Neuregulin activation of ErbB receptors in vascular endothelium leads to angiogenesis

KERRY STRONG RUSSELL,1 DAVID F. STERN,2 PETER J. POLVERINI,3 AND JEFFREY R. BENDER1

1Division of Cardiovascular Medicine and Molecular Cardiobiology, Boyer Center for Molecular Medicine, and 2Department of Pathology, Yale University School of Medicine, New Haven, Connecticut, 06536-0812; and 3Section of Pathology, University of Michigan School of Dentistry, Ann Arbor, Michigan, 48109-1078

Russell, Kerry Strong, David F. Stern, Peter J. Polverini, and Jeffrey R. Bender. Neuregulin activation of ErbB receptors in vascular endothelium leads to angiogenesis. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H2205–H2211, 1999.—The ErbB, or epidermal growth factor receptor (EGF-r), family of transmembrane tyrosine kinase receptors has been demonstrated to play an important role in growth regulation and intracellular signaling in a wide variety of cell types. Targeted deletion of neuregulin (an ErbB ligand) in mice results in endocardial cushion abnormalities, suggesting that these receptor-ligand interactions have important effects on vascular endothelial growth and development. To study the role of ErbB receptor signaling in vascular endothelium, we investigated the expression pattern of the various receptor family members and the effect of ErbB receptor stimulation in human umbilical vein endothelial cells (HUVEC). We demonstrate that ErbB2 (neu), ErbB3, and ErbB4 are highly expressed, whereas ErbB1 (EGF-r) is undetectable. Stimulation of HUVEC with recombinant neuregulin-1β (an ErbB3/4 ligand) induces rapid calcium fluxes, receptor tyrosine phosphorylation, and cell proliferation. We demonstrate marked in vitro and in vivo angiogenic responses to neuregulin-β, which are independent of vascular endothelial cell growth factor. These findings support an important role for the ErbB family of receptors in endothelial cell signaling and function, including neuregulin-induced angiogenesis.

epidermal growth factor receptor family; vascular biology; human endothelial cells; vascular endothelial cell growth factor; tyrosine kinase receptors

THE EPIDERMAL GROWTH FACTOR (EGF) family of receptors includes four closely related transmembrane tyrosine kinases: ErbB1 (HER1 or EGF receptor), ErbB2 (HER2 or neu), ErbB3 (HER3), and ErbB4 (HER4). These receptors can form both homo- and heterodimers in a manner that is dependent on the combination of receptors present, the stimulatory ligand, and the intracellular signaling pathways activated (27). This allows them to affect, positively and negatively, a wide variety of cellular functions, including proliferation, migration, differentiation, and cell survival.

The importance of overexpression of these receptors in cellular transformation and tumor metastasis has been elucidated by a number of in vitro and clinical studies (15). Furthermore, targeted deletion of ErbB2, ErbB3, ErbB4, or neuregulin-1 (a ligand for these receptors) in mice leads to developmental abnormalities that are severe in the nervous system and lethal in the cardiovascular system (8, 9, 17, 19, 21, 28). Cardiovascular abnormalities include aborted development of ventricular myocardial trabeculae and the endocardial cushion, the latter of which is dependent on mesenchymal cell growth and endocardial endothelia development.

The ligands for these receptors are primarily synthesized as transmembrane proteins, which can either be cleaved to form a soluble ligand or remain on the cell surface to mediate juxtacrine signaling. These ligands have been divided into three functional groups based on their pattern of ErbB receptor binding and activation. One group (including EGF, transforming growth factor-α, and amphiregulin) binds only ErbB1 and can transactivate the other receptors when heterodimerized with ErbB1. A second group (including betacellulin, epiregulin, and heparin-binding EGF) binds both ErbB1 and ErbB4 and transactivates ErbB2 and ErbB3 when heterodimerized with either of these receptors. The last group (including the neuregulins) can bind both ErbB3 and ErbB4 and transactivates ErbB2 and ErbB1 only when expressed in combination with one of these receptors (26).

The neuregulins (also known as heregulins, acetylcholine receptor-inducing activity, Neu Differentiation Factor, or glial growth factor) were originally described as splice variants derived from a single gene (36). More recently, two additional genes also encoding a variety of splice products have been described (3, 4, 37). These ligands play important roles in signaling not only in cardiac development but also in neuronal cell differentiation, signaling at neuromuscular junctions, and epithelial cell morphogenesis [e.g., mammary gland development and wound healing (5), and for a review see Ref. 2]. Recently, neuregulin-1 mRNA has been shown to be expressed by rat coronary microvascular endothelial cells, and neuregulin stimulation of rat primary cardiac myocyte cultures has been demonstrated to result in increased myocyte survival, hypertrophy, and proliferation (38).

Because of the role these receptors play in cardiovascular development, we investigated whether they are present and functional in human vascular endothelium. Here, we show that a subset of these receptors is present in human vascular endothelial cells (EC). EC receptor stimulation by neuregulin leads to prolifer-
tion and morphogenetic changes, which are consistent with an angiogenic response. Finally, using an in vivo assay, we demonstrate that neuregulin stimulation can lead to growth of new blood vessels.

**METHODS**

**Materials.** The EGF-like domain of human neuregulin-β3 (−7 kDa) was purified from a GST fusion construct (courtesy of Drs. M. Kraus and G. Carpenter, Vanderbilt University) by thrombin cleavage (1). Preparations were added to benzenedine-agarose beads (Sigma), 3–4 successive rounds, with a total binding capacity for thrombin =140–190-fold over the total amount of thrombin added for the cleavage. The control pGEX peptide was prepared from the empty pGEX-2T vector (Pharmacia Biotech) in an identical fashion. Neuregulin-β3 was obtained from Drs. M. Kraus and G. Carpenter, Vanderbilt University) by benzamidine-agarose beads (Sigma), 3–4 successive rounds, with a total binding capacity for thrombin =140–190-fold over the total amount of thrombin added for the cleavage. The control pGEX peptide was prepared from the empty pGEX-2T vector (Pharmacia Biotech) in an identical fashion. Neuregulin-β3 was obtained from Drs. M. Kraus and G. Carpenter, Vanderbilt University.)

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**Cell isolations and culture.** HUVEC were isolated from single donors as previously described (25). Cells were routinely passaged on gelatin-coated plates in medium 199 (M199) with 15% fetal bovine serum (FBS), bovine endothelial cell growth supplement (ECGS, 50 µg/ml), and heparin (100 µg/ml). BT474 cells were obtained from the ATCC and maintained in RPMI 1640 with 10% FBS. Reagents were added in M199 with 15% FBS and no ECGS.

**Expression of ErbB receptors.** Human umbilical vein EC (HUVEC) were evaluated for expression of ErbB2, ErbB2, ErbB3, and ErbB4 by immunoprecipitation and Western blotting. Figure 1A demonstrates that expression of ErbB2, ErbB3, and ErbB4 is easily detected in HUVEC. As noted in other cell types, several different molecular weight species are seen for ErbB4. An ErbB4-specific peptide (Santa Cruz) competes for antibody reactivity with both the higher and lower molecular weight species seen in ErbB4 blots (data not shown), suggesting that these represent alternative, perhaps differentially glycosylated, receptor isoforms. HUVEC lysates do not contain detectable ErbB1 (EGF-r). Because of receptor heterodimerization and apparent required complex formation for signal transduction, this combination of receptors in HUVEC makes their function likely. This pattern of ErbB receptor expression was also seen in lysates prepared from freshly isolated (never cultured) HUVEC, suggesting that the lack of ErbB1 expression is not an artifact of cell culture conditions.

**Growth curves.** HUVEC were plated at a density of 5,000 cells/well in 12-well plates in M199 with 5% FBS (no ECGS). The following day, reagents were added as indicated. New media and reagents were added on days 3, 5, 7, and 9. The anti-VEGF blocking antibody (MAB-293, R&D Systems) was used in concentrations reported (by the manufacturer) to block >80% of the activity of 10 ng/ml of recombinant human VEGF.

**Collagen tube formation.** HUVEC were plated in gelatin-coated 24-well plates in M199 with 15% FBS and ECGS. When confluent, monolayers were overlaid with 1.5 mg/ml rat tail collagen (type I, Collaborative Biomedical Products, Becton Dickinson). After neutralization, the collagen was allowed to solidify at 37°C for 30 min, after which the reagents were added in M199 with 15% FBS and no ECGS. Wells were monitored and photos taken at 16–48 h after reagent addition (35). All photos shown were taken at the same time point for comparison.

**Rat corneal angiogenesis.** Each reagent was embedded in Hydragel pellets (Interferon Sciences) and placed in the intracorneal pocket, 1–2 mm from the limbus, of anesthetized rats, as previously described (23). Sections were performed and data scored 7 days after the implantation. All animals were treated in accordance with institutional guidelines for animal care. Statistical differences were calculated using Fisher’s exact test.

**RESULTS**

**Expression of ErbB receptors.** Human umbilical vein EC (HUVEC) were evaluated for expression of ErbB1, ErbB2, ErbB3, and ErbB4 by immunoprecipitation and Western blotting. Figure 1A demonstrates that expression of ErbB2, ErbB3, and ErbB4 is easily detected in HUVEC. As noted in other cell types, several different molecular weight species are seen for ErbB4. An ErbB4-specific peptide (Santa Cruz) competes for antibody reactivity with both the higher and lower molecular weight species seen in ErbB4 blots (data not shown), suggesting that these represent alternative, perhaps differentially glycosylated, receptor isoforms. HUVEC lysates do not contain detectable ErbB1 (EGF-r). Because of receptor heterodimerization and apparent required complex formation for signal transduction, this combination of receptors in HUVEC makes their function likely. This pattern of ErbB receptor expression was also seen in lysates prepared from freshly isolated (never cultured) HUVEC, suggesting that the lack of ErbB1 expression is not an artifact of cell culture conditions.

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Effects of neuregulin on HUVEC tube formation in collagen gels. As suggested above, EC growth plays a central role in numerous physiological and pathological processes. For example, endothelial proliferation is required during the neovessel formation (angiogenesis) of wound healing. An in vitro tube formation assay was used to begin assessing whether neuregulin provides an angiogenic stimulus. Figure 4 demonstrates that within 24–36 h HUVEC grown in a two-dimensional collagen gel matrix form elongated tube structures in response to VEGF (Fig. 4B), neuregulin-β3 (Fig. 4D), and neuregulin-β1 (Fig. 4F) but not to control peptide (Fig. 4A). As with the EC proliferation assays, the neutralizing anti-VEGF antibody had no effect on neuregulin-induced tube formation (Fig. 4E), whereas it largely abrogated the VEGF response (Fig. 4C). These findings demonstrate that endothelial proliferative and in vitro angiogenic responses to neuregulin do not require VEGF activity.

In vivo angiogenesis induced by neuregulin. Although in vitro tube formation provides a useful method for testing the effects of defined factors on angiogenesis involving isolated EC, the relevance of this model to in vivo angiogenesis is less clear. Therefore, an established rat corneal angiogenesis model (23) was used, in which Hydron pellets containing various doses of neuregulin, VEGF, or control pGEX peptide were surgically placed in the eyes of rats, and corneal neovascularization was assessed 7 days later. As little as 10 ng of neuregulin induced corneal neovessels (Fig. 5B), with a marked angiogenic response to 50 ng (Fig. 5C), similar
to that seen with VEGF (25 ng) (Table 1). A dose response to neuregulin-β3 was observed (Table 1) with significant corneal inflammation observed only at the highest dose tested (200 ng). Thus the angiogenic response observed with lower neuregulin doses is not due to the elaboration of cytokines from invading inflammatory cells.

DISCUSSION

Receptor tyrosine kinases of the EGF-β family transduce mitogenic signals in numerous cell types. This has been most extensively studied in neoplastic cells and nervous system development. Our findings provide the first evidence that ErbB receptors (apart from EGF-β) transduce biochemical and functional signals in EC and that neuregulin is angiogenic. The presence of ErbB2, ErbB3, and ErbB4, but not ErbB1, in HUVEC is consistent with endothelial responses to neuregulin. Neuregulin-1 has been shown to bind to ErbB3 and ErbB4 and stimulate tyrosine kinase activity of ErbB2 and ErbB4 in an ErbB1-independent manner (27). In all other cell types previously studied, ErbB2 phosphorylation induced by neuregulin appears to occur through association with ErbB3 or ErbB4 (which preferentially

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**Fig. 2. Effects of neuregulin and epidermal growth factor (EGF) on HUVEC calcium fluxes.** HUVEC monolayers were loaded with fluo-3 in 96-well plates. Agonists (or controls) were added at indicated times (arrows). Representative curves are shown from 2 to 3 cells per field, as measured using an interactive laser cytometer. Curves were normalized to the background (≈1) fluorescence for each gated cell. Relative intracellular calcium levels (normalized fluorescence) were determined in response to recombinant human thrombin (1 U/ml) (A), neuregulin-β3 (500 ng/ml) (B), thrombin (1 U/ml) followed by neuregulin-β3 (100 ng/ml) in presence of thrombin inhibitor hirudin (100 U/ml, added just before start of each experiment, i.e., before time 0) (C), recombinant human EGF (500 ng/ml) followed by neuregulin-β3 (100 ng/ml) (D), and pGEX control peptide (500 ng/ml) (E).
Heterodimerize with ErbB2), because cells expressing only ErbB2 homodimers appear incapable of sufficient ligand binding to promote kinase activation (11, 27).

The downstream signaling effects of neuregulin in EC share several similarities with other signaling pathways, such as those activated by fibroblast growth factor (FGF) or VEGF, known to regulate angiogenesis. For example, the induction of calcium fluxes in EC after neuregulin treatment may be a critical part of angiogenic signaling, because inhibitors of ligand-stimulated Ca\(^{2+}\) influx have been shown to prevent FGF-induced angiogenesis (16). Furthermore, a recent study demonstrated that neutralizing antibodies directed against ErbB2 reduce tumor cell VEGF secretion, and expression of the oncogenic form of ErbB2 increases NIH/3T3 VEGF secretion (24). This raises the interesting possibility that neuregulin-ErbB signaling activates angiogenesis by inducing EC VEGF secretion and VEGF receptor signaling. However, our data show that anti-VEGF antibodies, in concentrations sufficient to block high levels of VEGF, had no effect on neuregulin-stimulated HUVEC growth or tube formation, demonstrating that these responses are VEGF independent.

Our findings provide the first evidence that ErbB receptors (apart from EGF-r) transduce biochemical and functional signals in EC and that neuregulin is angiogenic. This is particularly interesting in the context of recent studies demonstrating lethal cardiovascular abnormalities in neuregulin-1, ErbB2, ErbB3, or ErbB4 homozygous-deleted (null) mice (2). This information also supports the newly emerging concept that blood vessel and neuronal development share common signaling pathways, as has been shown for neuropilin-1, ELK, and CXCR4 receptor signaling (23, 29, 32, 34, 39). Furthermore, the finding that administration of nerve growth factor to human corneas results in neovascularization as well as healing of neurotrophic

Fig. 3. Effects of neuregulin on HUVEC proliferation. Medium control, neuregulin-β3 (50 ng/ml), or VEGF (50 ng/ml) were added to serum- and mitogen-depleted (1% FBS, no ECGS) HUVEC in the presence and absence of neutralizing anti-VEGF antibody (0.5 mg/ml) and harvested from single wells (in triplicate) for viable cell counts (trypan blue exclusion). Data points represent means ± SD of well counts.

Fig. 4. HUVEC tube formation in collagen gels. Control pGEX peptide (100 ng/ml) (A), vascular endothelial cell growth factor (VEGF, 50 ng/ml) (B), VEGF (50 ng/ml) + anti-VEGF (1 µg/ml) (C), neuregulin-β3 (50 ng/ml) (D), neuregulin-β3 + anti-VEGF (1 µg/ml) (E), or neuregulin-β1 (100 ng/ml) (F) were added in ECGS-free medium to confluent HUVEC monolayers overlaid with type I collagen (1.5 mg/ml). Photomicrographs were obtained 24 h after stimulus addition. Patches of detaching, round refractile cells, as well as areas of degenerating tubelike structures, are seen in A and C. Results are representative of 4 separate experiments. Magnification ×100.
ulcers suggests that there is overlap, or perhaps even cross talk, between pathways promoting nerve growth and angiogenesis in humans (18).

Data suggesting that receptors responsible for angiogenesis have opposing effects depending on the ligand present are emerging from several diverse fields. In the case of the neuropilin-1 receptor, stimulation by the ligand Semaphorin III appears to repel neuronal cell growth, whereas VEGF stimulation promotes blood vessel growth (13, 22, 32). This interesting dichotomy of responses also exists for the Tie2 receptor, which promotes angiogenesis when liganded by angiopoietin-1 and disrupts blood vessel formation when liganded by angiopoietin-2 (20). The current repertoire of ligands for the ErbB receptors includes EGF, amphiregulin, betacellulin, neuregulin, and others. Although these ligands have been shown to have differential effects on ErbB signaling, no such ligand-dependent opposing effects have been demonstrated for the mammalian ErbB receptor family. However, the Drosophila ErbB receptor homologue DER is positively regulated by its ligand Vein (a neuregulin homologue) and negatively regulated by its ligand Argos in a manner that is dependent on the EGF-like domain of the stimulatory ligand (30, 31). Although there is no currently known mammalian homologue of Argos, the high level of conservation of these signaling pathways raises the possibility that an anti-angiogenic ErbB receptor ligand exists in mammals. Pro- and anti-angiogenic regulation of this pathway may have important developmental and clinical implications.

Neuregulin mRNA has been shown to be present in cells from both the endocardial endothelium of mice and the coronary microvascular endothelium of rats (3, 37, 38). These sources of neuregulin may play an important role in paracrine and autocrine signaling in the heart, regulating both myocyte and endothelial cell structure and function. The finding that ErbB2 and ErbB4 expression is maintained in adult rat hearts (38) and our data showing that neuregulin can induce neovascularization in adult rats suggest that this signaling pathway may function both in embryonic and adult cardiovascular tissues.

Finally, neuregulin stimulation of the ErbB2, ErbB3, and ErbB4 receptors results in activation of several signaling pathways that are shared by other tyrosine kinase receptors involved in angiogenesis, such as the VEGF receptors (Flt-1 and Flk-1/KDR) and the Eph receptors (ELK and Eck), including the mitogen-activated protein kinase and c-Jun kinase pathways (10, 12, 33). These shared signaling pathways may provide valuable clues about the fundamental mechanisms regulating endothelial cell proliferation, migration, and angiogenesis.

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Address for reprint requests and other correspondence: J. R. Bender, Boyer Center for Molecular Medicine, 454C, Yale Univ. School of Medicine, 295 Congress Ave., New Haven, CT 06536-0812 (E-mail: jeffrey.bender@yale.edu).

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REFERENCES


11. Graus-Porta, D., R. Beerli, J. Daly, and N. Hynes. ErbB-2, the preferred heterodimerization partner of all erbB receptors, is a mediator of lateral signaling. EMBO J. 16: 1647–1655, 1997.


