Peritoneal Dissemination Requires an Sp1-Dependent CXCR4/CXCL12 Signaling Axis and Extracellular Matrix-Directed Spheroid Formation

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Abstract
Peritonitis carcinomatosa is an advanced and intractable state of gastrointestinal and ovarian cancer, where mechanistic elucidation might enable the development of more effective therapies. Peritoneal dissemination of this type of malignancy has been generally thought to initiate from “milky spots” of primitive lymphoid tissues in the peritoneal cavity. In this study, we offer evidence challenging this idea, based on the finding that tumor implantation and directional dissemination was not required for the presence of milky spots, but rather SCF/CXCL12-expressing niche-like cells located at the border regions of perivascular adipose tissue. Interestingly, we found that peritoneal cavity lavage fluid, which specifically contains peritoneal collagen type IV and plasma fibronectin, dramatically facilitated spheroid formation of murine and human colon cancer cells. Spheroid formation strongly induced the expression of CXCR4 in an Sp1-dependent manner to promote niche-directed metastasis. Notably, disrupting sphere formation or inhibiting Sp1 activity was sufficient to suppress tumor dissemination and potentiated chemosensitivity to 5-fluorouracil. Our findings illuminate mechanisms of peritoneal cancer dissemination and highlight the Sp1/CXCR4/CXCL12 signaling axis as a rational target for the development of therapeutics to manage this intractable form of malignancy. Cancer Res; 76(2); 347–57. ©2016 AACR.

Introduction
Peritonitis carcinomatosa is an advanced and intractable state of gastrointestinal and ovarian cancers (1). Because this pathology remains highly resistant to the current standard therapeutics, including chemotherapeutic agents, and shows a high mortality ratio, its underlying mechanism requires elucidation.

Once the cancer cells have detached from the primary tumor, they are thought to float in the ascites as single cells or as clusters. At the initial stage of the peritoneal dissemination, cancer cells and their clusters have been shown to attach and adhere to the mesothelial layer, and then to form disseminated colonies via proteolytic activity, (1) and it has generally been thought that the peritoneal tumor dissemination occurs randomly in the peritoneal cavity (2) In support of this idea, an analysis of 102 involved regions in human subjects revealed random peritoneal dissemination to the mesentery/small bowel (21.7%), pelvis (20.6%), omentum/transverse colon (19.6%), ileocecal area (17.6%), and so on (3). On the other hand, some recent studies have indicated that the microdistribution of peritoneally disseminated cancer cells may not be completely random: so-called “milky spots,” i.e., primitive lymphoid tissues in the peritoneal cavity of humans and animals, detected mainly in the greater omentum and the pelvic floor, have been suggested as the predominant sites for cancer dissemination (4–6). However, questions have arisen in regard to this milky spots hypothesis, as milky spots have been shown to be rare in the mesentery (5, 7), even though mesenteric dissemination is not rare in clinical and experimental settings. To consider an alternative mechanism, therefore, knowledge of the underlying tumor dissemination in the peritoneal cavity irrespective of the location of milky spots is needed.

A possible clue to such an alternative mechanism was first provided by Yasumoto and colleagues (2), showing the essential role of the CXCR4/CXCL12 axis during peritoneal dissemination using human gastric cancer cells; their results indicated that the CXCR4 antagonist AMD3100 (8) was effective to prevent peritoneal carcinomatosis and accumulation of ascites. However, their findings raised a number of further questions, including (i) the role of the alternative receptor, CXCR7, for CXCL12; (ii) whether the CXCR4/CXCL12 axis is truly not required for CXCR4-negative
tumor dissemination; (iii) how to explain the paradox between the milky spot hypothesis and the abundant expression of CXCL12 in the peritoneal mesothelial cells observed in their study.

In the current study, therefore, we extensively address the precise molecular and cellular mechanisms of peritoneal dissemination to understand the intractable nature of peritonitis carcinomatosa.

Materials and Methods

**Mice**

Male 7- to 8-week-old Balb/c and C57BL/6 mice were obtained from KFT Oriental Co., Ltd. [Charles River Grade]. Male 8-week-old C57BL/6-Tg (CAG-EGFP) mice were obtained from the Shizuoka Laboratory Animal Center. The mice were kept under specific pathogen-free and humane conditions. The animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee and by the Biosafety Committee for Recombinant DNA Experiments of Kyushu University. These experiments were also done in accordance with the recommendations for the proper care and use of laboratory animals and according to The Law (No. 105) and Notifications for the proper care and use of laboratory animals and experiments were also done in accordance with the recommendations of the lines, and PCR confirmed there was no mycoplasma contamination. Parental CT26 cells were transfected with the enhanced GFP expression simian lentiviral vector-GFP (10) and selected by single-cell cloning.

**Cell lines and culture conditions**

The murine (CT26) and human (Lovo, HCT116, Colo-201, and SW602) colon cancers were purchased from the ATCC. The HRA cells were provided by the Cell Resource Center for Biomedical Research at Tohoku University (Sendai, Japan; ref. 9). These cell lines were maintained in RPMI1640 medium supplemented with 10% FBS, penicillin, and streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. The growth and morphology of the lines were found to be consistent with the original descriptions of the lines, and PCR confirmed there was no mycoplasma contamination. Parental CT26 cells were transfected with the enhanced GFP expression simian lentiviral vector-GFP (10) and selected by single-cell cloning.

**Mouse model of tumor dissemination**

For the murine peritoneal metastasis model, CT26GFP cells (2 × 10⁶) were injected intraperitoneally with or without AMD3100 (0.25 mg/head; Sigma-Aldrich Japan), neutralizing anti-mouse CXCR4 (clone 2B11, rat IgG2b, 2 μg/head; Biolegend), anti-mouse CXCL12/SDF-1 antibody (Clone #79014, mouse IgG1, R&D Systems) or the appropriate isotype-matched IgGs in 200 μL PBS. CT26 cells were treated with or without mitomycin-C (MMC, 10 μg/mL for 1 hour) or aphidicolin (0.1 μg/mL for 24 hours) and rinsed with PBS before injection. Twenty-four hours after the injection, the peritoneae were cut on poly-L-lysine–coated slides and rinsed with PBS. The slides were mounted on DAPI-containing Vectashield (Vector Laboratories) and examined using a BZ-9000 microscope.

**CFSE assay**

CT26GFP cells were treated with or without MMC (10 μg/mL for 1 hour) or aphidicolin (0.1 μg/mL for 24 hours) and rinsed with RPMI medium. The cells were CFSE-labeled and cultured at 37°C in a humidified 5% CO₂ incubator for 24, 48, or 72 hours before analysis. These cells were analyzed using a FACSCalibur with CellQuest and FlowJo 7.6 software.

**Cell adhesion and clustering assay**

CT26GFP cells were cultured on a 24-well culture plate with or without PBS (control), AMD3100 (1.25 mg/ml), or anti-CXCR4 for 24 hours to determine the frequencies of cell adhesion. To assess the effects of AMD3100 and anti-CXCR4 for the clustering of CT26GFP cells, the cells were cultured on an MPC treatment plate (MD6 with Lipid-Cell Binding, Nagel Nunc International) with or without each reagent for 24, 48, or 72 hours.

**Quantitative reverse transcription-PCR**

Total RNA was extracted and cDNA was synthesized using a SuperscriptIII cells direct cDNA synthesis system (Invitrogen)
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according to the manufacturer's protocol. Real-time PCR was performed in a Step One Plus Universal Real Time PCR System (Life Technologies) using TaqMan Fast Universal PCR Master Mix (Life Technologies) according to the manufacturer's protocol. The specific primer and probe set used in this study was as follows: NRF1 (Mm01315606_m1), YY1 (Mm00456392_m1), HIF1α (Mm00468869_m1), NF-xb (Mm01310378_m1), FoxC1 (Mm01962704_s1), FoxC2 (Mm00546194_s1), SP1 (Mm00489039_m1), FoxO1 (Mm00490672_m1), Bcl2 (Mm00477631_m1), PAX3 (Mm00435491_m1), SP1 (Mm00679393_s1), α-tubulin (Mm02528102_g1). All experiments were carried out in triplicate, and the data were analyzed using Step One Software ver. 2.1 (Life Technologies). The messenger RNA expression levels were standardized using β-actin messenger RNA expression levels in each sample.

ELISA
The culture medium and cell lysate were used as samples. The protein amounts of type IV collagen, cFN, and FN were determined using a mouse specific ELISA kit (type IV collagen, E90149Mu; Uscn Life Science Inc.; cFN, BG-MUS10681: Bluegene, Shanghai, China, FN, ab108849: Abcam) according to the manufacturer's protocol. Three independent experiments were performed.

Sp1 gene silencing by siRNA
Transfection of CT26 cells with siRNA was done using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer's protocol. Briefly, siRNAs, including an siRNA targeting Sp1 (Mm_Sp1_5223_s/ as; Sigma-Aldrich) and a scrambled control (sc-37007; Santa Cruz Biotechnology), were used at a final concentration of 10 nmol/L, and transfection reagent was used at the dilution of 1:500 (v/v).

Chemotherapeutic sensitization against CT26 malignant ascites
Coll-1 (75 U/mL) administered twice, at the time of tumor cell injection and 6 hours later, significantly inhibited the spherical formation as well as mesenteric metastasis in vivo (see Fig. 5A). All mice that received higher doses (i.e., 150 and 300 U/mL) died within 24 hours due to lysis of the mesentery associated with massive intestinal necrosis, and therefore, 75 U/mL was selected as the optimal dose. The sensitivity of CT26 tumor cells to 5-fluorouracil (5-FU) and L-OHP under monolayer or sphere formation was examined in vitro. The design of the in vivo experiment is shown in Fig. 5B. Briefly, at the time of intraperitoneal inoculation of CT26 tumor cells, each chemical at each concentration or buffer was administered simultaneously. Six hours later, 5-FU (50 mg/kg) or buffer was administered in all groups except for Group C, and thereafter, 5-FU or buffer was administered weekly. Animal death was monitored daily and body weight was measured at 3-day intervals.

Sampling of the human mesentery
The experiments using human surgical sections were reviewed and approved by the Institutional Review Board of Kyushu University Graduate School of Medicine (Approval No. 25-341). Human mesentery samples from seven patients who provided written informed consent and who underwent surgical resection for primary cancers (2 females and 5 males; age range: 45–72 years; one case of gastrointestinal stromal tumor and six cases of colon cancer) were subjected to the organ culture study and to the identification of CAR niche-like cells.

Tissue processing of human mesentery and spheroid adhesion assay
Goat anti-human CXCL12 (sc-6193; Santa Cruz Biotechnology Inc.) and rabbit anti-human SCF (SA3500292; Sigma-Aldrich Japan) were used to identify CAR cells on the human mesentery samples. LoVo-GFP (positive for CXCR4) and HCT116-CSFE (negative for CXCR4 in monolayer) at 10⁴ cells were maintained for 24 hours to form spheroids on a 96-well plate that had been pretreated with MPC. Forty to fifty spheroids were added onto each medium of sliced human mesentery as organ culture that was placed on the 6-well plate. Two days later, each mesentery was placed onto the slide grass, and en face immunohistochemical analysis for human SCF was performed.

Statistical analysis
All data were expressed as the means ± SEM. The data were examined statistically by Dunnett test or one-way ANOVA with Scheffe adjustment. A probability value of P < 0.05 was considered statistically significant. Statistical analyses were determined using StatView software (SAS Institute).

Results
Milky spots are not necessary for tumor dissemination
To confirm the localization of so-called milky spots and their potential role in the peritoneal dissemination of cancers, we first identified milky spots in the omentum and mesentery of Balb/c (Fig. 1A) and C57BL/6j (data not shown) mice. As in the previous reports (4–6), we observed toluidine blue–stained cell aggregates mainly on the surface of narrow fat tissue stripes of the omentum (Fig. 1A, arrowheads), but not in the mesentery (Fig. 1B, top) in both animal strains. In addition, unlike in a previous study, (7) despite extensive observations we failed to identify fat-associated lymphoid clusters (FALC), which have been considered to be different from milky spots in the mesenteries of these animal strains under developed speciﬁc pathogen-free conditions. In contrast, intraperitoneal injection of OK-432, a streptococcal preparation, could induce FALC-like structures (Fig. 1B, bottom), suggesting that a proinflammatory reaction may be a cause of FALC formation.

Next, we assessed the distribution of disseminated tumor nodules in the omentum and mesentery by using stably enhanced GFP-labeled CT26 murine colon cancer cells that were established using a simian lentiviral vector (10) after single-cell selection (CT26GFP; Supplementary Fig. S1). Twenty-four hours after intraperitoneal administration of CT26GFP (2 × 10⁶ cells/head), the omentum and mesentery were examined under a dissecting fluorescent microscope (Fig. 1C and D). We also included a group simultaneously administered AMD3100, a specific inhibitor for CXCR4 (8), in these experiments. As expected, multiple GFP-positive tumor nodules were frequently observed in narrow fat tissue stripes of the omentum (5), but not in not other fat tissues (Fig. 1C, top), and their occurrence was diminished by AMD3100 (Fig. 1C, bottom), suggesting that milky spots may be the predominant sites for tumor dissemination mediated by CXCR4. However, even though there were no milky spots, frequent GFP-positive nodules that were sensitive to AMD3100 were also

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observed in the mesentery (Fig. 1D). These findings were also confirmed by measurements of the wet weight of narrow fat tissue stripes and mesenteries at 1 weeks after CT26GFP tumor cell inoculation (Fig. 1E and F).

Together, these results indicate that narrow fat tissue stripes in the omentum and mesentery are the predominant sites for CXCR4-dependent tumor dissemination; however, milky spots are not always necessary for peritoneal dissemination.

CXCR4-dependent tumor metastasis to the mesentery is directional to CXCL12-expressing niche-like cells

A previous study suggested that peritoneal cells abundantly express CXCL12 and that tumor dissemination might occur at random (2); however, our experiments shown in Fig. 1 unexpectedly revealed early tumor nodules at the border area of perivascular adipose tissue (Fig. 2A, arrowheads). In fact, subsequent quantitative examination revealed that more than 90% of these nodules were located at the border area of perivascular adipose tissue (Fig. 2A, graph) when nodules larger than 30 μm in diameter were counted. Unlike in the previous report (2), the expression of CXCL12 in parietal peritoneum samples obtained from the abdominal wall was much lower than those in the retroperitoneal fat, mesentery, and omentum narrow fat tissue stripes (Fig. 2B), suggesting that the parietal peritoneum may not be important for tumor dissemination.

Next, we determined the localization of CXCL12-expressing cells by en face observation using mesenteric tissue, and here we also examined the expression and localization of stem cell factor (SCF) simultaneously, because the CXCL12/CXCR4 axis is largely known for its niche-related proteins, which play roles in hematopoiesis and cancer metastasis (11–15). Cells in the mesentery, possibly mesothelium cells and perivascular adipocytes, abundantly expressed SCF, but in some cases, these cells also coexpressed CXCL12 (Fig. 2C). Importantly, the SCF+/CXCL12+ cells
were frequently located at the border regions of perivascular adipose tissue (Fig. 2D, inset C, arrows) but rather rarely seen in membranous regions (Fig. 2D, insets A and B, arrow), although single SCF$^+$ cells were abundant. We considered that these SCF$^+$/CXCL12$^-$CAR cells might be so-called "CXCL12-abundant reticular (CAR) cells," (16) and indeed, these cells were confirmed to have the reticular morphology typical of CAR cells (Fig. 2E, arrows).

We then examined the 10 mesenteric samples with CT26GFP tumor metastasis that were immunohistochemically determined to contain CAR-like cells, and approximately 85% of the nodules larger than 30 μm in diameter were located on the SCF$^+$ cells (Fig. 2F). These results indicated that CXCR4-dependent tumor metastasis to the mesentery is directional to CXCL12-expressing niche-like cells, but not random.

However, this raised two additional questions: (i) how was the visible size of mesenteric metastasis realized within 24 hours after tumor cell inoculation, and (ii) why was AMD3100 so effective, as shown in Fig. 1F, even though CT26GFP and its parent cells express very low levels of CXCR4?

Dramatic facilitation of spheroid formation in PCLF is mediated by the cooperative action of collagen type IV and plasma fibronectin

We first assessed the detailed process of mesenteric dissemination. To do this, we determined the time courses of the recovery of PCLF and mesenteric metastasis after intraperitoneal inoculation of CT26GFP cells; representative time courses are shown in Fig. 3A. Single cells as well as scattered cell
Clusters were recovered without apparent mesenteric metastasis within 1 and 3 hours after tumor inoculation. Importantly, apparent and grossly visible spheroids (arrowheads) as well as tumor nodules on the mesentery began to be observed from 6 hours after inoculation, and at 12 hours after inoculation, the size of spheroids was dramatically increased (arrows) and single cell recovery was very rare. The time course of the process of the spheroid formation in vivo seemed to be much earlier than that seen in vitro, suggesting that the peritoneal cavity might contain substances that facilitated sphere formation of the CT26 cells. To confirm this, a floating cultivation of CT26 cells was performed in fresh medium (fresh RPMI) as well as the medium that was used after intraperitoneal irrigation (PCLF-RPMI). C, cell cycle inhibition by aphidicolin or mitomycin C did not affect the formation of disseminated nodules. D, spontaneous spheroid formation and PCLF-mediated facilitation of spheroid formation were sensitive to collagenase. In both cases, CT26 cells could not form spheroids. E, Coll-IV and pFN cooperatively facilitated spheroid formation in vitro. Coll-IV, but neither Coll-I nor plasma/cellular fibronectins (pFN and cFN), facilitated the sphere formation (green squares), compared with baseline (blue squares). Importantly, the combination of Coll-IV and pFN showed the optimal effect (red squares). F, involvement of Coll-IV and pFN in the spheroid formation. E and F, measurement of Coll-IV and pFN in murine PCLF by ELISA (E), and immunohistochemical detection of Coll-IV and pFN in the CT26 spheroids (F) recovered from the peritoneal cavity.

Figure 3.
Facilitated spheroid formation of cancer cells in the peritoneal cavity is mediated by preexisting collagen type IV (Coll-IV) and plasma fibronectin (pFN). Each experiment was done more than three times and showed similar results. A, the representative time courses of the recovery of PCLF and mesenteric metastasis after intraperitoneal inoculation of CT26GFP cells. Single cells as well as scattered cell clusters were recovered without apparent metastasis within 1 and 3 hours after tumor inoculation. Grossly visible spheroids (arrowheads) as well as tumor nodules on the mesentery began to be observed from 6 hours after inoculation, and at 12 hours after inoculation, the size of spheroids was increased (arrows) and single cell recovery was very rare. B, PCLF facilitated sphere formation in vitro. Floating cultivation of CT26 was done in the fresh medium (fresh RPMI) as well as the medium that was used after intraperitoneal irrigation (PCLF-RPMI). C, cell cycle inhibition by aphidicolin or mitomycin C did not affect the formation of disseminated nodules. D, spontaneous spheroid formation and PCLF-mediated facilitation of spheroid formation were sensitive to collagenase. In both cases, CT26 cells could not form spheroids. E, Coll-IV and pFN cooperatively facilitated spheroid formation in vitro. Coll-IV, but neither Coll-I nor plasma/cellular fibronectins (pFN and cFN), facilitated the sphere formation (green squares), compared with baseline (blue squares). Importantly, the combination of Coll-IV and pFN showed the optimal effect (red squares). F, involvement of Coll-IV and pFN in the spheroid formation. E and F, measurement of Coll-IV and pFN in murine PCLF by ELISA (E), and immunohistochemical detection of Coll-IV and pFN in the CT26 spheroids (F) recovered from the peritoneal cavity.
To identify the protein(s) essential to the accelerated sphere formation, an in vitro spheroid formation assay was performed using two major ECM-sensitive proteases, collagenase and hyaluronidase (CT26 cells expressed hyaluronan receptor CD44 and collagen/laminin/RGD receptors integrin β1/α5; Supplementary Fig. S2A). Importantly, the sphere formation was completely abolished by collagenase (Fig. 3D), but was not sensitive to hyaluronidase or anti-integrin–neutralizing antibodies (Supplementary Fig. S2B and C), and these findings were common to all four human colon cancer cell lines tested (Supplementary Fig. S2D). An extensive spheroid formation assay also revealed that collagen type IV (Coll-IV), but neither Coll-I nor plasma/cellular fibronectins (pFN and cFN), facilitated the sphere formation (Fig. 3E, green squares). Important-ly, the combination of Coll-IV and pFN showed the optimal effect (Fig. 3D, red squares), indicating that Coll-IV is essential and pFN supports the formation of spheroids. In fact, considerable amounts of Coll-IV and pFN were found in murine PCLF (Fig. 3F), and these ECM proteins were certainly involved in the spheroids that were recovered from the peritoneal cavity (Fig. 3F), results that explain very well the clinical findings previously reported by our laboratory (17).

Role of spheroid formation on CXCR4 expression, involvement of Sp1

Next, we investigated the role of the CXCR4/CXCL12 axis during mesenteric tumor dissemination. For this purpose, we first assessed the expressions of CXCR4 and CXCR7, an alternative receptor for CXCL12, in response to inoculation with CT26GFP. The results showed that the expression of CXCR4 was very low (Supplementary Fig. S1b); however, administration of a CXCR4, a neutralizing antibody for CXCR4, either alone or together with a CXCR7 or AMD3100 or a CXCL12 antibody significantly inhibited the mesenteric microdissemination, while neither αCXCR7 alone nor control IgG showed a significant inhibition of mesenteric dissemination of tumor cells (Fig. 4A), indicating that the expression of CXCR4 in tumor cells, but not that of CXCR7, significantly contributed to peritoneal dissemination. These paradoxical data raise a question, namely, if CXCR4 plays an essential role in niche-directed tumor dissemination, why was...
the basal expression of CXCR4 on the CT26 monolayer so low? We hypothesized that sphere formation might accelerate the expression of CXCR4, and as shown in Fig. 4B, this was indeed confirmed by FACS and immunocytochemistry. Neither AMD3100 nor a CXCR4 affected the formation of the spheres themselves (Fig. 3c), indicating that spheroid formation itself may be a key event in the upregulation of CXCR4.

However, the inhibitor for FAK, a major signal molecule downstream of integrin signaling, did not affect the sphere-induced CXCR4 expression (Supplementary Fig. S3A), and FNs (Supplementary Fig. S3B, left) or Coll-I and/or Coll-IV (data not shown) also did not alter the expression of CXCR4. In addition, CXCR4 expression on the monolayer was not upregulated by either cultivation under a hypoxic condition, the addition of an inhibitor for hypoxia-inducible factor (HIF; Supplementary Fig. S3B, right), or the addition of an inhibitor for NF-kB (data not shown), unlike in previous reports (18, 19). Only two stress conditions, i.e., the presence of CoCl2 (20) and serum starvation (21), were identified as inducers of CXCR4 on the CT26 monolayer (Supplementary Fig. S3C and D). However, the antioxidant N-acetyl cysteine did not have any effect on spheroid-induced CXCR4 expression (Supplementary Fig. S3C, right). The latter had been coexpressed with insulin-like growth factor-1 receptor (IGFIR; Supplementary Fig. S3D), an inducer and transactivator of CXCR4 (21), however, picropodophyllin, an inhibitor of IGFIR, did not affect the expression level of CXCR4 (data not shown).

Therefore, we returned to a review of the transcriptional regulation of CXCR4, based on the structure of its promoter region (22). As no gene encoding a transcriptional factor that regulates CXCR4 exhibited a change in expression (Supplementary Fig. S3E and F), we focused on the Sp1 transcription factor, which targets GC boxes irrespective of its own transcriptional levels (23). As shown in Fig. 4D and E, spheroid-induced upregulation of CXCR4 was abrogated by the Sp1-specific inhibitor mithramycin A in a dose-dependent manner and by Sp1-specific siRNA. Importantly, mesenteric metastasis of GFPþ CT26 cells was significantly inhibited by the pretreatment with mithramycin A without any effect on spheroid formation in vivo (Fig. 4F), indicating that Sp1-mediated overexpression of CXCR4 plays an essential role in directional metastasis during tumor dissemination.

Chemotherapeutic sensitization against tumor dissemination via inhibition of Sp1, CXCR4, or spheroid formation

To assess the effect of inhibiting the CXCR4 axis as well as spheroid formation itself on the therapeutic effect of chemotherapy...
We first optimized the concentration of collagenase-L (Coll-L) as 75 U/ml required to inhibit spheroid formation of GFP CT26 cells in vivo (Fig. 5A).

Before determining the experimental design (Fig. 5B), the sensitivity of two independent chemotherapeutic agents that are the commonly used agents for colon cancers, namely 5-FU and L-OHP, was determined. Sphere formation made the cells significantly more resistant to both antitumor agents compared with the cells remaining in a monolayer (Fig. 5C, P < 0.05). As 5-FU was more effective than L-OHP when the drugs were used at lower concentrations, we selected 5-FU for use in the subsequent in vivo experiments (Fig. 5D).

All animals administered buffer as a control (Group A) were dead within 32 days, and 5-FU treatment (Group B) could not prolong their survival time significantly (P = 0.12). In contrast, early inhibition of sphere formation by Coll-L (Group C) as well as use of AMD3100 (Group D) or of mithramycin A (Group E) resulted in over 100 days of long-term survival in 3 (Groups C, D, and E) and 4 (Group E) of the 8 animals in each group. These results indicated that the inhibition of sphere formation in the peritoneal cavity and of directional sphere seeding might be a potential strategy to sensitize the disseminated tumor cells against chemotherapeutic agents.

Identification of CAR-like cell aggregates, accelerated sphere formation, and directional tumor metastasis in human tissue and cells

Finally, we addressed the question of the relevance of these findings to human tissue and cells.

A portion of the surgically resected human mesentery (Supplementary Fig. S4A, left) was subjected to en bloc immunohistochemistry for SCF, and en face observation under a dissecting fluorescence microscope demonstrated scattered SCF⁺ cells (Supplementary Fig. S4A, right, red staining, white arrows) on and around the perivascular adipose tissue (Supplementary Fig. S4A, right, yellow bipolar arrow). Double immunohistochemistry (Supplementary Fig. S4B) for SCF (red) and CXCL12 (green) was performed to identify CAR-like cells (white arrows) using cryosections, and doubly positive cells were frequently seen at the border area of perivascular adipose tissue (white arrows).

Next, we assessed the representative accelerated sphere formation and directional tumor dissemination in two independent human colon cancer cell lines, LoVo and HCT116, in the peritoneal cavity of nude mice. As shown in Supplementary Fig. S4C, spontaneous expression of CXCR4 was seen in the monolayer of LoVo cells, but not that of HCT116 cells, while CXCR4 expression was seen after spheroid formation (arrow). Murine PCLF facilitated the sphere formation of these human colon cancer cells in vitro (Supplementary Fig. S4d, red squares), but the formations were abolished when Coll-L was used (Supplementary Fig. S4d, green squares). These cells were intraperitoneally inoculated into the nude mice. Seven days after inoculation of GFP-labeled LoVo cells (LoVo-GFP) or CFSE-labeled HCT116 cells (HCT116-CFSE), the dissemination was mainly seen at the margin of the perivascular adipose tissue (Supplementary Fig. S4e, left, inset A and B), which was similar to the findings obtained by using CT26GFP cells. These disseminations were significantly abrogated by AMG3100 or mithramycin A (Supplementary Fig. S4e, right).

Surgically obtained mesenteric tissues were placed on a 6-well plate, and medium containing spheres of HCT116-CFSE was added. Two days later, these tissues were placed on a glass plate, and en bloc immunohistochemistry for SCF was done. En face findings demonstrated that LoVo-GFP (Supplementary Fig. S4F, top bottom) as well as HCT116-CFSE (Supplementary Fig. S4F, left bottom) spheres (green) were frequently present on the SCF⁺ cell sheet (red). Among the 39 and 35 green nodules counted, 26 (66.7%) and 23 tumors (65.7%) were located on the SCF⁺ cell sheet, respectively.

Subsequently, we asked whether the major findings obtained in this study using colon cancer cells were also representative to a HRA human ovarian cancer, an alternative malignant neoplasm that frequently induces peritoneal dissemination. As expected, sphere formation–induced spontaneous expression of CXCR4 (Supplementary Fig. S5a), PCLF-mediated acceleration of the sphere-forming process (Supplementary Fig. S5b), and inhibition of peritoneal dissemination of HRA cells in nude mice by AMD3100 as well as by mithramycin (Supplementary Fig. S5c) were also representative.

Together with these findings, intraperitoneal acceleration of sphere formation of cancer cells and CAR-like cell-directed dissemination of spheres could be common mechanisms in human tissue and cancers, at least in case of gastrointestinal and ovarian cancers.

Discussion

Mainly using CT26 murine colon cancer, that has been shown to share molecular features with aggressive, undifferentiated, recurrent human colorectal carcinoma cells (23), we have demonstrated the whole molecular and cellular mechanisms of peritoneal dissemination. The key findings of the current study were as follows: (i) intraperitoneally inoculated tumor cells can be implanted to narrow fat tissue stripes of the omentum with milky spots as well as to border regions of perivascular adipose tissue of the mesentery without milky spots, and both are sensitive to a CXCR4 inhibitor, AMD3100, suggesting that milky spots are not required to form disseminated cancer metastasis in the peritoneal cavity; (ii) SCF⁺/CXCL12⁺ CAR-like cells located at the border area of perivascular adipose tissue of the mesentery are a prominent disseminated dissemination site of tumor cells; (iii) peritoneal Coll-IV and pFN cooperatively facilitated the sphere formation of cancer cells within 3 to 6 hours; (iv) spheroid formation strongly promoted the expression of CXCR4 in an Sp1 transcription factor-dependent fashion (a schematic representation of a possible mechanism of tumor dissemination is provided in Supplementary Fig. S6). Also, (v) CAR-niche–directed and CXCR4/CXCL12 axis-dependent metastasis were representative in human cancer cells and mesentery.

Although a previous study simply demonstrated the essential role of the CXCR4/CXCL12 axis during tumor dissemination (2, 11–15), the current study made a related but apparently distinct contribution, i.e., the first demonstration of the global molecular and cellular mechanism of tumor dissemination. Specifically, this study was the first to demonstrate that Coll-IV and pFN-dependent facilitation of spheroid formation within the initial hours after dissemination was essential to induce CXCR4/CXCL12-dependent tumor dissemination, irrespective of the basal expression of CXCR4. First, it has been demonstrated that spheroids form tight junctions containing both E-cadherin and ZO-1 to protect themselves against anticancer agents (25). In
fact, cancer spheroids are much more resistant to irinotecan than cancer cells in a monolayer (26). Second, spheroid formation promptly induces antiapoptotic genes that might be involved in the mechanism of drug resistance (25). Other metabolic changes via the formation of spheroids, including hypoxia (27), starvation (28), anaerobic metabolism (27), proinflammatory potential (29, 30), as well as cancer stem cell–like potency (31, 32) may also contribute to the intractable potentials of disseminated cancer foci (Supplementary Fig. S7).

One important finding obtained in this study was that early and transient inhibition of sphere formation as well as of the CXCR4 axis rendered tumor cells in the peritoneal cavity significantly more sensitive to chemotherapeutic agents. In any case, these data indicated that the inhibition of the process of sphere formation as well as CXCR4-dependent directed dissemination might be effective to render tumor cells more sensitive to chemotherapeutic agents, and therefore these strategies need to be addressed in future clinical studies.

In conclusion, we here demonstrated the global mechanisms of peritoneal cancer dissemination at the molecular and cellular levels. Our results showed that the CXCR4-dependent interaction of tumor cells with the mesenteric CAR cell–like niche as well as the spheroid formation occurring before tumor dissemination is the critical mechanism underlying peritoneal carcinomatosis. The development of technologies that disrupt this process would be highly desirable for the treatment of patients with peritoneal carcinomatosis.

Disclosure of Potential Conflicts of Interest
T. Iwai is a researcher from Chugai Pharmaceuticals Inc. Y. Maehara and Y. Yonemitsu report receiving a commercial research grant. No potential conflicts of interest were disclosed by the other authors.

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