ risk of developing distant metastases, especially in patients in the early clinical stages of CRC whose primary tumors have not yet metastasized. Therefore, in this study, we investigated this hypothesis in a cohort of 172 patients with CRC in various stages of CRC.
based leukocyte-negative immunoselection was used to quantify CTCs. FACS data were analyzed using Kaluza software (Beckman Coulter).

Sera from the sampled blood were subjected to cytokine analyses using the human cytokine 27-Plex Panel Kit (Bio-Rad) with the Bio-Plex suspension array system (Bio-Rad) in accordance with the manufacturer’s protocol. Briefly, serum samples or known concentrations of standard samples were added to a mixture of Ab-conjugated beads and detector Ab-PE reagent. Fluorescent signals were read using a Luminex Machine (Bio-Rad). The analyte concentration was calculated using software provided by the manufacturer.

Cell culture
CT-26 mouse colon cells were engineered to express firefly luciferase and enhanced green fluorescent protein (EGFP). Cultured cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% FBS (Life Technologies). Cells were maintained in a humidified atmosphere at 37°C with 5% CO2.

Mouse cancer metastasis model
All procedures for mouse experiments were approved by the Ethical Committee of Animal Experimentation of National Yang-Ming University, Taipei, Taiwan. Six-week-old male BALB/c mice were purchased from the National Laboratory Animal Center. Mice were housed under specific pathogen-free, temperature-controlled conditions. Before implantation, mice were anesthetized with an intraperitoneal injection of pentobarbital (70 mg/kg) diluted in sterile saline. Mice were implanted, mice were anesthetized with an intraperitoneal injection of pentobarbital (70 mg/kg) diluted in sterilized water. A 1-cm longitudinal incision was made with a scalpel in the skin of the mouse’s left abdomen to expose the cecum. Then, 2.5 × 104 murine CT-26 colon cancer cells resuspended in 25 µL of HBSS (Invitrogen) and mixed with 25 µL of Matrigel (BD Bioscience) were injected into the submucosal layer of the cecum using a 29-gauge needle (28). Inoculation of 25 µL of Matrigel mixed with 25 µL HBSS was used as a control for the experiment.

Two days after inoculation, mice were anesthetized with isoflurane. Luciferin solution was then injected intraperitoneally (150 mg/kg). The mice were imaged using an in vivo imaging system (IVIS) Spectrum system (Caliper Life Sciences; IVIS) to confirm the success of cancer implantation. Mice that received leaky injections, which occurred in less than 5% of the experiments, were excluded from the study. Mice that received successful injections were monitored for tumor growth and metastasis under IVIS, and blood was sampled periodically on the days indicated. All mice were sacrificed on day 35 for internal organ dissection and microscopic examination of the tissues.

Mouse blood sampling and analyses
On days 4, 7, 11, 14, 18, 21, 25, 28, 32, and 35 after intracecal cancer implantation, mice anesthetized with pentobarbital (70 mg/kg) were subjected to retro-orbital blood sampling. Erythrocytes were removed using erythrocyte-lysis buffer, and nucleated cells were then incubated on ice for 30 minutes with a PE-Cy7–conjugated anti-CD45 Ab (BD Biosciences). FACS data were analyzed using Kaluza software (Beckman Coulter). We analyzed 8 or 10 mice in each group.

Sera from the sampled blood were subjected to cytokine analysis using a MILLIPLEX Mouse Cytokine/Chemokine Kit according to the manufacturer’s protocol (Millipore). Briefly, serum samples or known concentrations of standard samples were added to a mixture of Ab-conjugated beads and detector Ab-PE reagent. Fluorescent signals were read by using a Luminex xMAP device (Millipore). The analyte concentration was calculated using software provided by the manufacturer.

Interleukin-17A depletion and recombinant GM-CSF protein treatment
Ablation of interleukin (IL)-17A was performed using intraperitoneal injection of 100 µg of anti-IL-17A monoclonal Abs (MAB421; R&D Systems) or 100 µg of isotype control immunoglobulin G2Ab (MAB006; R&D Systems) on day 8. Mice were then treated on days 12, 15, 19, 22, 26, 29, and 33. For treatment with recombinant murine granulocyte macrophage colony-stimulating factor (rGM-CSF), mice were subjected to intraperitoneal injection with 5 mg/kg rGM-CSF protein (415-ML; R&D Systems) or the same amount of a control PBS solution on the days indicated.

Transwell assay
An in vitro cancer cell invasion assay was performed using 24-well BioCoat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer’s instructions. Membranes with 8-µm pores were used. In total, 5 × 104 CT-26 cells were added to the upper compartment of each well. The lower compartment was filled with standard cell culture medium with or without recombinant murine IL-17A (rIL-17A; 100 ng/mL). Cells were allowed to invade for 24 hours, after which the chambers were fixed for 10 minutes in 4% paraformaldehyde. After removal of the contents of the upper membrane surface, invasive cells were determined by counting the 4',6-diamidino-2-phenylindole (DAPI)-stained cells from 5 randomly selected microscopic visual fields. Each sample was assayed in duplicate, and 4 independent assays were performed. Mean values and SDs from the means were calculated from 4 independent experiments.

Immunohistochemical staining and quantification
Paraffin-embedded, formalin-fixed tissue sections (3-µm thickness) were deparaffinized and rehydrated. For antigen retrieval, slides were immersed in Trilogy (Cell Marque Corp.) and boiled for 30 minutes in a pressure cooker. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide (H2O2) for 30 minutes. Non-specific binding was blocked by incubation with 1% FBS and 5% BSA in PBS for 30 minutes. The slides were incubated with Abs against human IL-17A (R&D Systems), human CD4, RORc, and TCRγδ (BioLegend), or mouse CD3, inducible nitric oxide synthase (iNOS), and Arg-1 (Abcam) at 4°C overnight in a moist chamber.

www.aacrjournals.org  Clin Cancer Res; 20(11) June 1, 2014 2887

Published OnlineFirst March 27, 2014; DOI: 10.1158/1078-0432.CCR-13-2162
Diaminobenzidine tetrahydrochloride was used as the substrate, followed by counterstaining with hematoxylin. The stained slides were scanned using a Scanscope CS (Aperio Technologies).

Intumtral microvessel density was quantified according to the methods described by Weidner and colleagues (29). For each tissue section, neovascularization was defined by positive CD31 staining (brown) that had discrete microvessel patterns; the total area of neovascularization in one microscopic field was calculated. The mean neovascularization area of 4 randomly selected microscopic fields was quantified as the intumtral microvessel density for the tissue section examined.

The M1/M2 ratio was calculated as the ratio of staining intensity of iNOS to Arg-1 in the immediately adjacent slides of serial sections. iNOS and Arg-1 expression was quantified by calculating the number of positive staining signals (brown).

To quantify IL-17A staining in human tissue sections, a scoring system with the following features was applied: staining intensity (I, graded as 0, 1, 2, and 3 for no, mild, moderate, and strong signals, respectively) and distribution (D, graded as 0%–100% of the positively stained area per microscopic field). The composite Immunohistochemical (IHC) score was the product of D × I. All quantifications were performed using Definiens software (Aperio).

**Western blotting analysis and histology**

In total, 1 × 10⁶ CT-26 cells were exposed to increasing concentrations (0, 10, 50, and 100 ng/mL) of IL-17A for 12 hours. The total cell lysates were harvested with cell lysis buffer (Cell Signaling Technology). Western blotting analyses were performed by the standard protocol using antibodies against matrix metalloproteinase (MMP) 9 (Abcam) and β-actin (Biolegend). Histochemical analysis was performed using 3-μm paraffin-embedded sections of tumor xenografts. Hematoxylin and eosin staining was performed according to the manufacturer’s instructions (Dako).

**Flow cytometry analysis**

For flow cytometry analysis, 5 × 10⁵ erythrocyte-free cells were preincubated in PBS and stained for 30 minutes at 4°C using saturating concentrations of the following antibodies: FITC-conjugated anti-CD31 Abs, PE-conjugated anti-CD4 Abs, allophycocyanin (APC)-conjugated anti-CD8a Abs, PE-conjugated anti-CD49f (DX5) Abs (BD Biosciences), and PE-conjugated anti-IL-17RA Abs (eBiosciences). Cell viability was assessed by 7-AAD exclusion (BD Biosciences). FACS data were analyzed using Kaluza software (Beckman Coulter).

**Quantitative real-time PCR**

Total RNA was extracted using TRIzol Reagent (Life Technologies), and reverse transcription was performed using an SuperScript III First-Strand Synthesis System (Life Technologies) according the manufacturer’s instructions. Gene expression was quantified by real-time quantitative PCR using a SYBR Green PCR Kit and an StepOne Real-Time PCR System (Life Technologies). The primer sequences were as follows: hil-17a forward 5'-AACCGATTCACCTCACCCGTTG-3', hil-17a reverse 5'-TCTCTTCTGCGATGGGGAGCA-3', hmmmp9 forward 5'-GATGGGCGAGGGAAAGTGC-3', hmmmp9 reverse 5'-CCCAAGGCG ACTGCCGAGG-3', hVEGF-A forward 5'-CTGCGCTGCTGCACCC-3', hVEGF-A reverse 5'-CACACAGTGAGCTTGGAAG-3', mIL-17A forward 5'-CATCTCAGTGACACTGAGGACG-3', mIL-17A reverse 5'-TCTGTGCTGGAAGTGCTCC-3', mmmp9 forward 5'-ATCCTCAACTAGACTGAAGAGACG-3', mmmp9 reverse 5'-ACCTGAATGCTAAAGTGACGTCA-3', gapdh forward 5'-GGTATCCTGAGGAGACGAG-3', gapdh reverse 5'-ATGCCAGTCGCTCCTCGTGTCG-3'. All reactions were performed in triplicate.

**Quantification of serum IL-17A and grouping**

Serum IL-17A levels were determined using a human cytokine 27-Plex Panel Kit (Bio-Rad) with the Bio-Plex suspension array system (Bio-Rad) in accordance with the manufacturer's protocol. Serum IL-17A levels ranged from 0 to 27.7 pg/mL (6.3 ± 0.8 pg/mL). The optimum cut-off point was defined as the value that maximized the area under the receiver operating characteristic curve.

**Statistical analyses**

All data were statistically analyzed with SPSS software (v19.0) and GraphPad Prism (v5.0). Differences with P < 0.05 were considered statistically significant. The distributions of continuous variables were described as median values and ranges. The Mann–Whitney U test and the Wilcoxon-signed ranks test were performed to evaluate the differences between groups, as appropriate. Correlations between variables were determined by 2-tailed Pearson correlation test. Survival was estimated by the Kaplan–Meier method and was compared by the log-rank test. Receiver operating characteristic curve analysis was performed using MadCalc (v12.7).

**Results**

**Mesenteric CTCs increase and then decrease as CRC advances**

CTCs were defined as mononucleated cells with a surface immunophenotype of CD45dim/− and ESA+ (30, 31). We found that the number of CTCs detected in the blood of the inferior mesenteric vein (mesenteric CTCs) was higher than that of CTCs sampled from the forearm vein (forearm CTCs) in the same patient (Supplementary Fig. S1A). The number of forearm CTCs was positively correlated with the number of mesenteric CTCs (Supplementary Fig. S1B), and in individual patients, higher numbers of forearm CTCs were also associated with higher numbers of mesenteric CTCs (Supplementary Fig. S1C). In most cases, the number of mesenteric CTCs was 10 to 100 times higher than that of forearm CTCs. In total, 172 patients were recruited for this study; their clinical and pathologic characteristics are summarized in
Supplementary Table S1. The percentage of CTCs in the total mononucleated cell population ranged from 0% to 0.815%. When quantifying CTCs as a function of clinical stage, we noted that the average number of mesenteric CTCs was higher in stage II patients than in patients in stages I, III, and IV (Fig. 1A). This increasing-then-decreasing pattern from stages I to IV was not evident when forearm CTCs were analyzed because the number of cells was very low (Supplementary Fig. S1D). However, in patients with lung cancer, CTCs collected from the forearm vein, to which primary lung cancer cells drain directly, also exhibited a similar pattern, that is, high in stage II and low in stages III and IV (Supplementary Fig. S2, see Discussion).

CTC changes in a mouse CRC metastasis model

A mouse model was established to study the role of CTCs in cancer invasion and metastasis (28, 32, 33). Murine syngeneic CT-26 colon cancer cells labeled with EGFP and firefly luciferase were orthotopically implanted into the submucosal layer of the mouse cecum (Fig. 1B). Tumor growth at the inoculation site and metastasized nodules were monitored by IVIS. On day 2, the injected tumor cells were visible as a single spot under IVIS (Fig. 1C). Unsuccessful injection resulted in peritoneal seeding and was observed as multiple IVIS spots (Fig. 1C, left); these data were excluded. Bioluminescence images were comparable to the exploratory dissection findings (Fig. 1D). In addition to the inoculated tumor, metastatic nodules were noted on
day 15 in the colon away from the cecum and in the liver. On day 35, the mice were sacrificed and subjected to macroscopic dissection and microscopic examination (Fig. 1E); 85% (11/13) had metastatic nodules in the colon and 40% (5/13) had liver metastases.

Analysis of retro-orbital blood showed that the CTC population accounted for 0.009% of cells on day 4, and this proportion increased to 0.147% on day 14, decreased to 0.012% on day 28, and decreased further to 0.008% on day 35. Cumulative results from 10 independent experiments (Fig. 1G) also reflected such dynamics in CTCs; an increase was observed over the first 14 days, followed by a decrease during days 15 to 35.

**Changes in serum cytokines correlate with CTC dynamics**

To explore the potential relationship between changes in serum cytokine levels and CTC dynamics, we examined various serum cytokines on days 4, 7, 14, 18, 21, and 25. We found that the occurrence of metastasis did not correlate with tumor size at the implantation site (Fig. 2A). In fact, tumor volume was slightly larger in mice without metastases than in those with metastases (Fig. 2B), indicating that metastasis did not result from implanted tumor burden. Among the 32 cytokines examined, we found that IL-17A exhibited dynamics, including a transient increase, which correlated with the increase and decrease in the number of
CTCs in mice with metastases (Fig. 2C, red traces). In contrast, IL-17A and CTCs levels remained low in mice without metastases (Fig. 2C, blue traces). Serum levels of IL-17A were positively correlated with the number of CTCs (Fig. 2D). We also found that \( \text{il-17a} \) expression within the tumor at the implantation site and drainage lymph nodes increased more in mice with metastases than in mice without metastases by day 15 (Fig. 2E and F; \( P = 0.057 \) and 0.0057, respectively). Collectively, IL-17A levels were positively correlated CTC dynamics in mouse model.

In contrast, GM-CSF levels were low at first but gradually increased after day 14 in mice without metastases, whereas they stayed low in mice with metastases (Supplementary Fig. S3A). Therefore, serum GM-CSF levels were negatively correlated with the number of CTCs (Supplementary Fig. S3B). Dynamic changes in monokine induced by INF-\( \gamma \), KC (mouse homolog of IL-8), and IL-4 did not correlate with the appearance of CTCs or with the occurrence of metastasis (Supplementary Fig. S4A–S4F).

Ablation of IL-17A or administration of GM-CSF reduces the increase in CTCs and prevents metastasis

The corresponding changes in cytokine and CTC levels suggested that IL-17A and GM-CSF modulate the increase and decrease in CTCs. To test this hypothesis, we treated mice with neutralizing antibodies against IL-17A (nIL-17A Abs) or rGM-CSF (Fig. 3 and Supplementary Fig. S5). Isotype antibodies and PBS were used as controls for the nIL-17A Ab and rGM-CSF treatments, respectively. Both nIL-17A Abs and rGM-CSF effectively eliminated the increase in CTCs (Fig. 3B and Supplementary Fig. S5A) and then greatly reduced the number and volume of metastatic nodules in the colon and liver on day 35 (Fig. 3C and Supplementary Fig. S5B). Cumulative data from 8 or 10
independent experiments are shown in Fig. 3D–F. In the IL-17A–depleted group, only 10% (1/10) of mice had metastatic nodules in the colon and none had liver metastases; in the control mice, 87% (7/8) had colon metastases and 38% (3/8) had liver metastases. After rGM-CSF treatment, the prevalence of colon metastases decreased from 90% (9/10) in the control group to only 30% (3/10) in the treatment group, and that of liver metastases decreased from 40% (4/10) in the control group to 10% (1/10) in the treatment group (Supplementary Fig. S5C–S5E).

IL-17A promotes intravasation of cancer cells

Both protumor and antitumor effects have been attributed to IL-17A (34, 35). Recent studies have shown that IL-17A stimulates tumor progression and metastasis in several tumor types (36–38). Although most CT-26 cells contain IL-17A receptor α (IL-17RA; ref. 39 and Supplementary Fig. S6), treating these cells with murine IL-17A did not influence cell proliferation in vitro (data not shown). To determine the impact of IL-17A on CTCs, we performed real-time quantitative PCR to determine mmp-9 and mmp-2 expression in the IL-17A–depleted group and the control group. The IL-17A–depleted group presented reduced expression of mmp-9 and mmp-2 relative to the control group ($P = 0.021$ and 0.11, respectively; Supplementary Fig. S7). We then determined whether IL-17A increased cancer cell intravasation by promoting angiogenesis (40) through IHC staining of CD31, a microvasculature marker (41). Intratumoral microvasculature density (Fig. 4A) and the frequency of CD31$^+$ cells within the primary tumor (Fig. 4B) were profoundly reduced by IL-17A depletion relative to the control ($P = 0.004$ and 0.027, respectively). In addition, treatment of CT-26 cells with recombinant murine IL-17A protein increased MMP-9 expression in a concentration-dependent manner (Fig. 4C) and promoted cancer cell motility as measured by Boyden chamber invasion assay (Fig. 4D).

GM-CSF facilitates cancer cell clearance

In contrast to IL-17A, GM-CSF levels were correlated with the decline of CTCs in the mouse model. In cancerous tissues, tumor-associated macrophages can be polarized toward an antitumor M1 or a protumor M2 phenotype (42). Alternative activation or polarization toward M1 or M2 is governed by microenvironmental signals such as cytokines (43). To test whether and how GM-CSF affects the polarization of macrophages in tumor lesions, we performed IHC staining for iNOS and Arg-1, which indicate M1 and M2 polarization, respectively (Supplementary Fig. S8A). We found that GM-CSF treatment increased the ratio of M1 to M2 macrophages in tumor lesions (Fig. 4D).
of iNOS to Arg-1 staining, termed the M1/M2 ratio, and therefore polarized macrophages toward the M1 (antitumor) type compared with the control ($P = 0.011$). GM-CSF treatment also increased the number of CD8$^+$ and CD4$^+$ T cells and natural killer cells (Supplementary Fig. S8B; $P = 0.045, 0.186, \text{and} 0.128$, respectively).

**IL-17A serves as a prognostic marker for patients with stages I to III CRC**

Next, we determined whether the results obtained from the mouse model could be translated to patients by performing IL-17A IHC staining in human CRC tissue sections. IL-17A$^+$ cells were mainly found in the primary cancer tissue and in regions immediately adjacent to the tumor. A weak IL-17A signal in the normal tissue located more than 15 cm away from the tumor (Fig. 5A). Quantitative measurement of the IL-17A level showed that expression of IL-17A was significantly higher in CRC tissue than in the normal control (Supplementary Fig. S9A, $P = 0.0008$). To determine the source of the IL-17A, we stained serial tissue sections for markers of CD4$^+$ T helper cells, Th17 cells (RORc), and γδT cells (44, 45). We found that some IL-17A$^+$ cells also stained positively for CD4 and RORc, but not for γδTCR (Fig. 5B), suggesting that at least a portion of IL-17A–producing T lymphocytes were Th17 cells.

Next, serum IL-17A levels were determined in patients with CRC (Fig. 5C). From a total of 80 measurements, we found that IL-17A levels were significantly higher in stage II patients than in patients in stages III or IV CRC ($8.8 \pm 1.5 \text{pg/mL vs. } 4.4 \pm 1.1 \text{pg/mL and } 4.1 \pm 1.8 \text{pg/mL, } P = 0.023 \text{ and } 0.035$, respectively.) Patients with higher numbers of mesenteric CTCs showed a higher level of IL-17A in their serum than did patients with lower numbers of mesenteric CTCs ($0.069\% \pm 0.13\% \text{ vs. } 0.023\% \pm 0.037\%, P = 0.0032$). The IL-17A level in the serum was positively correlated with the number of mesenteric CTCs (Fig. 5D). In addition,
IL-17A expression positively correlated with MMP-9 and VEGF expression in CRC clinical samples (Supplementary Fig. S9B and S9C). Expression of IL-17A was significantly higher in patients with colon cancer with recurrence than in patients without recurrence (Supplementary Fig. S10, \( P = 0.0056 \)). Serum IL-17A levels could thus serve as a prognostic marker (Fig. 5E and Supplementary Table S2). Patients with CRC with higher levels of IL-17A than the cut-off value of 1.99 pg/ml had a poorer 5-year disease-free survival rate (66.7%) than did patients with IL-17A levels lower than the cut-off value (93.9%).

Discussion

In this study, we investigated the role of CTCs in the progression of CRC. We found that the number of CTCs exhibited a dynamic increase-then-decrease pattern as the cancer invaded and metastasized (Fig. 6). Our data show an early increase in CTCs in the mouse metastasis model and in patients with early stages (I–II) of CRC that resulted from progressive invasion, disruption of the matrix, and intravasation of tumor cells (red trace). Later, however, in contrast to the typical prediction in the field, the number of CTCs gradually decreased at later phases (days 14–35) in the mouse metastasis model and in patients with late stages (III–IV) of CRC (blue trace). We hypothesized that the decrease in CTCs resulted from CTC-induced immunosurveillance and host immunity, which gradually eliminate CTCs from the blood stream. Only a few CTCs escaping from such clearance mechanisms are able to reside at a distant site and grow into a metastatic nodule.

Although mesenteric CTCs and forearm CTCs were identified by the same set of surface markers, it is intriguing to note that the number of mesenteric CTCs was 1 to 2 orders of magnitude higher than the number of forearm CTCs obtained from the same patient with CRC. The number of peripheral CTCs was positively correlated with the number of mesenteric CTCs (Supplementary Fig. S1B and S1C). However, the number of forearm CTCs was typically very low and remained low as the disease advanced (Supplementary Fig. S1D). The extensive difference in the amounts of CTCs measured from the 2 sampled sites may have arisen because CTCs entering the venous blood of the colon were first drained via the portal system to the liver and then via the pulmonary circulation to the lung before reaching the forearm vein, where the number of forearm CTCs was measured. Thus, many CTCs could have been trapped in the liver or the lungs as they passed through these organs (24, 46), and they could also have been diluted by the large volume of peripheral blood (21). This notion is in agreement with the observation that the liver and lung are the first and second most frequent targets of distant CRC metastases, respectively (47). Our data indicate that only CTC measurements taken directly from the venous drainage of the primary tumor (i.e., the mesenteric CTC) precisely reflects

Figure 6. Dynamic changes in and modulation of CTCs as the primary tumor invades and metastasizes—a working model. When the primary tumor becomes invasive, cancer cells start to invade, disrupt, and penetrate through the basement membrane and enter the bloodstream, leading to a rise in CTCs. This invasion process occurs at the time when malignancy reaches clinical stage II, and during the early phases of mouse invasion and metastasis (red trace). IL-17A facilitates intravasation of cancer cells by promoting MMP9 expression, cancer cell motility, and angiogenesis. Once in the stroma and circulation, CTCs are continuously exposed to and attacked by the host immune system. Therefore, CTCs decline in clinical stage III/IV and during the later phases of mouse invasion and metastasis (blue trace). GM-CSF, by biasing macrophages to M1 polarity and mobilizing CD8 T cells and natural killer cells, is involved in clearing CTCs from the blood.
the level of primary cancer cell intravasation. As a result, the number of mesenteric CTCs, and not that of forearm CTCs, accurately predicted the risk of metastasis in patients with CRC. The number of mesenteric CTCs was, in turn, correlated with the risk of metastasis in patients with stages I to III CRC and with the patient survival rate. Patients with higher numbers of mesenteric CTCs had poorer prognoses than those with fewer CTCs (manuscript in preparation, see also ref. 5).

In contrast to the increase-then-decrease dynamics of mesenteric CTCs, the amount of forearm CTCs remained low as the disease advanced to stage IV CRC (Supplementary Fig. S1D) and most other solid tumor types (9–12, 16, 18), except for lung cancer. Our analysis showed that forearm CTC levels in patients with lung cancer are highest in stage II patients, similar to mesenteric CTCs in CRC cases (Supplementary Fig. S2). We reasoned that the level of CTCs was higher in patients with stage II lung cancer because primary lung cancer cells drain directly to the systemic (forearm vein) circulation without passing through the portal (liver) or pulmonary circulation. These results suggest that only CTC measurements obtained from the direct venous drain of the primary tumor can reflect the actual level of intravasation of primary cancer cells and therefore reflect the risk of metastasis.

Technical difficulties prevented us from sampling blood from the mouse’s mesenteric vein. Rather, we measured CTCs from the retro-orbital vessels. The retro-orbital CTC level was also high and exhibited an increase-then-decrease pattern in the mouse model similar to that of mesenteric CTCs in humans. In the mouse, the invasion and metastasis process occurred within a 35-day experimental period, whereas years or decades of time are needed for human CRC carcinogenesis and metastasis to develop. We believed that because the nonphysiologic ex vivo cecum tumor growth added an extremely large tumor burden to the portal and pulmonary "filters," the amount of retro-orbital CTCs in the mouse became indicative of implanted tumor cell intravasation. Therefore, retro-orbital CTCs in the mouse model could be used to predict the risk of cancer metastasis. In this study, most mechanistic evaluations of the role of CTCs and cytokines in metastasis were performed in mice or in vitro, and we were careful not to overextend our conclusions from the findings in our animal model to clinical diseases; thus, we re-examined the results of the animal experiments whenever possible using human subjects. For example, the notion that IL-17A promotes cancer cell intravasation and increases the risk of metastasis was first drawn from the mouse model (Figs. 2C and D and 3) and in vitro experiments (Fig. 4), but we also retested and confirmed these findings in humans (Fig. 5), demonstrating that the serum levels of IL-17A were positively correlated with the amount of mesenteric CTCs. Patients with elevated serum levels of IL-17A had a higher risk of recurrence than those without (Fig. 5E and Supplementary Table S2). Moreover, we noticed that more IL-17A+ cells were found in the intratumor and tumor-adjacent regions than in the normal colon. In fact, many IL-17A-producing cells were immune response cells, including Th17 cells, which reside within the tumor microenvironment. Our data indicate that IL-17A could serve as not only a prognostic marker but also a therapeutic target for CRC metastasis.

We found that tumor burden did not account for metastasis in the mouse model (Fig. 2A and B), and thus, there was no correlation between tumor size and metastasis (48). In the context of IL-17A depletion, the size of the primary tumor was reduced (Fig. 3D). According to Keiji and colleagues, inhibition of IL-17A significantly inhibited tumor growth in MC38 and B16 tumor models (49), and Kiyotetsu and colleagues reported that IL-17A promotes tumor progression in murine intestine (50). These results are consistent with the results of other studies. We also found that IL-17A depletion effectively suppressed the increase in CTCs (Fig. 3B) and distant metastasis (Fig. 3C). Reduction of tumor size might underlie the amelioration of the increase in CTCs and metastasis in IL-17A–depleted mice. To explore other possible mechanisms, we conducted a series of experiments to determine whether and how IL-17A alters CT-26 cell behavior. IL-17A stimulation significantly enhanced the invasive-ness of CT-26 cells in vitro (Fig. 4D) and increased the production of tumor invasion factors such as MMP-9 (Fig. 4C). In IL-17A–depleted mice, expression of MMP-9 was reduced relative to the control group (Supplementary Fig. S8), indicating that IL-17A directly affected primary tumor growth and CTC levels, thereby contributing to metastasis. The most intriguing observation in this study was the decline in CTCs that occurred in patients with stage II/IV CRC (compared with stage II patients) and during late-stage metastasis in the mouse model. Because surface ESA was used for identifying CTCs in patients with CRC, it is necessary to consider that the observed reduction in CTCs might have resulted from downregulation or redistribution of surface ESA through the epithelial-to-mesenchymal transition (51), rather than an actual decrease in cell number. The mouse model, however, shed some light on how the decrease in CTCs occurred. When using exogenous EGFP or luciferase, which permanently labeled the cancer cells as markers for detection and quantification CTCs, we still observed a reduction in the number of CTCs. These results indicate that the number of CTCs was reduced during late-phase invasion/metastasis, likely as a result of active elimination of CTCs from the blood. Multiple mechanisms may contribute to such CTC clearance. Here, we provide experimental evidence showing the involvement of CTC-induced immune-surveillance and host immunity, especially mechanisms facilitated by GM-CSF. Many strategies, including cancer cell vaccination (52), could be used to boost immunity and enhance the elimination of CTCs. Taken together, our data support that analyses of CTCs and serum levels of cytokines can be of clinical value for patients with CRC. However, CTC assessment has to be performed and interpreted with careful consideration, for example, only mesenteric CTCs rather than forearm CTCs measured in patients in the early clinical stages are indicative of the patients’ risk of metastasis. An integrated therapeutic plan after removal of the primary tumor, including treatments...
that aim to eradicate CTCs, may help to prevent the occurrence of distant metastases in patients with CRC who have not yet progressed to the metastatic state.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C.-Y. Yang, Y.-C. Wu, J.-K. Jiang, C.-H. Lin
Development of methodology: J.-Y. Tseng, S.-H. Yang, Y.-C. Wu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.-Y. Tseng, R.-S. Liu, J.-K. Lin, Y.-M. Chen, J.-K. Iinuma
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.-Y. Tseng, Y.-M. Chen, J.-K. Jiang, C.-H. Lin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.-Y. Yang, S.-C. Liang, S.-H. Yang, J.-K. Lin, Y.-C. Wu
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Acknowledgments

The authors thank Dr. O.K. Lee and N.-J. Chen for their helpful discussions during this study; the Flow cytometry Core Facility of National Yang Ming University for providing technical services; the Molecular and Genetic Imaging Core from the National Research Program for Genomic Medicine (NRPGM); and Taiwan Mouse Clinic from the National Research Program for Biopharmaceuticals (NRPB) Resource Center for technical support in the bioluminescent imaging experiment.

Grant Support

This work was supported by the National Science Council (NSC) (grants NSC101-2321-B-075-001, NSC101-2319-B-010-001, and NSC102-2319-B-010-001; to C.-H. Lin) and a grant, Aim for the Top University Plan, from the Ministry of Education in Taiwan.

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References


Interleukin-17A Modulates Circulating Tumor Cells in Tumor Draining Vein of Colorectal Cancers and Affects Metastases

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*Clin Cancer Res* 2014;20:2885-2897. Published OnlineFirst March 27, 2014.

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