CXCL12 and Vascular Endothelial Growth Factor Synergistically Induce Neoangiogenesis in Human Ovarian Cancers

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Abstract

Ovarian carcinomas have a poor prognosis, often associated with multifocal i.p. dissemination accompanied by intense neovascularization. To examine tumor angiogenesis in the tumor microenvironment, we studied malignant ascites and tumors of patients with untreated ovarian carcinoma. We observed that malignant ascites fluid induced potent *in vivo* neovascularization in Matrigel assay. We detected a sizable amount of vascular endothelial cell growth factor (VEGF) in malignant ascites. However, pathologic concentration of VEGF is insufficient to induce *in vivo* angiogenesis. We show that ovarian tumors strongly express CXC chemokine stromal-derived factor (SDF-1/CXCL12). High concentration of CXCL12, but not the pathologic concentration of CXCL12 induces *in vivo* angiogenesis. Strikingly, pathologic concentrations of VEGF and CXCL12 efficiently and synergistically induce *in vivo* angiogenesis. Migration, expansion, and survival of vascular endothelial cells (VEC) form the essential functional network of angiogenesis. We further provide a mechanistic basis for explaining the interaction between CXCL12 and VEGF. We show that VEGF up-regulates the receptor for CXCL12, CXCR4 expression on VECs, and synergizes CXCL12-mediated VEC migration. CXCL12 synergizes VEGF-mediated VEC expansion and synergistically protects VECs from sera starvation-induced apoptosis with VEGF. Finally, we show that hypoxia synchronously induces tumor CXCL12 and VEGF production. Therefore, hypoxia triggered tumor CXCL12 and VEGF form a synergistic angiogenic axis *in vivo*. Hypoxia-induced signals would be the important factor for initiating and maintaining an active synergistic angiogeneic pathway mediated by CXCL12 and VEGF. Thus, interrupting this synergistic axis, rather than VEGF alone, will be a novel efficient antiangiogenesis strategy to treat cancer. (Cancer Res 2005; 65(2): 465-72)

Introduction

Tumor angiogenesis is essential for the growth of primary and metastatic tumors. Tumors and metastases may originate as small avascular masses that induce the development of new blood vessels once they grow to a few millimeters in size (1, 2). One of the most well characterized angiogenic factors is vascular endothelial cell growth factor (VEGF). VEGF has angiogenic action in numerous *in vivo* and *in vitro* models (3, 4). Many antiangiogenic strategies have targeted VEGF activity. Some reports of tumor regression in experimental models of angiogenesis exist. The majority of studies show antiangiogenic therapy leads to an inhibition of tumor growth rather than a regression of established tumors (5, 6). Early clinical trials with antiangiogenic strategies, however, have not replicated the results observed from preclinical models (3, 7, 8). Previously identified angiogenic molecules β3 and β5 integrins have recently been shown not to support *in vivo* angiogenesis (9, 10). Angiogenic molecule basic fibroblast growth factor is found positively related to the prolonged survival of tumor patients (11). The reasons for these apparent discrepancies are that the extent of angiogenesis is determined by multiple factors in tumor microenvironment and each individual tumor may display a different angiogenic phenotype. Some other potential angiogenic factors may also have functionally been ignored in the designs of antiangiogenic strategies.

Ovarian carcinoma is the fifth leading cause of cancer among women and leading cause of mortality among cancers of the female reproductive system (12). Ovarian carcinomas have a poor prognosis, often associated with multifocal i.p. dissemination with potent neovascularization. The related mechanism remains poorly understood. Ovarian tumor cells produce a large amount of CXCL12 (13) and release into peritoneal cavity. In this report, we show that hypoxia importantly and synchronously induces CXCL12 and VEGF production by tumors, and CXCL12 and VEGF form a synergistic angiogenic axis to induce angiogenesis *in vivo*.

Materials and Methods

Human Subjects and Clinical Samples. We studied patients with ovarian carcinomas. Patients are given written informed consent. The study was approved by the local Institutional Review Board. No cancer patients received prior specific treatments. Ascites have been collected from consecutive patients with previously untreated ovarian carcinoma. Ascites were collected aseptically, and harvested cells by centrifugation over a Ficoll-Hypaque density gradient (13).

***In vivo* Matrigel Assay.** We established an *in vivo* Matrigel assay in mice (14–17). Briefly, 0.5 mL of iced Matrigel (Becton Dickinson, San Jose, CA) admixed with the relevant cytokines or ascites and heparin were injected into the right lower abdomen of female C57 mice (6-8 weeks). After 10 to 12 days (16), the Matrigel plugs were isolated and processed for quantifying microvessel density (18) with ImagePro Plus software (Image-Pro plus, Media Cybernetics, Silver Spring, MD). Microvessel density was expressed as mean percentage of microvessel surface area by confocal Leica TCS-NT SP microscope.

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Immunohistochemistry. Matrigel plugs were subjected to immunohistochemistry analysis with rabbit anti-human-vWF antibody (polyclonal, 1/100 dilution, DAKO, Carpinteria, CA), and further stained with goat anti-rabbit antibody (immunoglobulin G, 1/2,000 dilution, Molecular Probes, Eugene Oregon). Surface occupied by vascular endothelial cells (vWF$^+$ green cells) was quantified by confocal microscope as described above. Tumor tissues CXCL12 expression was analyzed by immunohistochemistry with 8-μm cryosections of acetone-fixed ovarian tumor tissues as we described previously (13, 19). Tumor tissues were incubated for 2 hours at room temperature with anti-CXCL12 antibody (clone K15C, IgG2a, 10 μg/mL), or control isotype. Antibody binding was detected with biotinylated anti-mouse antibodies and streptavidin conjugated to alkaline phosphatase (Biogenex, San Ramon, CA) using fast red substrate. Sections were counterstained with Mayler hematoxylin.

Reverse Transcriptase-PCR. CXCL12 and VEGF mRNA was detected by reverse transcriptase-PCR (RT-PCR) as we described (20). Briefly, β-actin was initially amplified and quantified with serial dilutions of cDNA from each sample. CXCL12 was then amplified in each sample containing identical amount of β-actin mRNA. β-Actin primers were sense 5′-gggtcagaaggattcctatg-3′ and antisense 5′-ggtctcaaacatgatctggg-3′. CXCL12 primers were sense 5′-gggctcctgggttttgtatt-3′ and antisense 5′-gtcctgagagtccttttgcg-3′. The identical technique and primers were used to amplify each VEGF splice forms as previously published (21).

Migration Assay. Fresh human umbilical vascular endothelial cells (HUVEC) were purified from human umbilical cords as we described (22). HUVECs were transferred into the upper chambers of 8-μm-pore transwell plates (Neuro Probe, Gaithersburg, MD). CXCL12 and VEGF (R&D System, Minneapolis, MN) were added to the lower chamber. After 40 hours at 37°C, migration was quantified by counting cells in the lower chamber and cells adhering to the bottom of the membrane (13).

Cell Proliferation. Fresh HUVECs (10^5/mL) were cultured with the indicated cytokines for 72 hours. ^3H thymidine was added at the last 16 hours, and cell proliferation was detected by thymidine incorporation as we described (13, 23, 24).

***In vitro* Apoptosis Assay.** Fresh HUVECs (5×10^5/mL) were cultured in 37°C with different concentrations of FCS in medium with the described conditions. After 24 hours, cells were harvested and stained with Annexin V and 7-AAD. Apoptosis of HUVEC was analyzed by fluorescence-activated cell sorting (13).

Hypoxia Experiments. Primary ovarian tumor cells (5×10^5/mL or 2×10^6/mL) were cultured in 37°C incubators with 1% oxygen (Coy Laboratory Products, Inc., Grass Lake, MI) or 21% oxygen with the described conditions. Cells were harvested for detecting VEGF and CXCL12.

ELISA. Cytokines/chemokines in cell supernatants and ascites were detected with commercial kits (R&D Systems). Hemoglobin content in Matrigel plugs was detected with a commercial kit (Sigma, St. Louis, MO).

Statistical Analysis. Differences in cell surface molecule expression were determined by χ^2 test, and in other variables by unpaired t test, with $P < 0.05$ considered significant.

Results

Malignant Ascites Fluid Induces Potent *In vitro* Angiogenesis. To determine the angiogenic factors in malignant ascites, we established an *in vivo* Matrigel assay model (16, 17). As expected, recombinant VEGF ($n = 8$) and tumor necrosis factor-α ($n = 8$) induced a significant angiogenesis (positive control; *, $P < 0.001$, compared with PBS). Interestingly, both malignant ascites fluid ($n = 12$) and nontumor ascites (idiopathic cirrhosis; $n = 6$) induced *in vivo* angiogenesis (*, $P < 0.001$, compared with PBS; Fig. 1). The percentage of microvessel surfaces (Fig. 1*A*) is correlated with the hemoglobin contents per Matrigel (Fig. 1*B*; refs. 14, 25). However, malignant ascites were thrice more powerful to induce angiogenesis *in vivo* than cirrhotic ascites (Fig. 1). These data showed that malignant ascites contained angiogeneic factor(s).

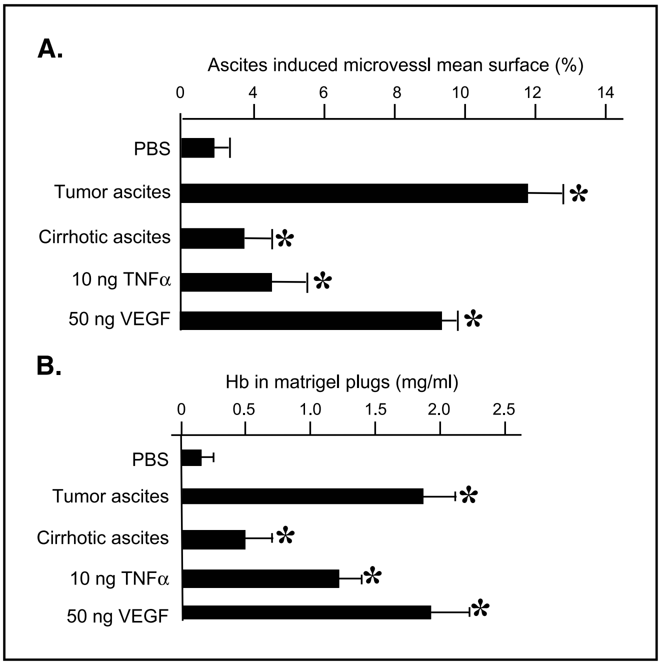

Figure 1. Malignant ascites fluid induces potent *in vivo* angiogenesis. C57 mice were inoculated with Matrigel plugs bearing malignant ascites fluid, cirrhotic ascites (0.5 mL), the indicated cytokines, or PBS. Day 12 Matrigel plugs were removed to study neovascularization as described in Methods. *A,* microvessel surface area in Matrigel plugs was quantified and expressed as % microvessel surface area as we described. *B, Hb* contents in Matrigel plugs were detected with a commercial kit. *, $P < 0.05$, compared with PBS.

CXCL12 in Malignant Ascites. To determine the angiogenic factors in malignant ascites, we screened malignant ascites for the important identified angiogenic factors. We observed significant amounts of VEGF (3.1 ng/mL), IL-8 (1.2 ng/mL), and moderate amounts of tumor necrosis factor-α (0.23 ng/mL), and MCP-1 (0.27 ng/mL) in malignant ascites ($n = 28$; Fig. 2*A*). Strikingly, we detected high level of CXCL12 (25 ng/mL) in malignant ascites ($n = 28$; *, $P < 0.001$, compared with other cytokines; Fig. 2*A*). Cirrhotic ascites ($n = 6$) contained moderate levels of these detected cytokines, suggesting that CXCL12 and VEGF may not be as critical as malignant ascites in inducing angiogenesis.

CXCL12 Expression in Tumor Tissues and Primary Tumor Cells. Consistent with our previous report (13), we observed that ovarian tumor tissues expressed potent CXCL12 mRNA ($n = 12$; Fig. 2*B*). Quantitative RT-PCR (20) revealed no significant difference of CXCL12 mRNA in tumors between FIGO stages I, II, III, and IV, as well as in tumors with different histology, including serous, mucinous, endometrioid, and undifferentiated ovarian tumors (Fig. 2*B*, data not shown). Immunohistochemistry analysis further confirmed that 100% ovarian tumor tissues expressed CXCL12 protein ($n = 72$; stage I, $n = 14$; stage II, $n = 12$; stage III, $n = 28$; stage IV, $n = 18$; Fig. 2*C*). No positive cells were observed in tissues stained with isotype antibody (data not shown). Notably, the level of CXCL12 protein expression was relatively variable and was not significantly different between different donors, between different disease stages, and between different tumor histologic types (Fig. 2*C*).

We further established 12 ovarian epithelial tumor cell lines from tumor ascites. We observed that all the primary tumor cell lines strongly expressed CXCL12 mRNA ($n = 12$; Fig. 2*D*). Intracellular

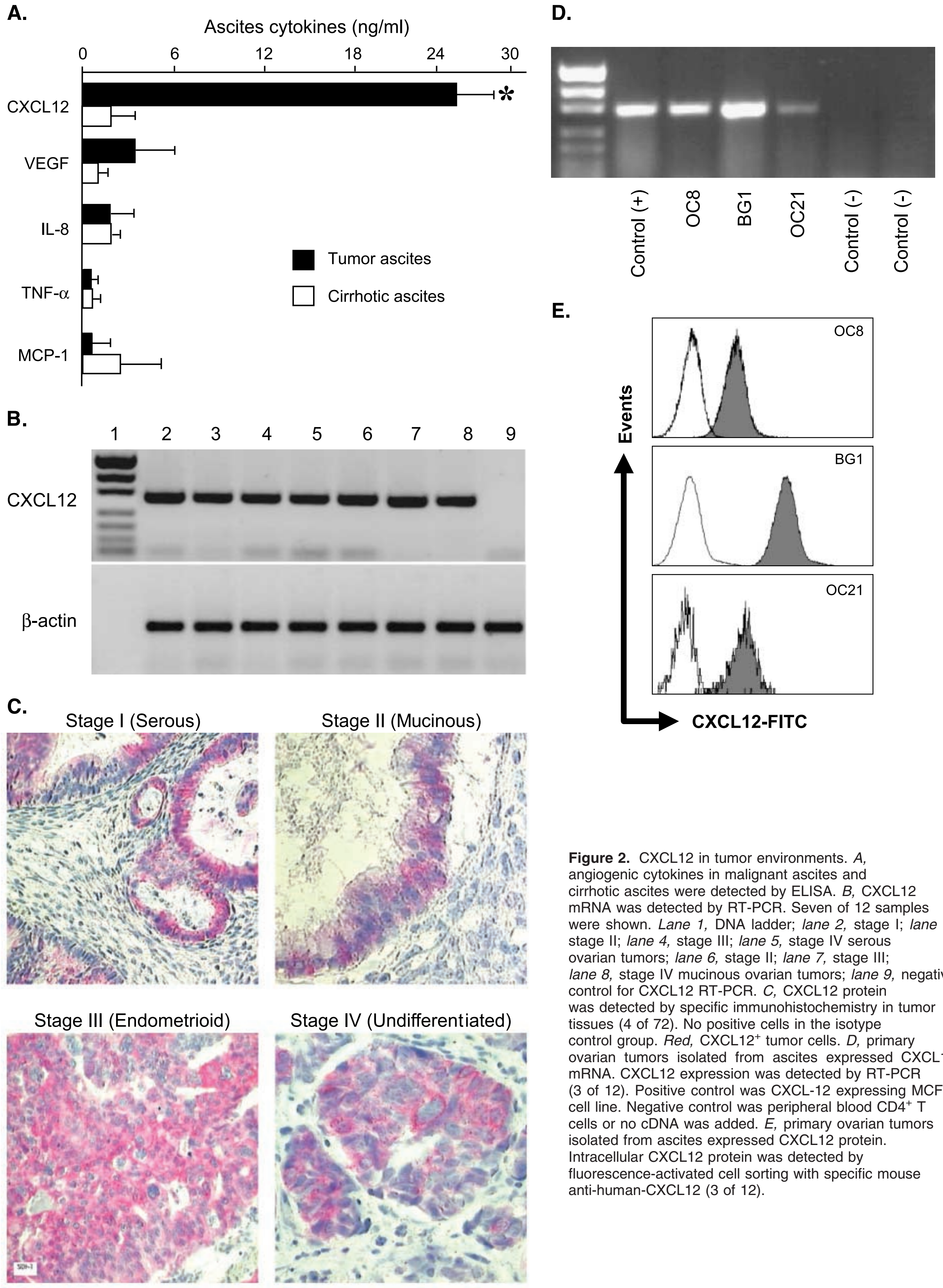

Figure 2. CXCL12 in tumor environments. *A,* angiogenic cytokines in malignant ascites and cirrhotic ascites were detected by ELISA. *B,* CXCL12 mRNA was detected by RT-PCR. Seven of 12 samples were shown. *Lane 1,* DNA ladder; *lane 2,* stage I; *lane 3,* stage II; *lane 4,* stage III; *lane 5,* stage IV serous ovarian tumors; *lane 6,* stage II; *lane 7,* stage III; *lane 8,* stage IV mucinous ovarian tumors; *lane 9,* negative control for CXCL12 RT-PCR. *C,* CXCL12 protein was detected by specific immunohistochemistry in tumor tissues (4 of 72). No positive cells in the isotype control group. *Red,* CXCL12^{+} tumor cells. *D,* primary ovarian tumors isolated from ascites expressed CXCL12 mRNA. CXCL12 expression was detected by RT-PCR (3 of 12). Positive control was CXCL-12 expressing MCF-7 cell line. Negative control was peripheral blood CD4^{+} T cells or no cDNA was added. *E,* primary ovarian tumors isolated from ascites expressed CXCL12 protein. Intracellular CXCL12 protein was detected by fluorescence-activated cell sorting with specific mouse anti-human-CXCL12 (3 of 12).

staining showed that these primary tumor cell lines actively expressed intracellular CXCL12 (n = 12; Fig. 2*E*). Therefore, ovarian tumor cells are the major cellular source in tumor environment.

CXCL12 and VEGF Synergistically Induced *In vivo* Angiogenesis. To determine the role of each individual cytokine in ascites-mediated *in vivo* angiogenesis (Fig. 1), we tested the *in vivo* angiogenesis in Matrigel assay with recombinant cytokines. CXCL12 induced a significant angiogenesis in a dose dependent manner (n = 7-10 for each point; Fig. 3*A*). Notably, the effective angiogenic concentrations of CXCL12 were superior to 25 ng/mL (Fig. 3*A*). We detected 25 ng/mL CXCL12 in malignant ascites (Fig. 2*A*). The data indicates that high concentration of CXCL12 is angiogenic *in vivo*, whereas pathologic concentration of CXCL12 is not.

As expected, recombinant VEGF induced a significant angiogenesis in a dose dependent manner (n = 7-10 for each point; Fig. 3*B*). However, consistent with other reports (14, 16), the effective angiogenic concentrations of VEGF were superior to 5 ng/mL in this experimental model (Fig. 3*B*). We detected 3.1 ng/mL VEGF in malignant ascites (Fig. 2*A*). The data indicates that pathologic concentration of VEGF is not able to induce a relevant *in vivo* angiogenesis in our model.

We hypothesized that tumor-derived VEGF and CXCL12 synergized to induce *in vivo* angiogenesis. We first examined whether VEGF synergized CXCL12-induced angiogenesis. Strikingly, 5 ng of VEGF significantly increased the angiogeneic effects of CXCL12 (5-50 ng/mL) in a dose dependent

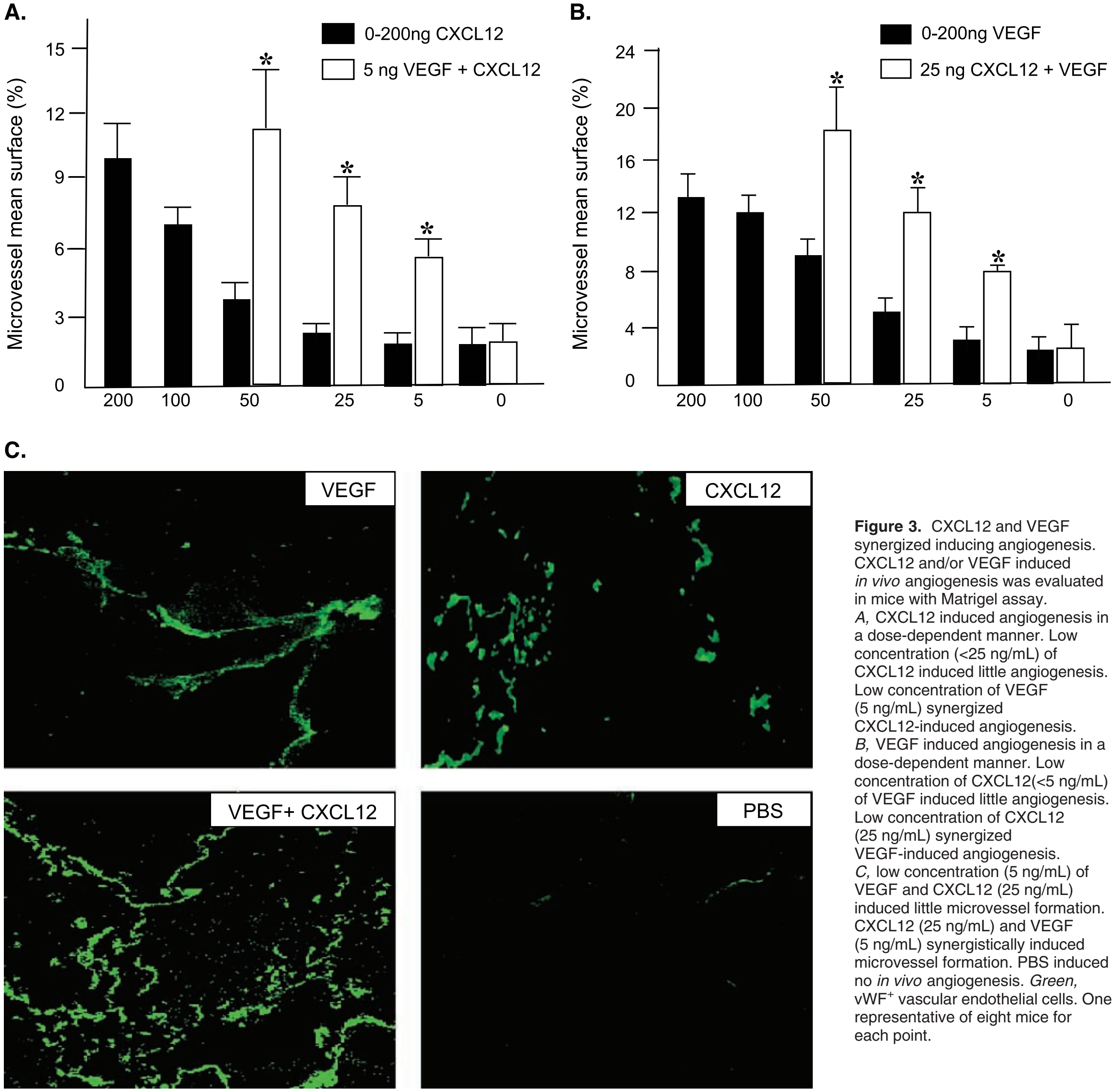

Figure 3. CXCL12 and VEGF synergized inducing angiogenesis. CXCL12 and/or VEGF induced *in vivo* angiogenesis was evaluated in mice with Matrigel assay. *A,* CXCL12 induced angiogenesis in a dose-dependent manner. Low concentration (<25 ng/mL) of CXCL12 induced little angiogenesis. Low concentration of VEGF (5 ng/mL) synergized CXCL12-induced angiogenesis. *B,* VEGF induced angiogenesis in a dose-dependent manner. Low concentration of CXCL12(<5 ng/mL) of VEGF induced little angiogenesis. Low concentration of CXCL12 (25 ng/mL) synergized VEGF-induced angiogenesis. *C,* low concentration (5 ng/mL) of VEGF and CXCL12 (25 ng/mL) induced little microvessel formation. CXCL12 (25 ng/mL) and VEGF (5 ng/mL) synergistically induced microvessel formation. PBS induced no *in vivo* angiogenesis. *Green,* vWF$^+$ vascular endothelial cells. One representative of eight mice for each point.

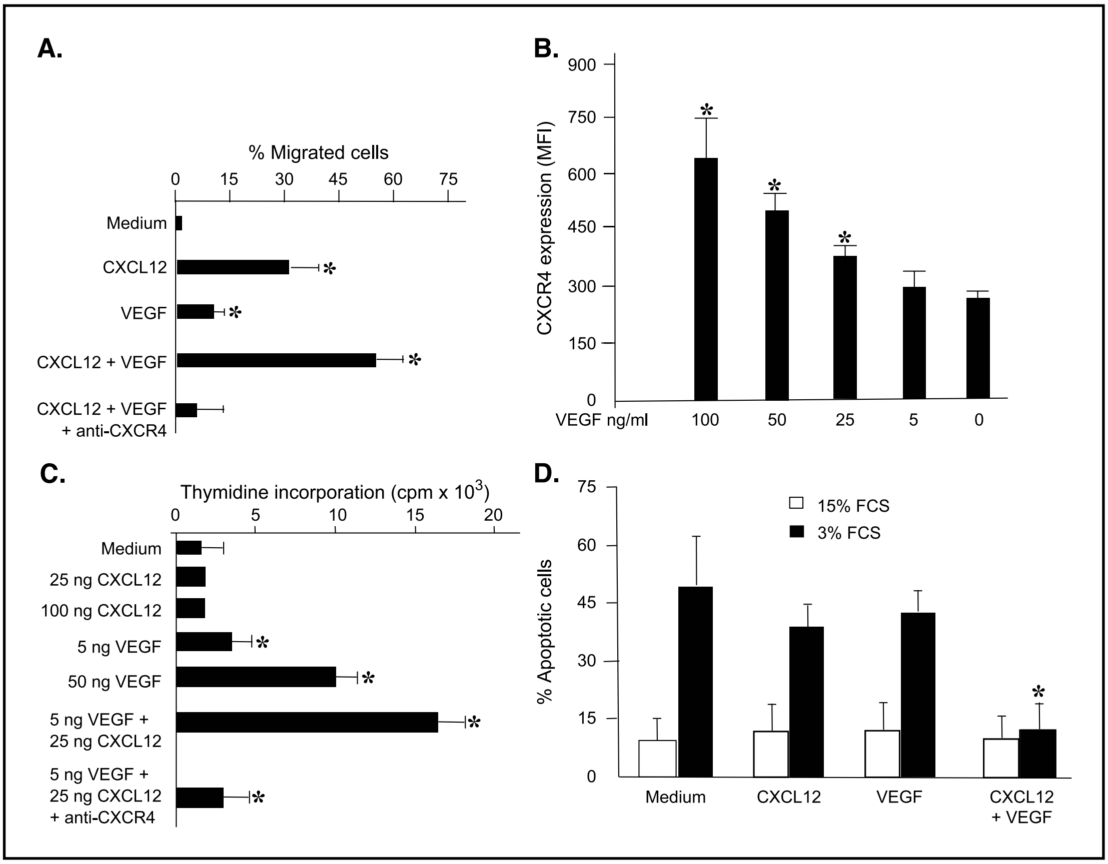

Figure 4. CXCL12 and VEGF synergistically promote angiogenic function of vascular endothelial cells. *A,* CXCL12 and VEGF synergistically induced HUVEC migration. CXCL-12, 100 ng/mL; VEGF, 5 ng/mL. *B,* VEGF up-regulated CXCR4 expression on HUVEC. CXCR4 expression was analyzed by fluorescence-activated cell sorting. 100% cells express CXCR4. *MFI,* mean fluorescence intensity. One representative of six. *C,* CXCL12 synergized VEGF-mediated HUVEC proliferation. Cell proliferation was detected by thymidine incorporation (cpm). *D,* CXCL12 and VEGF synergistically protect HUVEC apoptosis. HUVEC were cultured with medium containing 3% and 15% FCS for 24 hours. CXCL12 (100 ng/mL) and VEGF (5 ng/mL) were added into the culture. Cellular apoptosis was analyzed by annexin V and 7-AAD staining and expressed as % apoptotic cells.

manner ($n = 8$ for each point; *, $P < 0.001$, compared with CXCL12 alone; Fig. 3*A*). The data indicate that pathologic concentration of VEGF synergized CXCL12-induced angiogenesis.

We next examined whether CXCL12 synergized VEGF-induced angiogenesis. Strikingly, 25 ng of CXCL12 significantly increased the angiogeneic effects of VEGF (5-50 ng/mL) in a dose-dependent manner ($n = 8$ for each group; *, $P < 0.001$, compared with VEGF alone; Fig. 3*B*). The data indicate that pathologic concentration of CXCL12 synergized VEGF-induced angiogenesis. Histologic analysis showed significant vascular channel formation and tortuous neovessels in Matrigel plugs containing 25 ng of CXCL12 plus 5 ng of VEGF (Fig. 3*C*; ref. 17), but few microvessel formation in Matrigel plugs containing 25 ng of CXCL12 or 5 ng of VEGF. No microvessel formation was observed in Matrigel plugs containing PBS ($n = 8$ for each group; Fig. 3*C*; ref. 25). Therefore, tumor-derived CXCL12 and VEGF likely form a synergistic angiogeneic pathway *in vivo*.

CXCL12 and VEGF Synergize to Promote Angiogenic Function of Vascular Endothelial Cells. We next examined the synergistic mechanism by which CXCL12 and VEGF induced angiogenesis. Vascular endothelial cell migration is a critical step of tumor angiogenesis (4). We studied the directional migration of HUVEC. Both CXCL12 and VEGF induced a notable migration of HUVEC ($n = 6$; *, $P < 0.0001$, compared with medium for all; Fig. 4*A*). Interestingly, CXCL12-mediated migration was significantly more efficient in the presence of low concentration of VEGF (5 ng). Preincubation with a neutralizing antibody against CXCR4 completely disabled CXCL12-mediated HUVEC migration in the presence of VEGF, confirming the involvement of CXCR4. Further experiments showed that VEGF increased CXCR4 expression on HUVEC ($n = 5$; *, $P < 0.001$, compared with medium; Fig. 4*B*), indicating that VEGF sensitized CXCL12-mediated migration of vascular endothelial cells through up-regulating CXCR4.

Vascular endothelial cell growth is important for tumor angiogenesis. We examined whether CXCL12 and VEGF could synergize to stimulate HUVEC proliferation. Unexpectedly, 25 to 100 ng of CXCL12 induced little HUVEC proliferation. VEGF induced HUVEC proliferation in a dose-dependent manner ($n = 8$; *, $P < 0.001$, compared with medium or CXCL12 alone). Interestingly, pathologic concentrations of VEGF (5 ng) and CXCL12 (25 ng) were significantly more efficient to induce vascular endothelial cell proliferation than VEGF alone ($n = 5$; *, $P < 0.001$, compared with 5 ng VEGF; Fig. 4*C*). Again, preincubation with anti-CXCR4 completely cancelled the VEGF-mediated proliferation, which was sensitized by CXCL12 (Fig. 4*C*). Thus, tumor-derived CXCL12 sensitizes VEGF-mediated vascular endothelial cell expansion.

Survival of vascular endothelial cells is critical for forming stable neovascularization. Deprivation of nutrients results in vascular endothelial cell apoptosis (14). We show that tumor environmental CXCL12 protects plasmacytoid dendritic cells from apoptosis (13). We hypothesize that CXCL12 and VEGF synergistically protect vascular endothelial cell apoptosis. To test this hypothesis, fresh HUVEC were cultured with different concentrations of FCS medium. FCS medium (3%) induced 50% apoptotic cells ($n = 6$; $P < 0.01$, compared with 15% medium; Fig. 4*D*). CXCL12 and VEGF independently and marginally decreased the percentage of apoptotic cells induced by sera starvation ($n = 6$; $P < 0.05$, compared with 15% FCS; Fig. 4*D*). Strikingly, CXCL12 plus VEGF efficiently reduced the percentage of apoptotic cells induced by 3% FSC medium

($n = 6$; $P < 0.001$, compared with VEGF or CXCL12 alone; Fig. 4*D*), suggesting that VEGF and CXCL12 synergistically protected vascular endothelial cell apoptosis. The data indicate that multiple mechanisms are implicated in the *in vivo* synergistic angiogenic induction of CXCL12 and VEGF.

Hypoxia Triggered CXCL12 and VEGF Production. After determining that CXCL12 and VEGF form a synergistic angiogeneic pathway, we next examined the potential regulatory mechanisms for this synergistic pathway. It is well documented that hypoxia induces VEGF production by tumor cells (4, 26, 27). We confirmed this finding. We showed that 4 to 6 hours after exposure to hypoxia, the level of VEGF165 and VEGF121 mRNA was 50- and 2.5-fold higher, respectively, in hypoxia-treated tumor cells than normoxia-treated tumor cells ($n = 4$; Fig. 5*A*). Hypoxia particularly triggered the expression of VEGF121 and VEGF165, but not VEGF189 and VEGF206 (Fig. 5*A*). The hypoxia-induced VEGF was maintained for >24 hours (data not shown). As confirmation, hypoxia-treated ovarian tumor cells released more VEGF protein than normoxia ($n = 8$, $P < 0.001$; Fig. 5*B*). Furthermore, 4 hours after exposure to hypoxia, CXCL12 mRNA was significantly induced ($n = 12$; Fig. 5*C*). At 6 hours, the level of CXCL12 mRNA was 100-fold higher in hypoxia than normoxia ($n = 12$; Fig. 5*C*). We observed similar results in one commercialized ovarian tumor cell line (BG-1) and three primary ovarian tumors cell lines (OC8, OC21, and OC38) established in the laboratory. As confirmation, intracellular staining showed that the level of CXCL12 protein was significantly higher in tumor cells exposed to hypoxia than normoxia ($n = 8$, *, $P < 0.001$, compared with normoxia; Fig. 5*D*). Therefore, hypoxia activates the synergistic angiogeneic pathway between VEGF and CXCL12 through synchronously triggering VEGF and CXCL12 production.

Discussion

CXCL12 was originally isolated from murine bone marrow stromal cells (28), and described for its activity as a chemotactic cytokine for leukocytes (29, 30), $CD34^+$ progenitor cells (31–34), platelets (35, 36), and stem cells (33, 37). We previously showed that human ovarian epithelial tumor cells express high levels of CXCL12. Tumor-derived CXCL12 contributes to plasmacytoid dendritic cell trafficking and accumulation in tumor microenvironment (13). Tumor environmental plasmacytoid dendritic cells induce neoangiogenesis through IL-8 and tumor necrosis factor-α (25). We now show for the first time that pathologic concentration of

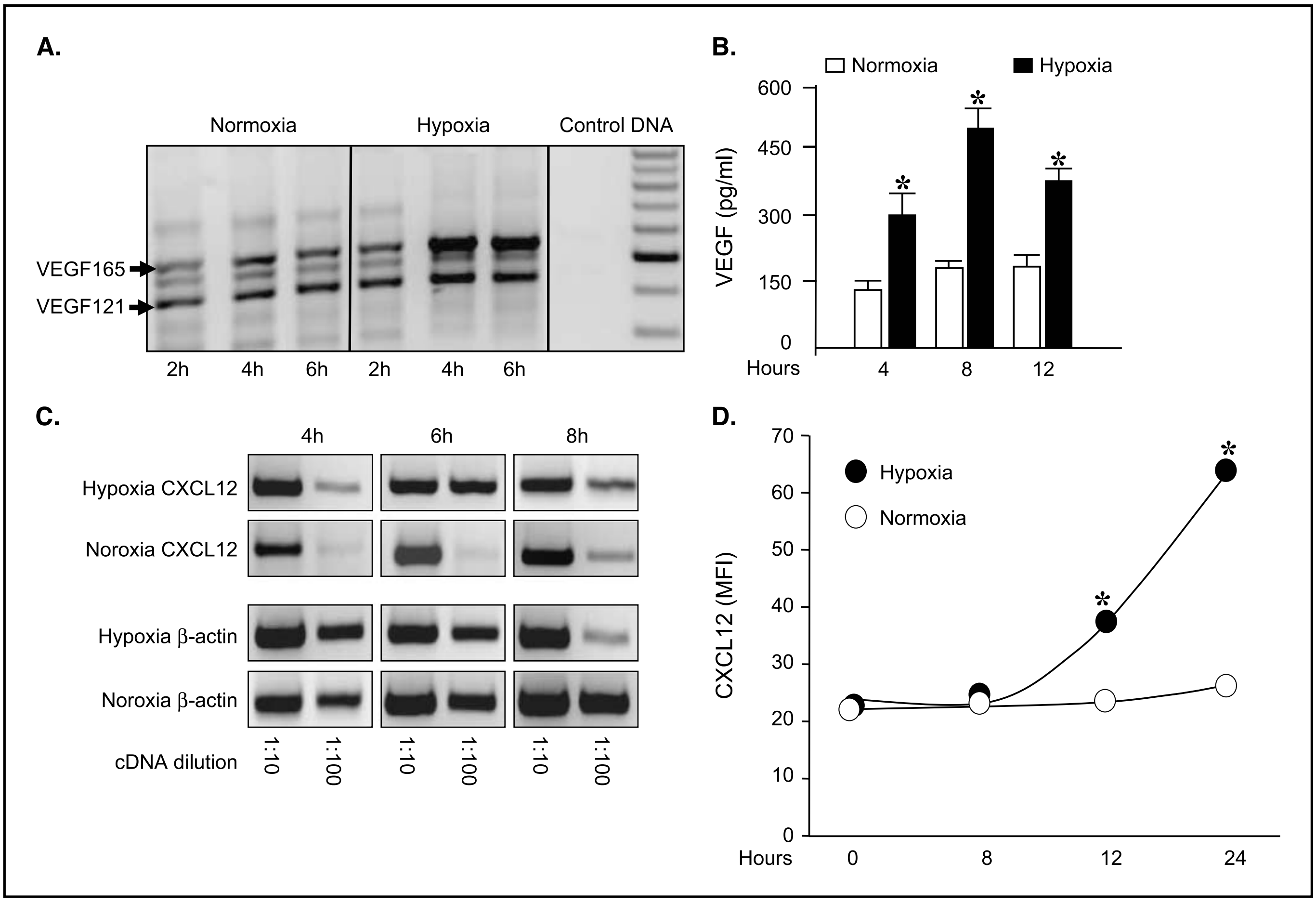

Figure 5. Hypoxia induces CXCL12 and VEGF production. Ovarian tumor cells (*OC8*) were exposed to normoxic (21% O_2) or hypoxic (1% O_2) for different times. *A,* hypoxia induced VEGF mRNA expression in tumor cells. Nested RT-PCR was done as described in Materials and Methods. VEGF165, 572 bp; VEGF121, 440 bp. *Control,* no DNA was added; *DNA,* DNA ladder. One of four representatives. *B,* hypoxia induced VEGF production by tumor cells. VEGF was detected in the culture supernatants by ELISA kit. *, $P < 0.001$, compared with normoxia. *C,* hypoxia induced CXCL12 mRNA expression in tumor cells. RT-PCR was done as described in Materials and Methods. One of six representatives. *D,* hypoxia induced CXCL12 protein by tumor cells. CXCL12 was analyzed by fluorescence-activated cell sorting. *MFI,* mean fluorescence of intensity of CXCL12 expression.

tumor-derived CXCL12 plus VEGF synergistically induce potent neovascurization *in vivo*. The data suggest that this synergistic pathway would predominantly contribute to tumor vascularization *in vivo* in a real situation. The notion is supported by four lines of evidence: (1) High concentrations, but not pathologic concentrations of VEGF or CXCL12 induce angiogenesis in our *in vivo* model. (2) Pathologic concentrations of CXCL12 plus VEGF synergistically induce angiogenesis *in vivo*. (3) Hypoxia synchronously triggers both VEGF and CXCL12 production by tumor cells. (4) CXCL12 and VEGF synergistically promote vascular endothelial cell function, including migration, expansion, and survival. In support of the *in vivo* synergic effects of tumor-derived CXCL12 and VEGF in angiogenesis that we report here, the interaction between recombinant CXCL12 and VEGF has been described in previous *in vitro* studies, including *in vitro* cultured umbilical vein endothelial cells (38, 39), lymphohematopoietic cells (40), and breast cancer cell lines (41). However, direct evidence showing the *in vivo* synergistic angiogenesis between VEGF and CXCL12 is missing.

CXCL12 does not contain the Nh_2-terminal Glu-Leu-Arg (ELR) motif with angiogenic function (42). The angiogenic potentiality of CXCL12 has been suggested in different settings of *in vitro* experiments (39, 43–46). Our *in vivo* data indicate that high concentration of CXCL12 directly induce angiogenesis. In support, mice lacking CXCL12 or CXCR4 have defective vascular system development (47, 48). S.c. injection of high concentration recombinant CXCL12 induced formation of local small blood vessels (38). However, our *in vitro* and *in vivo* data indicate that pathologic concentrations of CXCL12 and VEGF are not able to induce a pronounced *in vivo* angiogenesis, whereas pathologic concentrations of CXCL12 and VEGF induce potent *in vivo* angiogenesis in a synergistic manner. Apart from tumors (13), CXCL12 is constitutively expressed in stromal cells, vascular endothelial cells, osteoclast, and some epithelial cells, suggesting that CXCL12 would be important in different physiologic angiogenesis settings.

Anti-CXCR4 treatment significantly decreases the progression and metastasis of cancers in mice (49). The current explanation is that anti-CXCR4 blocks CXCR4 expressing tumor migration to CXCL12 expressing tissues (49–52). Our current data indicate that CXCL12/CXCR4 system is significantly involved in tumor angiogenesis by synergizing with VEGF. Thus, additional explanation is that anti-CXCR4 blocks tumor angiogenesis mediated by CXCL12 and VEGF synergistic pathway and in turn reduces tumor metastasis. In further support of this notion, VEGF has been reported to be an autorine survival factor and protects breast cancer cells from apoptosis induced by serum deprivation (53, 54). Hypoxia induces tumor VEGF (53). We now show for the first time that hypoxia triggers tumor CXCL12 expression. It may be a common mechanism in many human tumors that hypoxia synchronously induces VEGF and CXCL12, and VEGF and CXCL12 in turn synergistically protect tumor cell or vascular endothelial cell apoptosis from hypoxia in tumor environment and synergistically promote tumor vascularization and growth. Ovarian tumor cells produce a large amount of CXCL12 and VEGF, which are released into peritoneal cavity. The synergistic pathway between CXCL12 and VEGF would explain, at least partially the extensive tumor vascularization and metastasis in peritoneal cavity in most advanced ovarian cancers.

Therapeutically, the synergic axis between CXCL12 and VEGF has been ignored in prior antitumor angiogenesis strategies. Importantly, early human clinical cancer treatment trials with antiangiogenic molecules have not shown significant benefits predicted from preclinical models (3, 7, 8). More strikingly, recent reports (55–57) suggest that certain angiogenesis inhibitors (or antagonists) alone, by depriving tumors of oxygen, could have an unintended effect: promotion of tumor metastasis by increasing CXCR4 expression. These results reflect our growing understanding of the complexity of the tumor angiogenic process and suggest that blocking both CXCR4 and VEGF will be a novel, efficient strategy to treat human cancers.

In summary, we show in this report that tumors produced functional CXCL12 and VEGF, and tumor-derived CXCL12 and VEGF formed a synergistic angiogenesis axis *in vivo*, and hypoxia activates this axis through synchronously triggering tumor CXCL12 and VEGF production. The study suggests that CXCL12 and VEGF formed synergistic angiogenic pathway is critical for tumor neovascularization, and targeting both CXCL12 and VEGF signals may be a novel, efficient strategy for treating human cancers.

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