Introduction

Signal transduction mediated by tyrosine kinase growth factor receptor is one of the important pathways for cellular proliferation. Many oncogenes are included in this signal transduction, leading to the idea that inhibitors of these signaling molecules can be used for cancer therapy. On the other hand, growth factor itself has not been applied to clinical medicine for a long time even though it has strong biologic activity. Growth factors have been thought to have deleterious effects for promoting tumor formation and proliferation for clinical use. However, these effects have recently been proven to be minimal because of the downregulation of its receptor, although several specific growth factors such as erythropoietin and granulocyte colony-stimulating factor have started to be used clinically, and their effects were proved to be significant and remarkable. Thinking of their specificity and strong effects, growth factors are expected to be used for further clinical applications.

Neurons and cardiomyocytes are nonproliferative cells; however, these cells are known to require growth signals for their survival. In neurons, a substance called neurotrophin is reported to be essential for their survival; however, in cardiomyocytes, it is unknown what signal is essential for their survival. Cardiomyocytes stop their proliferation soon after birth and their number does not change much throughout life. This limited proliferation ability causes difficulty in repair of heart muscles after injuries such as ischemia-reperfusion injury. We need to investigate how the proliferation and growth of cardiomyocytes may be induced. Several investigators including ourselves suggest that heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) is the potential candidate for cell growth and wound healing.

In this review, we summarize the function of HB-EGF, which is one of the EGF family of growth factors,
especially for the metabolism of cardiomyocytes and discuss the probability of the clinical application of HB-EGF in heart failure.

**HB-EGF and Signaling of ErbB Family Receptors**

EGF growth factor family molecules including HB-EGF have ErbB family as their receptors. The ErbB family of receptor tyrosine kinases (RTKs) has fundamental roles in development, proliferation, and differentiation of various organs. There are 4 members of the RTK ErbB family, EGFR/ErbB1/HER1, ErbB2/HER2/neu, ErbB3/HER3, and ErbB4/HER4. EGF family ligands bind to and activate their receptors by inducing the formation of either homodimers or heterodimers, resulting in autophosphorylation of specific tyrosine residues within the cytoplasmic domain. The phosphorylated tyrosine residues bind to the adapter proteins, which are instrumental in mediating downstream signaling pathways that determine the biologic activity of EGF growth factor family molecules.

In vertebrates, the EGF family of ligands binds to ErbB receptors with some degree of preference. EGF, transforming growth factor-α (TGF-α), and amphiregulin bind to ErbB1; HB-EGF, epiregulin, and betacellulin bind to both ErbB1 and ErbB4; NRG-1 (neuregulin/hergulin/NDF) and NRG-2 bind to ErbB3 and ErbB4; NRG-3 and NRG-4 bind to ErbB4 but not to ErbB3. Although no ligand for ErbB2 has yet been described, ErbB2 is active as a signaling receptor by forming heterodimers with other ErbB receptors.

HB-EGF is synthesized as a type I transmembrane protein (proHB-EGF) composed of signal peptide, heparin-binding, EGF-like, juxtamembrane, transmembrane, and cytoplasmic domains. The membrane-bound proHB-EGF is cleaved at the juxtamembrane domain, resulting in the shedding of soluble HB-EGF. A similar mechanism regulates other EGF family ligands and determines the specific role at their target site. The full-length proHB-EGF is biologically active as a juxtacrine growth factor that transmits the signals to neighboring cells in a nondiffusible manner. ProHB-EGF forms complexes with CD9 on the cell membrane. ProHB-EGF is also the receptor for diphtheria toxin (DT), and mediates the entry of DT into the cytoplasm. Soluble HB-EGF is a potent mitogen and chemoattractant for a number of cell types including vascular smooth muscle cells (VSMCs), fibroblasts, and keratinocytes. HB-EGF has been implicated in many physiologic and pathologic processes, which include wound healing, cardiac hypertrophy, smooth muscle cell hyperplasia, kidney collecting duct morphogenesis, blastocyst implantation, pulmonary hypertension, and oncogenic transformation.

Gene-targeted mouse studies indicate that the ErbB RTKs are critical for normal heart development. ErbB1 null mice with a CD1 background, and ErbB1 mutant (waved-2) mice exhibit semilunar valve enlargement. ErbB2 null mouse embryos die from a defect in trabeculae formation. The phenotype of ErbB4 knockout mice is also embryonically lethal due to a similar abnormal trabeculae formation as are mice lacking NRG, the ErbB4 ligand. Disruption of the ErbB3 gene causes heart valve malformation, whereas trabeculae formation is not affected. The ErbB family is required also for the maintenance of normal heart function in the adult. Ventricle-specific conditional ErbB2 knockout mice have severe heart failure with dilated ventricles and decreased contractility function. These symptoms resemble human dilated cardiomyopathy. These lines of evidence strongly suggest the tight relationship between EGF growth factor signal and cardiac development.

**HB-EGF and Cardiac Hypertrophy**

The important role of HB-EGF on cardiomyocytes was first disclosed by an accidental discovery during the screening of metalloprotease inhibitors for drugs to treat diabetic mice. One of the novel metalloprotease inhibitors called KBR-7785 strongly inhibited cardiac hypertrophy in vivo. KBR-7785 also inhibited cardiac hypertrophy done by aortic banding of mice. These data suggested that metalloprotease activity is involved in cardiac hypertrophy. At the same time, Prenzel et al. reported that ErbB1 phosphorylation by endothelin was mediated by cleaved HB-EGF through membrane-anchored metalloprotease in tumor cells. In this report, metalloprotease inhibitor completely inhibited ErbB1 phosphorylation by G protein coupled receptor (GPCR) agonists such as endothelin or thrombin. They concluded that GPCR agonists activated cell membrane metalloprotease and cleaved HB-EGF and released HB-EGF that binds to ErbB1. This ErbB1 phosphorylation by GPCR agonists is called transactivation because GPCR
agonists do not directly bind to ErbB1. The transactivation of EGFR by GPCR agonists was first reported in 199637. However, it was surprising that this transactivation was mediated by cleaved growth factor from cell membrane. Since GPCR agonists were also known to induce cardiac hypertrophy38, metalloprotease KBR-7785 was thought to inhibit GPCR agonist-induced cardiac hypertrophy by preventing cleavage of HB-EGF.

Substantial evidence indicates the existence of RTKs, including ErbB1, in the heart24,35,39,40. Using rat neonatal cardiomyocytes, EGFR phosphorylation by GPCR agonists was examined and it was observed that HB-EGF phosphorylates its receptor (ErbB1). At the same time, GPCR agonists phosphorylate ErbB1. This transactivation was completely blocked by the metalloprotease inhibitor KBR-7785 or neutralizing antibody for HB-EGF. Both KBR-7785 and the neutralizing antibody for HB-EGF also attenuated the hypertrophy of cardiomyocytes mediated by either aortic banding or GPCR agonists in vivo. These data suggest that GPCR agonists utilize HB-EGF-mediated transactivation as a common pathway for cardiac hypertrophy. Moreover, A disintegrin and metalloprotease (ADAM) 12 was identified as a major metalloprotease for HB-EGF cleaving in cardiomyocytes24. This unique signaling mechanism is illustrated in Figure 1. In this model, once extracellular stimuli are incorporated into the cell, then the inside-out signal as growth factor shedding occurs. Shedded growth factor binds and activates EGFR from outside of the cell membrane. This inside-out signaling seems rather ineffective; however, this mechanism may be more plausible than the intracellular transactivation of EGFR by GPCR agonists. EGF ligands including HB-EGF bind to EGFR from outside the cell membrane, and dynamically change the conformation of EGFR, leading to the autophosphorylation of EGFR dimer41–43. Thinking of the necessity of complex conformation change, activation of EGFR without the ligand binding is rather unlikely. Instead, shedding of membrane-anchored ligand is a more likely mechanism of transactivation of EGFR after the stimulation of GPCR. GPCR agonist regulates rapid response of cell activity such as contractility mediated by transient change of intracellular signal such as calcium44. Growth signaling is a rather slow cellular response. EGFR transactivation may be a shearing mechanism to handle different cellular responses to GPCR agonists.

Figure 1. G protein coupled receptor (GPCR) agonists activate membrane-anchored A disintegrin and metalloprotease (ADAM) 12, which is a cleaved enzyme for membrane-anchored heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF). Cleaved HB-EGF binds and activates EGFR receptor from outside of the cell membrane. Growth signal mediated by downstream signal such as ERK-1,2 induces cardiac hypertrophy. Metalloprotease inhibitor KBR-7785 inhibits ADAM12, resulting in unsuccessful cleaving of HB-EGF from cell membrane after GPCR ligand stimulation.
HB-EGF, Transactivation, and Cardiac Hypertrophy

ErbB Receptor Transactivation and Ectodomain Shedding of HB-EGF

Indeed, many of the mitogenic effects of GPCR agonists are mediated through transactivation of EGFR. Besides the heart, the inhibition of EGFR kinase using the selective inhibitor AG1478 abolishes GPCR agonist-mediated downstream signaling such as activation of ERK-1,2 in several cell types, including VSMCs and cardiac myocytes, and growth and migration of VSMCs. Thus, the transactivation of EGFRs by GPCRs explains the majority of growth-promoting responses. EGFR, an RTK, is endogenously expressed in numerous cell types and is an important factor in the control of many fundamental cellular processes, including cellular migration, metabolism, and survival, in addition to cellular proliferation and differentiation. RTKs possess an extracellular ligand-binding domain connected to the cytoplasmic domain by a single transmembrane helix. The cytoplasmic domain contains a conserved protein kinase core and additional regulatory sequences that are subjected to autophosphorylation and phosphorylation by other protein kinases. Overexpression of EGFR often results in uncontrolled growth and tissue remodeling. Not surprisingly, EGFR and its ligands are frequently upregulated in human cancers. Consequently, blocking the proliferative effects of EGFR activation has potential therapeutic applications in cancer and aberrant tissue growth. Then, a part of the effect of GPCR agonists might be mediated by EGFR kinase activated by truncated EGFR ligand family.

Ectodomain shedding of proHB-EGF is induced by various stimuli including phorbol esters, calcium ionophore, and lysophosphatidic acid. Multiple signaling cascades such as the mitogen-activated protein kinase and protein kinase C pathways appear to regulate HB-EGF shedding. Metalloproteases were implicated as the protease for shedding of proHB-EGF since various metalloprotease inhibitors inhibited ectodomain shedding of HB-EGF efficiently. Matrix metalloprotease (MMP) 3, MMP7, ADAM9, ADAM10, ADAM12, and ADAM17 have been implicated as responsible proteases. The specific proteases involved in the shedding process of HB-EGF depend on cell type and biologic environment. For example, MMP7 appears to be involved in the shedding process in epithelial cells, especially on the apical surface of cells. On the other hand, ADAM12 seems to be responsible for HB-EGF shedding in cardiomyocytes. Pro148-Val149 and Glu151-Asn152 have been suggested as the shedding sites of proHB-EGF. Highly specific HB-EGF shedding inhibitors have been found by a screening of hydroxamic acid-based compounds. They inhibit cutaneous wound healing and cardiac hypertrophy in mice and submandibular gland development in organ culture, suggesting that ectodomain shedding of HB-EGF is essential for various pathophysiologic processes and that its inhibition might be a novel therapeutic strategy.

Cardiac Phenotype of HB-EGF Gene-targeting Mouse

To further clarify the in vivo role of HB-EGF in cardiac metabolism, a gene-targeting mouse of HB-EGF was established. One of the HB-EGF gene aberrant mice was designed to make a mouse in which HB-EGF was not cleaved from cell membrane (HB-EGFuc/uc). An uncleavable form of proHB-EGF was generated by creating double point mutations in the juxtamembrane domain. HB-UC (a product of HBuc) was also resistant to ectodomain shedding in response to various shedding-inducing stimuli, while the other biologic properties of HB-UC were similar to those of WT proHB-EGF. To assess the biologic significance of proHB-EGF ectodomain shedding, mutant mice expressing HB-UC instead of WT proHB-EGF were also generated by targeted replacement of the proHB-EGF gene with HBuc complementary deoxyribonucleic acid. Homozygous mice (HBuc/uc) were born at the predicted Mendelian frequency. Northern blotting of the transcripts obtained from adult mice indicated that the WT and HBuc alleles were expressed equally in the heart, lung, and kidney. Uncleavability of HB-EGF was also confirmed in vivo. HB-EGFuc/uc has a relatively short life and showed remarkable cardiac specific phenotype even though HB-EGF was replaced by an uncleavable form in the whole body. At 12 weeks, the hearts of HB-EGFpro/pro mice were dilated and showed diminished movement. Histologically, the cardiomyocytes of HB-EGFuc/uc mice were degraded and replaced with fibrosis. Echocardiography showed that the ejection fraction of HB-EGFuc/uc mice was severely diminished. These phenotypic changes are quite similar to human dilated cardiomyopathy.

HB-EGF null mice (HB-EGFdel/del) were also produced by gene targeting and they showed similar heart failure. As with HB-EGFuc/uc mice, HB-EGF null mice...
showed obvious phenotype only in the heart, even though HB-EGF was absent in the whole body. Although studies of HBuc/uc mice indicated that ectodomain shedding of proHB-EGF and release of sHB-EGF are necessary for proper HB-EGF function in vivo, the physiologic importance of the control of proHB-EGF ectodomain shedding remains unclear.

To address this issue, another mouse mutant that only expresses sHB-EGF was designed. The transmembrane domain-truncated mutant (HB tm) was generated by insertion of a stop codon in the major site for proHB-EGF processing. Mitogenic activity was similar between HB-TM (product of HB tm) and WT sHB-EGF derived from proHB-EGF shedding, but HB-TM is secreted at much higher levels than WT sHB-EGF. The targeting construct for HB tm is similar to that for HBuc. One allele of the proHB-EGF gene in embryo stem (ES) cells was replaced with HB tm through homologous recombination. Chimeric mice carrying HB tm were generated from these ES clones. Most chimeric founders exhibited abnormally small bodies and thickened skin. Morphologic abnormalities were also found in the heart of HB tm mice. Histologic specimens showed ventricular hypertrophy in hearts from HB tm E16.5 embryos. The cardiac muscle fibers in chimeric mice were loose, with enlarged nuclei. These results suggest that the expression of HB tm reduces cardiomyocyte differentiation or terminates proliferation. These cardiac developmental abnormalities may be the primary cause of early death in HB tm mice. The phenotype observed in HB tm mice is likely due to deregulated secretion of sHB-EGF. Under normal conditions, a portion of proHB-EGF molecules is converted to sHB-EGF, but the majority of proHB-EGF molecules on the cell surface seem to be internalized without shedding. In the case of HB tm, most synthesized molecules would be secreted without shedding, resulting in oversecretion of sHB-EGF even though the native HB-EGF promoter regulates gene expression. Therefore, deregulated secretion of sHB-EGF would result in severe cardiac hypertrophy. The present study thus confirms the notion that ectodomain shedding of proHB-EGF must be strictly controlled in vivo. Also, HB-EGF is an important growth regulator of cardiomyocytes. HB-EGF and its cleavage from cell membranes are important not only for cardiac hypertrophy by GPCR agonists but also fundamental metabolism for cardiomyocytes. HB-EGF might have the role of a survival factor, like neurotrophin in neurons, by exerting growth signal in cardiomyocytes.

### EGFR in Cardiomyocytes and HB-EGF

Transactivation of ErbB1 by GPCR agonists was stated above; however, there are 4 different subtypes of EGFRs that exist genetically. To understand the precise physiologic roles of HB-EGF, it is essential to examine which receptor subtypes are used in cardiomyocytes. Binding affinity and capacity for phosphorylation of EGFRs are different for each EGF family growth factor such as EGF and TGF-α. HB-EGF can bind to ErbB1, 3, and 4 and phosphorylates ErbB1, 2, and 3. ErbB4 is weakly phosphorylated by HB-EGF. Since ErbB3 is rarely expressed in cardiomyocytes, cardiac function mediated by HB-EGF is mainly mediated by ErbB1, 2, and 4. Indeed gene-targeting ErbB2 null mice showed the severe cardiac phenotype, suggesting that HB-EGF signal is partly mediated by ErbB2. Since ErbB2 does not have physiologic ligands, ErbB2 forms heterodimer with ErbB1 or 4. Therefore, ErbB2 is phosphorylated by binding of EGF family growth factor to ErbB1 or 4. In cardiomyocytes, HB-EGF can enhance the phosphorylation of ErbB1, 2, and 4. The in vivo phosphorylation of HB-EGF null mice (HB-EGFedel/del) heart showed that endogenous ErbB2 and 4 phosphorylation is attenuated. We are now generating mice expressing receptor-specific ligands in cardiomyocytes to rescue HB-EGF null mice to clarify which EGFRs are important for cardiac metabolism.

### Future Clinical Application of HB-EGF

There is clinical evidence implying the important role of HB-EGF in cardiac metabolism. Humanized antibody against ErbB2 (trastuzumab) is clinically used for the treatment of advanced breast cancer all over the world. In an early clinical trial, the combined therapy of trastuzumab with anthracycline worsened heart function in 28% of patients. Trastuzumab possibly enhanced the cardiac toxicity of anthracycline. Since ErbB2 is an important signaling molecule for HB-EGF, blocking of ErbB2 signal by trastuzumab might cause the impairment of HB-EGF signal, resulting in the enhancement of cardiac toxicity. Anthracycline is reported to induce cardiac toxicity by oxiradical. Cardiomyocytes damaged by this oxiradical might require more HB-EGF to maintain functional metabolism. ErbB2 antibody trastuzumab is now prohibited for combined use with anthracycline. In such cases, HB-EGF
administration could improve the cell damage. Also, in case of other damage of cardiomyocytes such as ischemia or myocarditis, HB-EGF could help in the recovery of surviving cardiomyocytes. Growth factor therapy still has some difficult aspects, especially concerning tumor progression; however, its beneficial effect to tissue is potent. Clinical trials using growth hormone or insulin-like growth factor for heart failure is already in progress. We are still examining the biochemical property of EGF signaling using HB-EGF-targeting mice, seeking the possibility of growth factor therapy for heart failure.

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References