Up-regulation of Vascular Endothelial Growth Factor C in Breast Cancer Cells by Heregulin-β1

A CRITICAL ROLE OF p38/NF-kB SIGNALING PATHWAY*

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Vascular endothelial growth factor C (VEGF-C) is a critical activator of tumor lymphangiogenesis that recently has been strongly implicated in the tumor metastasis process. In this study, we identified that HRG-β1 stimulated up-regulation of VEGF-C mRNA and protein of human breast cancer cells in a dosage- and time-dependent manner and that this up-regulation was de novo RNA synthesis-dependent. The HRG-β1-induced increase in VEGF-C expression was effectively reduced by treatment with Herceptin, an antibody specifically against HER2. Also, when HER2 was overexpressed in MCF-7 cells that resulted in an evident increase in the VEGF-C level, suggesting an essential role of HER2 in mediating VEGF-C up-regulation by HRG-β1. NF-κB has been shown to be probably involved in interleukin-1β- or tumor necrosis factor-α-induced VEGF-C mRNA expression in human fibroblasts. Here we found that HRG-β1 could stimulate NF-κB nuclear translocation and DNA-binding activity via the IkBα phosphorylation-degradation mechanism. Blockage of the NF-κB activation cascade caused a complete inhibition of the HRG-β1-induced elevation of VEGF-C. In promoter-reporter assay, the luciferase activities of the reporter constructs, including the putative NF-κB site deleted and mutated form were significantly reduced after HRG-β1 treatment as compared with the 1.5-kb VEGF-C promoter. Although investigating the upstream kinase pathways involved in HRG-β1-elicited NF-κB activation and VEGF-C up-regulation, we found that HRG-β1 could activate extracellular signal-regulated protein kinase 1/2, phosphatidylinositol 3-kinase, and p38 mitogen-activated protein kinase (MAPK) in MCF-7. However, only SB203580 (a specific inhibitor of p38 MAPK), not PD98059 nor LY294002, blocked the up-regulation of VEGF-C by HRG-β1. A similar inhibition in VEGF-C expression was obtained by cell transfection with dominant-negative p38 (p38AF). Interestingly, the HRG-β1-induced NF-κB activation cascade was also effectively blocked by SB203580 treatment or p38AF transfection. Our data thus suggests that HRG-β1 stimulated a NF-κB-dependent up-regulation of VEGF-C through the p38 MAPK signaling pathway in human breast cancer cells.

Tumor progression and metastasis rely mainly on both vascular and lymphatic systems by which cancer cells can spread widely into regional or distant tissues. The high tumor metastasis is strongly associated with short disease-free survival periods and poor prognosis in cancer patients. In the past decades, many subsets of molecules have been reported to be critically involved in regulating the blood microvesSEL formation in tumor development (1–3). Little is known about how cancer cells can migrate to regional lymph nodes or promote the proliferation of lymphatic vessel. Recent evidence showed that VEGF-C and VEGF-D, two members of the VEGF family, are the ligands for VEGF receptor(3)3, which can stimulate the lymphatic vessel growth (lymphangiogenesis) and also enhance lymphatic metastasis in animal model (4–8). VEGF-C displays a high degree of similarity to VEGF-A, including conservation of the eight cysteine residues involved in intra- and intermolecular disulfide binding. The cysteine-rich COOH-terminal region increases the half-life of the VEGF-C protein relative to the other members of the family (9, 10). VEGF-C mRNA is first translated into a 58-kDa precursor from which the 29/31-kDa mature ligand is formed by a general proteolytic process independent of the cell type (11, 12). The secreted 29/31-kDa VEGF-C polypeptide specifically binds to the VEGF receptor(3)3, which is predominantly expressed on lymphatic endothelium in adults, and triggers survival and proliferative activity in the lymphatic endothelium via p42/44 MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways (13–16). Supportively, VEGF-C expression has been detected in most of the important human cancers analyzed (17), and its expression level correlated with lymph node metastases in especially thyroid, gastric, colorectal, lung, prostatic, and breast cancers (18–23). Clinical and experimental findings strongly suggest a potential role for VEGF-C-mediated lymphangiogenesis in human cancer metastasis. However, little is known about the regulation of VEGF-C in tumor cells to date.

HRG-β1, a member of the epidermal growth factor-like growth factor family, is secreted from mesenchymal cells and acts as a combinatorial ligand for the HER3 and HER4 receptors in breast cancer cells (24–26). The binding of HRG-β1 to its

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‡ The abbreviations used are: VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; HRG-β1, heregulin-β1; HER, human epidermal growth factor receptor; NF-κB, nuclear factor-κB; IkB, inhibitor of κB; ERK, extracellular signal-regulated protein kinase; PI3K, phosphatidylinositol 3-kinase; DN, dominant-negative; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; IKK, IκB kinase; NIK, NF-κB-inducing kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; EMSA, electrophoretic mobility shift assay.
receptors activates a diversified signaling pathway by which many biological functions, including proliferation (27, 28), differentiation (27, 29), apoptosis (30, 31), and migration (32, 33), occur. These biological alterations induced by HRG-β1 result in the invasive and metastasis-related properties in human breast cancer (25, 34, 35). HRG-β1 Up-regulates VEGF-C in Breast Cancer Cells

This study was undertaken to investigate whether HRG-β1 would regulate the expression of VEGF-C in human breast cancer cells. Here we demonstrate that HRG-β1, but not other growth factors, is a potent agent to induce VEGF-C up-regulation. Using NF-κB decoy or transfection with dominant-negative (DN)-IκBα to specifically abrogate the HRG-β1-mediated NF-κB activation including nuclear translocation and DNA-binding activity that resulted in an inhibition of VEGF-C up-regulation. Searching the upstream kinase regulator of NF-κB signaling, we found that SB203580, a p38 MAPK inhibitor, but not PD98059 or LY294002, strongly abolished HRG-β1-stimulated NF-κB activation as well as the subsequent VEGF-C up-regulation. A similar result was obtained using transfection with DN-p38 (p38AP). Our current data demonstrate for the first time that HRG-β1 potently up-regulates the VEGF-C gene through the activation of the p38 MAPK/NF-κB signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Cultures—Human breast cancer cell lines (MCF-7, SKBr3, and T47D), MCF-7 HER2/neu stable cell line expressing Her2/neu (a kind gift from Dr. Ruey-Long Hung, Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan), and HBL-100 immortalized human breast cell line were maintained in Dulbecco’s modified Eagle’s medium supplemented 10% fetal bovine serum with 2 mM l-glutamine (Invitrogen), 100 μg/ml streptomycin, and 100 units/ml penicillin in a humidified 5% CO2 atmosphere.

Transfections—p38AP dominant-negative mutant (generously provided by Dr. Ching-Chow Chen, Department of Pharmacology, College of Medicine, National Taiwan University Hospital, Taiwan), dominant-negative 3236A mutated form of IκBα (kindly provided by Dr. Shuang-En Chuang), and vector were transfected into MCF-7 cells using transfection reagent FuGENE-6 (from Roche Molecular Biochemicals) per manufacturer’s protocol. Twenty-four hours after transfection, cells were serum-starved for a further 24 h and stimulated with HRG-β1, and then cells were lysed for analysis. Each experiment was repeated with three independent transfections, and transfection efficiency varied between 20 and 30%.

Reagents—Recombinant human heregulin-β1, human interleukin-1β, human interleukin-8, human tumor necrosis factor-α, and human polyclonal VEGF-C antibody were purchased from R&D Systems (Minneapolis, MN). The kinase inhibitors LY294002, PD98059, and SB203580 were obtained from Sigma. The epidermal growth factor receptor inhibitor PD153035 and PMA (phorbol-12-myristate-13-acetate) were purchased from Calbiochem. Antibodies to human Her2/neu, phospho-p38, p38, phospho-ERK1/2, ERK1/2, NF-κB (p65), NF-κB (p50), proliferating cell nuclear antigen, &-tubulin, and secondary horseradish peroxidase-conjugated antibodies were obtained from Santa Cruz Biotechnology. Antibodies to phospho-AKT and AKT were purchased from the Upstate Biotechnology. [α-32P]dCTP was obtained from Amersham Biosciences. Anti-Her-2 monoclonal antibody, Herceptin (Trastuzumab), was provided by Dr. Chih-Hsin Yang (Department of Oncology, National Taiwan University Hospital, Taipei, Taiwan).

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction—A total of 8 breast carcinoma patients’ tissue RNA were obtained from the Department of Pathology, National Taiwan University Hospital, Taipei, Taiwan. Total RNA was isolated using RNA-Beek™ reagent (Tel-Test, Inc.) as recommended by the manufacturer’s instructions. Total RNA (1 μg) was reverse-transcribed into single-stranded cDNA with Moloney murine leukemia virus Reverse Transcriptase and random hexamers (Promega, Madison, WI). Amplification of growth factor cDNAs and β-actin cDNA as an internal control in each reaction was carried out by polymerase chain reaction with the primers as described below. VEGF-C cDNA PCR were 5′-CAGTATGCGCTCCGTCTGAC-3′ (forward) and 5′-GGACACATGGAGGTATAAGAAG-3′ (reverse). The primer sequences for VEGF-D cDNA PCR are 5′-TGCGTACCTCTGGCGGT-3′ (forward) and 5′-GATGATGATCGCCGGAAC-3′ (reverse). The primer sequences were as follows: VEGF-A cDNA PCR are 5′-AGACTCTCCTGATCCAACTGCC-3′ (forward) and 5′-TGGATCTAAGACCCCCCCTCC-3′ (reverse). PCRs were carried out using specific primers for β-actin and GAPDH (β-actin: forward, 5′-GATGATGCATGCCCCGCCT-3′, reverse, 5′-TGCGTACCTCTGGCGGT-3′; GAPDH: forward, 5′-CCACCATGGCCAACTACTGAGGA-3′, reverse, 5′-TCTAGACCGCACTGGCTACCC-3′). Primers were used at a final concentration of 0.5 μM. Reaction mixture was first denatured at 95 °C for 10 min. The PCR condition was as follows: 95 °C for 1 min, and 72 °C for 1 min, for 30 cycles, followed by 72 °C for 10 min. Polymerase chain reaction products were visualized by ethidium bromide staining after agarose gel electrophoresis. Mammary tumors from HER2/neu transgenic mice were generously provided by Dr. Ricci SM (Ovarian and Breast Cancer, The University of Texas M.D. Anderson Cancer Center, Houston, TX). For the quantitative real-time PCR analysis of human VEGF-C and GAPDH mRNA levels, a Light Cycler system and reagents (Roche Molecular Diagnostics) were used with a double-stranded DNA binding dye, SYBR Green 1, according to the procedure provided by the manufacturer. The real-time PCR program for VEGF-C consisted of 30 cycles with denaturation at 95 °C for 15 s, annealing at 66 °C for 5 s, and extension at 72 °C for 10 s. For GAPDH, it consisted of 30 cycles with denaturation at 95 °C for 15 s, annealing at 66 °C for 5 s, and extension at 72 °C for 20 s.

Western Blot Analysis—Cells were starved in serum-free medium overnight then incubated with HRG-β1 for different times or for various concentrations. Cells were washed two times with ice cold phosphate-buffered saline and lysed in buffer (50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) for 15 min on ice. The lysates were centrifuged in an Eppendorf at 4 °C for 1 min. The equal amounts of protein from the cell lysates were resuspended in gel sample buffer, resolved by 10% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane (Millipore). Membranes were blocked for 1 h in 5% (w/v) dry milk, phosphate-buffered saline, and 0.1% Tween 20. Primary antibodies as indicated were incubated with membranes for 1 h, and the membranes were washed in phosphate-buffered saline with 0.1% Tween 20. Renaissance® (NEN™ Life Science Products) enhanced chemiluminescence reagent was used to detect membrane-bound protein by luminesceny. This light is captured on Kodak X-OMAT Blue Autoradiography film.

NF-κB/Rel-specific Decoy Oligodeoxynucleotides—We used a phosphorothioate double-stranded oligodeoxynucleotide carrying the NF-κB/Rel-consensus sequence 5’-CTTGAAGGGATTTCCCTCC-3’. The mutated (scrambled) form 5’-CCTGTACATGGTGTACCC-3’ was used as a control (41). The 1 μM oligodeoxynucleotide was mixed with 5 μg/ml of TransFast™ (Promega) for 15 min at room temperature, then the mixture was added to MCF-7 cells in serum-free medium. After 5 h of incubation, the cells were treated with HRG-β1 for further appropriate times.

Electrophoretic Mobility Shift Assay—Confluent MCF-7 cells cultures were serum-starved overnight followed by stimulation with or without HRG-β1 (50 ng/ml) for various time points. Nuclear extracts were prepared by using a nonionic detergent method as described previously (42). In brief, nuclei were extracted from breast cancer cells in extraction buffer (420 mM KCl, 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.2 mM EDTA, and 20% glycerol) plus protease inhibitors (0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). After centrifugation at 14,000 rpm in a microcentri-
Analysis was performed as described under growth factors for various times as indicated. RT-PCR and Western blot analysis was performed as described under “Experimental Procedures.” A, HRG-β1-induced VEGF-C mRNA expression in MCF-7 cells. RT-PCR and Western blot analysis was performed as described under “Experimental Procedures.”

RESULTS

HRG-β1 Up-regulates VEGF-C in Breast Cancer Cells—Initially, we used the semi-quantitative RT-PCR technique to analyze VEGF-C and -D mRNA expression in human breast cancer cells treated with different growth factors for 6 h. B and C, HRG-β1-mediated up-regulation of VEGF-C mRNA and protein expression in MCF-7 cells is time- and concentration-dependent. MCF-7 cells were treated with 50 ng/ml HRG-β1 for 0, 3, 6, 9, 12, or 24 h or with 0, 0.1, 1, 10, 50, or 100 ng/ml HRG-β1 for 6 h (RT-PCR) and 9 h (Western blot analysis). The 58-kDa VEGF-C precursor and the 29/31-kDa mature VEGF-C protein could be increased by HRG-β1 (50 ng/ml, 9 h) treatment. D, RT-PCR and Western blot analysis of VEGF-C mRNA and protein in MCF-7 cells pretreated with either 4 μg/ml actinomycin D or 30 μg/ml cycloheximide for 1 h and then incubated with 50 ng/ml HRG-β1 for 6 or 9 h.

Fig. 1. HRG-β1 up-regulates VEGF-C expressions through transcription regulation in MCF-7 breast cancer cells. Total RNA and protein were collected from serum-starved MCF-7 breast cancer cells treated with various dosages of HRG-β1 or other cytokines and growth factors for various times as indicated. RT-PCR and Western blot analysis was performed as described under “Experimental Procedures.”
HRG-1 appears to be dosage-dependent. An initial increase of HRG-1, which the activity of the HER2 receptor is critical for HRG-1/H9252 upper panel VEGF-C mRNA (middle panel) and protein level (lower panel) was further investigated. As shown in Fig. 1B, upper panel, the VEGF-C mRNA level was elevated with a peak in the 3–9 h time period after 50 ng/ml HRG-1 treatment. Western blot analysis revealed that the 58-kDa VEGF-C precursor protein was significantly increased in the MCF-7 cells at 9 h and was sustained for up to 24 h post-HRG-1 treatment (Fig. 1B, lower panel). Fig. 1C reveals that the HRG-1-induced increase in VEGF-C mRNA (upper panel) and protein level (middle panel) appeared to be dosage-dependent. An initial increase of VEGF-C mRNA and protein was observed at a dose of 0.1 ng/ml HRG-1, and the maximal induction was found at a dose of 50 ng/ml. Fig. 1C, lower panel, shows that not only the 58-kDa VEGF-C precursor but also the 29/31-kDa mature VEGF-C protein could be increased by HRG-1. It is strongly suggested that VEGF-C protein induction by HRG-1 is functionally active in breast cancer cells. We next examined whether VEGF-C mRNA up-regulation by HRG-1 is due to increased transcription or increased RNA stability. To this end, we pretreated MCF-7 cells with 4 μg/ml actinomycin D, a RNA synthesis inhibitor, or 30 μg/ml cycloheximide, a protein synthesis inhibitor, for 1 h before HRG-1 was added to the culture medium. Fig. 1D shows that actinomycin D completely abolished HRG-1-induced VEGF-C up-regulation, whereas cycloheximide had no effect on VEGF-C induction by HRG-1. This finding suggests that HRG-1-induced VEGF-C up-regulation requires de novo RNA synthesis but not new protein synthesis. The effect of HRG-1 on VEGF-C mRNA expression in different breast cancer cell lines was further investigated. As shown in Fig. 2A, HRG-1 had a broad capacity to induce a VEGF-C mRNA increase (around 3–4-fold) in various human breast cancer cell lines, including SKBr3, T47D, and MCF-7. In support of a previous study (33), we found that VEGF-A mRNA was also markedly elevated in breast cancer cell lines treated with HRG-1. Interestingly, among these cell lines, HBL-100 cells had a more abundant HRG-1 level and displayed higher levels of VEGF-C and -A mRNA, suggesting that endogenous HRG-1 is capable of inducing the VEGF-C mRNA expression. As previously described (26), HRG-1 preferentially binds to HER3 and HER4 receptors and then transactivates HER2 by forming a HER3/HER2 or HER4/HER2 heterodimer, which in turn elicits the signaling pathways for certain malignant properties. Here we asked whether the HER2 receptor is required for HRG-1-induced VEGF-C expression. Fig. 2B, left panel, shows that HER2-overexpressed MCF-7 cells displayed much more abundant levels of VEGF-C mRNA and protein as compared with the vector control cells. In addition, HRG-1-induced up-regulation of the VEGF-C mRNA or protein was completely blocked by treatment with Herceptin (an antibody against HER2 function) or PD153035 (an inhibitor of HER2 kinase) (Fig. 2B, right panel). This evidence strongly suggests that the activity of the HER2 receptor is critical for HRG-1-mediated VEGF-C gene expression. To demonstrate the relationship between HER2 and VEGF-C in breast carcinoma, eight breast tumor RNA samples were detected with HER2 and VEGF-C mRNAs by using RT-PCR. Fig. 2C, upper panel, reveals that in HER2/neu overexpressing tumors, the VEGF-C is also overexpressed. Because human tumors are quite heterogeneous, we obtained mammary tumors from HER2/neu transgenic mice and isolated RNA for examining VEGF-C mRNA by using real-time RT-PCR. As expected, we found that the level of VEGF-C mRNA is significantly elevated in HER2-positive tumors compared with normal tissues (Fig. 2C, lower panel). This result suggests that HER2/neu overexpression correlates with increased expression of VEGF-C in human breast cancers. Activation of NF-κB Is Required for VEGF-C Expression—Transcription factor NF-κB is one of the important factors activated by HRG-1 in human breast cancer cells (43, 44). A putative NF-κB-binding site was found to be located within the VEGF-C gene promoter region. This site may be implicated in the induction of VEGF-C mRNA by interleukin-1β and tumor
necrosis factor-α (45, 46). We thus tested if NF-κB activation would be involved in HRG-β1-induced VEGF-C mRNA expression. To this end, MCF-7 cells were exposed to 50 ng/ml HRG-β1, nuclear extracts were prepared, and NF-κB protein translocation as well as NF-κB-binding activities were assessed respectively, by Western blotting and EMSA using an oligonucleotide corresponding to the putative NF-κB-binding site within the VEGF-C promoter. As shown in Fig. 3A, the p65 and p50 NF-κB subunits appeared in nuclear fractions at 30–120 min after HRG-β1 treatment. The nuclear proliferating cell nuclear antigen protein level is an internal control to ensure equal amounts of total nuclear protein. Consistently, the DNA-binding activity of NF-κB increased significantly at 30–120 min (Fig. 3B, upper panel, lanes 2–4) after HRG-β1 treatment, and the induction of DNA-binding activity was completely attenuated by specifically competing with the non-radiolabeled probe (Fig. 3B, upper panel, lanes 5 and 6). To quantify the NF-κB activity, we transiently transfected a NF-κB luciferase reporter into MCF-7 cells. The data of luciferase activity (Fig. 3B, lower panel) was correlated with the DNA-binding activity of NF-κB in gel shift assay. Super-shift assays were done to confirm the presence of p65 and p65 binding to the NF-κB-binding site, showing that the specific protein-DNA-binding activity was super-shifted by the addition of anti-p65 or anti-p50 antibodies (data not shown). We next employed a NF-κB decoy oligonucleotide, which sequenced as transcription factor decoys to inhibit NF-κB binding to the native DNA sites, to specifically block HRG-β1-induced activation of the NF-κB pathway. Fig. 3C reveals that the NF-κB decoy effectively blocked p65 subunit nuclear translocation (upper panel) as well as abolished the DNA-binding activity of NF-κB (lower panel) induced by HRG-β1. In contrast, the scrambled NF-κB oligonucleotide had no effect on the nuclear translocation and DNA-binding activity of NF-κB by HRG-β1 treatment (Fig. 3C). As shown in Fig. 3D, treatment with decoy NF-κB completely inhibited HRG-β1-mediated VEGF-C mRNA up-regulation, suggesting that NF-κB activation is important for VEGF-C gene up-regulation by HRG-β1. To identify whether the NF-κB site is actually involved in transcriptional regulation of VEGF-C by HRG-β1, we transiently transfected the 1.5-kb VEGF-C promoter-reporter containing NF-κB site, a NF-κB-deleted promoter plasmid, 1.4-kbΔNF-κB VEGF-C, and a NF-κB site mutated promoter plasmid, 1.5-kbmutNF-κB VEGF-C (see “Experimental Procedures”) into MCF-7 cells and then examined their luciferase activities after HRG-β1 treatment. Fig. 3E shows a 4.2-fold induction of a 1.5-kb VEGF-C promoter activity by treatment with 50 ng/ml HRG-β1. However, HRG-β1 had no effect on the luciferase activity of control pGL3-basic vector. When the NF-κB-deleted and -mutated promoter-reporter, pGL3–1.4-kbΔNF-κB VEGF-C and pGL3–1.5-kbmutNF-κB VEGF-C, were transfected into MCF-7 cells, their luciferase activities were decreased around 60% in response to HRG-β1 when compared with pGL3–1.5-kb VEGF-C. These experiments demonstrate that the NF-κB site is indeed important for HRG-β1-mediated VEGF-C gene up-regulation.

The NF-κB activation is intimately associated with the IκBα phosphorylation and degradation. We thus tested whether HRG-β1 could induce endogenous IκBα phosphorylation and degradation. Western blot analysis shows that an evident IκBα protein phosphorylation and a subsequent degradation occurred in the MCF-7 cells after HRG-β1 treatment (Fig. 4A). A DN-IκBα vector, which is resistant to phosphorylation and degradation, was transfected and overexpressed in the MCF-7 cells. Overexpression of DN-IκBα significantly repressed the NF-κB-DNA-binding activity (Fig. 4B) and NF-κB nuclear translocation in MCF-7 cells after exposure to HRG-β1 (data not shown). Under the same experimental conditions, we further examined whether DN-IκBα expression would modulate the HRG-β1-induced VEGF-C gene expression. The data shown in Fig. 4C reveals that the HRG-β1-mediated increase in VEGF-C mRNA (upper panel) and protein (lower panel) was strongly abolished in DN-IκBα overexpressed cells but not in the control vector-transfected cells. These results suggest that IκBα degradation and subsequent NF-κB activation were required for VEGF-C gene expression induced by HRG-β1.

p38 MAPK Is Involved in HRG-β1-induced VEGF-C Gene Up-regulation—Many of the kinase signaling pathways have been shown to be activated and involved in HRG-β1-induced diversified cellular functions (44, 47). Therefore, we examined which of the kinase signaling pathways is/are required for HRG-β1-induced up-regulation of the VEGF-C gene. To address this issue, MCF-7 cells were pre-treated with SB203580 (p38 MAPK inhibitor), PD98059 (MEK inhibitor), or LY294002 (PI3K inhibitor) for 1 h and followed by treatment with HRG-β1 for 6 or 9 h, and total RNA and protein were used to determine VEGF-C expression. Fig. 5A reveals that 20 μM of SB203580 completely reduced the increased level of VEGF-C mRNA (upper panel) and protein (lower panel) induced by HRG-β1, whereas two other kinase inhibitors, PD98059 and LY294002, had no effect on the up-regulation of VEGF-C. As shown in Fig. 5B, HRG-β1 indeed activated these three kinase pathways, p38 MAPK, ERK1/2, and PI3K, in MCF-7 cells as evidenced by the elevation of their phosphorylated form using their specific antibodies. Our data also demonstrated that these three signaling pathways were effectively blocked in MCF-7 cells when treatment with pharmacological inhibitors at the dose indicated (Fig. 5B). It is suggested that those pharmacological inhibitors were functionally active in inhibiting the specific signaling pathways. Because SB203580 was demonstrated to significantly abolish VEGF-C gene up-regulation by HRG-β1, we thus used another approach to test the importance of p38 MAPK by establishing a MCF-7 cell line expressing dominant-negative p38 (p38AF) and examined the expression level of VEGF-C mRNA and protein. Fig. 5C, upper panel, we demonstrated that HRG-β1-stimulation of the phosphorylation of p38 MAPK was totally reduced in p38AF expressed cells but not in the control vector expressed cells. Blocking the p38 MAPK pathway by p38AF also significantly diminished the HRG-β1-induced up-regulation of VEGF-C mRNA (Fig. 5C, middle panel) and protein (Fig. 5C, lower panel). In brief, these results suggest the possible involvement of p38 MAPK, but not ERK1/2 or PI3K, in the regulation of VEGF-C gene expression in breast cancer cells induced by HRG-β1.

p38 MAPK has recently been shown to regulate NF-κB activity in certain cell systems (48, 49), and we therefore explored the possible link between p38 MAPK and NF-κB in HRG-β1-mediated VEGF-C gene expression. To this end, we determined the NF-κB DNA-binding activity and NF-κB transcriptional activity in cells treated with different pharmacological inhibitors. Fig. 6A shows that the HRG-β1-stimulated increase in NF-κB DNA-binding activity and NF-κB reporter activity were nearly abolished by SB203580 but not affected by PD98059 and LY294002. Consistently, HRG-β1 treatment neither induced the nuclear translocation of NF-κB p65 (Fig. 6B, upper panel) nor elevated the NF-κB DNA-binding activity (Fig. 6B, lower panel) in p38AF-expressed cells as compared with control vector cells.

Our results suggest that blockage of p38 MAPK could prevent NF-κB nuclear translocation, NF-κB DNA-binding activity, and NF-κB reporter activity by HRG-β1. We further examined whether the level of phosphorylated IκBα in cells.
expressing p38AF or treated with SB203580 would be changed. As expected, Western blot analysis showed that HRG-β1 induced the elevation of phosphorylated IκB and its degradation was nearly completely abolished in cells treated with SB203580 (Fig. 6C, upper panel) or in cells expressing p38AF (Fig. 6C, lower panel). Together, the data obtained here strongly suggests that p38 MAPK may act as an up-stream kinase to activate the NF-κB-dependent up-regulation of VEGF-C in response to HRG-β1.

### DISCUSSION

In this study we demonstrated that activation of a novel signaling pathway from p38 MAPK to subsequent NF-κB is a potential requirement for HRG-β1-mediated up-regulation of the lymphangiogenic factor VEGF-C in human breast cancer cells. Although VEGF-C has been shown to be highly expressed in a vast array of malignant tissues, its up-regulation in cancer cells by which factor(s) is completely unknown. Few, if any, investigations have found that pro-inflammatory cytokines
such as interleukin-1β and tumor necrosis factor-α could strongly induce VEGF-C gene expression in human fibroblasts (46). Here we show that neither IL-1β nor TNF-α were capable of up-regulating VEGF-C in human breast cancer cells (as can be seen in Fig. 1A). Importantly, this is the first time it has been demonstrated that HRG-β1 is a strong up-regulator of the VEGF-C gene in human breast cancer cells. In addition, our current data and that from others (15) suggest that the regulatory mechanism for the VEGF-C gene varies in different cellular contexts or in different pathological processes.

Accumulating evidence showed that overexpression of HRG-β1 in human breast cancer cells resulted in the more aggressive phenotypes, i.e., enhancement in cell migration and invasion abilities (32, 50). Many of the downstream effector genes, such as urokinase plasminogen activator (37, 51), metalloproteinases (36), paxillin (38), and autocrine motility factor (52), have been identified and may partially account for the role of HRG-β1 in the progression of human breast cancer.

**Fig. 4.** Effect of HRG-β1 on IκBα phosphorylation and degradation. A, cytosol extracts were prepared from serum-starved MCF-7 cells treated with 50 ng/ml HRG-β1 for 0, 30, 60, or 120 min then subjected to Western blotting with indicated antibodies. Phosphospecific antibody recognizes IκBα phosphorylated at serine 32. B, MCF-7 cells were transiently transfected with dominant-negative IκBα. The transfected were treated with 50 ng/ml HRG-β1 for 60 min. Active NF-κB was determined by its [α-32P]DNA-binding activity by EMSA analysis. C, the level of VEGF-C mRNA and protein in DN-IκBα-expressing MCF-7 cells and vector control transfected cells after treatment with 50 ng/ml HRG-β1 for 6 or 9 h, as measured by RT-PCR and Western blot analysis.

**Fig. 5.** The p38 MAPK kinase pathway is required for the induction of VEGF-C by HRG-β1. A, MCF-7 cells were incubated with or without 75 μM PD98059, 37.5 μM LY294002, or 20 μM SB203580. HRG-β1 (50 ng/ml) was added to the media, cell lysates were collected after 9 h for Western blot analysis of VEGF-C expression, and total RNA was prepared from cells for RT-PCR analysis of VEGF-C mRNA after 6 h of HRG-β1 treatment. B, to test the efficacy of inhibitors, MCF-7 cells were starved overnight, pretreated with the inhibitors at the concentrations as in Fig. 5A for 1 h, and stimulated by 50 ng/ml HRG-β1 for 5 min. Cell lysates were prepared and subjected to Western blot analysis using antibodies against p-p38, p-ERK1/2, and p-AKT. C, MCF-7 cells were transfected with vector or p38AF and serum-starved for 24 h and treated with HRG-β1 (50 ng/ml) for 6 or 9 h, analyzed on Western blotting and RT-PCR.
HRG-β1 Up-regulates VEGF-C in Breast Cancer Cells

Figure 6. p38 MAPK inhibitor and dominant-negative p38 reduced HRG-β1-mediated NF-κB activation. A, upper panel, the effect of inhibitors on HRG-β1-induced NF-κB activation in MCF-7 cells. MCF-7 cells were pretreated with the p38 MAPK inhibitor SB203580 (20 μM), ERK/MAPK inhibitor PD98059 (75 μM), or PI3K inhibitor LY294002 (37.5 μM) for 1 h. Subsequently, cells were incubated with HRG-β1 (50 ng/ml) for 60 min, and nuclear extracts were subjected to EMSA with NF-κB probe. A, lower panel, after transfected with p-NF-κB-Luc, MCF-7 cells were treated with the same experimental conditions as mentioned in the upper panel, then the luciferase activity was measured. The data were the representative of three independent experiments. B, MCF-7 cells were transiently transfected with p38AF then treated with 50 ng/ml HRG-β1 for 60 min. Active NF-κB was determined by Western blotting. C, NF-κB phosphorylation-degradation level in these cytosol extracts was measured by Western blotting.

However, the detailed mechanism underlying how HRG-β1 induces these downstream effector genes to coordinate with one another and arrange the aggressive phenotype is largely unknown. Here we provide evidence that shows the lymphangiogenic factor, VEGF-C, is induced in human breast cancer cells by HRG-β1. The induction of VEGF-C does not require protein synthesis, suggesting that it may be mediated by activation of certain existing transcription factor(s) by post-translational modification or relocation. Consistent with this notion, we detected that the transcription factor NF-κB is activated and critically required for HRG-β1-induced VEGF-C up-regulation as evidenced by NF-κB decoy treatment (Fig. 3C) and DN-IκBα transfection (Fig. 4C). Our data further points out that HRG-β1-stimulation of NF-κB DNA-binding activity is mainly through a mechanism involving phosphorylation-degradation of IκBα and the subsequent translocation of NF-κB into the nucleus. Supportively, the VEGF-C promoter-reporter assay clearly demonstrated that the putative NF-κB site in the VEGF-C promoter region is critical for HRG-β1-induced VEGF-C up-regulation (Fig. 3E). Although the sequential activation of NF-κB is commonly observed, this is the first time it has been shown that HRG-β1 is capable of activating this mechanism in human breast cancer cells. NF-κB activation plays a key role in drug resistance and metastasis in human breast cancer (53–55). Here we report that the lymphangiogenic factor, VEGF-C, acts as one of the downstream effector genes of NF-κB signaling. This provides a new aspect of NF-κB in the pathogenesis of human breast cancer.

Many protein kinases including PI3K/Akt, ERK1/2, and p38 MAPK have been found to integrate into the activation process of NF-κB in different cell systems in a distinct way (56–58). Our data demonstrates that in MCF-7 cells these three signaling pathways are all activated by HRG-β1 (Fig. 5B). Using a pharmacological and genetic inhibition approach, we found that only p38 MAPK signaling is specifically and dominantly involved in HRG-β1-stimulated NF-κB-dependent VEGF-C up-regulation, although a possible cross-talk may occur between the p38 signaling and NF-κB activation process. Currently, the mechanism for the interplay between these two pathways is not yet understood and may vary in different cell models. A recent study (57) revealed that the p38 pathway did not affect NF-κB recruitment into a subset of gene promoters in response to inflammatory stimuli (57). Distinct from that, in our cell model HRG-β1-stimulated p38 signaling could promote IκBα phosphorylation and degradation. Phosphorylation of IκBα is carried out by the multisubunit IκB kinase (IKK), which is in turn activated by the NF-κB-inducing kinase (NIK) or by the mitogen-activated protein kinase (MEKK1) (59, 60). How could p38 kinase signaling connect with the NF-κB activation process? One possibility could be through phosphorylation of the IKK molecule. Several reports showed that not only NIK and MEKK1 but also Akt could...
phosphorylate IKK (61, 62), suggesting that IKK could be as a key molecule to receive various kinase signaling and transmit into NF-κB activation cascade. Supporting this hypothesis, we found that p38 kinase is closely associated with IKK in response to HRG-β1 as demonstrated by co-immunoprecipitation using the anti-p38 antibody (data not shown). Alternatively, it is also possible that there may be a critically intermediate kinase connecting p38 signaling and IKK.

Ectopic overexpression of VEGF-C in different human breast cancer cells including MCF-7 and MDA-MB-435, all potently increased intratumoral lymphangiogenesis, resulting in significantly enhanced metastasis in the regional lymph nodes (5, 7, 63). The 29/31-kDa mature form of VEGF-C, which specifically activates VEGFR-3, was obviously detected in both cell lines. Using a soluble form VEGFR-3 fusion protein inhibited the tumor growth and tumor-associated lymphangiogenesis by blocking the interaction between the mature form of VEGF-C and its receptor. Under the same MCF-7 cell system, we demonstrated that HRG-β1-treated MCF-7 cells indeed produced a large amount of mature VEGF-C either in the total cell lysate (Fig. 1C) or in conditioned media (data not shown). This strongly suggests that the enhancement of lymphangiogenesis may partially account for HRG-β1-mediating a more aggressive phenotype of human breast cancers. Our current data for cell lines, clinical specimens, and transgenic mice also show that HER2 receptor overexpression caused an increased expression of VEGF-C, thus suggesting that the level of HER2 receptor is a switch for promoting lymphangiogenesis in breast cancer. In agreement with our data, Yang et al. (64) have demonstrated that the expression of VEGF-A, -C, and -D was positively correlated with HER2/neu expression in human breast carcinomas (64).

In summary, our findings identified for the first time that HRG-β1 potently induces the up-regulation of VEGF-C mRNA and protein in human breast cancer MCF-7 cells through a novel signaling pathway from HER2 receptor, p38 MAPK, to the subsequent activation of NF-κB cascade (Fig. 7). Our study also provides a therapeutical rationale for the inhibition of breast tumor lymphangiogenesis using pharmacological inhibitors to block this signaling cascade or using Herceptin to inhibit HER2 activity.

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Up-regulation of Vascular Endothelial Growth Factor C in Breast Cancer Cells by Heregulin-β1: A CRITICAL ROLE OF p38/NUCLEAR FACTOR-κB SIGNALING PATHWAY

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