Vascular Endothelial Growth Factor Up-regulation via p21-activated Kinase-1 Signaling Regulates Hereregulin-β1-mediated Angiogenesis*

Received for publication, July 12, 2000, and in revised form, August 24, 2000
Published, JBC Papers in Press, August 30, 2000, DOI 10.1074/jbc.M006150200

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Hereregulin-β1 promotes the activation of p21-activated kinase 1 (Pak1) and the motility and invasiveness of breast cancer cells. In this study, we identified vascular endothelial growth factor (VEGF) as a gene product induced by heregulin-β1. The stimulation by heregulin-β1 of breast cancer epithelial cells induced the expression of the VEGF mRNA and protein and its promoter activity. Heregulin-β1 also stimulated angiogenesis in a VEGF-dependent manner. Herceptin, an anti-HER2 antibody inhibited heregulin-β1-mediated stimulation of both VEGF expression in epithelial cells and angiogenesis in endothelial cells. Because the activation of Pak1 and VEGF expression are positively regulated by heregulin-β1, we hypothesized that Pak1 regulates VEGF expression, and hence explored the role of Pak1 in angiogenesis. We provide new evidence to implicate Pak1 in angiogenesis and to increased metastasis in human breast cancer (1). HER family receptors are transactivated by receptors binding to more than a dozen isoforms of the heregulins, and each can activate the HER-2 receptor via interactions among heterodimeric interactions (3–6). Accordingly, an anti-HER2 mAb, Herceptin, has been shown to block heregulin-triggered interactions between HER3 and HER2 interactions, and some of the biological effects of heregulin-β1 (HRG) (7, 8).

The physiologic significance of HRG was established through targeted deletion studies in which mice lacking HRG had developmental abnormalities in the nervous and cardiovascular systems (9, 10). The most prominent cardiovascular abnormalities included a lack of the endocardial cushion, which requires mesenchymal cell growth for its development. Heregulin supports angiogenesis (11). Accumulating evidence suggests that HRG, a paracrine growth factor secreted from mesenchymal cells, regulates the progression of breast cancer cells to the invasive phenotype (12–14). Recently, we confirmed that in the absence of HER-2 overexpression, HRG-stimulated activation of breast cancer cells promotes the development of more aggressive phenotypes; this development includes the formation of lamellipodia and an increase in cell motility through activation of p21-activated kinase-1 (Pak1) (7).

Additional evidence that Pak1 may play a role in HRG-mediated invasion of breast cancer cells was demonstrated by the use of kinase-dead Pak1 mutants that promoted cell spreading and the stabilization of focal points, reducing cell invasion (15, 16). Despite the widely acknowledged role of HRG in angiogenesis and breast cancer progression, the molecular mechanism by which HRG affects angiogenesis and the potential role that Pak1 signaling plays in angiogenesis remain poorly understood.

Several recent studies suggest that tumor growth and progression are closely linked to angiogenesis. The neovascularization or angiogenesis, which provides nutrient flow to solid tumors, is critical to the initial rapid tumor growth and the growth of metastases (17). The onset of tumor angiogenesis depends on the production of angiogenic factors by the tumor cells or tumor microenvironment that stimulate host organ vascular endothelial cell growth and chemotaxis (18).

This paper is available on line at http://www.jbc.org

* This study was supported in part by National Institutes of Health Grants CA80066 and CA65746, by the Breast and Ovarian Research programs of the University of Texas M. D. Anderson Cancer Center, and by Bristol-Myers Squibb research funds (to R. K.).
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1 The abbreviations used are: HER, human epidermal growth factor receptor; HRG, heregulin-β1; Pak1, p21-activated kinase; VEGF, vascular endothelial growth factor; mAb, monoclonal antibody; HUVEC, human umbilical vein endothelial cells; HMVEC-1, human lung microvascular endothelial cells; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.
HER2 receptors in human tumor cell lines is associated with increased expression of VEGF and angiogenesis (23).

Overexpression of HER2 occurs in 20–25% of breast cancer patients. The remainder of breast cancer patients, who have no overexpression of HER-2 receptor levels, often exhibit metastasis. Thus, it is important to explore the potential regulation of VEGF by mesenchymal growth factor HRG in the absence of HER2 overexpression. The results of this study show that HRG regulates the expression of the VEGF promoter, VEGF mRNA, and VEGF protein and its secretion and that HRG stimulates angiogenesis in a VEGF-dependent manner. Our results implicate Pak1 signaling in HRG regulation of VEGF expression. Using dominant-negative or dominant-active Pak1 mutants, we discovered that Pak1 activity was required for the transcriptional expression of VEGF and thereby influenced the level of VEGF secretion.

MATERIALS AND METHODS

Cells Culture and Reagents—MCF-7, MDA-MB231, and MDA-MB435 human breast cancer cell lines (7, 15, 16, 24) were maintained in Dulbecco’s modified Eagle’s medium/F12 (1:1) supplemented with 10% fetal calf serum. Human umbilical vein endothelial cells (HUVEC), purchased from ATCC (Manassas, VA), were maintained in F12 and supplemented with 2 mM glutamine, 100 μg/ml heparin, 30–50 μg/ml endothelial cell growth supplement, and 10% fetal bovine serum. Human lung microvascular endothelial cells (HMVEC-L) were purchased from the Clontics Corp. and cultured according to the manufacturer’s instructions. Antibodies against HER3 (25) and VEGF and recombinant heregulin-beta1 were obtained from Neomarkers Inc. (Fremont, CA). Antibodies against HER2 and epidermal growth factor receptors have been described previously (7). Recombinant VEGF was purchased from R & D Systems (Minneapolis, MN).

Metabolic Labeling and Immunoprecipitation of VEGF—Breast cancer cells were grown to 50% confluency and then incubated in serum-free conditions for 24 h. Cells were metabolically labeled with 20 μCi/ml of [35S]methionine for 24 h in a methionine-free medium containing 2% dialyzed fetal bovine serum in the absence or presence of HRG (50 ng/ml) (13). Conditioned media with equal trichloroacetic acid perceptible counts were immunoprecipitated with the desired or control antibody, resolved on SDS-PAGE, and analyzed using autoradiography.

Measurement of VEGF Protein—Breast cancer cells were cultured under serum-free conditions for 24 h and treated in the presence or absence of HRG (50 ng/ml); conditioned medium was harvested after 24 h. The amount of secreted VEGF was measured in the conditioned medium with a VEGF-specific sandwich-ELISA assay (R & D Systems) according to the manufacturer’s instructions. VEGF protein levels were normalized to the number of cells.

Northern Analysis—Total cytoplasmic (RNA 20 μg) was analyzed by Northern blot analysis. Northern blots were probed with [3P] labeled 473 bp VEGF cDNA fragment (provided by Dr. Abraham Judith). Glyceraldehyde 3-phosphate dehydrogenase levels were used to control for RNA loading (25).

In Vitro Kinase Assay—The Pak1 kinase assay was performed as described (4). Briefly, MCF-7 cells expressing HA-tagged T423E Pak1 were treated with doxycycline (1 μg/ml) for 24 h to induce the expression of active Pak1, and T423E Pak1 was immunoprecipitated with HA monoclonal antibody. Immunocomplexes were washed three times with lysis buffer and two times with kinase buffer containing 20 mM HEPES (pH 7.4), 1 mM dithiothreitol, 10 mM MnCl2, and 10 mM MgCl2. The kinase reaction was carried out in kinase buffer supplemented with myelin basic protein as a substrate and 10 μCi of [γ-32P]ATP at 30 °C for 30 min. The kinase reaction was terminated by the addition of 5× SDS-PAGE sample buffer followed by autoradiography. To study Pak kinase activity in tissues from transgenic mice, endogenous Pak1 was immunoprecipitated from tissues lysates using Pak1 antibody (Santa Cruz Biotechnology).

In Vitro Angiogenesis Assay—In vitro angiogenesis in collagen gels was quantitated using spheroids of microvascular endothelial cells (27). To generate these spheroids, 1 × 104 cells were suspended in culture medium and seeded in 96-well plates coated with 0.5% (w/v) agarose. HMVEC-L spheroids were generated overnight and then embedded into collagen gels. A collagen stock solution was prepared prior to use by mixing acidic collagen extract of rat tails (equilibrated at 4 °C to 3 mg/ml with 10× Dulbecco’s modified Eagle’s medium (8:1)) with 0.1 N NaOH to adjust the pH to 7.4. A 0.5-mL sample of this stock solution was mixed at room temperature with 0.5 ml of medium containing 10% fetal calf serum and 50 ng/ml HRG in the presence or absence of 100 nM Herceptin. The results are representative of four independent experiments.

FIG. 1. HRG regulation of VEGF secretion in epithelial cancer cells. A, MCF-7 cells were treated with HRG (50 ng/ml) and labeled with [35S]methionine. After 24 h, conditioned media were resolved on SDS-PAGE and analyzed by autoradiography. B and C, [32P]methionine-labeled conditioned media as described for panel A or from MDA-MB435 cells were immunoprecipitated (IP) with an anti-VEGF antibody, resolved on SDS-PAGE, and analyzed by autoradiography. D, enhancement of MDA-MB435 cell invasion by HRG as measured by a Boyden chamber. Con, control. E, ELISA measurement of secreted VEGF level in the conditioned media (24-h collection) from control and HRG (50 ng/ml)-treated MCF-7 and MDA-MB435 cells. The results are representative of four independent experiments.
were treated with cycloheximide (CHX) were placed in the Boyden chambers. Cells (10^5) suspended in Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin (BSA) were added to the upper chamber. Conditioned medium from mouse fibroblast NIH3T3 cells was used as a source of chemoattractant and was placed in the lower compartment of the Boyden chambers. After 24 h of incubation, the invaded cells are counted as described above.

Murine Angiogenesis Assay—Angiogenesis was assayed in terms of the growth of blood vessels from subcutaneous tissue into a solid gel of reconstituted basement membrane (i.e. Matrigel) containing the test sample as described (28). Matrigel rapidly solidified at body temperature, thereby trapping the factor, assuring its slow release, and prolonging exposure of surrounding tissues to it. After 2 weeks, mice were killed, and Matrigel plugs were photographed.

Transgenic Studies—A breeding pair of HRG transgenic mice was kindly provided by Dr. Philip Leder (29). Genotype of the animals was confirmed by Southern blotting of tail DNA. Animal breeding and maintenance was performed according to IACUC guidelines. Approximately 50% of the transgenic offspring showed hyperplasia of the harderian gland as reported earlier (29). The hyperplastic harderian gland was dissected and processed for RNA extraction using the TRIZOL reagent. Total RNAs were treated with RNase-free DNase (100 U/ml) in the absence or presence of actinomycin D (ACD, 5 μg/ml) or cycloheximide (CHX, 50 μg/ml). Total RNAs (20 μg) from transgenic lines and normal harderian gland from wild type animals was subjected to Northern blotting; subsequently, the blot was reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Quantification of VEGF mRNA is shown in the bottom panel. B, MCF-7 cells were treated with cycloheximide (CHX, 50 μg/ml) or actinomycin D (ACD, 5 μg/ml) in the presence or absence (Con, control) of HRG (50 ng/ml). Quantitation of VEGF mRNA is shown in the bottom panel. The results are representative of three separate experiments.

Nonmigrating cells were scraped off, and the cells that had migrated to the lower surface of the filter inserts were fixed with 100% methanol for 10 min and stained with hematoxylin-eosin. Six randomly selected fields on each filter were then counted.

To test the invasion behavior of HRG-treated MDA-MB435 cells, 8-μm filters were coated with Matrigel (20 μg filter). The coated filters were placed in the Boyden chambers. Cells (10^6) suspended in Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin were added to the upper chamber. Conditioned medium from mouse fibroblast NIH3T3 cells was used as a source of chemoattractant and was placed in the lower compartment of the Boyden chambers. After 24 h of incubation, the invaded cells are counted as described above.

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Sections of the harderian glands were stained with CD34 monoclonal antibody (NeoMarkers, CA). CD34 is a differentiation antigen expressed by most endothelial cells in vivo; expression of CD34 has been shown to be up-regulated by endothelial cells during angiogenesis (30, 31). Sections were deparaffinized and rehydrated. Endogenous peroxidase was inactivated with 3% H₂O₂, washed in phosphate-buffered saline followed by preincubation with goat serum for 30 min, and then incubated with a mouse monoclonal antibody against CD34 (dilution 1:50) for 60 min. After three washes, the sections were incubated with biotinylated anti-mouse IgG antibodies for 10 min, washed, and incubated with streptavidin-peroxidase for 10 min before the addition of 3-amino-9-ethyl-cabazole. Meyer’s hematoxylin was used for counter-staining.

RESULTS AND DISCUSSION

Enhancement by HRG of VEGF Protein Production in Epithelial Cells—During an earlier study to characterize the nature of secreted proteins from HRG-stimulated [³⁵S]methionine-labeled MCF-7 cells, we discovered that HRG enhanced the accumulation of several newly synthesized proteins in the conditioned medium, including a protein with a molecular mass of approximately 20 kDa (Fig. 1A, arrowhead). In the present study, we investigated whether this secreted 20-kDa protein is a VEGF. Immunoprecipitation of the conditioned medium from control (−) and HRG-stimulated (+) labeled MCF-7 with an anti-VEGF mAb revealed that HRG enhanced the secretion of a 20-kDa VEGF protein (Fig. 1B, arrow). To understand the correlation between VEGF production and the invasiveness of cells, we examined the effect of HRG on the secretion of VEGF and the invasiveness of highly metastatic MDA-MB435 cell (15). Consistent with the invasive nature, MDA-MB435 cells secreted higher levels of VEGF than did MCF-7 cells (Fig. 1C) and were more invasive than MCF-7 cells. As shown in Fig. 1C, however, MDA-MB435 cells responded to HRG with further induction of a 20-kDa VEGF

![Fig. 2. HRG induces the expression of VEGF mRNA in breast cancer epithelial cells.](image)

![Fig. 3. Anti-HER receptor antibodies block heregulin-stimulation of VEGF promoter activity.](image)
protein band similar to that of VEGF 165 and a weaker induction of a protein similar to VEGF 121 (32). HRG treatment also promoted the ability of MDA-MB435 cells to invade through a porous membrane as measured by a Boyden chamber assay (Fig. 1D). Quantitation of the VEGF in the conditioned medium using a very sensitive ELISA assay confirmed that HRG stimulation of MCF-7 cells tripled the level of VEGF protein over 24 h. The amount of VEGF protein secreted into the conditioned media from MDA-MB435/CMV cells was significantly more HER-1 than is present on MCF-7 and MDA-MB231 cells (Fig. 3). The observed HRG-mediated up-regulation of VEGF promoter activity in MCF-7 and MDA-MB435 cells was effectively suppressed by pretreating the cells with the anti-HER-2 mAb Herceptin, which inhibits the formation of HER-3/HER-2 interaction (7), and with anti-HER-3 mAb, which competes with the HRG binding site on the HER-3 receptor (25, 33). Anti-epidermal growth factor receptor mAb C225 was more effective than MCF-7 cells in blocking the stimulatory effect of HRG on the VEGF promoter in MDA-MB-231 cells, suggesting that C225 may also prevent HRG-induced interactions between HER-3 and HER-2 receptors. This idea is supported by results showing that anti-HER3 antibody (Ab3) was more inhibitory than Herceptin in MDA-MB435 cells (34). The observed HRG-stimulated increase in VEGF mRNA expression in epithelial cells—We performed a Northern blot analysis to determine whether the HRG-mediated increase in the level of expression of VEGF protein was accompanied by an increase in the level of expression of VEGF mRNA. VEGF mRNA level increased in response to HRG stimulation. HRG treatment continuously increased the steady-state levels of VEGF mRNA by 2–6-fold at 1–9 h after treatment (Fig. 2A). Treatment of cultures with actinomycin D, an inhibitor of transcription, completely inhibited the HRG-mediated induction of VEGF mRNA (Fig. 2B). To study translational regulation, we utilized cycloheximide, a translational inhibitor. Treatment of cells with cycloheximide stabilized the levels of VEGF mRNA expression; treatment with HRG, however, superinduced the expression of VEGF mRNA (Fig. 2B). Together, these results suggest that HRG regulate VEGF at a pretranslational level.

**Effect of Anti-HER-blocking Antibodies on HRG-mediated Stimulation of VEGF Promoter Activity—**To further confirm the role of HRG in the transcriptional regulation of the VEGF gene, cells were transiently transfected with a chimeric luciferase gene fused with the 5’ region of the VEGF promoter (26), and the activity of the promoter was assayed in the presence or absence of HRG. HRG treatment stimulated VEGF promoter activity in three breast cancer cell lines, all of which had normal levels of HER receptors (Fig. 3). The observed HRG-mediated up-regulation of VEGF promoter activity in MCF-7 and MDA-MB435 cells was effectively suppressed by pretreating the cells with the anti-HER-2 mAb Herceptin, which inhibits the formation of HER-3/HER-2 interaction (7), and with anti-HER-3 mAb, which competes with the HRG binding site on the HER-3 receptor (25, 33). Anti-epidermal growth factor receptor mAb C225 was more effective than MCF-7 cells in blocking the stimulatory effect of HRG on the VEGF promoter in MDA-MB-231 cells, suggesting that C225 may also prevent HRG-induced interactions between HER-3 and HER-1 receptors. This idea is supported by results showing that anti-HER3 antibody (Ab3) was more inhibitory than Herceptin in MDA-MB231 cells (Fig. 3B) and because MDA-MB231 cells expressed significantly more HER-1 than is present on MCF-7 and MDA-MB435 cells (34).

**Effect of p21-activated Kinase-1 Signaling on Expression and Secretion of VEGF—**Recent studies from this laboratory showed that Pak1 plays a role in HRG-mediated stimulation of leading edge formation and invasiveness of noninvasive breast cancer cells (7) and in the maintenance of motile/invasive phenotypes of MDA-MB435 cells (15). Because the activation of Pak1 (7) and VEGF (this study) expression are positively regulated by heregulin-β1 and because VEGF expression can be regulated by phosphatidylinositol 3-kinase (22), a HRG-inducible kinase that is the upstream regulator of Pak1 (7), we hypothesized that Pak1 regulates VEGF expression and therefore explored the role of Pak1 in angiogenesis. To test this possibility, we examined the effect of the dominant-negative K299R Pak1 mutant (7, 15) on HRG-induced activation of the
VEGF promoter. Transient co-transfection of MCF-7 cells with pGL3-VEGF and kinase-dead K299R Pak1, but not with control vector, completely suppressed the ability of HRG to stimulate transcription from the VEGF reporter system (Fig. 4A).

To further validate the involvement of Pak1 signaling on the level of VEGF expression, we next used well characterized MDA-MB435 cells expressing either K299R Pak1 mutant (435-K16 and 435-K17 cells) or control vector (435-CMV cells) (15). The 435-K17 cells represent a different clone with properties similar to 435-K16 cells. The inhibition of Pak1 signaling was accompanied by a significant reduction (50–60%) in the level of VEGF promoter activity (Fig. 4B) and VEGF mRNA expression (Fig. 4C) and by a 35% reduction in secretion of VEGF protein in the conditioned medium (Fig. 4D) compared with the levels in the control cells. The observed discrepancy between a significant reduction of VEGF mRNA level (60% compared with control cells) and a modest suppression of VEGF accumulation in the conditioned medium (35% compared with control cells) could be due to the possible detection of more than one isoform of VEGF using a radioimmunoassay kit. However, HRG treatment was not able to rescue or induce the observed reduction in the basal VEGF secretion, suggesting the possible requirement of a functional Pak1 pathway during HRG-mediated up-regulation of VEGF.

Effect of Expression of Kinase-active T423E Pak1 on Expression and Secretion of VEGF—To study the effect of dominant-active Pak1 on the expression of VEGF, we used MCF-7 clones expressing kinase-active T423E Pak1 under the control of an inducible tetracycline promoter (16). As expected, expression of HA-tagged T423E-Pak1 increased Pak kinase activity compared with the level in vector control cells (Fig. 5A). Interestingly, the increased expression of kinase-active T423E Pak1 in MCF-7 cells was accompanied by increased VEGF promoter activity (Fig. 5B) and enhanced secretion of VEGF protein in the conditioned medium, as determined by immunoprecipitation of $^{35}$S-labeled VEGF (Fig. 5C) and the results of the ELISA assay (Fig. 5D). Taken together, these results suggest that Pak1 signaling regulates the expression of VEGF and that Pak1 may be an important mediator of HRG regulation of VEGF expression.

Stimulation by HRG of Vascular Tube Formation, Endothelial Cell Migration, and Angiogenesis in Vivo—We tested the ability of HRG to stimulate angiogenesis in cells embedded in collagen gels. In the presence of HRG or VEGF, capillary sprouts originated from HMVEC-L spheroids embedded in collagen gels in the presence of 10% fetal calf serum (Fig. 6A). Inclusion of anti-HER-2 receptor mAb Herceptin or anti-VEGF mAb (but not control antibody; data not shown) suppressed sprouting of vascular endothelial cells in HRG-treated cultures (Fig. 6A).

Because basement membrane can stimulate differentiation, HUVEC were also plated onto a gel composed of reconstituted basement membrane proteins, to which HUVEC attach rapidly. After 24 h, elongated processes appeared in the control cells that had been cultured in the absence of serum. In contrast, the addition of HRG and VEGF to HUVEC promoted the formation of networks of branching and anastomosing cords of cells (commonly known as tube formations) (Fig. 6B). This effect of HRG was significantly blocked by Herceptin or anti-VEGF mAb (Fig. 6B) but not by the control antibody (data not shown).

The effect of HRG on the migration of HUVEC and HMVEC-L was also analyzed using the Boyden chamber assay. Endothelial cells migrated in response to a chemotactic gradient of VEGF and HRG. In this assay, HUVEC were more responsive to HRG than were HMVEC-L. As expected from Fig. 6, A and B, HRG tripled the migration rate of HUVEC and doubled that of HMVEC-L cells (Fig. 6C). However, anti-VEGF mAb and Herceptin each suppressed HRG stimulation of cell migration (Fig. 6C).

The angiogenic activity of HRG was also tested in a murine angiogenesis model involving subcutaneous injection of Matri-
HRG up-regulates Pak1 activity, VEGF expression, and angiogenesis in a transgenic model. To evaluate the HRG modulation of angiogenesis, Pak1 kinase activity, and VEGF expression in vivo, we used a murine mammary tumor virus-driven HRG transgenic model that develops mammary adenocarcinomas and hardieran tumors (29). Because hardieran tumors can usually be detected by 3 weeks of age as opposed to the 12–16 months required for detection of mammary gland tumors, we used the hardieran tumors to establish the proof-of-principle of our hypothesis of HRG regulation of angiogenesis in vivo. Hardieran glands from wild type (WT) and HRG-transgenic (HRG-TG) mice were analyzed by RT-PCR for the expression of HRG and by RT-PCR followed by Southern blotting for VEGF. HRG-transgenic mice have significantly elevated levels of HRG transcript compared with wild type mice (Fig. 7B). Interestingly, overexpression of HRG in hardieran tumors was accompanied by increased Pak1 kinase activity (Fig. 7D) as well as VEGF expression (Fig. 7C). Furthermore, we also discovered a significant increase in angiogenesis as determined by immunostaining with CD34 antigen (a specific angiogenic marker, Refs. 30 and 31; shown by brown stain) in the hardieran gland tumors as compared with wild type tissues (Fig. 7E). Together, these results imply a close relationship among the expression of HRG, Pak1 activity, VEGF expression and angiogenesis in vivo.

In summary, the results presented here demonstrate that: 1) HRG regulates the expression and secretion of VEGF from breast epithelial cancer cells; 2) Pak1 signaling is an important regulator of VEGF expression and functions; 3) HRG stimulates angiogenesis; and 4) VEGF may play a role in the angiogenic effects of HRG on endothelial cells. These data provide evidence that up-regulation of the VEGF pathway in breast cancer epithelial cells by HRG, which is secreted from mesenchymal cells, may have functional implications for the enhancement of invasiveness of breast cancer cells. Any potential up-regulation of VEGF by HRG in breast cancer epithelial cells is likely to sustain and perhaps further promote the ability of tumor cells to survive and metastasize by supporting neovascularization. In addition, because HRG is a potent angiogenic factor (this study) and receptors for HRG are also present on endothelial cells (11), we propose a model in which HRG interacts with both mammary epithelial tumor cells and endothelial cells and in which induced secretion of VEGF from tumor cells may further amplify the angiogenic responses of endothelial cells to HRG.
Acknowledgments—We are grateful to Philip Leder for providing heregulin transgenic mice. We thank Amjad Taludker and Liana Adam for their help in the initial part of this project, Judith Abraham (Scios Inc.) for providing VEGF-promoter (via L. Ellis) and VEGF cDNA probe (via D. Mukhopadhyay), Genentech Inc. for Herceptin, and ImClone Inc. for C225.

REFERENCES
1. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., and Ullrich, A. (1989) Science 244, 707–712.
2. Hynes, N. C., and Stern, D. F. (1994) Biochim. Biophys. Acta 1198, 165–184.
3. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Levy, R. B., and Yarden, Y. (1992) Cell 69, 205–216.
4. Wen, D., Peles, E., Cupples, R., Suggs, S., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Hu, S., Silbiger, S. M., and Levy, R. B. (1992) Cell 69, 559–572.
5. Graus-Ponta, D., Beerli, R. R., Daly, J. M., and Hynes, N. E. (1997) EMBO J. 16, 1647–1655.
6. Alroy L., and Yarden, Y. (1997) FEBS Lett. 410, 83–86.
7. Adam, L., Vadlamudi, R., Kondapaka, S. B., Chernoff, J., Mendelsohn, J., and Kumar, R. (1997) J. Biol. Chem. 272, 28238–28246.
8. Kumar, R., Mandal, M., and Vadlamudi, R. (2000) Semin. Oncol., in press.
9. Kramer, R., Bucay, N., Kane, D., Martin, L., Tarpley, J., and Theill, L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4833–4838, 1996.
10. Lee, K., Simon, H., Chen, H., Bates, B., Hung, M., and Hauser, C. (1995) Nature 378, 394–398.
11. Russell, K. S., Stern, D. F., Polverini, P. J., and Bender, J. R. (1999) Am. J. Physiol. 277, H2205–H2211.
12. Pietras, R. J., Arboleda, J., Reese, D. M., Wongvipat, N., Pegram, M. D., Ramos, L., Gorman, C. M., Parker, M. G., Sliwkowski, M. X., and Slamon, D. J. (1995) Oncogene 10, 2435–2446.
13. Kumar, R., Mandal, M., Ratzkin, B. J., Liu, N., and Lipton, A. (1995) J. Cell. Biol. 124, 102–112.
14. Tang, C. K., Perez, G., Grunt, T., Waibel, C., Cho, C., and Lupe, R. (1996) Cancer Res. 56, 3350–3358.
15. Adam, L., Vadlamudi, R., Mandal, M., Chernoff, J., and Kumar, R. (2000) J. Biol. Chem. 275, 36238–36244.
16. Vadlamudi, R., Adam, L., Wang, R-A., Mandal, M., Nguyen, D., Sahin, A., Chernoff, J., Hung, M.-C., and Kumar, R. (2000) J. Biol. Chem. 275, 36238–36244.