Overexpression of Both CXC Chemokine Receptor 4 and Vascular Endothelial Growth Factor Proteins Predicts Early Distant Relapse in Stage II-III Colorectal Cancer Patients

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Abstract

Purpose: CXC chemokine receptor 4 (CXCR4) and vascular endothelial growth factor (VEGF) are implicated in the metastatic process of malignant tumors. However, no data are currently available on the biological relationship between these molecules in colorectal cancer. We studied whether CXCR4 and VEGF expression could predict relapse and evaluated *in vitro* the contribution of CXCR4 in promoting clonogenic growth, VEGF secretion, and intercellular adhesion molecule-1 (ICAM-1) expression of colorectal cancer cells.

Experimental Design: CXCR4 and VEGF were studied in colorectal cancer tissues and in Lovo, HT29, and SW620 colorectal cancer cell lines by immunohistochemistry. Correlations with baseline characteristics of patients and tumors were analyzed by χ^2 test. VEGF secretion induced by CXCL12 was measured by ELISA. The effect of CXCL12 on ICAM-1 expression was evaluated by flow cytometry. Clonogenic growth induced by CXCL12 was determined by clonogenic assays. Functional effects induced by CXCL12 were prevented by the administration *in vitro* of AMD3100, a bicyclam noncompetitive antagonist of CXCR4.

Results: Seventy-two patients, seen between January 2003 and January 2004, were studied. CXCR4 was absent in 16 tumors (22.2%); it was expressed in ≤50% of cells in 25 (34.7%) tumors and in >50% of cells in 31 (43.0%) tumors. VEGF was absent in 17 (23.6%) tumors; it was expressed in ≤50% of cells in 16 (22.2%) tumors and in >50% of cells in 39 (54.2%) tumors. There was a significant association between CXCR4 expression and lymph nodal status ($P = 0.0393$). There were significant associations between VEGF and tumor invasion ($P = 0.0386$) and lymph nodal involvement ($P = 0.0044$). American Joint Committee on Cancer stage ($P = 0.0016$), VEGF expression ($P = 0.0450$), CXCR4 expression ($P = 0.0428$), and VEGF/CXCR4 expression ($P = 0.0004$) had a significant prognostic value for disease-free survival with univariate analysis. The predictive ability of the American Joint Committee on Cancer stage and of the concomitant and high expression of VEGF and CXCR4 was confirmed by multivariate analysis. Prognosis is particularly unfavorable for patients whose primary tumors express CXCR4 and VEGF in >50% of cells (median disease-free survival in relapsed patients, 5.8 months; hazard ratio of relapse, 8.23; 95% confidence interval, 7.24-14.29). In clonogenic assays, CXCL12 (20 ng/mL/d) significantly increased the number of clones in SW620, HT29, and Lovo cells at 7 and 14 days. Again, CXCL12 was able to stimulate VEGF secretion in SW620, HT29, and Lovo cells as well as up-regulated ICAM-1. These effects were prevented by the administration of AMD3100 (1 μmol/L).

Conclusions: We have shown that concomitant and high expression of CXCR4 and VEGF is a strong and independent predictor of early distant relapse in colorectal cancer. CXCR4 triggers a plethora of phenomena, including stimulation of clonogenic growth, induction of VEGF release, and ICAM-1 up-regulation. These data support the inhibition of CXCR4 to prevent the development of colorectal cancer metastasis.

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Colorectal cancer (CRC) is one of the three leading causes of cancer-related death among men and women worldwide. The neoplasm frequently metastasizes to the liver, so that hepatic metastases are present in 30% to 40% of patients at early stages of the disease. In advanced disease, the liver is involved in up to 95% of patients and the mortality of CRC is principally attributable to the development of hepatic metastases. Nevertheless, 50% of patients develop extrahepatic metastases, most often in the lungs, lymph nodes, and peritoneum (1). Knowledge of the factors involved in CRC metastases is largely lacking. Several studies have revealed that the establishment of metastasis is the final outcome of a series of phenomena such as tumor cell invasion of distant organs, clonogenic growth, and neoangiogenesis. Each step of this multistep process is essential for tumor cell survival and establishment and is regulated by inter-actions of cancerous cells with the host microenvironment (2, 3).

The occurrence of cellular clones with metastatic phenotypes is a rare event and the identification of biological and/or molecular characteristics accounting for the metastatic behavior could provide new targets for anticancer therapies, biomarkers of disease, and predictors of patient outcome.

Recent evidence indicates an important role for CXCL12 (a CXC chemokine) and its receptor (CXCR4) in the ''metastatic homing'' of tumor cells (4). Numerous authors have reported on the involvement of the CXCR4/CXCL12 axis in promoting metastatic phenotype in tumors (5–24). CXCR4 has been identified as a prominent chemokine receptor by microarray screening of tissues from CRC patients and cellular extracts from liver parenchyma have high concentrations of CXCL12 (25) that could provide a specific homing target and promoting factor for CXCR4-expressing CRC cells.

Vascular endothelial growth factor (VEGF) is the most important and best-characterized angiogenic factor. It belongs

Table 1. Characteristics of patients and tumors according to CXCR4 and VEGF expression

	No. (%)	CXCR4 expression			*P*	VEGF expression			*P*
		Negative	Low	High		Negative	Low	High	
Median age (range), y	55 (34-82)								
Age, y									
<70	44 (61.1)	10	13	21		13	9	22	
≥70	28 (38.9)	6	12	10	0.4820	4	7	17	0.3313
Gender									
Male	38 (52.7)	6	15	17		10	9	19	
Female	34 (47.3)	10	10	14	0.3544	7	7	20	0.7465
Location									
Colon	57 (79.2)	15	19	23		3	1	11	
Rectum	15 (20.8)	1	6	8	0.4495	14	15	28	0.3191
Tumor invasion									
pT_1	9 (12.5)	1	6	2		3	2	4	
pT_2	20 (27.8)	4	7	9		4	3	13	
pT_3	36 (69.2)	10	10	16		5	11	20	
pT_4	7 (13.4)	1	2	4	0.4543	5	0	2	0.0386
Lymph nodal status									
pN_0	40 (55.5)	13	16	11		16	9	15	
pN_1	21 (29.2)	2	6	13		1	4	16	
pN_2	11 (15.3)	1	3	7	0.0393	0	3	8	0.0044
AJCC stage									
II	39 (54.2)	12	14	13		11	9	19	
III	33 (45.8)	4	11	18	0.0954	6	7	20	0.5339
Tumor size, cm									
<2	6 (8.3)	1	4	1		3	2	1	
2-5	53 (73.6)	13	14	26		10	13	30	
>5	13 (18.1)	3	6	4	0.2473	5	1	7	0.1606
Lymphovascular invasion									
None	57 (79.2)	15	18	24		14	11	32	
Present	15 (20.8)	0	8	7	0.0620	3	5	7	0.5081
Histologic variant									
Colonic	60 (83.3)	12	20	28		13	15	32	
Mucinous	12 (16.7)	4	5	3	0.3516	4	1	7	0.3921
Pathologic grade									
Well	6 (8.3)	2	2	2		2	0	4	
Moderate	63 (87.5)	14	22	27		14	16	33	
Poor	3 (4.2)	0	1	2	0.8129	1	0	2	0.5588

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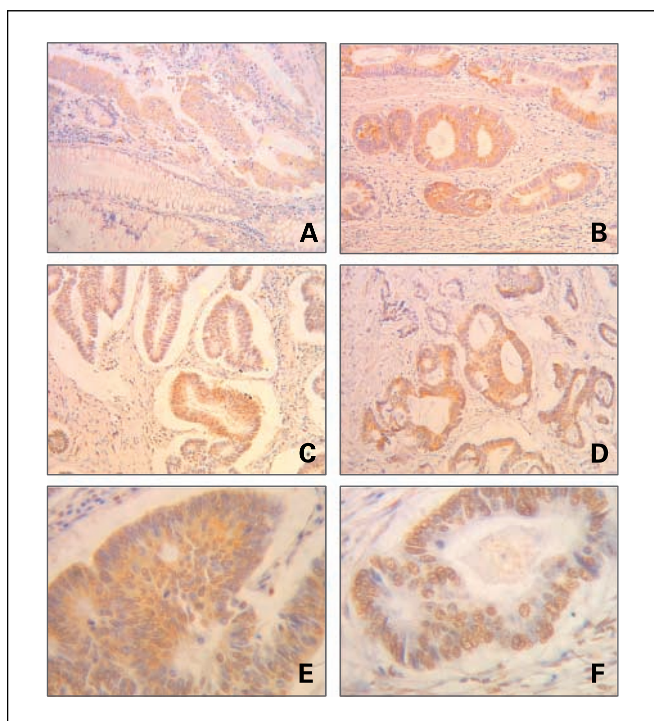

Fig. 1. Immunohistochemical expression of VEGF and CXCR4 in colorectal cancer tissues. *A,* VEGF expression in <50% of neoplastic cells (×40); *B,* VEGF expression in >50% of neoplastic cells (×40); *C,* CXCR4 expression in <50% of neoplastic cells (×40); *D,* CXCR4 expression in >50% of neoplastic cells (×40); *E,* cytoplasmatic expression of CXCR4 (×63); *F,* nuclear expression of CXCR4 (×63).

to the platelet-derived growth factor superfamily of growth factors. VEGF potently increases vascular permeability and promotes the formation of new blood vessels by stimulating endothelial cells to migrate and divide. VEGF is overexpressed by the vast majority of solid human cancers. Members of the VEGF family include VEGF-A, -B, -C, -D, -E, and placental growth factor. Interestingly, the amount of VEGF expressed by cancer cells has been found to correlate with poor prognosis in many types of tumors, including carcinomas of the breast, kidney, colon, brain, ovary, cervix, thyroid, bladder, esophagus, and prostate, and in osteoid and soft tissue sarcomas and pediatric tumors (26, 27).

Intercellular adhesion molecules (ICAM) are members of the immunoglobulin superfamily that is characterized by the presence of immunoglobulin-like domains. ICAM-1 plays an important role in cell-cell and cell-extracellular matrix interactions. It is expressed in hemopoietic cells and vascular endothelium where it promotes adhesion and leukocyte transmigration (28–30). ICAM-1 expression is frequently enhanced in tumors and it is speculated that ICAM-1 expression in tumor cells may have a functional significance by increasing the migration/invasiveness of cells (31).

In the present study, we tested the hypotheses that CXCR4 and VEGF expression could have a prognostic role in CRC and that CXCL12 could promote the malignant phenotype by favoring the clonogenic growth and regulating VEGF and ICAM-1 expression in CRC cells.

Materials and Methods

Patient management and follow-up. Seventy-two consecutive, unselected, stage II-III CRC patients were seen at the Division of Surgery C (surgical approach) and at the Division of Medical Oncology B (adjuvant intervention) of the National Cancer Institute (Naples, Italy) from January 2003 to January 2004. The histologic sections were reviewed by two expert pathologists (F.T. and R.F.) to verify the histologic diagnosis. All patients underwent curative-intent surgery for stage II-III CRC [according to the American Joint Committee on Cancer (AJCC) criteria] after anesthesiologic examination. Then, therapeutic strategies were applied according to stage of disease and presumed risk of relapse. Patients with stage II disease underwent follow-up based on history, physical examination, complete blood count, liver function tests, ultrasound scan of the abdomen, and carcinoembryonic antigen monitoring every 3 months. Total body computed tomography scan and colonoscopy were done once a year. Patients with stage II high-risk disease (pT$_4$ and/or gross volume tumors, perforation, obstruction, poorly differentiated histology, long-lasting symptoms, elevated carcinoembryonic antigen preoperatively, blood or lymphatic vessel invasion) were encouraged to undergo adjuvant chemotherapy. If no contraindications were present, patients with stage III disease underwent 6 months of fluorouracil-based adjuvant chemotherapy, then were followed up. Fifty-two patients received fluorouracil-based adjuvant chemotherapy. Twenty stage II patients did not receive adjuvant interventions.

Tissue samples, immunohistochemistry, and immunocytochemistry. Formalin-fixed, paraffin-embedded 4-μm tissue sections of primary carcinomas and hepatic metastases were immunostained using a biotin-streptavidin-peroxidase method (YLEM kit, Rome, Italy). Sections were subjected to routine deparaffinization and rehydration. Slides were then immersed in 10 mmol/L sodium citrate buffer (pH 6.0) for CXCR4 staining or Dako target retrieval solution, high pH, for VEGF staining, incubated for 10 minutes on a hot plate (95-99°C) or boiled, and then allowed to cool for 20 minutes. Sections were then incubated for 10 minutes in 3% hydrogen peroxide in distilled water, washed in PBS thrice for 5 minutes, and incubated with 10% normal horse serum in PBS for 30 minutes.

Table 2. Correlations between combined expression of CXCR4 and VEGF and lymph nodal status, primary tumor extension, presence of metastases, and stage of disease at diagnosis

	High CXCR4 and high VEGF	Other CXCR4/VEGF combinations	*P*
Lymph nodes			
pN$_0$	5	35	0.0006
pN$_1$	10	11	
pN$_2$	7	4	
Tumor			
pT$_1$	2	7	0.9316
pT$_2$	6	14	
pT$_3$	12	24	
pT$_4$	2	5	
Metastases			
Yes	10	6	0.0045
No	12	44	
Stage (AJCC)			
II	9	30	0.2146
III	13	20	

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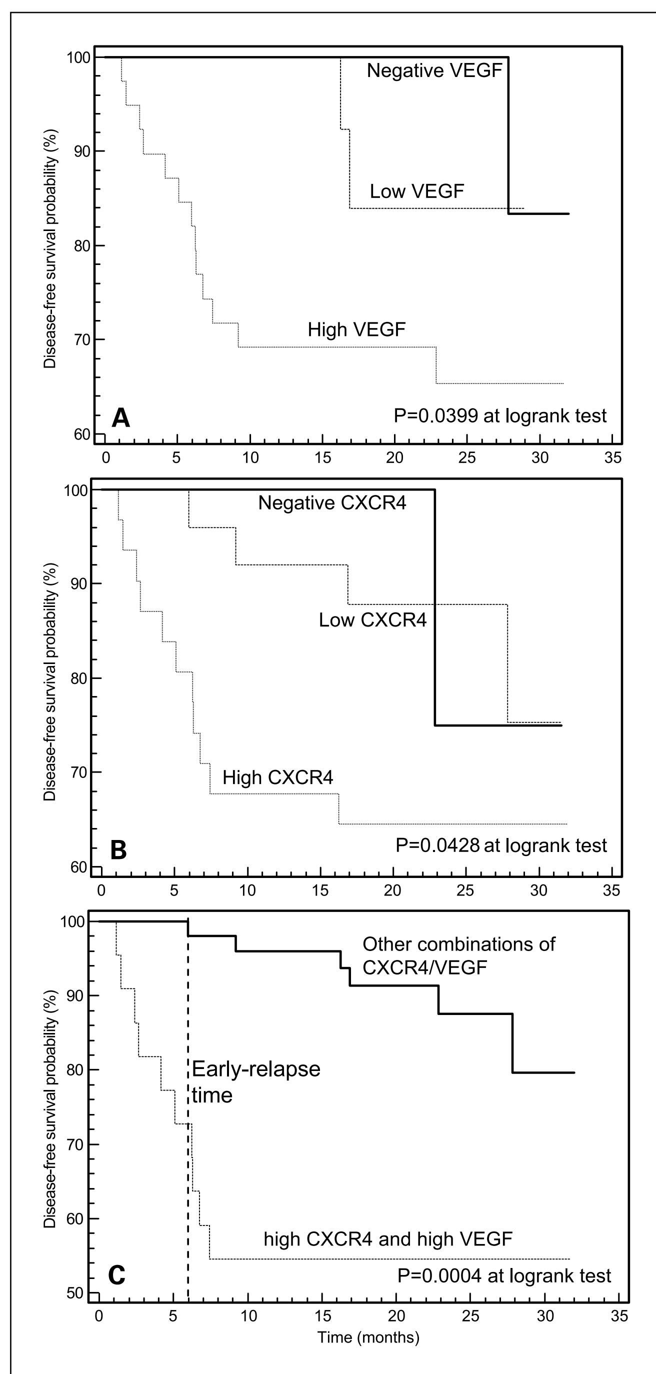

Fig. 2. Disease-free survival curves according to VEGF expression (*A*), CXCR4 expression (*B*), and VEGF/CXCR4 combinations (*C*).

After three washes in PBS buffer, the sections were incubated for 2 hours at room temperature with primary monoclonal antibodies, anti-CXCR4 antibody (MAB 172, clone 44716, R&D Systems, Inc., Minneapolis, MN) or anti-VEGF (VG1 clone, Dako, Milan, Italy). The sections were then incubated with biotin-labeled secondary antibody (1:30) and streptavidin-peroxidase (1:30) for 20 minutes each. Slides were stained for 5 minutes with 0.05% 3,3′-diaminobenzidine tetrahydrochloride freshly prepared in 0.05 mol/L Tris-HCl buffer (pH 7.6) containing 0.024% hydrogen peroxidase, and then counterstained with hematoxylin, dehydrated, and mounted in Diatex. All series included positive controls (melanoma and breast cancer tissues). Negative controls were obtained by substituting the primary antibody with a mouse myeloma protein of the same subclass at the same concentration as the monoclonal antibody. All controls gave satisfactory results. Staining for CXR4 and VEGF was categorized into three semiquantitative classes based on the rate of stained (positive) tumor cells: absence of staining, ≤50% positive cells (low), and >50% positive cells (high). CXCR4 and VEGF expression were evaluated in tumor cells. Semiquantitative classes were chosen by our pathologists (internal pathologists from Naples: R.F., G.B., F.T.; external pathologist from Benevento: A.D.B.) after consensus discussion and careful revision of all slides.

For immunocytochemical staining, cells were washed with PBS and fixed with 3.7% formaldehyde in PBS, rinsed, and, when indicated, permeabilized with 0.5% Triton X-100 in PBS. Samples were blocked with 1% bovine serum albumin and cells were labeled with primary antibodies anti-VEGF, anti-CXCR4, and anti–carcinoembryonic antigen (clone II-7, Dako) for 2 hours at 37°C. Slides were stained for 10 minutes with 3-amino, 9 ethyl-carbazole chromogen (DAKO Cytomation, Milan, Italy) and then counterstained with hematoxylin, washed, and mounted in water fluid. Data on expression of VEGF by CRC clones were normalized as the VEGF staining index, defined as the number of positive clones in an experimental group divided by the number of positive clones in control groups.

Cell lines and cell culture. SW620, Lovo, HT29 human colorectal cancer cell lines, and MDA-MB-231, a human breast cancer cell line, were purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI supplemented with 10% (v/v) heat-inactivated FCS.

AMD3100. This competitive inhibitor of CXCR4 was obtained from Sigma-Aldrich, Inc. (St. Louis, MO).

Flow cytometry for ICAM-1 expression. To evaluate the expression of ICAM-1 (CD54), adherent cancer cells at subconfluency (50-70% confluent) were detached with 2 mmol/L EDTA in PBS, washed, resuspended in ice-cold PBS, and incubated for 30 minutes at 4°C with anti-CD54-PE antibody (clone HA58, BD PharMingen, San Diego, CA). After three washes in PBS, the cells were analyzed by fluorescence-activated cell sorting (Becton Dickinson, Milan, Italy). Negative controls were obtained by substituting the primary antibody with a mouse myeloma protein of the same subclass at the same concentration as the monoclonal antibody.

Evaluation of VEGF secretion. The concentration of VEGF in the medium obtained from SW620, Lovo, HT29, and MDA-MB-231 cells after CXCL12 stimulation in 0.5% FCS medium was measured using a commercially available sandwich ELISA kit (R&D Systems) according to the instructions of the manufacturer. Assays were done in quadruplicate. Results were normalized for the number of producing cells and reported as picograms of VEGF per 10^6 cells per 24 hours.

Clonogenic assays. The effect of CXCR4 stimulation/inhibition on clonogenic growth was determined. In brief, 500 tumor cells were plated for each group into four-well, 60-mm dishes, then incubated at 37°C with 5% CO_2 in RPMI-0.5% FCS to allow macroscopic colony development. In treatment groups, CXCL12 was added daily at 20 ng/mL with or without 1 μmol/L AMD3100. At 7 and 14 days, cells were fixed and stained with hematoxylin and total cell clone numbers (>50 cells) were counted. All experiments were done at least thrice in triplicate dishes per experimental point.

Statistical analyses and data presentation. Associations between immunohistochemical scores and clinicopathologic variables of tissue specimens were evaluated by χ^2 test. $P < 0.05$ was considered statistically significant. Graphs show the mean ± SD of data from a representative experiment; data are representative of at least three experiments with comparable results. Student's t test was used for comparing the means and $P < 0.05$ was considered statistically significant.

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Table 3. Univariate and multivariate analysis for disease-free survival

Covariate	Univariate analysis		Multivariate analysis	
	Events/patients	*P*	Hazard ratio (95% confidence interval)	*P*
Age (<70 vs ≥70 y)	(12/44 vs 4/28)	0.1779	0.65 (0.50-3.34)	0.3745
Gender (male vs female)	(8/38 vs 8/34)	0.6940	0.73 (0.48-3.91)	0.5531
AJCC stage (II vs III)	(3/39 vs 13/33)	0.0016	6.42 (5.21-16.15)	0.0025
VEGF expression (negative vs positive)	(1/17 vs 15/55)	0.0450	3.23 (0.49-6.76)	0.1613
CXCR4 expression (negative vs positive)	(1/16 vs 15/56)	0.0428	3.01 (0.88-5.21)	0.0991
High CXCR4 and high VEGF expression (present vs not present)	(10/22 vs 6/50)	0.0004	8.23 (7.24-14.29)	0.0033

Disease-free survival was defined as the time elapsed from the date of the initial diagnosis to the appearance of local relapse or distant metastasis. Early relapse was defined as a clinically detectable tumor developing in remote sites (lymph nodes, liver, lung) within 6 months of the initial diagnosis. The Kaplan-Meier product limit method was applied to graph disease-free survival. Univariate analysis was done with the log-rank test. Cox proportional hazards regression was used to analyze the effect of several risk factors on disease-free survival. Risk factors (covariates) were considered dichotomous (male versus female; age <70 versus ≥70 years; AJCC stage II versus stage III; CXCR4 negative versus positive; VEGF negative versus positive; high CXCR4 and high VEGF versus other combinations). Ninety-five percent confidence intervals of hazard ratios were also reported.

Results

Characteristics of patients and tumors. Seventy-two patients seen between January 2003 and January 2004 were studied. Characteristics of all patients are summarized in Table 1. Median age was 55 years; 28 patients were ≥70 years old. Genders were equally represented. Fifteen tumors originated in the rectum. The majority of lesions presented with a pT_2 or pT_3 extent of invasion. Thirty-three patients presented with pN+ disease. Thirty-nine patients presented with stage II disease, 33 with stage III. Three fourths of patients had tumors between 2 and 5 cm. Lymphovascular invasion was described in 15 tumors. The most common histology was colonic adenocarcinoma. The majority of patients (87.5%) had moderate-grade disease (Table 1).

CXCR4 was absent in 16 tumors (22.2%); it was expressed in ≤50% of cells in 25 (34.7%) tumors and in >50% of cells in 31 (43.0%) tumors. Staining was observed predominantly in the cytoplasm of tumor cells. VEGF was absent in 17 tumors (23.6%); it was expressed in ≤50% of cells in 16 (22.2%) tumors and in >50% of cells in 39 (54.2%) tumors. Staining was observed in the cytoplasm of neoplastic cells. Figure 1 shows some examples of CXCR4 and VEGF staining of CRC tissues. There was a significant association between CXCR4 expression and lymph nodal status (P = 0.0393). There were

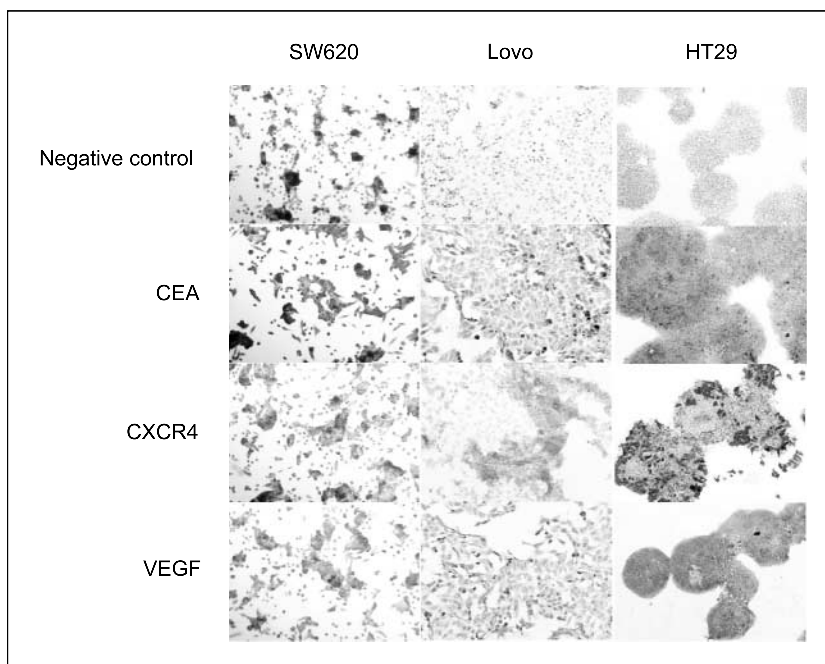

Fig. 3. Representative immunocytochemical staining of CXCR4 and VEGF in SW620, HT29, and Lovo cells. CXCR4, VEGF, and carcinoembryonic antigen (*CEA*) are expressed in SW620, HT29, and Lovo cells. Different degrees of expression are observed. Negative controls obtained by substituting the primary antibody with a mouse myeloma protein of the same subclass are also shown. Magnification, ×40.

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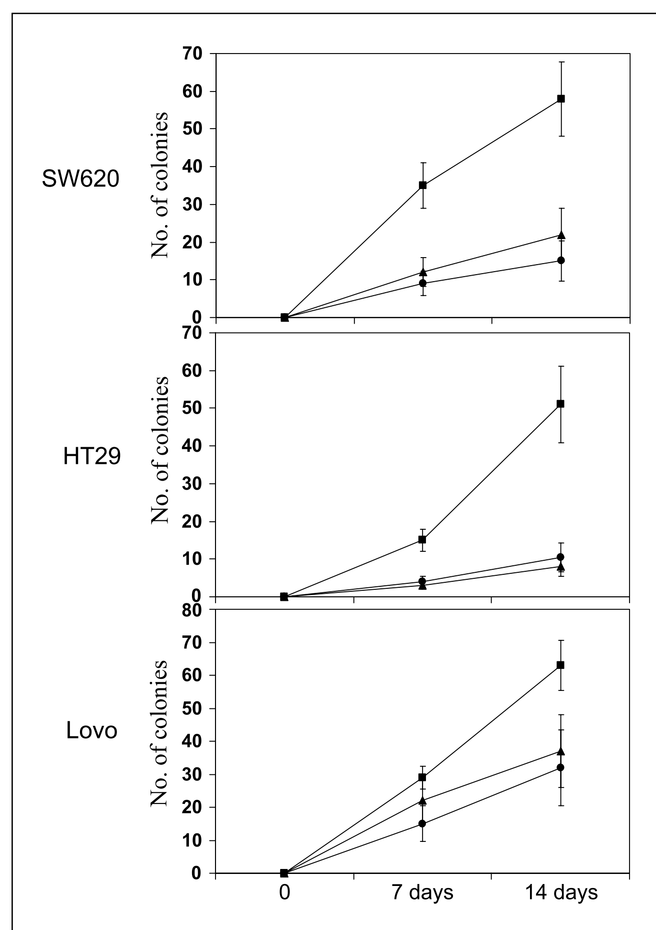

Fig. 4. CXCL12 promotes the clonogenic growth of CRC cell lines. SW620, HT29, and Lovo cells were plated for clonogenic cell growth in RPMI supplemented with 0.5% FCS and 20 ng/mL/d (■) of CXCL12 or 20 ng/mL/d of CXCL12 + 1 μmol/L AMD3100 (▲) or RPMI 0.5% + 1% bovine serum albumin (●) were added. Colonies were counted after 7 and 14 days. Points, mean; bars, SD.

significant associations between VEGF and tumor invasion (P = 0.0386) and lymph nodal involvement (P = 0.0044; Table 1). Notably, high CXCR4 and high VEGF expressions, when compared with other combinations, were significantly associated with the lymph nodal status (P = 0.0006) and the presence of distant metastases (P = 0.0045; Table 2).

Eleven patients developed metastases in the liver, four in the peritoneum, and one in the ovary. CXCR4 expression was detected in 12 of 16 (75%) metastases and VEGF in 13 (81.2%) metastases (data not shown).

Overexpression of both CXCR4 and VEGF strongly predicts early relapse of CRC. As of August 2005, after a median follow-up for alive patients of 23 months, 16 patients (22.2%) had suffered tumor progression and one had died. Median disease-free survival in the whole series was not reached. Analysis of prognostic factors for disease-free survival is summarized in Table 3. AJCC stage, VEGF expression, CXCR4 expression, and VEGF/CXCR4 combined evaluation had a significant prognostic value with univariate analysis. The predictive ability of the AJCC stage and of the VEGF/CXCR4 expression was confirmed with multivariate analysis (Table 3) adjusted by age, sex, AJCC stage, and expression of VEGF and CXCR4. Both hazard ratios of relapse and graphic pattern of Kaplan-Meier estimated curves (Fig. 2) suggest that prognosis is particularly unfavorable for patients whose primary tumors express both CXCR4 and VEGF in >50% of neoplastic cells (median disease-free survival in relapsed patients, 5.8 months; hazard ratio of relapse 8.23; 95% confidence interval, 7.24-14.29).

CXCL12 promotes clonogenic growth of CRC cells. Intrigued by the previous results, we examined the expression of CXCR4 and VEGF in SW620, Lovo, and HT29 colon cancer cells by immunocytochemistry. All the examined cell lines stained positive for both proteins (Fig. 3). Tumor growth and metastasis require the generation of a cell population from a single cell so that clonogenicity is one of the fundamental features of malignant cells. Clonogenic assays were done to test

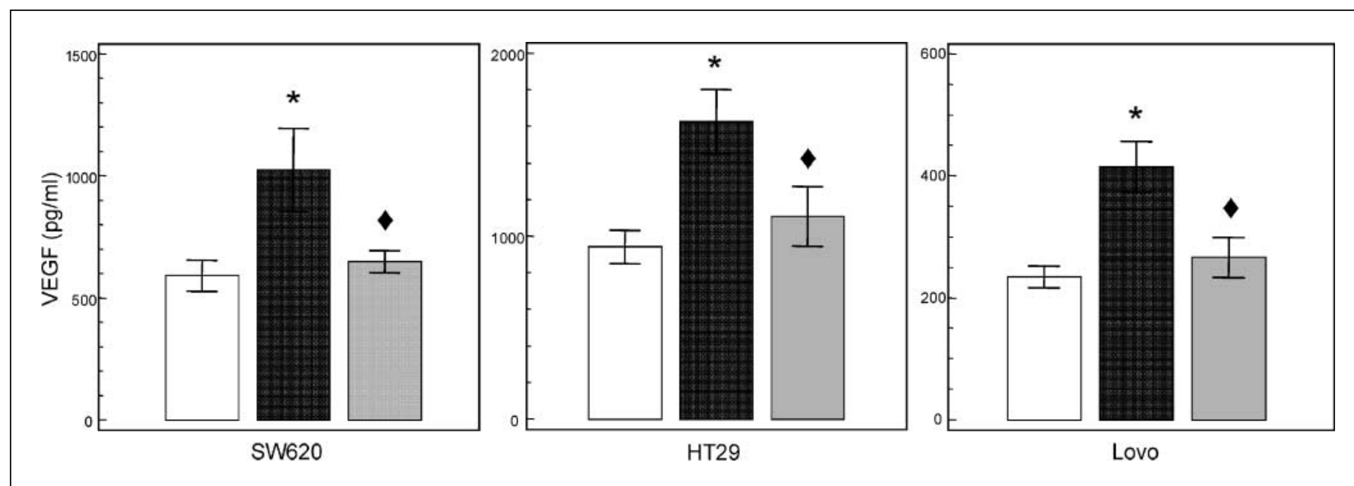

Fig. 5. Effect of CXCL12 on VEGF secretion in CRC cell lines. SW620, HT29, and Lovo cells were cultured for 24 hours in RPMI supplemented with 0.5% FCS (□) or 0.5% FCS + 200 ng/mL of CXCL12 (■). Supernatants were assayed for VEGF content by ELISA. When indicated (▨), 1 μmol/L AMD3100 was added 5 minutes before the stimulation with CXCL12. Results were normalized for the number of producing cells and reported as picograms of VEGF per 10^6 cells per 24 hours. Columns, mean; bars, SD. *, $P < 0.05$, versus control (t test); ♦, $P < 0.05$, significant inhibition by AMD3100 (t test).

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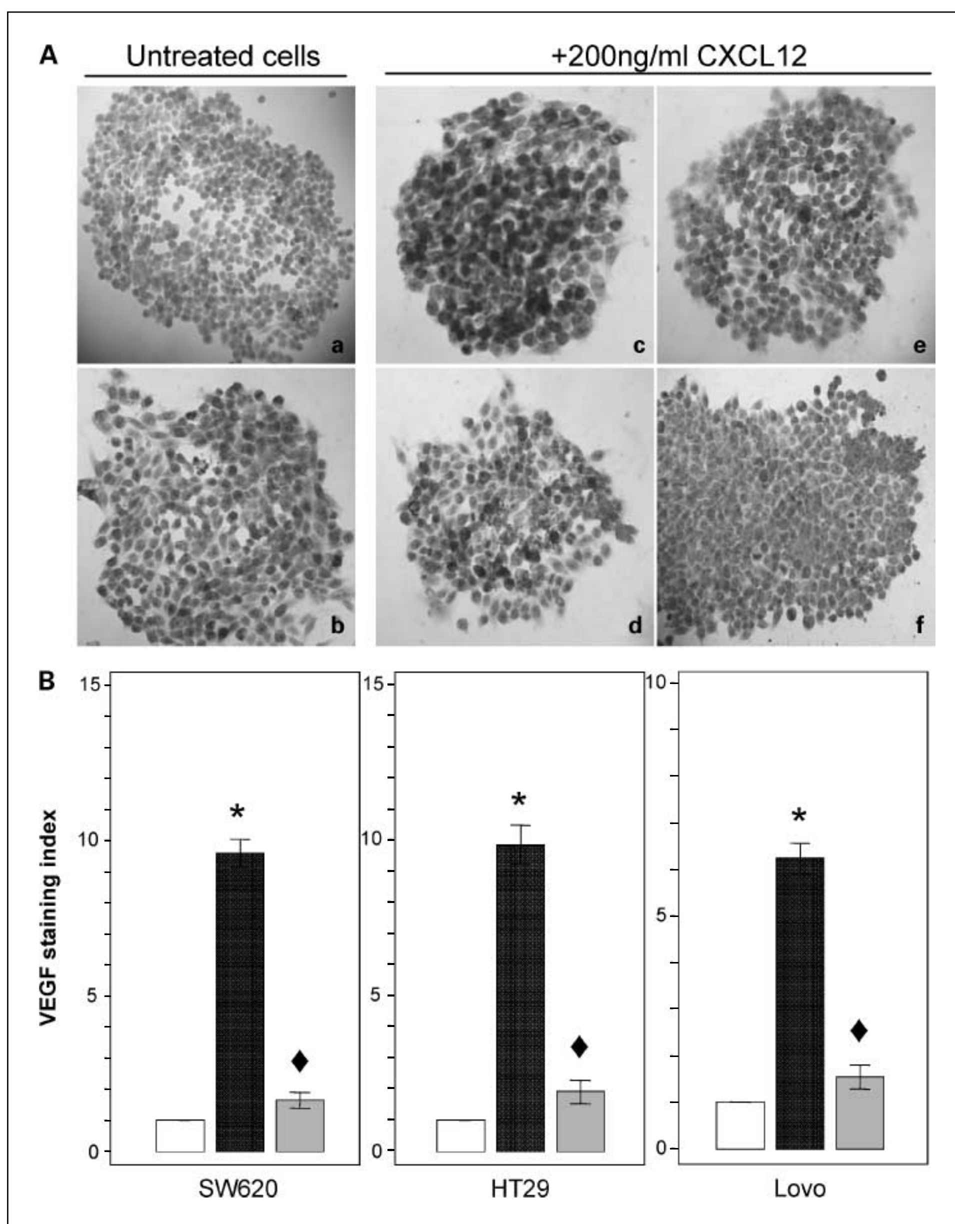

Fig. 6. CXCL12 promotes VEGF secretion in CRC clones. CRC clones from SW620, HT29, and Lovo cell lines were treated with 200 ng/mL of CXCL12, and VEGF expression was evaluated by immunocytochemistry after 24 hours. *A,* two examples of negative staining (*a* and *b*) and four examples of positive staining (*c-f*) for VEGF in SW620 are shown. *B,* the staining index of VEGF (see Materials and Methods) after 24 hours of stimulation with CXCL12 (■). When indicated (▨), 1 μmol/L AMD3100 was added 5 minutes before the stimulation with CXCL12. Columns, mean; bars, SD. *, $P < 0.05$, versus control (*t* test); ♦, $P < 0.05$, significant inhibition by AMD3100 (*t* test).

the effects of long-term stimulation with CXCL12 on the clonogenic potential of colon cancer cells. Daily addition of CXCL12 at 20 ng/mL into culture medium significantly increased the number of clones in SW620, HT29, and Lovo cells at 7 and 14 days. This effect was abrogated by treatment with 1 μmol/L AMD3100 (Fig. 4).

CXCL12 stimulates VEGF in CRC cells. Previous studies have provided evidence that the VEGF pathway could play an important role in the promotion of cancer growth and neoangiogenesis. To test the hypothesis that CXCL12 could trigger VEGF secretion by colon cancer clones, we first treated SW620, HT29, and Lovo cells with 200 ng/mL of CXCL12, showing stimulation of VEGF secretion after 24 hours by ELISA assay. The stimulation was reduced by treatment with 1 μmol/L AMD3100 (Fig. 5). Next, we determined the expression of VEGF in colon cancer clones by immunocytochemistry after 24-hour stimulation with 200 ng/mL of CXCL12. Interestingly, the number of VEGF-positive clones was significantly higher in the CXCL12 treated group compared with the untreated one. Treatment with 1 μmol/L AMD3100 significantly reduced the number of VEGF-positive clones (Fig. 6).

CXCL12 up-regulates ICAM-1. ICAM-1 expression has been implicated in tumor progression and metastases. We tested the effects of CXCL12 on ICAM-1 expression of CRC cells. Interestingly, there was a slight but definite up-regulation in SW620, Lovo, and HT29 cells treated with 200 ng/mL, which was reduced by CXCR4 antagonism with 1 μmol/L AMD3100 (Fig. 7; Table 4).

Discussion

In the present study, we show that the concomitant expression of CXCR4 and VEGF in >50% of neoplastic cells is

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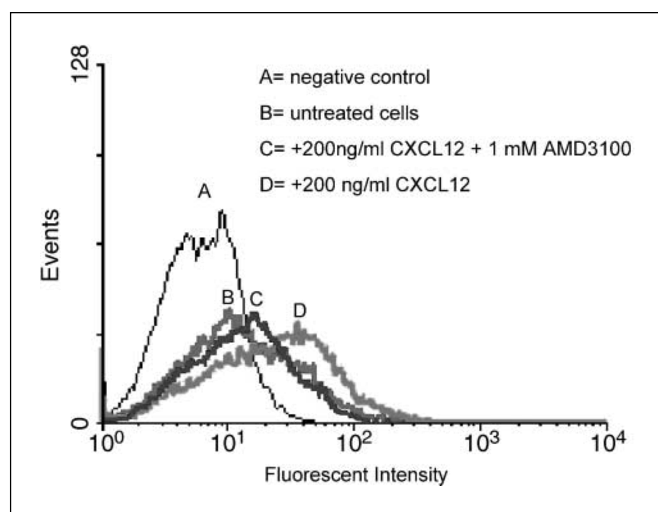

Fig. 7. CXCL12 promotes ICAM-1 expression in CRC cells. ICAM-1 expression has been evaluated with anti-ICAM-1-PE antibodies by flow cytometry. As indicated by *A*, *B*, *C*, and *D* histograms, CXCL12 stimulates ICAM-1 expression; treatment with AMD3100 prevents ICAM-1 up-regulation. Shown is one of three representative experiments in SW620 cells.

an independent predictor of early metastases in CRC patients and that CXCL12 stimulates ICAM-1 and VEGF expression and clonogenic growth of CRC cells, which are all important phenomena in the establishment of metastatic cells. ICAM-1 is expressed in CRC tissues and cell lines and is up-regulated by inflammatory cytokines (IFN-α, tumor necrosis factor α, or interleukin 1β; ref. 32). Interestingly, ICAM-1 is shed by CRC cells and soluble ICAM-1 can be detected in the cell culture supernatants. In fact, CRC patients show significantly higher serum levels of soluble ICAM-1 compared with healthy controls and there is a significant association between the serum levels of this molecule, disease stage, and the presence of both lymph node and distant metastases. Serum levels of soluble ICAM-1 decrease significantly after radical resection of the tumor (33). The increase in ICAM-1 by the CXCR4/CXCL12 pathway might contribute to the attachment of CRC cells to the endothelium and to CRC cell establishment in distant organs (liver, lung, and lymph nodes).

The expression of VEGF is associated with poor prognosis and increased resistance to therapeutic interventions in several neoplasms (25, 26). In particular, inhibition of VEGF by antibodies (Avastin, bevacizumab) is pursued as a therapeutic target in advanced CRC. Interestingly, compared with chemotherapy alone (irinotecan, bolus fluorouracil, and leucovorin), the association with bevacizumab increases the progression-free survival, the overall response rate, and the median duration of response in advanced CRC patients (34). For these reasons, bevacizumab has become the first U.S. Food and Drug Administration–approved anti-VEGF therapy. Other strategies to inhibit VEGF, including binding of the VEGF protein and blockade of the VEGF receptor signaling, have recently undergone clinical testing (35). Our results show that stimulation of CXCR4 on CRC cells by CXCL12 increases VEGF expression. CXCL12 is highly expressed in the environment of metastatic sites (liver, lymph nodes, and lungs); thus, it might contribute to the establishment and progression of metastasis by promoting VEGF. Concomitant inhibition of CXCR4 and VEGF could have a synergistic action on reducing CRC-derived VEGF.

Notably, a recent study has identified CXCR4 as a prominent chemokine receptor by microarray screening of tissues from CRC patients. The analysis of primary CRC tumors from all stages of disease and liver metastases by quantitative reverse transcription-PCR showed significantly higher CXCR4 expression in the liver metastases. In stage I and II patients, 6 of 30 patients with tissues expressing high CXCR4 experienced local (n = 2) or distant (n = 4) recurrence whereas none of 27 patients with low-CXCR4-expressing tissues had recurrence during a median follow-up time of 28 months. In multivariate analysis adjusted by clinicopathologic characteristics, CXCR4 expression was a significant prognostic factor for disease-free survival in stage I-II patients and for overall survival in stage IV patients (24). In addition, we previously found higher expression of CXCR4 by immunohistochemistry in primary and secondary CRC compared with normal mucosa and adenomas and showed that the CXCR4/CXCL12 pathway was functional in CRC cell lines (36). Recently, Zeelenberg et al. (23) showed that CXCR4 is up-regulated by the microenvironment of distant sites and that blocking CXCR4 function in CT-26 CRC cells greatly reduced metastasis of these cells to liver and lungs by impairing their outgrowth. Furthermore, Schimanski et al. (37) reported that human CRC tissues and cell lines expressed CXCR4 and CCR7 with variable intensities but only expression of CXCR4 was significantly associated with stage, lymph node metastasis, and distant metastasis and was correlated with a reduced 3-year survival rate. In the present study, we have shown that both CXCR4 and VEGF were able to predict lymph node and distant metastases, thus confirming that CXCR4- and VEGF-positive cells are more prone to involve the draining lymph nodes and the distant organs.

CXCR4 staining in our series mainly involved cytoplasms. However, in three cases, it was found exclusively in the nucleus. This phenomenon has already been described and might

Table 4. Effects of CXCL12 and AMD3100 on ICAM-1 expression in CRC cells

	ICAM-1 MFI		
	Untreated cells	+ 200 ng/mL CXCL12	+ 200 ng/mL CXCL12 + 1 μmol/L AMD3100
SW620	12 ± 8	41 ± 11	16 ± 3
Lovo	11 ± 3	31 ± 8	17 ± 4
HT29	18 ± 7	40 ± 5	21 ± 4

NOTE: Means of fluorescence intensity (MFI) of ICAM-1 expression as compiled from three independent experiments are reported for SW620, Lovo, and HT29 cells. Relative SDs are reported for each value.

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represent a functional status of the receptor but we cannot reach any conclusions about this different localization because the number of examined events is too small.

Several studies have shown diverse methods for inhibiting the CXCR4 receptor: peptides, antibodies, small molecules (38, 39). We previously reported on the ability of anti-CXCR4 antibodies to inhibit CXCL12-induced phenomena in CRC cells (35). Here, we report the ability of AMD3100, a bicyclam derivative, to inhibit some CXCR4-mediated activities (clonogenic growth, VEGF, and ICAM-1 expression) in CRC cells. AMD3100 has already been tested in phase I clinical trials and it has been shown to be safe and active in inhibiting CXCR4-mediated signaling (40, 41).

Although surgical resection is still the only curative treatment for CRC patients, chemotherapy in the adjuvant setting has been shown to improve survival in patients with node-positive disease (stage III) and could potentially be beneficial for high-risk stage II patients (42). However, the morbidity related to the side effects of chemotherapy remains a common concern so that novel and less toxic molecular and biologically oriented agents are warranted. Interestingly, when focusing the analysis on stage II patients in our series, only those with tumors expressing high CXCR4 and high VEGF suffered progression ($P = 0.0008$; data not shown). Both CXCR4 and VEGF could be evaluated in an adjuvant clinical setting (*i*) to identify stage II-III patients at higher risk for relapse who may benefit from more aggressive adjuvant interventions, and (*ii*) to test the efficacy of novel therapies aimed at their inhibition. In conclusion, our study suggests that CXCR4 and VEGF are predictors of early relapse in CRC patients and supports a rationale for the inhibition of CXCR4 and VEGF in the prevention of development of metastatic clones.

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Overexpression of Both CXC Chemokine Receptor 4 and Vascular Endothelial Growth Factor Proteins Predicts Early Distant Relapse in Stage II-III Colorectal Cancer Patients

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