Autocrine Induction of Invasive and Metastatic Phenotypes by the MIF-CXCR4 Axis in Drug-Resistant Human Colon Cancer Cells

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Abstract
Metastasis and drug resistance are major problems in cancer chemotherapy. The purpose of this work was to analyze the molecular mechanisms underlying the invasive potential of drug-resistant colon carcinoma cells. Cellular models included the parental HT-29 cell line and its drug-resistant derivatives selected after chronic treatment with either 5-fluorouracil, methotrexate, doxorubicin, or oxaliplatin. Drug-resistant invasive cells were compared with noninvasive cells using cDNA microarray, quantitative reverse transcription-PCR, flow cytometry, immunoblots, and ELISA. Functional and cellular signaling analyses were undertaken using pharmacologic inhibitors, function-blocking antibodies, and silencing by retrovirus-mediated RNA interference. 5-Fluorouracil- and methotrexate-resistant HT-29 cells expressing an invasive phenotype in collagen type I and a metastatic behavior in immunodeficient mice exhibited high expression of the chemokine receptor CXCR4. Macrophage migration-inhibitory factor (MIF) was identified as the critical autocrine CXCR4 ligand promoting invasion in drug-resistant colon carcinoma HT-29 cells. Silencing of CXCR4 and impairing the MIF-CXCR4 signaling pathways by ISO-1, pAb FL-115, AMD-3100, monoclonal antibody 12G5, and BIM-46187 abolished this aggressive phenotype. Induction of CXCR4 was associated with the upregulation of two genes encoding transcription factors previously shown to control CXCR4 expression (HIF-2α and ASCL2) and maintenance of intestinal stem cells (ASCL2). Enhanced CXCR4 expression was detected in liver metastases resected from patients with colon cancer treated by the standard FOLFOX regimen. Combination therapies targeting the CXCR4-MIF axis could potentially counteract the emergence of the invasive metastatic behavior in clonal derivatives of drug-resistant colon cancer cells. Cancer Res; 70(11): 4644–54. ©2010 AACR.

Introduction
Distinct classes of cytotoxic drugs have been developed in cancer chemotherapy, including antimetabolites, DNA alkylating and intercalating agents, mitotic inhibitors, and other molecular interventions targeting oncogenic signaling pathways (1). A major limitation in the clinical efficacy of anticancer chemotherapy is drug resistance (2, 3). Drug resistance is associated with the activation of oncogenic pathways inducing survival pathways and convergent inhibitors of apoptosis. Studies on chemoresistant cancer cell lines brought to evidence the activation of epidermal growth factor receptor, hedgehog, and Wnt/β-catenin pathways and alterations in ceramide metabolism. Such properties are essential features of metastatic cancer cells and cancer cell progenitors (2).

We have previously reported that the HT-29 subclone 5M21, derived from a subpopulation resistant to methotrexate (MTX), displayed a constitutive invasive phenotype in type I collagen gels, and a metastatic behavior in immunodeficient mice. 5-Methylcytosine DNA methylation (5mC) promotes the expression of the transcription factor HIF-1α and ASCL2 in colorectal carcinoma cells. Therefore, HIF-2α and ASCL2 might be involved in the autocrine induction of invasive and metastatic phenotypes by the MIF-CXCR4 axis in a subset of drug-resistant colon carcinoma cells.
mice (4). A comparative transcriptome analysis showed that the gene transcripts encoding the chemokine (C-X-C motif) receptor 4 (CXCR4) were upregulated 11-fold in MTX-resistant HT-29 5M21 cells versus parental HT-29 cells. CXCR4 is the receptor of stromal cell-derived factor-1 (CXCL12/SDF-1α). CXCR4 promotes tumor progression at different levels of malignancy including tumor growth, angiogenesis, metastatic dissemination, and homing in CXCL12-enriched cellular niches in metastasis target tissues (5–10). CXCR4 expression is a prognostic marker in various types of cancer including acute myelogenous leukemia, breast, and colon carcinomas (11–14).

The purpose of this work was to identify the major mechanisms responsible for the emergence of invasive and aggressive drug-resistant human colon cancer cells HT-29 selected by chronic treatment with the cytotoxic drugs 5-fluorouracil (5-FU) and MTX, with special reference to CXCR4 (4, 15). We have extrapolated our data by subsequent studies on chemotherapeutic resistance observed in HT-29 cells exposed to the clinical anticancer agents oxaliplatin (OXA) and doxorubicin (DOX).

Materials and Methods

Human colorectal carcinoma cell lines and patient tissue samples

Parental HT-29 cell line, 5-FU (10 μmol/L) and MTX (10 μmol/L)–resistant HT-29 cell subpopulations (HT-29 FU and HT-29 MTX) and their 5-FU–resistant (5F7 and 5F31), and MTX-resistant (5M21) clonal derivatives were cultured as described (16, 17). HT-29 cell subpopulations resistant to DOX (HT-29 DOX, 30 nmol/L; ref. 18) and OXA (HT-29 OXA, 2 μmol/L; ref. 19) were from Dr. A. Bosia and Dr. LM. Ellis, respectively. Liver metastases of colon adenocarcinoma from 23 patients who underwent surgical resection of metastases were processed by the tumor cell and tissue bank of the Regional Reference Cancer Center of Lille. After hepatic resection, fragments were taken from macroscopic metastases and adjacent hepatic tissue, snap-frozen in liquid nitrogen, and stored at −80°C. The whole remaining tissue was fixed in 10% formalin and several other fragments were taken from the fixed metastases and adjacent hepatic tissue and embedded in paraffin. Hematoxylin, eosin, saffron, and astra blue–stained sections were examined by an expert pathologist to establish the histologic diagnosis and the morphologic characteristics of the frozen fragments. Fragments used in this study contained at least 60% malignant cells. Informed consent was obtained from all patients.

Collagen invasion assay

Invasion assays were performed as previously described (4). Invasion index is expressed as the percentage of invading cells versus total number of cells. Data are means ± SD, and significance was assessed by the unpaired Student’s t test.

Immunohistochemistry

Paraffin-embedded sections were pretreated in CC1 buffer (pH 8) at 98°C for 36 minutes for macrophage migration-inhibitory factor (MIF) labeling and 8 minutes for CXCR4 labeling and processed with the MIF antibody (dilution 1:200, incubation 32 minutes) or the CXCR4 antibody (dilution 1:100, incubation 28 minutes) using a Benchmark XT Ventana equipment. Labeling was visualized by the Ultraview-DAB system and slides were counterstained with hematoxylin. Specificity was checked by control staining performed in the absence of primary antibody and with several positive tissue controls. Staining intensity was evaluated as low (+), moderate (++), and high (+++). The Ki-67 proliferation index was assessed in 1,000 cells at the tumor periphery, i.e., in areas associated with the highest Ki-67 nuclear signal.

DNA microarray analysis

Human and mouse Whole Genome Agilent 44K 60-mer oligonucleotide Microarrays were performed according to the Two-Color Microarray-Based Gene Expression protocol (Agilent Technologies). Microarrays were scanned using the Agilent scanner G2505C and Feature Extraction software (v10.5). Data were processed with the GeneSpring (v10) for normalization, filtering, and statistical analysis. The genes upregulated or downregulated with statistical significance (P < 0.05) were sorted using asymptotic P value computation and Benjamini Hochberg FDR multiple testing corrections. Microarray data are available through GEO (accession no. GSE20147).

Flow cytometry and Western blot

Cells were detached with trypsin-EDTA, rinsed, resuspended at 10^5 cells per milliliter in PBS-1% bovine serum albumin, incubated for 60 minutes at room temperature with anti–CXCR4-PE and analyzed using a Beckman-Coulter Epics XL-MCL4 cytometer. Immunoblotting was performed as previously described using purified membrane fractions for CXCR4 (20).

Results

HT-29 clones selected for 5-FU and MTX resistance differ in their invasive metastatic behavior and CXCR4 expression

According to our previous report (15), the HT-29 5M21 clone, selected by MTX resistance, displayed a constitutive invasive phenotype in type I collagen, whereas parental HT-29 cells were not spontaneously invasive in this extracellular matrix component (Fig. 1A). To further address the effect of drug resistance on the aggressive phenotype and expression of CXCR4, we analyzed the invasive potential of four HT-29 clones selected for resistance to 5-FU, the most commonly used anticancer drug. As shown in Fig. 1A, collagen gels were invaded by 5-FU–resistant 5F7 cells (P < 0.0001). In contrast, 5-FU–resistant 5F31 cells were not invasive. Quantitative reverse transcription-PCR (qRT-PCR) showed that CXCR4 mRNA levels were increased 135 ± 21-fold and 41 ± 11-fold in 5F7 and 5M21 cells versus 5F31 cells, respectively (Fig. 1B). Flow cytometry confirmed a weak CXCR4 expression in 5F31 cells, as compared with
5F7 and 5M21, the highest CXCR4 expression being detected in 5F7 cells (Fig. 1C). The two other 5-FU–resistant clones (5F35 and 5F12) showed a constitutive invasive behavior and enhanced expression of CXCR4 relative to parental HT-29 cells (data not shown).

The 5F7 and 5F31 clones were then examined for their metastatic behavior, using the metastatic 5M21 xenografts as control (4). Cells were first injected s.c. in the flank of nude mice and the primary tumors were reimplanted into severe combined immunodeficiency (SCID) mice for the analysis of metastasis (Supplementary Fig. S1A and B). 5F7 and 5M21 xenografts developed lung micrometastases (four of four mice), whereas 5F31 xenografts were ineffective at spreading into the lungs (zero of four mice). These data revealed that chronic treatment of the parental HT-29 cell line by the anticancer drugs 5-FU and MTX led to the emergence of invasive cell populations characterized by a strong expression of CXCR4.

Transcriptome analysis of the 5-FU– and MTX-resistant HT-29 clones and their corresponding tumor xenografts

We compared the invasive clones 5M21 and 5F7 to the noninvasive 5F31 clone, using the 44K Whole Human Genome Oligo Microarray (Table 1; Supplementary Table S1). In coherence with our data (Fig. 1), CXCR4 seemed strongly expressed in invasive cells with a very high fold change (123.5). Other genes highly expressed in the invasive clones 5F7 and 5M21 were BAMBI (99.8-fold), GRB10 (96.1-fold), and SYT13 (93.7-fold). We also observed a higher expression of several ABC transporters, i.e., ABCB2 (8.4-fold), ABCB6 (6.9-fold), ABCC6 (6.9-fold), ABCG5 (4.5-fold), ABCA2 (2.5-fold), and ABCC2 (4.5-fold), and of the receptor activator of nuclear factor-κB TNFRSF11A/RANK (7.4-fold). The transcription factors Achaete scute-like 2 (ASCL2) and hypoxia-inducible factor E2F1 (encoding HIF-2α), known to induce CXCR4 transcription, are upregulated in the invasive 5F7 and 5M21 clones versus the noninvasive 5F31 clone (3.8-fold and 1.3-fold, respectively; Supplementary Table S1). Next, we analyzed the corresponding xenografts to incriminate CXCR4 in metastasis. CXCR4 overexpression persisted in the metastatic 5F7 and 5M21 tumor xenografts (by 61-fold; Table 2). Transcripts highly expressed in invasive 5F7 and 5M21 cells in vitro were also overexpressed in their corresponding xenografts, including BAMBI (118.1-fold), IGFBP2 (63.9-fold), GRB10 (47.2-fold), and SYT13 (19.4-fold). In addition, some transcripts showing low variations in invasive versus noninvasive cells (Table 1; Supplementary Table S1) seemed highly overexpressed in the metastatic versus nonmetastatic tumor xenografts (Table 2; Supplementary Table S2). These transcripts included the matrix metalloproteases MMP1 (19.6-fold) and MMP10 (8.3-fold), the membrane serine protease hepsin (11.4-fold), and endothelin 2 (18.8-fold), all involved in invasive tumor growth and metastasis (Table 2; Supplementary Table S2; refs. 21–23). In metastatic tumor xenografts, we also noticed a stronger induction of human ASCL2 (12-fold).

Because these microarrays were established from tumor xenografts containing human cancer cells and murine tumor stroma cells, we performed a comparative mouse pan-genomic microarray on the same RNAs using the 44K Whole Mouse Genome Oligo Microarrays. Comparison of metastatic 5M21 and 5F7 xenografts versus nonmetastatic 5F31 xenografts did not produce overexpressed genes with high fold change as obtained with the human microarray (Table 2). Concerning murine Cxcr4, only a 5-fold increase was observed, supporting the notion that the transcripts...
detected in the metastatic 5F7 and 5M21 xenografts are mainly associated with CXCR4 hyperexpression in human colon cancer cells.

**Implication of CXCR4 in the invasive and metastatic behavior of 5-FU-resistant HT-29 clones**

As shown in Fig. 2A, the constitutive invasive phenotype of 5F7 cells in collagen type I gels was abolished by the anti-CXCR4 12G5 antibody and the CXCR4 antagonist AMD3100. Downstream of CXCR4, receptor-activated G-protein subunits were shown to signal with PLCβ. It is well known that these signaling networks are involved in cancer cell invasion and metastasis. In agreement, the 5F7 invasive phenotype was alleviated by the Goi inhibitor PTx, the new G protein signaling inhibitor BIM-46187 targeting Go subunits downstream activated G protein–coupled receptor (GPCR; ref. 25), and by pharmacologic inhibitors targeting several signaling effectors, including the ras oncogene, the phospholipase Cβ (PLCβ)/calcium/PKC cascade, and the Rho-Rho-kinase (ROK) axis, respectively (24). Additionally, G(13) subunits released from CXCR4-activated heterotrimeric subunits were shown to signal with PLCβ. It is well known that these signaling networks are involved in cancer cell invasion and metastasis.

### Table 1. Upregulated genes (fold-change >10) in the invasive 5M21 and 5F7 cells vs. the noninvasive 5F31 cells and their corresponding GenBank accession numbers

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<td>CHRD</td>
<td>H. sapiens chordin</td>
<td>10.0</td>
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Reduced by 83% to 96% in flow cytometry (Fig. 2B and C). CXCR4 mRNA levels were silenced by RNA interference (Supplementary Methods). CXCR4 resistant HT-29 cells. We silenced in the tumorigenicity and metastatic behavior of 5-FU–resistant HT-29 cells. Of note, silencing of CXCR4–silenced subclones were selected by qRT-PCR and flow cytometry showed that drug resistance was associated with the notion that CXCR4-mediated migration of carcinoma cells is controlled by phosphoinositide-3-kinase/Akt-dependent and p42/44-independent pathways (26).

Next, we examined whether CXCR4 plays a direct role in the tumorigenicity and metastatic behavior of 5-FU-resistant HT-29 cells. We silenced CXCR4 in invasive 5F7 cells by RNA interference (Supplementary Methods). CXCR4-silenced subclones were selected by qRT-PCR and flow cytometry (Fig. 2B and C). CXCR4 mRNA levels were reduced by 83% to 96% in CXCR4-silenced XN6 and XN3 cells, respectively, as compared with 5F7. Flow cytometry confirmed that CXCR4 protein levels were depleted by 94% to 97% in CXCR4-silenced cells. Of note, silencing of CXCR4 abrogated the invasive potential of the XN6 and XN3 sublines, whereas the nonsilenced XP6 clone remained invasive (data not shown). Next, the two CXCR4-silenced subclones XN3 and XN6 and the control XP6 were inoculated in SCID mice. The depletion of CXCR4 had no significant effect on tumor growth as the median volume of the tumors at day 62 postinjection was 1,331 mm$^3$ in control 5F7 xenografts versus 1,338 and 1,130 mm$^3$ in the CXCR4-depleted XN3 and XN6 xenografts, respectively. At the histologic level (Fig. 2D), tumors appeared as carcinomas with cell rows and glands containing mucus in their lumen. The Ki-67 cell proliferation marker was found at comparably high levels in xenografts established from parental 5F7 cells (77%) and their CXCR4-depleted clonal derivatives XN3 (83%) and XN6 (78%). Metastases and/or micrometastases in lungs were observed in four of four mice inoculated with the CXCR4-positive control XP6 xenografts versus 1,338 and 1,130 mm$^3$ in the CXCR4-depleted XN3 and XN6 xenografts, respectively. Of note, silencing of CXCR4–silenced subclones were selected by qRT-PCR and flow cytometry showed that drug resistance was associated with the notion that CXCR4-mediated migration of carcinoma cells is controlled by phosphoinositide-3-kinase/Akt-dependent and p42/44-independent pathways (26).

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Enhanced expression of CXCR4 in drug-resistant human colon cancer cells

To substantiate the relationship between drug resistance and CXCR4, we analyzed CXCR4 expression in HT-29 cell subpopulations resistant to clinical anticancer drugs. Flow cytometry showed that drug resistance was associated with CXCR4 expression in HT-29 cell subpopulations resistant to clinical anticancer drugs. Flow cytometry showed that drug resistance was associated with

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**Table 2. Uregulated genes (fold-change >10) in the metastatic 5M21 and 5F7 primary tumors vs. the nonmetastatic 5F31 xenografts and their corresponding GenBank accession numbers**

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<th>GenBank no.</th>
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<td>NM_012342</td>
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<td>1.87E-02</td>
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a higher percentage of CXCR4-positive cells (Fig. 3A). The percentage of CXCR4-positive cells was 3% in parental HT-29 cell population. This percentage increased to 17% and 49% in OXA- and DOX-resistant cells, and to 68% and 73% in 5-FU− and MTX-resistant cells, respectively. Western blot analysis confirmed the higher expression levels of CXCR4 in drug-resistant cancer cells, especially in the DOX-, 5-FU−, and MTX-resistant HT-29 cells (Fig. 3B). The increase in CXCR4 protein was paralleled by an increase in CXCR4 transcript, as shown by qRT-PCR (Fig. 3B). Of note, OXA- and DOX-resistant HT-29 cells also display an invasive behavior (ref. 19; data not shown).

Next, we examined CXCR4 expression in established liver metastases from colon cancer patients under the FOLFOX regimen (27) within the 6-month period prior to liver metastasis resection. Control patients were not exposed to any anticancer drug within the 6-month period prior to surgery. This control group includes patients who never received any chemotherapy and patients previously treated with a 5-FU-based chemotherapeutic regimen before the 6-month preoperative period. Quantification of CXCR4 expression by qRT-PCR showed enhanced CXCR4 mRNA levels in FOLFOX-treated patients (median ratio, 3.8; \( P < 0.05 \); Fig. 3C). CXCR4 expression was further analyzed by immunohistochemistry in human liver colon metastases from FOLFOX-treated and untreated patients (Fig. 3D). In all cases, CXCR4 staining was detected in cancer epithelial cells and tumor stroma cells. In metastases from untreated patients, CXCR4 staining in cancer cells was weak/moderate and localized in the cytoplasm and nucleus. In FOLFOX-treated patients, the CXCR4 signal in cancer cells was strong and mainly localized in the nucleus. It is

![Figure 2](image.png)

**Figure 2.** Implication of CXCR4 in the invasive and metastatic behavior of 5-FU−resistant 5F7 cells. A, collagen type I invasion assays were performed in the presence and absence (control) of chemical inhibitors or neutralizing antibodies targeting CXCR4 (mAb 12G5, dilution 1:100; ADM3100, 10 \( \mu \)g/mL), GPCR signaling (BIM-46187, 1 \( \mu \)mol/L), Gα/o/i subunits (PTx, 0.2 \( \mu \)g/mL), PKA (KT5720, 500 nmol/L), phosphoinositide-3-kinase (wortmannin, 10 nmol/L), PLCβ (U73122, 1 \( \mu \)mol/L), PKC (Gö6976, 1 \( \mu \)mol/L), ROCK (Y27632, 10 \( \mu \)mol/L), Rho-GTPase (C3T exoenzyme, 5 \( \mu \)g/mL), and ROCK (Y27632, 10 \( \mu \)mol/L), p38 (SB203580, 10 \( \mu \)mol/L), and p42/44 (PD098059, 50 \( \mu \)mol/L). Columns, mean from three independent experiments; bars, SD. qRT-PCR analysis (B) of CXCR4 mRNA expression and flow cytometry analysis (C) of CXCR4 protein levels in 5F7 cells submitted to CXCR4 silencing by ARN interference. Data are representative of three independent experiments. D, histologic analysis (hematoxylin, eosin, saffron, and astra blue, HESAB) of primary tumor xenografts (XN3, XN6, and XP6) and lung metastases (XP6, white arrows) after inoculation in SCID mice (magnification, ×200; except for XP6, ×400). The presence of metastases containing colon cancer cells with mucus secretion was detected in the lungs of mice bearing control XP6 tumor xenografts.
noteworthy that CXCR4 nuclear localization is associated with metastasis (28, 29). Consistently, a nuclear localization sequence mediates the transfer of CXCR4 to the nucleus after interaction with its ligand CXCL12/SDF-1 (30).

**MIF is an autocrine proinvasive factor in 5-FU and MTX-resistant HT-29 cells**

CXCL12 is the canonical CXCR4 ligand involved in different malignant processes, including tumor cell growth, angiogenesis, and metastatic spread (10, 14). However, CXCL12 was not detected in the native and concentrated (CM, 20×) conditioned culture media prepared from parental HT-29 cells and the 5-FU–resistant clones (data not shown), in agreement with CXCL12 promoter hypermethylation in this model (31). The expression of the MIF was therefore investigated. This cytokine produced by intestinal cells (32) was recently described as a functional CXCR4/CXCR2 ligand (33). High MIF levels were detected by immunoblotting in cell lysates and CM prepared from parental and drug-resistant HT-29 cells (Fig. 4A; data not shown). These CM contained high levels of vascular endothelial growth factor whereas tumor necrosis factor-α, interleukin-1β, interleukin-4, interleukin-7,

![Figure 3](image_url)

**Figure 3.** Enhanced expression of CXCR4 in drug-resistant human colon cancer cells and clinical liver metastases. A, CXCR4 expression by flow cytometry in DOX-, OXA-, 5-FU–, and MTX-resistant HT-29 cells in comparison with parental HT-29 cells (Ct). Drug-resistant HT-29 subpopulations were cultured for 4 weeks in the presence of drugs before analysis. Representative data from two independent experiments are shown for each drug. B, CXCR4 expression by Western blot (43 kDa) and qRT-PCR in drug-resistant HT-29 subpopulations after 3 months of treatment with drugs compared with parental HT-29 cells. Annexin A2 was used as a loading control (35 kDa). Data are representative of two independent experiments. C, qRT-PCR analysis of CXCR4 expression in human colon liver metastases resected from FOLFOX-treated patients within the 6-month period prior to surgery (▼) versus untreated patients (▼) and patients who had received 5-FU–based chemotherapy after the resection of the primary tumor, but not subsequently treated within the 6-month period prior to surgery (▲). Individual values and median values relative to untreated patients (bars) are shown (*, P < 0.05). D, representative immunohistochemistry of CXCR4 in human liver metastases from FOLFOX-treated patients within the 6-month period before surgery (n = 5 specimens), relative to untreated patients (n = 6 specimens). CXCR4 staining (brown) is revealed by counterstaining with hematoxylin (blue; magnification, ×200).
interleukin-11, IFNα, IFNβ, and IFNγ were not detected (data not shown). MIF expression was analyzed by immunohistochemistry in liver metastases resected from FOLFOX-treated and untreated patients. For each group of patients, we observed a cytoplasmic staining with moderate intensity (++) in cancer cells as well as MIF staining in tumor stroma, including immune cells (Fig. 4B). Immunohistochemistry was also performed in tumor xenografts established in SCID mice following inoculation of parental HT-29 cells and their clonal derivatives 5F31, 5F7, and 5M21. MIF staining (brown) is shown after counterstaining with hematoxylin (blue; magnification, ×200). C, collagen invasion assays using 5F7 invasive cells treated by the MIF inhibitor ISO-1 (10 and 100 μmol/L), the MIF-neutralizing pAb FL-115 (dilution 1:20) and its rabbit control isotype (***, P < 0.001). D, effects of the combinations of 5-FU (10−5 mol/L) with the CXCR4 and/or MIF inhibitors (AMD3100 10 μg/mL and ISO-1 0.1 mmol/L, respectively) on the proliferation of 5-FU-resistant HT-29 FU cells. Cells in six-well plates were cultured for 6 days in the presence or absence of the indicated drugs, and then numbered. Treatments with AMD3100, ISO-1, and 5-FU as single agents did not bring any significant difference in cell number as compared with 5-FU-resistant cells cultured under control conditions (without any drug). In contrast, significant decreases in cell number were observed when 5-FU was combined with AMD3100, ISO-1, or their association, as compared with control conditions (*, P < 0.05).
AMD3100 or the MIF inhibitor ISO-1 (Fig. 4D). Whereas AMD3100, ISO-1, and 5-FU were ineffective as single agents, combinations of 5-FU with AMD3100 or ISO-1 led to a significant decrease in cell number as compared with 5-FU-resistant cells cultured in control conditions. Simulta-
neous addition of AMD3100 and ISO-1 with 5-FU did not have an additive or synergistic effect on cell growth. Consis-
tently, both MIF and CXCR4 acted as upstream activators and integral components of the same signaling pathway. In all conditions tested, flow cytometry showed that the percentage of cells at the sub-G1 fractions was below 1%, suggesting that the MIF/CXCR4 axis plays a role in the prolif-
eration of established 5-FU-resistant colon cancer cells, without affecting their survival. Of note, a strong amplifica-
tion of the TYMS gene encoding thymidylate synthetase was detected in the CXCR4-negative noninvasive variant 5F31 (8.8-fold in 5F31 cells versus 2.2-fold in 5F7 cells; data not shown). Therefore, amplification of the TYMS gene might be involved at different degrees in the resistance of HT-29 cells to 5-FU.

Discussion

An important advance at the origin of this work was the emergence and characterization of drug-resistant CXCR4-positive and invasive HT-29 colon cancer cells selected by the clinical anticancer agents 5-FU, OXA, DOX, and MTX. We report that induction of the GPCR CXCR4 acts as a major mechanism underlying the aggressiveness of 5-FU-resistant HT-29 cells in vitro and in vivo. Consistently, silencing of CXCR4 and treatments by several inhibitors targeting canonical CXCR4 and GPCR pathways (25) markedly reversed cellular invasion and the development of lung metastases induced by 5-FU-resistant xenografts.

We identified the human colon cancer cell–derived MIF as the critical autocrine CXCR4 ligand promoting the invasion of several drug-resistant colon carcinoma HT-29 cells. The receptor for MIF signaling was identified as a molecular scaffold involving CD74 (MHC class II invariant chain) and CD44 coreceptor (34, 35). Both CD74 and CXCR2, as well as the CXCR4 agonist CXCL12, were not expressed in the 5-FU-resistant clones 5F7 and 5M21 (data not shown), thus supporting the activation of a MIF-dependent autocrine loop via CXCR4 in the present study. MIF, originally known as a key mediator in the regulation of immune and inflammatory responses, exerts a pivotal role in oncogenic transformation and tumor progression. The generation of MIF knockout mice showed that MIF-deficient cells exhibit a resistance to oncogenic transformation (36). In the APCMin/+ mouse model of intestinal tumorigenesis, homozygous MIF deletion was associated with a reduced tu-
morogenesis (37). High MIF levels were found in tumors and sera of patients with different types of cancer, and consist-
tently, MIF production has been associated with aggressive-
ness and metastatic potential of human tumors (38–40). MIF was reported to promote tumor growth, angiogenesis, inva-
sion, and metastasis through the induction of metallopro-
teases and vascular endothelial growth factors (40, 41).

Both MIF and vascular endothelial growth factors are constitu-
tively expressed in parental HT-29 cells and their drug-resistant derivatives. MIF exerts a tautomerase activ-
ity in vitro (42). We found that the invasive phenotype of 5-FU-resistant HT-29 cells is inhibited by the MIF neutraliz-
ing antibody and the MIF tautomerase inhibitor ISO-1. Although the tautomerase activity might be dispensable in the biological functions of MIF (43), its inhibition reduced tumor progression in murine CT26 colon carcinoma models (40). We have therefore shown that chemokine receptor CXCR4 functions as a dominant invasion promoter and metastasis progression signal in 5-FU- and MTX-resistant cells, in a MIF-dependent manner. It is likely that the in-
vasive and metastatic phenotype of CXCR4-positive and drug-resistant cancer cells is also regulated in a paracrino-
manner by MIF and CXCL12/SDF-1 derived from tumor stroma cells. In the present study, the CXCR4 antagonist AMD3100 and the MIF inhibitor ISO-1 significantly decreased the growth of 5-FU-resistant HT-29 cells maintained under the 5-FU pressure, suggesting that CXCR4 and its noncognate ligand MIF are also connected with cell proliferation signaling pathways in this model of drug resistance.

CXCR4 expression is known to be downregulated by the translation inhibitor miR-146 (44). Specific qRT-PCR revealed that miR-146a was downregulated in all drug-resistant HT-29 cells, independently of their CXCR4 status and invasive potential (data not shown). CXCR4 expres-
sion is also induced at the transcriptional level by the oncogenic nuclear factor-κB pathways, the transcription fac-
tors ASCL2 and the hypoxia-inducible factors, HIF-1α and HIF-2α (45–47). Recent lines of evidence indicate that a small population of cancer cells harboring several traits inherent to cancer progenitor stem cells may be more resis-
tant to chemotherapy (2). Interestingly, ASCL2 was recently identified as a critical factor involved in the maintenance of intestinal stem cells (48). In 5-FU-resistant HT-29 cells, CXCR4 induction was associated with EPAS1 and ASCL2 upregulation. Thus, we cannot exclude the possibility that miR-146a depletion and ASCL2 overexpression could con-
tribute, together with other mechanisms, to the aggressive phenotypes observed in CXCR4-positive drug-resistant HT-29 cells. The selective nuclear factor-κB inhibitor GS143, which inhibits IκBα ubiquitinylation (49), decreased the ability of CXCR4-positive 5F7 cells to invade collagen gels (data not shown). It is therefore conceivable that chronic drug pressure on parental HT-29 cells leads to the selective survival and selection of CXCR4-proficient HT-29 cell subpopulations characterized by a constitutive invasive phenotype and metastatic behavior. Finally, a recent report established that CXCR4 transcripts are upregulated in tu-
mors from patients with rectal cancer treated with the vas-
cular endothelial growth factor antibody bevacizumab (50), associating CXCR4 upregulation to treatment by another class of therapeutic agents.

We believe that transient or chronic induction of the CXCR4 signaling cascades by MIF autocrine and paracrine loops might account for the important mechanisms associat-
ed with drug resistance, invasion, and metastasis in human
colon cancer cells. Consistently, human liver metastases established in colon cancer patients treated with the FOLFOX standard regimen prior to surgery also exhibited higher levels of CXCR4 transcripts. Thus, targeting the MIF/CXCR4 pathways in combination therapies might exert beneficial effects to overcome the deleterious emergence of CXCR4 in clonal derivatives of drug-resistant cancer cells. Improved clinical outcome might be obtained in subsets of colon cancer patients that exhibit constitutive or transient activation of the MIF-CXCR4 axis associated with intrinsic or acquired resistance to chemotherapeutic agents during adjuvant and neoadjuvant therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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Autocrine Induction of Invasive and Metastatic Phenotypes by the MIF-CXCR4 Axis in Drug-Resistant Human Colon Cancer Cells

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