

# CXCL10 Promotes Invasion-Related Properties in Human Colorectal Carcinoma Cells

Adi Zipin-Roitman,<sup>1</sup> Tsipi Meshel,<sup>1</sup> Orit Sagi-Assif,<sup>1</sup> Bruria Shalmon,<sup>2</sup> Camila Avivi,<sup>2</sup> Raphael M. Pfeffer,<sup>3</sup> Isaac P. Witz,<sup>1</sup> and Adit Ben-Baruch<sup>1</sup>

<sup>1</sup>Department of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel and

<sup>2</sup>Department of Pathology and <sup>3</sup>Oncology Institute, Sheba Medical Center, Tel-Hashomer, Israel

## Abstract

**CXCL10 was recently shown to exert antimalignancy functions by influencing the tumor microenvironment. Here, we have taken a different approach, investigating the effects of CXCL10 directly on tumor-promoting functions in colorectal carcinoma (CRC) cells. CXCL10 expression was detected in preferred metastatic sites of CRC (liver, lungs, and lymph nodes), and its CXCR3 receptor was expressed by eight CRC cell lines (detected: reverse transcription-PCR and/or flow cytometry). Detailed analysis was done on two cell lines derived from primary CRC tumors (SW480, KM12C) and their metastatic descendents (SW620 and KM12SM). The three known variants of CXCR3 (CXCR3-A, CXCR3-B, and CXCR3-alt) were detected in all four cell lines. CXCR3 expression was also observed on colorectal tumor cells in biopsies of CRC patients (immunohistochemistry). CXCL10 and CXCR3 expression were potently induced in CRC cells by Interferon  $\gamma$  and all four CRC cell lines responded to CXCL10 by extracellular signal-regulated kinase 1/2 dephosphorylation. The chemokine did not affect tumor cell growth or angiogenesis-related functions in the tumor cells, such as CXCL8 and vascular endothelial growth factor secretion. Importantly, CXCL10 significantly up-regulated invasion-related properties in CRC cells: It promoted matrix metalloproteinase 9 expression and induced CRC cell migration. Of note, CXCL10-induced migration was detected only in the two metastatic cells and not in their primary counterparts. Also, CXCL10 promoted the adhesion of metastatic cells to laminin. These results suggest that CXCL10 can be exploited by CRC cells toward their progression, thus possibly antagonizing the antimalignancy effects of the chemokine on the tumor microenvironment. Therefore, care should be taken when considering CXCL10 as a therapeutic antitumor modality for CRC treatment. [Cancer Res 2007;67(7):3396–405]**

## Introduction

Colorectal carcinoma (CRC) is the fourth most abundant type of cancer in the world.<sup>4</sup> Most of the CRC patients die from metastases, predominantly in the liver, lungs, and lymph nodes (1). Metastasis results from a complex cascade of events, necessitating the involvement of numerous tumor cell–derived factors and micro-environmental molecules, including specific chemokines (2–5).

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

**Requests for reprints:** Adit Ben-Baruch, Department of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel. Phone: 972-3-6407933; Fax: 972-3-6422046; E-mail: aditbb@tauex.tau.ac.il

©2007 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-06-3087

In addition to their activities in the hematopoietic context, chemokines regulate tumor growth and metastasis in different ways—either by promoting or inhibiting these processes (3, 4, 6, 7). The possible role of chemokines in metastasis has motivated us to investigate the hypothesis that chemokines, which are expressed at preferred metastatic sites of CRC, may not only affect the tumor microenvironment, but also directly regulate malignancy-associated properties in the tumor cells. Accordingly, in this study, we focused on chemokines that are expressed at preferred metastatic sites of CRC, whose corresponding receptors are expressed by CRC cells.

An initial screen has extended published analyses on the expression of chemokines in organs that constitute preferred metastatic sites of CRC such as liver, lungs, and lymph nodes (e.g., refs. 8–11). In parallel, the expression of the corresponding receptors for these chemokines by CRC cells was studied. Based on the results obtained in the initial screen, we have focused in the present study on the CXCL10 chemokine and its CXCR3 receptor.

CXCL10 is a member of the subfamily of Interferon  $\gamma$  (IFN $\gamma$ )–inducible, non-ELR CXC chemokines and is expressed by a large variety of cell types. It functions as a major chemoattractant for activated T cells and natural killer cells (12–14). Recently, numerous studies on the roles played by IFN $\gamma$ -inducible CXCR3 ligands in malignancy, primarily CXCL10, have been gathered into the concept of “immunoangiostasis” (15). This term reflects the ability of CXCR3 ligands to exert concomitantly two tumor-inhibiting properties on cells of the tumor microenvironment: (a) recruitment of CXCR3-expressing mononuclear cells with anti-tumor activities into tumor sites and (b) antagonizing the vascularizing effects of powerful angiogenic factors (12–16).

Although the antimalignancy activities of CXCL10 were determined in several tumor systems, only a limited number of studies analyzed its role in CRC, primarily in murine model systems (17–21). Moreover, these analyses did not address the highly important issue of whether the chemokine affects the expression of malignancy-related properties by CRC cells, from murine or human sources.

In this study, we have taken a different approach, analyzing the direct activities of CXCL10 on CRC cells that express CXCR3, the receptor for CXCL10. The findings reported in this study indicate that CXCL10 acts directly on CRC tumor cells to promote their promalignancy/prometastatic characteristics, and suggest that the tumor cells exploit the chemokine for promoting their invasive capacities. Moreover, our study dissociates between primary and metastatic cells and shows that the two cell types respond differently to the tumor-supporting activities of CXCR3 ligands.

<sup>4</sup> <http://www.iarc.fr>

The identification of such effects for CXCL10 is important because it suggests that at CRC tumor sites, the chemokine has two opposing effects: antimalignancy functions, which are exerted on the tumor microenvironment, versus promalignancy activities, which are mediated by its ability to act directly on the tumor cells. Overall, the present observations emphasize the need for a cautious approach when considering CXCL10, and possibly other IFN $\gamma$ -inducible CXCR3 ligands, as therapeutic measures in malignancy, and call for further elucidation of the roles played by CXCR3 ligands in CRC and in malignancy in general.

## Materials and Methods

**CRC cell lines.** The SW480 and SW620 CRC cell lines were obtained from the American Type Culture Collection (Manassas, VA). The SW480 cell line was derived from a primary Duke B CRC, whereas the SW620 line was derived from a lymph node metastasis of the same patient (22). The KM12C and KM12SM CRC cell lines were kindly provided by Dr. I.J. Fidler (Department of Cancer Biology, M.D. Anderson Cancer Center, Houston, TX). The KM12C cell line was derived from a Duke B2 CRC primary tumor, whereas the KM12SM cell line was originated in liver metastasis that developed in BALB/c nude mice inoculated with KM12C cells to cecum (23). The study included four additional CRC cell lines (primary, 474 and 1203; liver metastasis, 1086; lymph node metastasis 0485), kindly provided by Dr. D.L. Morton (John Wayne Cancer Institute, Santa Monica, CA).

**RNA preparation.** Total cellular RNA was isolated from serum-deprived CRC cell lines using the EZ-RNA kit, according to the manufacturer's instructions (Biological Industries, Beit Haemek, Israel). Chemokine expression by human liver was determined on human liver polyadenylic acid [poly(A)] RNA purchased from Ambion (Austin, TX). Murine lungs, livers, and lymph nodes were harvested, washed with ice cold PBS, and then immediately put on dry ice. Small pieces of the frozen tissues were taken for RNA isolation, as described above.

**Reverse transcription-PCR and product sequencing.** Total RNA (2  $\mu$ g) derived from CRC cell lines or 100 ng of human liver poly(A) RNA samples were used for generation of first-strand cDNA synthesis. The first-strand cDNA was subjected to PCR reaction, as detailed in the Supplementary Material. Reactions without cDNA and reactions without reverse transcriptase ruled out DNA contamination in all samples. PCR products were sequenced and aligned with the corresponding published sequences.

**Flow cytometry.** CRC cells were grown in serum-free medium [containing 0.5% bovine serum albumin (BSA)] with or without recombinant human (rh) IFN $\gamma$  (PeproTech, Rocky Hill, NJ) for 48 h, then stained by primary antibodies directed against CXCR3 (R&D Systems, Minneapolis, MA) or against IFN $\gamma$  receptor (Serotec, Oxford, United Kingdom). This was followed by incubation with FITC-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratory, West Grove, PA). Baseline staining was obtained by adding cell sorter medium to the cells instead of primary antibody. Additional negative controls included irrelevant isotype-matched antibodies that confirmed the specificity of the staining (data not shown).

**Immunohistochemistry of CXCR3 and CD3 expression in CRC tumors.** CRC patients were enrolled to Sheba Medical Center (Tel-Hashomer, Israel). The study was approved by the Helsinki Committee of Sheba Medical Center. In brief, formalin-fixed colorectal tumors from five CRC patients were dehydrated, embedded in paraffin, and sectioned at 4  $\mu$ m. For CXCR3 staining, antigen retrieval was done using microwave at 98°C for 20 min in citrate buffer (pH 6). Subsequently, an endogenous peroxidase block was done for 10 min in 3% H<sub>2</sub>O<sub>2</sub>/methanol. After rinses in TBS, sections were incubated with antibodies against CXCR3 (R&D Systems; 1:40) for 90 min at room temperature. Detection was done with the Envision Dual link, horseradish peroxidase (HRP)-labeled (Dakocytomation, Copenhagen, Denmark), for 30 min at room temperature. After TBS rinses, antibody binding was visualized with the substrate-chromogen AEC and counterstained with hematoxylin. For all tumors analyzed, negative controls included sections in which the primary antibody was replaced by PBS, as

well as sections stained by an irrelevant isotype-matched antibody. No staining was detected in these negative control slides (data not shown). For CD3 staining, antigen retrieval was done using a pressure cooker at 110°C for 3 min in citrate buffer (pH 6). Subsequently, antibodies to CD3 (Dakocytomation; 1:100) were used according to routine procedures at the Department of Pathology, Sheba Medical Center, in an automated immunostainer (NEXES, Ventana Medical Systems, Tucson, AZ) using an I View DAB detection kit (Ventana Medical Systems).

**Extracellular signal-regulated kinase 1/2 phosphorylation.** CRC cell lines were grown in serum-free medium (as above) with or without rhCXCL10 (PeproTech) for 24 h. Cell lysates were subjected to SDS-PAGE and Western blotting with rabbit polyclonal antibodies against phosphorylated extracellular signal-regulated kinase 1/2 (Erk1/2; R&D Systems). As loading control, rabbit polyclonal antibodies against Erk2 (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Detection was done by secondary HRP-conjugated goat antibodies against rabbit IgG (Jackson ImmunoResearch Laboratory). Bands were visualized by chemiluminescence-enhanced reaction (Amersham, Buckinghamshire, United Kingdom).

**Matrix metalloproteinase expression.** CRC cell lines were grown in serum-free medium (as above) with or without rhCXCL10 (PeproTech) for 48 h. Cell supernatants were subjected to SDS-PAGE and Western blotting using monoclonal mouse antibodies against pro/active human matrix metalloproteinase 9 (MMP9; 8  $\mu$ g/mL, MAB936, R&D Systems), followed by HRP-conjugated goat antibodies against murine IgG (Jackson ImmunoResearch Laboratory). Protein loading was determined by Ponceau staining.

**ELISA assays.** For detection of CXCL8 and vascular endothelial growth factor (VEGF), CRC cell lines were grown in serum-free medium (as above) with or without rhCXCL10 (PeproTech) for 48 h. Supernatants were collected, and CXCL8 and VEGF expression was determined by ELISA detection kit according to the manufacturer's instructions (R&D Systems and PeproTech, respectively; data not shown). For detection of CXCL10, CRC cell lines were grown in the same medium, with or without rhIFN $\gamma$  for 24 h. Supernatants were collected, and CXCL10 expression was determined by ELISA detection kit according to the manufacturer's instructions (PeproTech). Statistical analysis was done by Student's *t* test.

**Cell growth.** CRC cell lines were grown in serum-free medium (as above) with or without rhCXCL10 (PeproTech) for 72 h in a 96-well plate. Proliferation was determined by an endogenous alkaline phosphatase (ALP) assay, by the addition of an ALP substrate buffer, containing 3 mg/mL *p*-nitrophenyl phosphate disodium (Sigma-Aldrich, St. Louis, MO). Preliminary calibration of the ALP assay indicated that it provides a reliable measurement of cell proliferation (data not shown). Absorbance was measured at 405 nm. Statistical analysis was done by Student's *t* test.

**Migration.** The migration of serum-deprived CRC cells was assessed by a 48-well modified Boyden chamber technique. Briefly, the lower compartment of the chamber was loaded with serum-free medium or rhCXCL10 (PeproTech) diluted in a similar medium, whereas the upper compartment of the chamber was loaded with  $4 \times 10^5$ /mL cells (resuspended in a similar medium). The two compartments were separated by a 10  $\mu$ m pore-sized PVDF polycarbonate membrane coated either by 30  $\mu$ g/mL laminin (Sigma-Aldrich) for SW480 and SW620 cells, or by 50  $\mu$ g/mL rat collagen type I (Collaborative Biomedical Products, Bedford, MA) for KM12C and KM12SM cells. Following 6-h incubation at 37°C, cells on the upper side of the membrane were removed, and the cells on the lower side were fixed and stained with a Diff-Quik kit (Dade Behring, Beerfield, IL). The migrated cells in several high-power fields (in each of the triplicates done) were counted by light microscopy. The statistical significance of the number of cells migrating in response to stimuli versus to medium was evaluated by Student's *t* test.

**Adhesion assays.** Non-tissue culture 96-well plates were coated with or without rhCXCL10 (PeproTech) at room temperature for 1 h. Following washes with PBS, the plates were coated with 30  $\mu$ g/mL laminin (Sigma-Aldrich) for an additional 1 h at room temperature. Following additional washes, the plates were coated by 1% heat-inactivated BSA for 30 min at room temperature. The plates were washed twice by PBS, then 100  $\mu$ L of  $4 \times 10^5$ /mL serum-starved SW620 cells were added. The plate was centrifuged for 7 or 10 min at room temperature. Nonadherent cells were

removed by washings with ice-cold PBS. The number of adhering cells was evaluated by the ALP test described above. Statistical analysis was done by Student's *t* test.

## Results

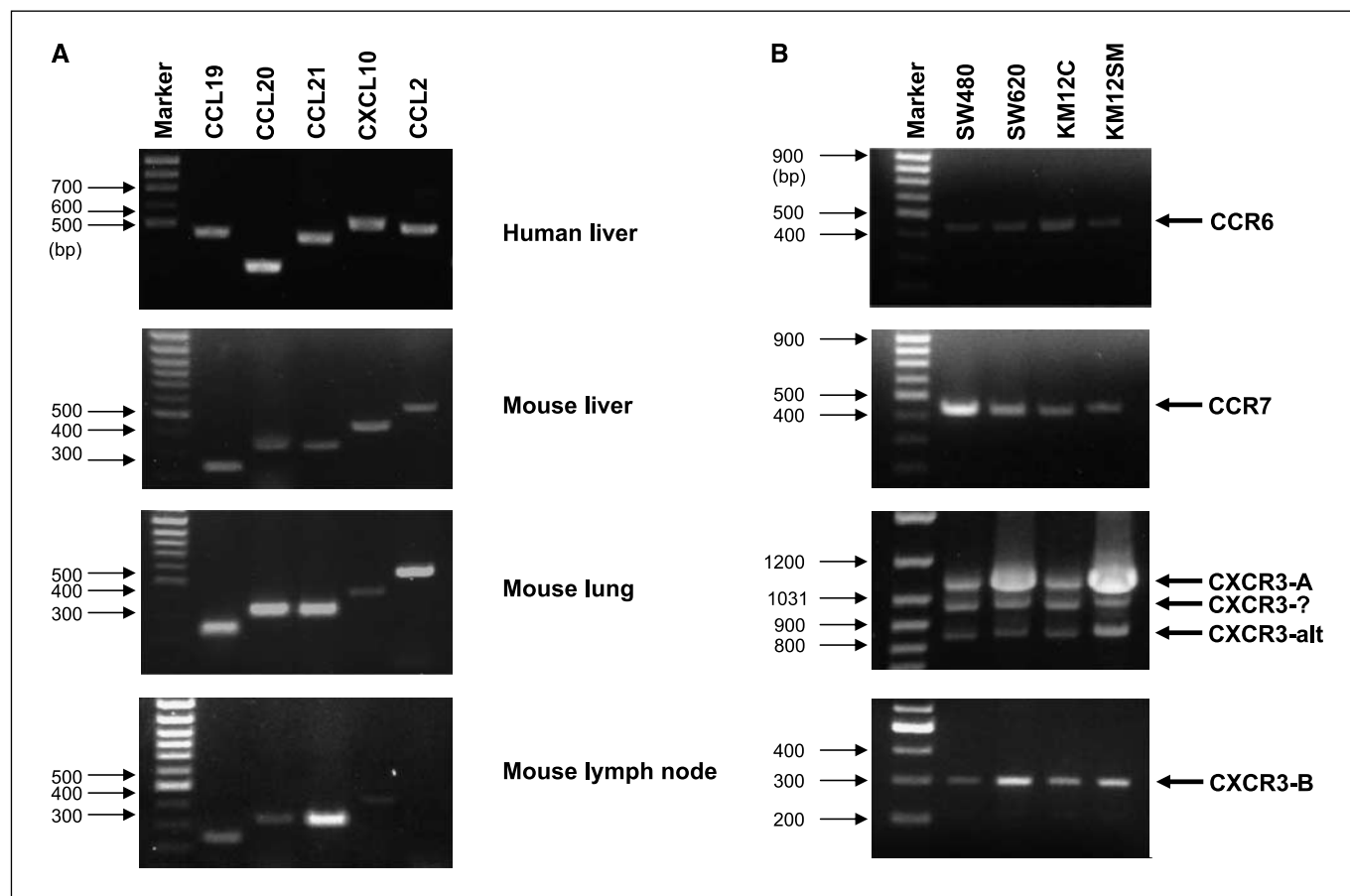
**Expression of chemokines in metastatic organs of CRC and expression of chemokine receptors by CRC cells.** The aim of the present research was to gain better knowledge on the roles played by chemokines and their corresponding receptors in CRC metastasis. Colon carcinoma cells metastasize mainly to the liver, lungs, and lymph nodes, sites in which the expression of several chemokines was already detected, for example, the expression of CCL19 and CCL21 in lymph nodes and of CCL20 in the liver (8–11).

Analyses that were done on CCL19, CCL20, and CCL21 expression in liver, lungs, and lymph nodes from human and/or murine sources have shown that all these chemokines were expressed by the three CRC metastatic sites (Fig. 1A), confirming and expanding results obtained by other investigators (8–11). The analysis was further extended to inflammatory chemokines at these sites, based on publications showing that such chemokines may be constitutively expressed, at least to some extent, at sites such as liver (e.g., CXCL10; refs. 24–27), thus providing a first line of defense against infections. As shown in Fig. 1A, in contrast to CCL2 whose expression was limited to only several of the sites, CXCL10 was

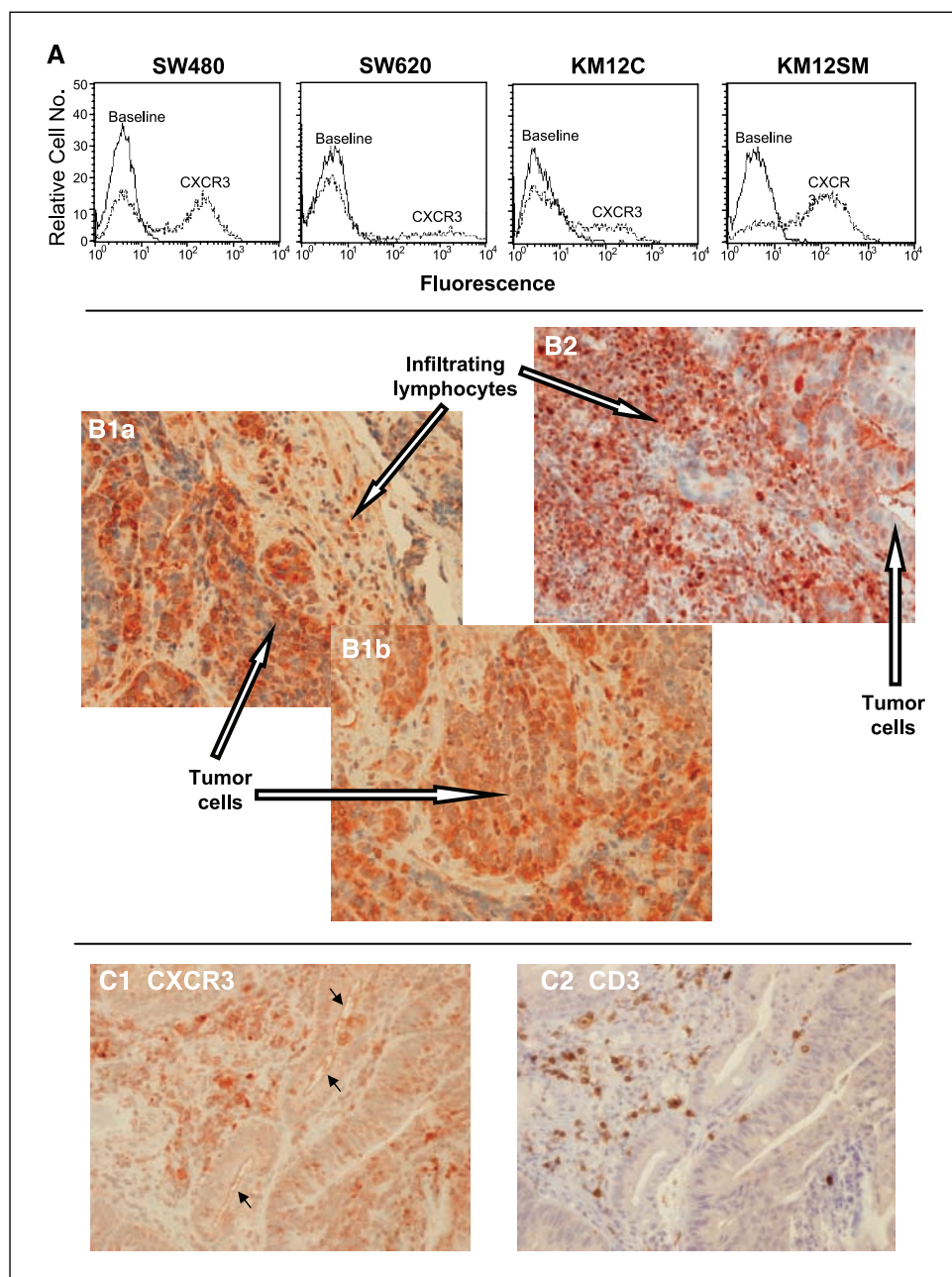
expressed at all three CRC metastatic sites: liver, lungs, and lymph nodes.

In view of the possibility that metastasis is regulated by an interaction between chemokines secreted from cells in the target sites of tumor spread, and the corresponding receptors expressed by the tumor cells, we measured the expression of receptors for the above chemokines by CRC cells. To this end, we have used two model systems, which are based on the working hypothesis that tumor cells that have successfully formed metastases are equipped with unique properties that distinguish them from their counterparts at the primary site. As this may be true also for the responses of the two different cell types to chemokines, we compared in the present study cells that were derived from the primary tumor site and their metastatic descendants. The two pairs of primary and metastatic cells used in our study were as follows: (a) SW480 and SW620, respectively (22); (b) KM12C and KM12SM, respectively (23) (see also Materials and Methods). In each pair, the primary and the metastatic cells originated from the same patient, thus having the same genetic background.

In these four cell types, we measured the expression of receptors for chemokines that are expressed by all CRC metastatic sites, namely CCL19, CCL20, CCL21, and CXCL10. Using reverse transcription-PCR (RT-PCR), the three corresponding receptors—CCR6, CCR7, and CXCR3—were detected on all four CRC cells



**Figure 1.** The expression of chemokines at CRC metastatic organs and of chemokine receptors by CRC cells. *A*, RT-PCR analysis of chemokine expression by CRC metastatic organs. The expression of CCL19, CCL20, CCL21, CXCL10, and CCL2 by CRC metastatic organs was determined by RT-PCR using human liver RNA or murine liver, lung, and lymph node RNA. Sequencing of the resulting bands confirmed their identity. Negative controls included reactions without cDNA and reactions without reverse transcriptase (not shown). *B*, RT-PCR analysis of CCR6, CCR7, and CXCR3 expression by CRC cells. Sequencing of the resulting bands confirmed their identity. Negative controls included reactions without cDNA and reactions without reverse transcriptase (not shown).



**Figure 2.** CXCR3 is expressed by CRC cells in culture and by colorectal tumor cells in biopsies of CRC patients. **A**, cell surface expression of CXCR3 by CRC cell lines. The expression of CXCR3 by CRC cells was determined by flow cytometry, using specific antibodies to CXCR3, followed by secondary antibodies. *Baseline*, cells stained by secondary antibodies only. Negative controls also included irrelevant isotype-matched antibodies (not shown). Representatives of five to seven independent experiments done for each cell line. **B**, CXCR3 expression in biopsies taken from human colorectal tumors. Magnification,  $\times 400$ . The expression of CXCR3 was determined by immunohistochemistry by using specific CXCR3 antibodies. Two negative controls were included (not shown): (1) sections stained by an irrelevant isotype-matched antibody; (2) sections stained by PBS instead of antibodies against CXCR3. Both controls were negative. *B1a* and *B1b*, sections taken from the same CRC patient. *B2*, a section taken from a different CRC patient. CXCR3 expression was detected in tumor cells of all CRC biopsies that were analyzed. **C**, consecutive staining of CXCR3 (*C1*) and CD3 (*C2*) in serial sections, in the same CRC tumor. *Black arrows*, membranous apical expression of CXCR3 by the tumor cells. Magnification,  $\times 400$ .

(Fig. 1B). Whereas the expression of CCR6 and CCR7 by CRC cells was already described (28, 29), the finding on the expression of CXCR3 is novel.

Analyzing the expression of the three chemokine receptors at the protein level (by flow cytometry), it was found that only CXCR3 was expressed at the cell membrane of the four CRC cell lines mentioned above (Fig. 2A), as well as by four additional CRC cell lines (474, 1203, 1086, and 0485; data not shown). In contrast, no cell surface expression of CCR6 or of CCR7 was detected by the different CRC cells (data not shown). The expression of the CXCR3 receptor by a large number of CRC cells suggests that this receptor is a general characteristic of CRC cells.

In view of the fact that CXCL10 is expressed at the microenvironment of metastatic CRC cells, as well as at primary tumor sites (30), and in view of the novel finding that CXCR3 is expressed by

CRC cells, we focused on CXCL10-CXCR3 interactions in CRC cells and on the effects induced by CXCL10 on the malignancy phenotype of these cells.

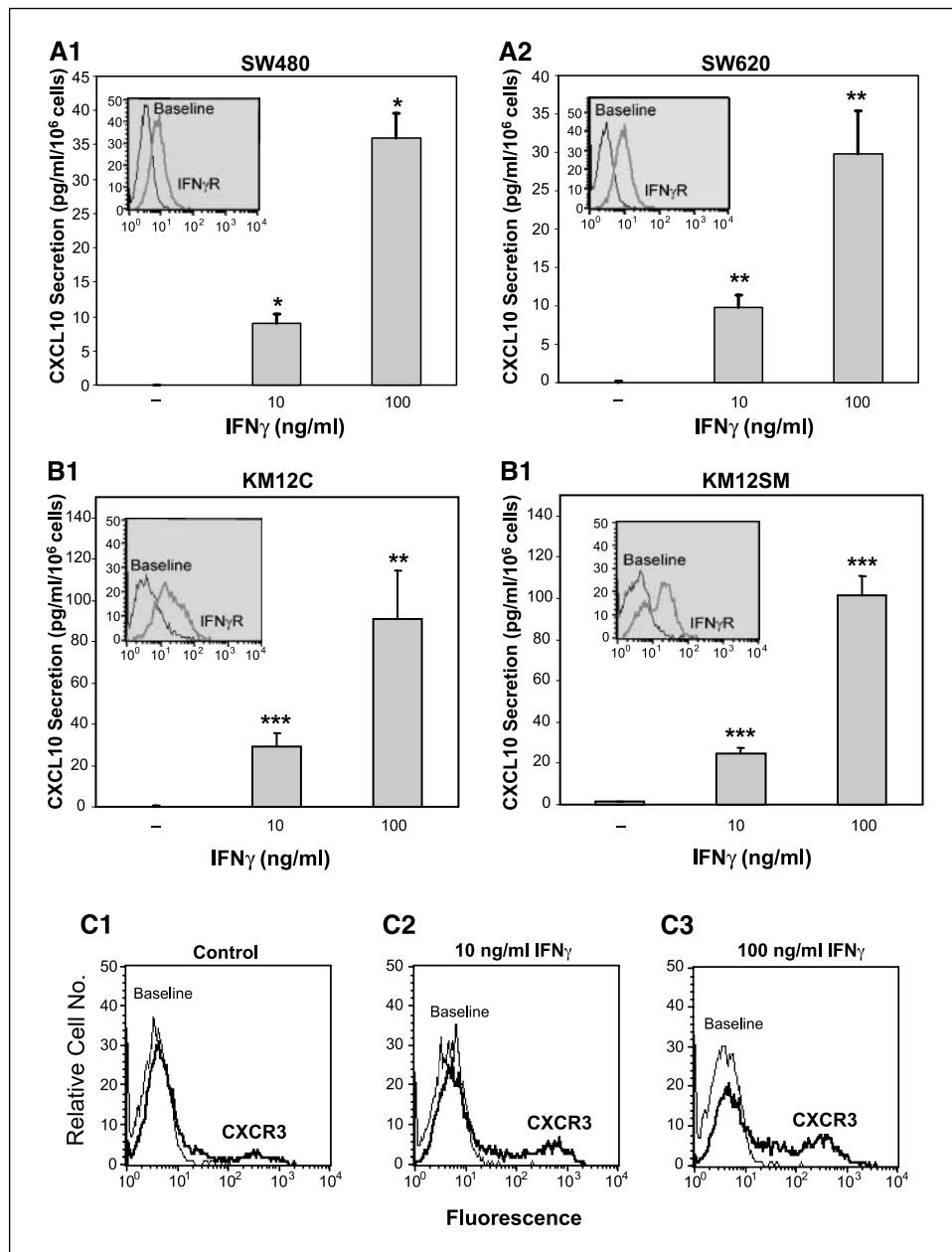
**CRC cell lines express all three known CXCR3 variants.** Recently, it was shown that CXCR3 has three variants: CXCR3-A, CXCR3-B, and CXCR3-alt (31, 32). The expression of the different CXCR3 variants by the SW480, SW620, KM12C, and KM12SM CRC cell lines was analyzed by RT-PCR. The results shown in Fig. 1B indicate that the three CXCR3 variants are expressed by all four cell types. Sequencing of the RT-PCR products confirmed the identity of these receptor variants. In all four CRC cells, another band was detected between the CXCR3-A and CXCR3-alt bands. This band, termed CXCR3-?, was generated by using specific primers for CXCR3-A and CXCR3-alt. Partial sequencing showed homology to CXCR3; however, the identity of this receptor remains to be fully determined.

**Tumor biopsies of CRC patients reveal the expression of CXCR3.** In view of the possible importance of CXCR3 expression by CRC cells, we have extended our analyses and determined the expression of CXCR3 by colorectal tumor cells in biopsies of patients. Our immunohistochemical analysis provides the first evidence to the expression of the CXCR3 receptor by CRC cells in tumors (Fig. 2B and C1). In line with published findings on CXCR3 expression by lymphocytes (33), significant staining of CXCR3 was detected also by the tumor-residing lymphocytes. Consecutive staining of serial sections by antibodies against CXCR3 and CD3 has shown the membranous expression of CXCR3 by CRC tumor cells (Fig. 2C), which were clearly distinguishable from the infiltrating lymphocytes. More specifically, the figure shows carcinoma glands with apical membranous CXCR3 expression by the tumor cells (Fig. 2C1, black arrows). Taken together with our

observations on CRC cell lines, these *in situ* results support the possibility that CXCR3 plays a role in CRC malignancy.

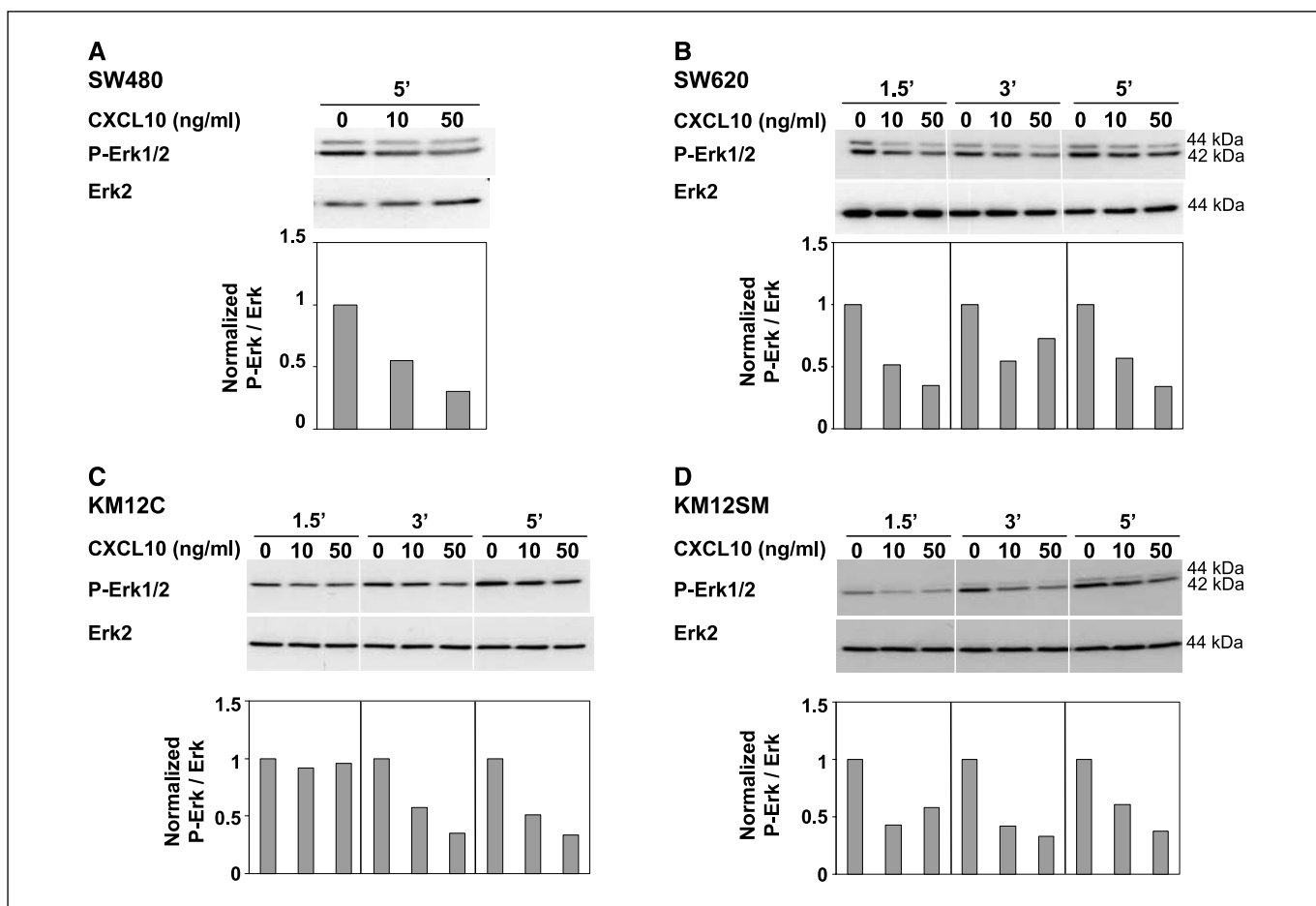
**IFN $\gamma$  stimulates CXCL10 and CXCR3 expression by CRC cells.** The expression of CXCL10 at the microenvironment of metastatic and primary CRC tumors (our current results and refs. 24–27, 30) suggests that the chemokine may act on CRC cells at these sites, and affects their phenotype. Such paracrine activities of CXCL10 can be complemented by CXCL10 secreted from the tumor cells themselves, either constitutively or after exposure to micro-environmental stimuli.

As shown in Fig. 3A and B, the two primary cells and their metastatic descendents did not secrete CXCL10 constitutively. In view of the fact that CXCL10 has been well characterized to be up-regulated by IFN $\gamma$  (12–14), we determined the effects of IFN $\gamma$  on CXCL10 secretion by the CRC cells, which express IFN $\gamma$  receptors



**Figure 3.** IFN $\gamma$  up-regulates CXCL10 secretion and CXCR3 expression by CRC cell lines. A and B, CXCL10 expression in supernatants of CRC cells after stimulation by IFN $\gamma$ . A1, SW480 cells; A2, SW620 cells. B1, KM12C cells; B2, KM12SM cells. CXCL10 expression was detected by ELISA assay before and after stimulation by IFN $\gamma$  for 24 h. \*,  $P < 0.02$ ; \*\*,  $P < 0.008$ ; \*\*\*,  $P < 0.0008$ . Insets, expression of IFN $\gamma$  receptors by the cells. *Baseline*, cells stained by secondary antibodies only. Negative controls also included irrelevant isotype-matched antibodies (not shown). In all parts of the figure, the results are representatives of at least three independent experiments. C1 to C3, CXCR3 expression by SW620 cells after stimulation by IFN $\gamma$ . CXCR3 expression was determined by fluorescence-activated cell sorting analysis before (C1) and after stimulation by IFN $\gamma$  (C2, 10 ng/mL; C3, 100 ng/mL) for 48 h. In all parts of the figure, the results are representatives of at least three independent experiments.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/67/7/3396/2581006/3396.pdf by guest on 23 April 2025



**Figure 4.** CXCL10 down-regulates Erk1/2 phosphorylation in CRC cell lines. Determination of Erk1/2 phosphorylation in CRC cell lines [SW480 (A), SW620 (B), KM12C (C), and KM12SM (D)], stimulated by CXCL10 for the indicated time points. Representatives of three to four independent experiments done for each cell line.

(Fig. 3A and B). Exposure of the four CRC cell lines to IFN $\gamma$  induced a potent CXCL10 secretion from these cells in a dose-dependent manner (Fig. 3A and B). Moreover, analysis of the SW620 cells indicated that IFN $\gamma$  up-regulated not only CXCL10 expression by the cells, but also CXCR3 expression, in a dose-dependent manner (Fig. 3C).

These results raise the possibility that IFN $\gamma$  produced by infiltrating T cells up-regulates the secretion of CXCL10 by the tumor cells, and also of CXCR3. The tumor cell-derived CXCL10, joining forces with microenvironmentally derived CXCL10, may then act directly on the tumor cells that were induced to express elevated levels of CXCR3, giving rise to amplification of the effects of CXCL10 on the malignancy phenotype of CRC cells.

#### CXCL10 regulates intracellular signaling in CRC cell lines.

The expression of CXCR3 receptors by CRC cells has led us to determine the ability of the corresponding CXCL10 ligand to affect signaling in CRC cells. To this end, we focused on Erk phosphorylation as this signaling component is regulated by G protein-coupled receptors, including CXCR3 (34, 35).

The SW480, SW620, KM12C, and KM12SM CRC cells were exposed to 10 or 50 ng/mL of CXCL10 for different time points. Figure 4 shows that CXCL10 induced a down-regulation of Erk1/2 phosphorylation in the primary CRC cells as well as in their metastatic descendants, to the following extents: 28% to 67% reduction in SW480 cells (three independent experiments), 29% to

68% in SW620 cells (four independent experiments), 47% to 77% in KM12C cells (four independent experiments), and 35% to 82% down-regulation of Erk1/2 phosphorylation in KM12SM cells (four independent experiments).

Together, these results indicate that CXCL10 is functionally active on CRC cells and that one of the signaling components it may affect is Erk phosphorylation. These results suggest that various signaling pathways induced by the chemokine can regulate the ability of CRC cells to express promalignancy activities.

**CXCL10 does not regulate angiogenesis-related properties and growth of CRC cells.** In line with the possibility mentioned above, we have determined the ability of CXCL10—as a chemokine that is expressed at the microenvironment of CRC tumors, and also by the tumor cells upon stimulation by IFN $\gamma$ —to act directly on CRC cells and to affect their tumorigenicity- and metastasis-related functions. The chemokine, used at the 10 to 700 ng/mL concentration range, did not affect the secretion from the tumor cells of the highly potent angiogenic factors VEGF and CXCL8 (data not shown). Also, CXCL10 at 50 and 500 ng/mL did not affect CRC cell growth (data not shown).

These results indicate that CXCL10 does not affect the growth of CRC cells and does not modify their ability to secrete angiogenic factors playing key roles in CRC growth and metastasis.

**CXCL10 promotes promalignancy and invasive activities in CRC cells.** Next, we analyzed the ability of CXCL10 to affect

invasion-related functions by CRC cells. First, we have determined CXCL10 effects on MMP9 expression, as this matrix-degrading enzyme is a well-characterized contributor to CRC malignancy (36). This analysis showed that CXCL10 up-regulated the secretion of MMP9 from CRC cells. As shown in Fig. 5, CXCL10 induced the expression of the ~95 kDa pro-active form of MMP9 in SW480 cells. Also, CXCL10 promoted in SW620 and in KM12C cells the expression of the ~64 kDa c-truncated form of MMP9, which was identified to have impaired sensitivity to inhibition by tissue inhibitors of MMP (37) and therefore is highly important in cancer. Similar analyses in KM12SM cells did not produce consistent results (data not shown).

We next assayed the ability of CXCL10 to induce CRC cell migration, being a prerequisite for interstitial motility of tumor cells at metastatic sites (38). Figure 6A and B shows that the metastatic SW620 and KM12SM cells migrated potently to CXCL10. However, in contrast to the metastatic cells, CXCL10 did not induce reproducible migration of their primary counterparts, SW480 and KM12C (Fig. 6A and B).

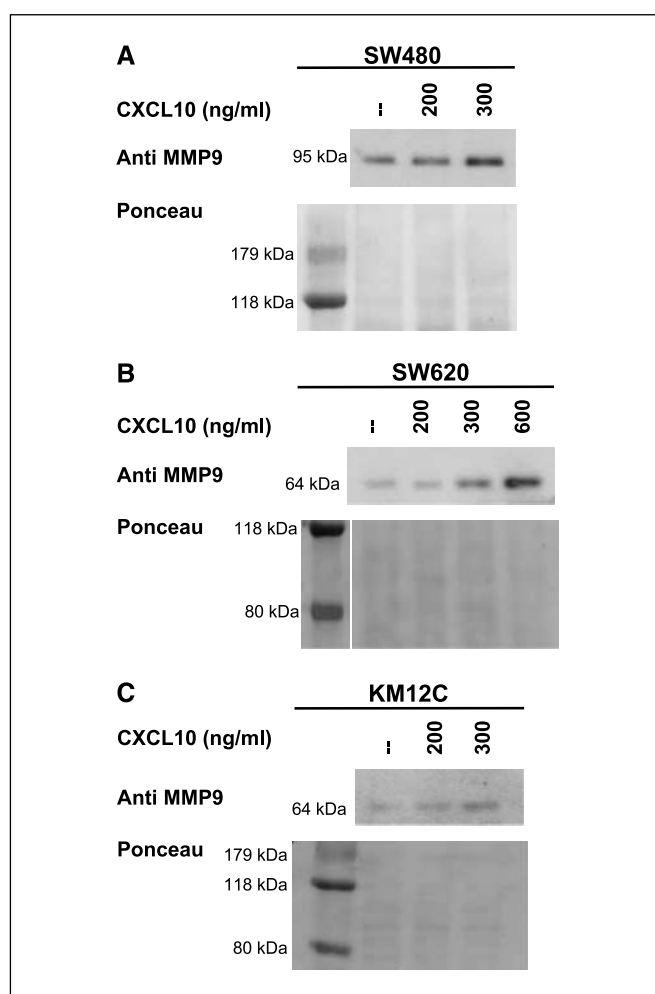
An increased malignancy phenotype is accompanied by an augmented capacity of tumor cells to adhere to components of the extracellular matrix. The experiment shown in Fig. 6C indicates that CXCL10 promotes the adhesion of SW620 metastatic CRC cells to laminin. Because adhesion is fundamental for migration, the adhesion-promoting activity of CXCL10 may enhance the motility of metastatic CRC cells at CXCL10-expressing microenvironments, such as CRC metastatic sites.

These results led us to hypothesize that by augmenting MMP secretion from CRC cells, by inducing the migration of CRC cells and by promoting CRC cell adhesion to extracellular matrix molecules, CXCL10 contributes to invasion and metastasis formation by CRC cells. The fact that metastatic CRC cells are better equipped to respond to the migratory signals of CXCL10 than the primary CRC cells supports this hypothesis. Evidently, these tumor-promoting functions of CXCL10 may be active side by side with the tumor-inhibiting functions of the chemokine, which are exerted on the tumor microenvironment. To answer the question which of the two dominates, one has to perform *in vivo* experiments. However, as discussed below, this is not a trivial task in view of the existence of three CXCR3 receptors in human cells (and an additional one in the CRC cells used in our study, which are of human origin).

## Discussion

The findings of this study indicate that the chemokine CXCL10 can act directly on CRC cells and promote their ability to express invasion-related properties. These observations are of interest in view of the well-documented antimalignancy functions of CXCL10 and other CXCR3 ligands, primarily by acting on nontumor cells at the tumor microenvironment (12–16), including in CRC of murine origin (17–21). However, these studies neither addressed the question if the chemokine directly regulates malignancy-related properties of CRC cells nor have they been expanded to investigations of human CRC systems.

Based on immunohistochemical analyses of CRC biopsies and on flow cytometry of CRC cell lines, our findings indicate that CXCR3 expression is a general characteristic of human CRC cells. The expression of CXCR3 by the tumor cells raises the possibility that they interact with the CXCL10 chemokine, which is found at their microenvironment, and as our findings also show is expressed by IFN $\gamma$ -stimulated CRC cells.



**Figure 5.** CXCL10 up-regulates MMP expression by CRC cell lines. SW480 (A), SW620 (B), and KM12C (C) cells were stimulated by CXCL10 for 48 h, and MMP9 expression was determined by Western blotting. Ponceau staining was used as a loading control. Representatives of three to four independent experiments done for each cell line.

Indeed, our findings indicate that CRC cells can use CXCL10 for their own benefit, as the chemokine promotes invasion-related properties, such as the expression of matrix-degrading enzymes, in primary as well as in metastatic CRC cells. This function of CXCL10 may come into effect at the primary and metastatic tumor sites, as the chemokine is actually expressed in these loci (our current results and refs. 24–27, 30). This function of CXCL10 is complemented by more specific effects that are restricted to the metastatic cells, as indicated by the ability of the chemokine to enhance the migration of the metastatic CRC cells, but not of their primary counterparts. Therefore, CXCL10 may promote interstitial motility and adherence (as shown in our results) in CRC cells that have reached the metastatic loci.

The malignancy-enhancing functions of CXCL10 on CRC cells, observed at the level of MMP production, migration, and adhesion, suggest that the chemokine acts differentially on the tumor microenvironment and on the tumor cells: Although the chemokine increases the infiltration of immune cells and reduces angiogenesis, thus exerting antimalignancy functions, it may, on the other hand, increase tumorigenicity and metastasis by acting directly on the tumor cells to promote their tumor-supporting characteristics.

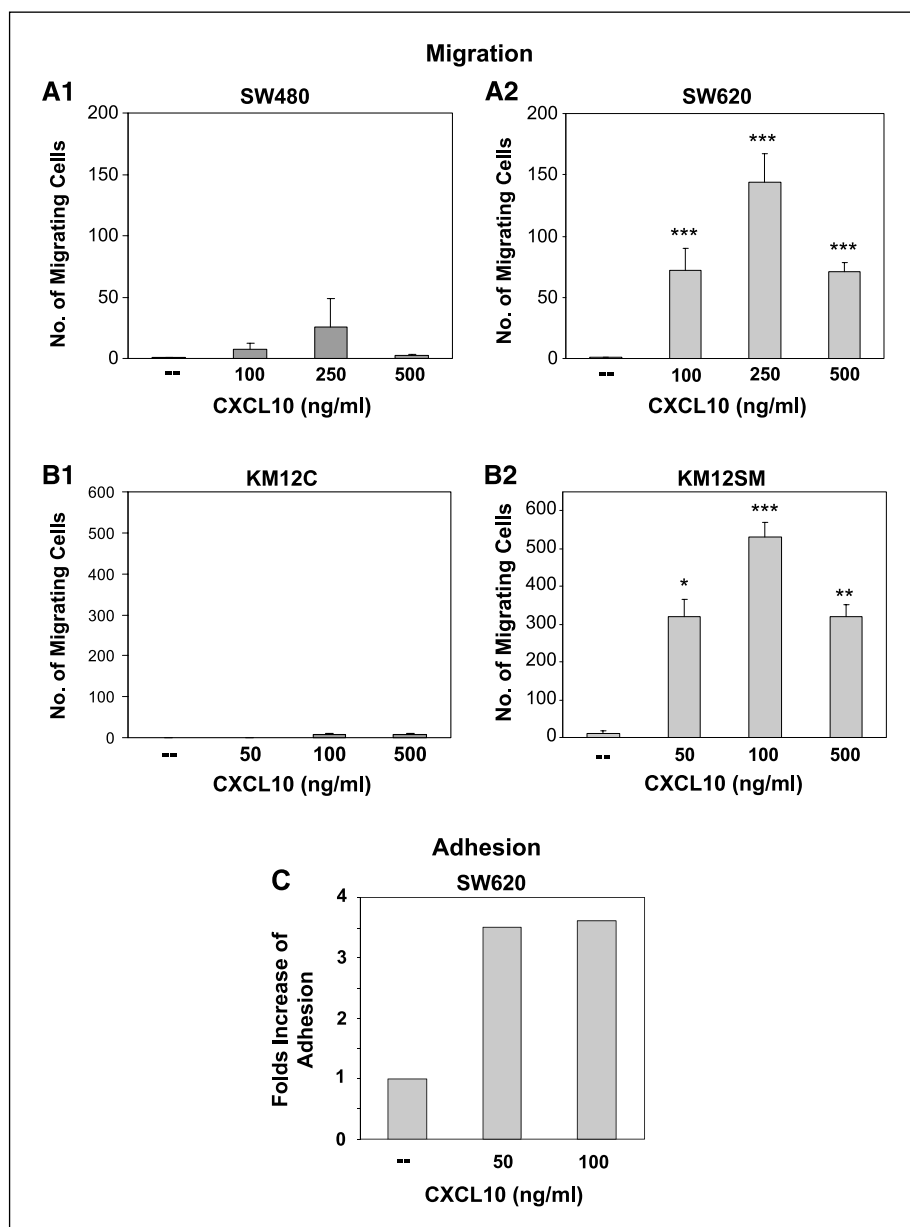
However, it is also possible that these two activities of CXCL10 are connected and interdependent, in the following manner: This study and others (24–27, 30) have indicated that CXCL10 is expressed constitutively at CRC primary and metastatic sites. The expression of this chemokine at these sites may induce the recruitment of Th1 cells to these loci. Th1 cells that localize at the tumor site may exert antitumor activities (e.g., by virtue of the release of IFN $\gamma$ ). However, it is possible that this cytokine not only potentiates tumor-inhibiting immune functions but also has other effects, such as inducing up-regulation of CXCL10 release by the tumor cells and by other cells at the tumor microenvironment. More so, the elevated levels of IFN $\gamma$  may up-regulate the expression of CXCR3 by the CRC cells, thus equipping them with improved ability to respond to CXCL10. Together, the exacerbated levels of CXCL10, which are present at the tumor microenvironment, joined by increased CXCR3 expression by the tumor cells, may enable potentiated activities of CXCL10 on the tumor cells. Eventually,

such promoted activities of CXCL10 on CRC cells may amplify their promalignancy activities, thus supporting their metastatic potential.

The ability of CXCL10 to induce Th1 infiltration to tumor sites, followed by the secretion of IFN $\gamma$  with its potential deleterious effects, is of major interest. It suggests that Th1 cells may turn into detrimental components at the tumor site, as they may be skewed to a tumor-promoting entity. This is in marked contrast to the possible role of CD8 $^+$  cells, which were recently suggested to be associated with good prognosis in CRC (39–41). Taken together with our findings, these observations suggest that the content and the actual phenotype of lymphocytes that are present at the tumor site may play a role in dictating the fate of the tumor cells and of metastasis.

The above-suggested model exemplifies a possible scenario of CXCL10 activities in shaping the malignancy phenotype of CRC by acting directly on the tumor cells. However, as described above, the

**Figure 6.** CXCL10 induces migration and adhesion of metastatic CRC cells. The migration of SW480 (A1) and SW620 (A2) cells was assessed in a 48-well modified Boyden chamber. For SW620 cells, the results are of one representative experiment of three to four independent experiments. Columns, mean; bars, SD. \*\*\*,  $P < 0.005$ , difference between CXCL10-stimulated and unstimulated cells. In SW620 cells, 250 ng/mL of CXCL10 induced migration in all four experiments done (100 and 500 ng/mL, in two of three experiments). For SW480 cells, the results are of four independent experiments. Columns, mean; bars, SE. The migration of KM12C (B1) and KM12SM (B2) cells was assessed in a 48-well modified Boyden chamber. \*,  $P < 0.02$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ , difference between CXCL10-stimulated and unstimulated cells. For KM12SM cells, the results are of one representative experiment of three independent experiments. Columns, mean; bars, SD. For KM12C cells, the results are of two independent experiments. Columns, mean; bars, SE. C, CXCL10 promotes the adhesion of SW620 cells to laminin. Representatives of nine readouts, obtained for CXCL10-induced adhesion in three independent experiments, all showing significance for CXCL10 effects ( $P < 0.01$ – $P < 0.0001$  in the different experiments).





chemokine also restrains tumor progression by acting on the tumor microenvironment. These two contradicting activities of CXCL10 may come into effect at different stages of the disease. The question of which of the two activities dominates, and at what phase of tumor progression, remains open but warrants a serious consideration. Because of the dual activity of CXCL10, and its possible opposing effects on the progression of CRC, such a question cannot be addressed by neutralizing CXCL10. Rather, one should down-regulate CXCR3 in CRC cells and determine the malignancy phenotype of the cells in xenograft model systems. This approach is challenging when studying human cells because human cells express three CXCR3 variants (and another one is expressed in the CRC cells used in our study). As based on published data, each of the CXCR3 receptors may have its own specific activities, as was already suggested for breast cancer and multiple myeloma cells (31, 42–44). Therefore, to provide a conclusive understanding of the roles played by CXCR3 in the progression of CRC, a separate *in vivo* study should be done in which each of the different CXCR3 variants alone, and in varying combinations, will be knocked down. Only such an analysis will enable to determine which of the CXCR3 variant(s) is(are) responsible for the promalignancy effects of CXCL10 on CRC cells and what are the implications of such CXCL10 activities on the malignancy phenotype of these tumor cells.

Of note, the findings emerging from our study, demonstrating that CXCL10 directly supports metastasis-promoting activities in CRC cells, join other studies that have recently debated the absolute view of CXCR3 and its ligands as antimalignancy factors. For example, research done on melanoma, multiple myeloma, and breast cancer has shown that IFN $\gamma$ -inducible CXCR3 ligands have malignancy-promoting functions. Specifically, the different studies showed that

tumor cell lines express CXCR3, that CXCR3-expressing tumor cells respond to its ligands, and that the expression of the receptor by tumor cells in clinical samples may be linked to tumor progression (45–50).

The significance of the above-mentioned studies and ours is that they offer a paradigm shift. Until recently, CXCR3 ligands were considered as potent, and absolute, antimalignancy factors. Our study as well as those mentioned above suggest that CXCR3 ligands can have opposing effects on malignancy. Because this notion is not fully substantiated as yet in the field, and in view of the emerging debate on the roles played by CXCR3 and its ligands in tumor progression, it is imperative to consider findings such as ours when aiming at getting a comprehensive view of the effects of IFN $\gamma$ -inducible chemokines and of CXCR3 on malignancy. In particular, our findings and others call for a cautious approach when considering the use of these chemokines as therapeutic modalities in the treatment of CRC, and possibly in other cancer diseases.

## Acknowledgments

Received 8/24/2006; revised 1/30/2007; accepted 1/30/2007.

**Grant support:** The Jacqueline Seroussi Memorial Foundation for Cancer Research; The Ela Kodesz Institute for Research on Cancer Development and Prevention, Tel Aviv University; The chief Scientist Office of the Ministry of Health, Israel (grant no. 6082); Federico Foundation; The Fainberg Family Fund (Orange County, CA); The Fred August and Adele Wolpers Charitable Fund (Clifton, NJ); Bonnie and Steven Stern (New York, NY); Arnold and Ruth Feuerstein (Orange County, CA); The Pikovsky Fund (Jerusalem, Israel); and Natan Blutingier (West Orange, NJ).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. I. Yron for her contribution.

## References

- Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* 2002;2:563–72.
- Zlotnik A. Chemokines in neoplastic progression. *Semin Cancer Biol* 2004;14:181–5.
- Mantovani A, Allavena P, Sozzani S, Vecchi A, Locati M, Sica A. Chemokines in the recruitment and shaping of the leukocyte infiltrate of tumors. *Semin Cancer Biol* 2004;14:155–60.
- Balkwill F. Cancer and the chemokine network. *Nat Rev Cancer* 2004;4:540–50.
- Witz IP. Presence and functions of immune components in the tumor microenvironment. *Adv Exp Med Biol* 2001;495:317–24.
- Salcedo R, Oppenheim JJ. Role of chemokines in angiogenesis: CXCL12/SDF-1 and CXCR4 interaction, a key regulator of endothelial cell responses. *Microcirculation* 2003;10:359–70.
- Ben-Baruch A. The multifaceted roles of chemokines in malignancy. *Cancer Metastasis Rev* 2006;25:357–71.
- Witz G, Hopken UE, Lipp M. The impact of CCR7 and CXCR5 on lymphoid organ development and systemic immunity. *Immunol Rev* 2003;195:117–35.
- Randolph GJ. Dendritic cell migration to lymph nodes: cytokines, chemokines, and lipid mediators. *Semin Immunol* 2001;13:267–74.
- Schuttyser E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev* 2003;14:409–26.
- Hieshima K, Imai T, Opdenakker G, et al. Molecular cloning of a novel human CC chemokine liver and activation-regulated chemokine (LARC) expressed in liver. Chemotactic activity for lymphocytes and gene localization on chromosome 2. *J Biol Chem* 1997;272:5846–53.
- Singh UP, Singh S, Iqbal N, Weaver CT, McGhee JR, Lillard JW, Jr. IFN- $\gamma$ -inducible chemokines enhance adaptive immunity and colitis. *J Interferon Cytokine Res* 2003;23:591–600.
- Neville LF, Mathiak G, Bagasra O. The immunobiology of interferon- $\gamma$  inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. *Cytokine Growth Factor Rev* 1997;8:207–19.
- Lazzeri E, Romagnani P. CXCR3-binding chemokines: novel multifunctional therapeutic targets. *Curr Drug Targets Immune Endocr Metabol Disord* 2005;5:109–18.
- Strieter RM, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA. Cancer CXC chemokine networks and tumour angiogenesis. *Eur J Cancer* 2006;42:768–78.
- Romagnani P, Annunziato F, Lasagni L, et al. Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity. *J Clin Invest* 2001;107:53–63.
- Narvaiza I, Mazzolini G, Barajas M, et al. Intratumoral coinjection of two adenoviruses, one encoding the chemokine IFN- $\gamma$ -inducible protein-10 and another encoding IL-12, results in marked antitumoral synergy. *J Immunol* 2000;164:3112–22.
- Mendiratta SK, Quezada A, Matar M, et al. Combination of interleukin 12 and interferon  $\alpha$  gene therapy induces a synergistic antitumor response against colon and renal cell carcinoma. *Hum Gene Ther* 2000;11:1851–62.
- Zilocchi C, Stoppacciaro A, Chiodoni C, Parenza M, Terrazini N, Colombo MP. Interferon  $\gamma$ -independent rejection of interleukin 12-transduced carcinoma cells requires CD4<sup>+</sup> T cells and granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1998;188:133–43.
- Li G, Tian L, Hou JM, et al. Improved therapeutic effectiveness by combining recombinant CXC chemokine ligand 10 with cisplatin in solid tumors. *Clin Cancer Res* 2005;11:4217–24.
- Yao L, Pike SE, Setsuda J, et al. Effective targeting of tumor vasculature by the angiogenesis inhibitors vasostatin and interleukin-12. *Blood* 2000;96:1900–5.
- Kubens BS, Zanker KS. Differences in the migration capacity of primary human colon carcinoma cells (SW480) and their lymph node metastatic derivatives (SW620). *Cancer Lett* 1998;131:55–64.
- Morikawa K, Walker SM, Nakajima M, Pathak S, Jessup JM, Fidler IJ. Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. *Cancer Res* 1988;48:6863–71.
- Wang H, Gao X, Fukumoto S, Tadamoto S, Sato K, Hirai K. Differential expression and regulation of chemokines JE, KC, and IP-10 gene in primary cultured murine hepatocytes. *J Cell Physiol* 1999;181:361–70.
- Patzwahl R, Meier V, Ramadori G, Mihm S. Enhanced expression of interferon-regulated genes in the liver of patients with chronic hepatitis C virus infection: detection by suppression-subtractive hybridization. *J Virol* 2001;75:1332–8.
- Ren X, Kennedy A, Colletti LM. CXC chemokine expression after stimulation with interferon- $\gamma$  in primary rat hepatocytes in culture. *Shock* 2002;17:513–20.
- Barsig J, Flesch IE, Kaufmann SH. Macrophages and hepatocytic cells as chemokine producers in murine listeriosis. *Immunobiology* 1998;199:87–104.
- Gunther K, Leier J, Henning G, et al. Prediction of lymph node metastasis in colorectal carcinoma by expression of chemokine receptor CCR7. *Int J Cancer* 2005;116:726–33.
- Dellacasagrande J, Schreurs OJ, Hofgaard PO, et al. Liver metastasis of cancer facilitated by chemokine receptor CCR6. *Scand J Immunol* 2003;57:534–44.
- Dwinell MB, Luger N, Eckmann L, Kagnoff MF. Regulated production of interferon-inducible T-cell

- chemoattractants by human intestinal epithelial cells. *Gastroenterology* 2001;120:49–59.
31. Lasagni L, Francalanci M, Annunziato F, et al. An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *J Exp Med* 2003;197:1537–49.
32. Ehlert JE, Addison CA, Burdick MD, Kunkel SL, Strieter RM. Identification and partial characterization of a variant of human CXCR3 generated by posttranscriptional exon skipping. *J Immunol* 2004;173:6234–40.
33. Berencsi K, Meropol NJ, Hoffman JP, et al. Colon carcinoma cells induce CXCL11-dependent migration of CXCR3-expressing cytotoxic T lymphocytes in organotypic culture. *Cancer Immunol Immunother* 2007;56:359–70.
34. Gutkind JS. The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J Biol Chem* 1998;273:1839–42.
35. Bonacchi A, Romagnani P, Romanelli RG, et al. Signal transduction by the chemokine receptor CXCR3: activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes. *J Biol Chem* 2001;276:9945–54.
36. Turpeenniemi-Hujanen T. Gelatinases (MMP-2 and -9) and their natural inhibitors as prognostic indicators in solid cancers. *Biochimie* 2005;87:287–97.
37. Ries C, Lottspeich F, Dittmann KH, Petrides PE. HL-60 leukemia cells produce an autocatalytically truncated form of matrix metalloproteinase-9 with impaired sensitivity to inhibition by tissue inhibitors of metalloproteinases. *Leukemia* 1996;10:1520–6.
38. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* 2003;3:362–74.
39. Menon AG, Janssen-van Rhijn CM, Morreau H, et al. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. *Lab Invest* 2004;84:493–501.
40. Chiba T, Ohtani H, Mizoi T, et al. Intraepithelial CD8<sup>+</sup> T-cell-count becomes a prognostic factor after a longer follow-up period in human colorectal carcinoma: possible association with suppression of micrometastasis. *Br J Cancer* 2004;91:1711–7.
41. Pages F, Berger A, Camus M, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* 2005;353:2654–66.
42. Romagnani P, Maggi L, Mazzinghi B, et al. CXCR3-mediated opposite effects of CXCL10 and CXCL4 on TH1 or TH2 cytokine production. *J Allergy Clin Immunol* 2005;116:1372–9.
43. Datta D, Flaxenburg JA, Laxmanan S, et al. Ras-induced modulation of CXCL10 and its receptor splice variant CXCR3-B in MDA-MB-435 and MCF-7 cells: relevance for the development of human breast cancer. *Cancer Res* 2006;66:9509–18.
44. Giuliani N, Bonomini S, Romagnani P, et al. CXCR3 and its binding chemokines in myeloma cells: expression of isoforms and potential relationships with myeloma cell proliferation and survival. *Haematologica* 2006;91:1489–97.
45. Kawada K, Sonoshita M, Sakashita H, et al. Pivotal role of CXCR3 in melanoma cell metastasis to lymph nodes. *Cancer Res* 2004;64:4010–7.
46. Monteagudo C, Martin JM, Jorda E, Llombart-Bosch A. CXCR3 chemokine receptor immunoreactivity in primary cutaneous malignant melanoma: correlation with clinicopathologic prognostic factors. *J Clin Pathol*. Epub 2006 Apr 28.
47. Suyama T, Furuya M, Nishiyama M, et al. Up-regulation of the interferon  $\gamma$  (IFN- $\gamma$ )-inducible chemokines IFN-inducible T-cell  $\alpha$  chemoattractant and monokine induced by IFN- $\gamma$  and of their receptor CXCR3 in human renal cell carcinoma. *Cancer* 2005;103:258–67.
48. Pellegrino A, Antonaci F, Russo F, et al. CXCR3-binding chemokines in multiple myeloma. *Cancer Lett* 2004;207:221–7.
49. Jones D, Benjamin RJ, Shahsafaei A, Dorfman DM. The chemokine receptor CXCR3 is expressed in a subset of B-cell lymphomas and is a marker of B-cell chronic lymphocytic leukemia. *Blood* 2000;95:627–32.
50. Walsler TC, Rifat S, Ma X, et al. Antagonism of CXCR3 inhibits lung metastasis in a murine model of metastatic breast cancer. *Cancer Res* 2006;66:7701–7.