Vascular Endothelial Growth Factor C Expression and Lymph Node Metastasis Are Regulated by the Type I Insulin-like Growth Factor Receptor

Yucheng Tang, Donglei Zhang, Lucia Fallavollita, and Pnina Brodt

Departments of Surgery [Y. T., D. Z., L. F., P. B.] and Medicine [P. B.], McGill University Health Center, the Royal Victoria Hospital, Montreal, Quebec, H3A 1A1 Canada

Abstract

Vascular endothelial growth factor (VEGF-C) is a lymphangiogenic factor implicated in lymphatic metastasis. In this study, we investigated the role of the type I insulin-like growth factor receptor (IGF-IR) in the regulation of VEGF-C expression. We used Lewis lung carcinoma subline M-27 cells transfected with human IGF-IR cDNA. These cells, but not the wild-type cells, expressed VEGF-C mRNA, produced a M, 58,000 VEGF-C precursor protein, and secreted a M, 29,000 processed form in response to IGF-I. In vivo, they acquired a lymph node metastasizing potential. VEGF-C induction was abolished in cells expressing an IGF-IR with tyrosine-phenylalanine substitutions in the kinase domain, but not in the COOH-terminal domain. The induction was phosphatidylinositol 3'-kinase dependent and, to a lesser extent, mitogen-activated protein kinase signaling dependent, as determined by the use of the respective inhibitors LY294002 (84.6% reduction) and PD98059 (38% reduction). The results identify the IGF-IR as a positive regulator of VEGF-C expression and implicate it in the control of lymphatic metastasis.

Introduction

The regional lymph nodes draining primary tumors are generally the first, and by far the most common, site of metastasis for some of the major human malignancies including carcinomas of the breast, colon, and prostate. Until recently, tumor cell dissemination to the regional lymph node was generally believed to be a passive process involving tumor cell spread via preexisting afferent lymphatic channels and following natural routes of lymphatic drainage. Recent evidence of de novo formation of intratumoral lymphatic capillaries (lymphangiogenesis) raised the possibility that cells within primary tumors can contribute actively to lymphatic dissemination through the induction of a lymphangiogenic process (1). The evidence suggests that this process is driven by tumor-derived VEGFs3 VEGF-C and VEGF-D (2). VEGF-C is synthesized as a disulfide-linked prepropeptide, M, 59,000–61,000 dimer, which is proteolytically processed to a M, 21,000 homodimer. In this form, it is the ligand for two receptors, VEGFR-2 and VEGFR-3 (flt-4), whereas the partially processed forms can also bind with high affinity to VEGFR-3 (3). In normal adult tissues, VEGFR-3 is expressed predominantly on lymphatic endothelial cells, but expression was also noted on tumor-associated blood vessels (4, 5). When overexpressed in the skin of transgenic mice or applied onto the chick chorioallantoic membrane, VEGF-C stimulated lymphangiogenesis exclusively (6–8). Overexpression of VEGF-C in MCF-7 cells promoted tumor lymphangiogenesis and tumor metastasis via lymphatic vessels (9). Moreover, transgenic mice with targeted pancreatic β-cell VEGF-C expression (Rip-VEGF-C) that were crossed with Rip1-Tag2 mice that spontaneously develop nonlymphangiogenic and nonmetastatic pancreatic β-cell tumors formed tumors that were surrounded by well-developed lymphatics and frequently metastasized to the regional lymph nodes (10). Finally, in various human cancers, a positive correlation was observed between VEGF-C expression in the primary tumor and lymph node metastasis, implicating VEGF-C in the progression of clinical disease (11). It should be noted, in this regard, that recent observations suggest that lymphatic metastasis can also occur in the absence of functional, intratumoral lymphatics (12), probably via preexisting vessels on the tumor margin. This raises the possibility that the dependency of the process on de novo-generated lymphatic vessels may be variable.

The IGF-IR has been implicated in the induction and maintenance of the malignant phenotype and in the control of cellular functions that impact on invasion and metastasis (13). We have recently shown that this receptor regulates the synthesis of MMP-2 (14, 15). Receptor overexpression has been documented in many human malignancies, and high plasma IGF-I levels were identified as a potential risk factor for several carcinomas, including breast and colon carcinomas that metastasize via the lymphatics (13, 16). Recently, IGF-IR was implicated in the regulation of VEGF-dependent, tumor-induced neovascularization (17). Here, we investigated whether the IGF-IR/IGF-I axis plays a role in the regulation of VEGF-C expression and thereby in the control of lymphatic metastasis. We used a murine carcinoma model consisting of cell lines with divergent potentials to metastasize to regional lymph nodes from local sites that correlate with their IGF-IR expression levels (14, 18, 19).

Materials and Methods

Cells. The origin and metastatic phenotypes of murine Lewis lung carcinoma sublines H-59 and M-27 have been described previously. M-27 cells express low IGF-IR levels, respond poorly to IGF-I, and do not metastasize to regional nodes from primary s.c. sites (15, 18–20). H-59 cells are highly responsive to IGF-I and metastasize primarily to the lymph nodes and liver from local s.c. sites (18–21). The protocols used to generate IGF-IR mutants and to produce M-27 cells expressing WT (M-27IGF-IR) or mutated receptors, as well as the invasive/metastatic phenotypes of the transfected cells, have been described in detail elsewhere (15). All cells were maintained in RPMI 1640 supplemented with 10% FCS and antibiotics (18). IGF-IR transfectants were maintained in medium containing 200 μg/ml G418. The cells are routinely monitored for common infectious agents and were free of infection during the course of this study.

Reagents. The polyclonal goat anti-VEGF-C antibody (C-20) was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to MAPK (ERK), phospho-p44/p42 MAPK, Akt, and phospho-Akt were from Cell Signaling (Beverly, MA). The PI3K inhibitor LY294002 and the MAPK inhibitor PD98059 were from Calbiochem (San Diego, CA).

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2 To whom requests for reprints should be addressed, at The Division of Surgical Research, Royal Victoria Hospital, Room H6.25, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1. Phone: (514) 842-1231, ext. 36692; Fax: (514) 843-1411; E-mail: pnina.brodt@muhc.mcgill.ca.

3 The abbreviations used are: VEGF, vascular endothelial growth factor; ERK, extra-cellular response kinase; IGF-I, type I insulin-like growth factor; IGF-IR, IGF-I receptor; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; PI3K, phosphatidylinositol 3'-kinase; VEGFR, VEGF receptor; RT-PCR, reverse transcription-PCR; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; WT, wild-type.
and 20-

Western Blot Analysis. For detection of VEGF-C, cells cultured in serum-free medium for 24 h were stimulated with 10 ng/ml IGF-I and solubilized in a lysis buffer (50 mM HEPES (pH 7.6), 150 mM NaCl, 10 mM EDTA, 10 mM Na3P2O7, 2 mM sodium orthovanadate, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 100 IU/ml aprotinin, 20 μM leupeptin, and 1% (v/v) Tween 20) for 15 min at 4°C. The lysates were clarified by centrifugation, and 50 μg of protein were loaded per lane of a 10% SDS-polyacrylamide gel, separated by electrophoresis, transferred onto a nitrocellulose membrane, and probed with the goat anti-VEGF-C antibody diluted 1:100 and a HRP-conjugated goat antirabbit antibody (Jackson ImmunoResearch Labs, Westgrove, CA) diluted 1:2000.

Lymph Node Metastasis Assay. Mice were injected s.c. with 2 × 10^5 tumor cells. Tumors appeared 6 days later and were allowed to progress until the mean tumor volume calculated as length (mm) × width (mm)^2/2 reached 3500–4000 mm^3 (as per the McGill Animal Care Committee regulations). The animals were sacrificed, and the draining axillary lymph nodes were removed, fixed in 10% formalin, and embedded in paraffin. Tissue sections were stained with H&E using standard procedures.

Results

VEGF-C Synthesis Is Regulated by IGF-IR. To analyze the role of IGF-IR in the regulation of VEGF-C production, we used IGF-I nonresponsive carcinoma M-27 cells that were transfected with the full-length human IGF-IR (M-27IGF-IR). We reported previously that these cells express approximately 39,000 receptors/cell (an increase of 2.0-fold relative to nontransfected M-27 cells; Ref. 15). Stimulation of these cells but not of parental M-27 cells with IGF-I resulted in the induction of VEGF-C mRNA synthesis in a time- and dose-dependent manner, as measured by RT-PCR. Maximal induction was seen when serum-deprived cells were stimulated with 10 ng/ml IGF-I (Fig. 1A). At this concentration, an increase in VEGF-C mRNA levels was observed at 2 h, and it reached maximal levels by 6 h (Fig. 1B).

VEGF-C Induction Is Dependent on an Intact Kinase Domain but Is Not Affected by Y-F Substitutions in Residues 1250 and 1251 of the COOH-terminal Domain. We have shown previously that various IGF-IR-regulated cell functions that can impact metastasis have distinct patterns of dependency on tyrosines located in different regions of the IGF-IR β subunit. Here, we used M-27 cells expressing IGF-IRs in which tyrosines in the kinase domain or COOH-terminal domain were substituted with phenylalanine to assess

![Fig. 1. Regulation of VEGF-C synthesis by IGF-I. Serum-starved M-27IGF-IR cells were stimulated with increasing concentrations of IGF-I (A and C) and subsequently stimulated with 10 ng/ml IGF-I for the duration indicated (B). Total RNA was extracted and analyzed by RT-PCR (A and B), or tumor cell lysates (top) and tumor cell-conditioned media (bottom) were analyzed by Western blotting (C) using 100 μg protein per lane of a 10% polyacrylamide gel. Probing was performed with a goat anti-VEGF-C antiserum (C-20) and a HRP-conjugated goat IgG antibody, and band visualization was performed using the ECL system. Shown in A and B are the results of a representative experiment of three RT-PCR analyses performed. Results in the bar graph are expressed as the ratios of VEGF-C/glyceraldehyde-3-phosphate dehydrogenase mRNA relative to the control (nonstimulated) cells, which were assigned a value of 1.

![A](image1.png) ![B](image2.png) ![C](image3.png)

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their role in VEGF-C regulation. RT-PCR analysis showed that Y1131F/Y1135F/Y1136F but not Y1250F/Y1251F substitutions inhibited VEGF-C induction in response to IGF-I (Fig. 2A).

Western blot analysis confirmed these results. After treatment with 10 ng/ml IGF-I for 48 h, the M2, 58,000 band corresponding to the VEGF-C propeptide and the M1, 29,000 processed form were detected in cell lysates and conditioned media, respectively, of M-27 cells expressing the WT IGF-IR and those expressing IGF-IR with Y1250F/Y1251F substitutions, but no protein was detectable in cell lysates or conditioned media of nontransfected or IGF-IR Y1131F/Y1135F/Y1136F-transfected M-27 cells (Fig. 2B). In all of these analyses, the highly metastatic, IGF-I-responsive H-59 carcinoma cells (14, 15) were used as a control, and they showed the pattern of expression seen with M-27 IGF-IR cells.

**IGF-I-induced VEGF-C Expression is PI3K Dependent.** The PI3K/Akt and the Ras/Raf/MAPK pathways have been implicated in IGF-IR signaling (22). Their involvement in transcriptional control appears to be cell context and transcript dependent (23). In M-27 IGF-IR cells, both pathways are activated in response to IGF-I, as evidenced by increases of 13.8 ± 4.8-fold (n = 3; P = 0.044) and 3.86 ± 2.2-fold (n = 3; P = 0.046) in the levels of phospho-Akt and phospho-ERK, respectively, as detected by Western blotting (Fig. 3, A and B).

To identify the signaling pathway involved in IGF-IR-mediated induction of VEGF-C mRNA synthesis, in this model, we used kinase-specific inhibitors. We found that cells pretreated with LY294002 under conditions that inhibited IGF-I-induced Akt phosphorylation (Fig. 3A) blocked VEGF-C induction (84.6 ± 3.7% reduction; n = 3; P = 0.006; see Fig. 3C). Pretreatment with PD98059, under conditions that blocked MAPK activation (Fig. 3B), caused only a partial inhibition of VEGF-C induction, reducing it by 38.2 ± 3.8% (n = 3; P = 0.003) relative to untreated controls, whereas a combination of the two inhibitors completely abolished VEGF-C mRNA synthesis (Fig. 3C). Interestingly, we found that although IGF-I-induced Akt phosphorylation was abolished in cells expressing the Y1131F/Y1135F/Y1136F receptor mutant, it was not reduced in cells expressing the Y1250F/Y1251F mutant (Fig. 3A). Constitutive and IGF-I-induced MAPK phosphorylation were also abolished in cells expressing IGF-IR Y1131F/Y1135F/Y1136F but not in those expressing IGF-IR Y1250F/Y1251F.

**Tumor Cells Expressing IGF-IR Acquire a Lymph Node Metastasizing Potential.** To assess whether the up-regulated expression of VEGF-C affected the ability of the tumor cells to metastasize to regional draining nodes, the tumor cells were injected s.c., and the lymph nodes (n = 10) were removed and measured 23 days later. Results shown in Fig. 4A confirm the expression of VEGF-C in vivo in a s.c. M-27 IGF-IR tumor, but not in a M-27 tumor. As noted previously (24), there was no significant difference in the growth rates of these tumors in vivo, and the mean tumor volumes/group as measured on the day the animals were sacrificed were 3700 ± 750 and 3900 ± 500 mm³ in animals bearing M-27 and M-27 IGF-IR tumors, respectively. Axillary nodes derived from all mice bearing M-27 IGF-IR tumors were significantly enlarged (mean volume, 25.4 mm³) due the presence of metastases, and this was also confirmed by histology. Consistent with our previous reports on this tumor line (18, 19), lymph nodes derived from mice bearing M-27 tumors were not enlarged (mean size, 5.1 mm³) and showed no evidence of metastases when analyzed by histology (Fig. 4, B and C).

**Discussion**

Our results identify the IGF-IR as a positive regulator of VEGF-C synthesis and thereby implicated the IGF-IR/IGF-I axis in the control of lymphangiogenesis and lymphatic metastasis. The results further suggest that both PI3K and ERK signaling play a role in this regulation, with the former having the predominant role. Other reports identified epidermal growth factor and platelet-derived growth factor, two factors that activate PI3K signaling, as positive regulators of VEGF-C in lung fibroblasts and fibrosarcoma cells. Interestingly, in these reports, overexpression of Ras was shown to have no effect on VEGF-C production (25).

The VEGF-C promoter has putative bindings sites for the transcription factor AP-2, multiple binding sites for Sp1, proximal to the AP-2 consensus site, and no TATA box (26). AP-2 and Sp1, or closely related sequences, have also been identified in the promoter regions of other IGF-IR-regulated proteins such as VEGF (27) and MMP-2 (14, 28), identifying them as common regulatory elements downstream of IGF-I signaling. Moreover, Sp1 was identified as a possible mediator of IGF-IR/estrogen receptor cross-talk in breast cancer (29), suggesting that VEGF-C induction may be linked to malignant progression in this disease.

PI3K has been identified as a major transducer of the IGF-IR signal in various cellular systems. Among others, its activity was shown to be critical for cell survival, a function mediated through Akt and Bax activation, and it was implicated in mitogenesis, protein synthesis, and differentiation (reviewed in Refs. 13, 22, and 30). The MAPK pathway was found to be equally important for some of these functions (31) or to play a more minor role for others (32). The degree to which different cells use common pathways to convey the IGF-IR signals may be cell context dependent and may be determined by the level of expression of downstream substrates such as IRS or Shc (22, 23, 30). Recent studies suggest that cellular epidermal growth factor receptor levels may be one of the factors determining which of the signaling pathway predominates (33).

We reported previously that M-27 cells expressing an IGF-IR in which tyrosines 1250/1251 in the COOH-terminal domain have been substituted with phenylalanines cannot be induced by IGF-I to produce the MMP MMP-2 and have a reduced ability to spread and invade through Matrigel-coated filters (15). Here, IGF-I-mediated VEGF-C induction was not inhibited in the same cells. This suggests that despite similarities in the promoter regions of these proteins and their common dependency on the PI3K pathway, their transcriptional activation by IGF-I also involves distinct mechanisms. It appears that for MMP-2 induction, spreading, migration, and invasion, a second,
COOH-terminal domain-dependent signal, possibly involving integrin signaling and the cytoskeleton (34), may also be required. RACK1, a molecule recently identified as an IGF-IR-binding protein and a positive regulator of cell spreading and focal adhesion kinase phosphorylation, has been shown to require an intact COOH terminus domain for binding (35, 36) and may be one protein involved in transmitting this "second signal."

M-27 cells that overexpress IGF-IR acquired the ability to metastasize to the regional nodes from s.c. sites. This was also observed with breast carcinoma MCF-7 cells that were transfected with VEGF-C (9). Interestingly, IGF-IR was identified as a marker of progression in breast carcinoma, and high serum IGF-I levels were found to be associated with increased risk for this disease (13). Moreover, IGF-I was identified as a major growth-promoting factor produced by activated stromal cells in primary breast carcinomas (37), and we have recently shown that lymph node-derived stromal cells can promote breast carcinoma cells through the elaboration of IGF-1 (38). In our model, IGF-IR expression was previously shown to regulate several cellular functions that impact metastasis, including survival, MMP-2 synthesis, and invasion.

Fig. 3. IGF-I-induced VEGF-C expression is PI3K and MAPK signaling dependent. Tumor cells were serum-starved overnight, treated or not treated with 20 μM LY294002 (A) or PD98059 (B) for 5 h and then stimulated with 10 ng/ml IGF-I for 15 min, and lysed, and the lysate proteins were analyzed by Western blotting. Membranes were probed first with an antibody to Akt (A) or ERK (B) and then stripped and reprobed with an antibody to phospho-Akt (A) or phospho-ERK (B), respectively. Bands were subjected to densitometry, and the results are expressed as the ratio of phosphorylated:total protein. To analyze the effect of the inhibitors on VEGF-C induction (C), the cells were preincubated with the inhibitor for 5 h and stimulated with 10 ng/ml IGF-I for 6 h, and total RNA was extracted and analyzed by RT-PCR. A and C show the results of a representative experiment of three experiments performed, and B shows the results of two separate experiments (I and II), each performed twice. Results of densitometry are depicted in the bar graphs.
The involvement of IGF-IR in the regulation of invasion and metastasis was also demonstrated recently in the RIP1-Tag2 mouse model of pancreatic islet tumorigenesis (39). Taken together, the present results suggest that in addition to its involvement in invasion, IGF-IR expression can contribute to the acquisition of an aggressive phenotype by inducing the expression of the lymphangiogenic factor VEGF-C, thereby facilitating lymphatic metastasis.

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**References**

REGULATION OF VEGF-C BY THE IGF-IR


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