Chapter 1

POLYPEPTIDE GROWTH FACTORS AND THEIR RECEPTORS

Roles in Signaling and Cancer Therapy

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1. INTRODUCTION

Cellular proliferation and survival are tightly controlled processes. Extracellular stimuli, such as cytokines and growth factors, provide signals to target cells, which regulate cell cycle transition and also protect cells from undergoing apoptosis. Cytokines are polypeptide growth factors that could be either secreted or membrane-bound and regulate the growth, differentiation, and activation of various cell types. On the target cells, cytokines bind to its receptors, which are often composed of two or more subunits. Binding of the cytokines to their cognate receptors activates downstream signaling events that result in the required biological response. Although cytokine receptors do not possess intrinsic kinase activity, they signal in analogous fashion to receptor tyrosine kinases. Epidermal growth factor (EGF) is one of the well-studied prototype polypeptide growth factor with a role in mitogenesis. Since the epidermal growth factor receptor (EGFR) was the first receptor tyrosine kinase to be discovered and remains the most investigated, with most of the mechanistic principles of receptor tyrosine kinases first established with EGFR as a model, this review will focus on EGF/EGFR and its family members as prototypes to elaborate the role of growth factor/cytokine ligands and receptors in cancer.

Recent advances in molecular and cellular biology led to identification of several structurally and functionally related molecules now collectively called the EGF family growth factors, each encoded by a distinct gene. A

common feature among the EGF family of polypeptides is the presence of six spaced cystines (Xn**C**X7**C**X2-3GX**C**X10-13 **C**X**C**X3YXGXR**C**X4LXn) in the EGF domain. These cystine residues form three disulfide bonds and thus, provide a specific secondary structure that is essential for the biological activity of the polypeptides (1). Currently, the EGF family of growth factors consists seven members- EGF, transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epiregulin (ER), and heregulins (HRG). The EGF family of ligands binds to transmembrane receptor tyrosine kinases, commonly known as HER receptors (**H**uman **E**pidermal growth factor **R**eceptor).

The EGFR family comprises four distinct receptors: EGFR/ErbB-1, HER2/ErbB-2, HER3/ErbB-3 and HER4/ErbB-4. HER1 is a single pass transmembrane receptor with two extracellular, cysteine-rich regions involved in ligand binding, and intervening region important for receptor dimerization, an intracellular tyrosine kinase domain, and a number of intracellular sites for autophosphorylation, phosphorylation by other kinases, and docking of intracellular signaling components. A range of growth factors serves as ligands for these receptors with the exception of HER2. No ligand has been identified for the HER2 receptor. HER receptors exist both as monomers and dimers, either homo- or heterodimers. The regulation of HER family members by the EGF family of ligands is complex, as binding of ligands to these receptors can lead to the formation of multiple distinct homodimers or heterodimers among the HER receptors and thus presumably, engagement of distinct signaling pathways. Ligand binding to HER1, HER3 or HER4 induces rapid receptor dimerization, with a marked preference for HER2 as a dimer partner (2). HER-2-containing heterodimers are characterized by extremely high signaling potency because HER-2 dramatically reduces the rate of ligand dissociation, allowing strong and prolonged activation of downstream signaling pathways.

The key role of the HER family of receptors in cancer has been widely acknowledged. Overexpression, activating mutations and gene amplification of members of the ErbB family is frequently found in malignant situations, which would suggest that they play some part in tumorigenesis and also in the transition from early disease to more aggressive forms. Studies demonstrated that HER kinases transform cells by enhancing cell-cycle progression by modulating the function of cyclin D1 and CDK inhibitors, p21Cip1/WAF1 and p27Kip1, via Akt and MAPK signaling pathways, respectively (3,4). Therapeutic strategies designed to target and inhibit HER activation are in clinical development and is the subject of a number of ongoing clinical trials.

2. EGFR AND HER3 DOMAIN STRUCTURE

In common with other receptor tyrosine kinases, the HER family receptors are cell surface allosteric enzymes consisting of a single transmembrane domain that separates an intracellular kinase domain from an extracellular ligand-binding domain. Numerous theories have been postulated regarding the stoichiometry of ligand and receptor in various receptor dimers, the mechanism underlying the preferred HER2 heterodimerization among HER family members and domains involved in ligand binding. Elucidation of the crystal structure of HER3 (5) and EGFR (6,7) provided the critical evidence necessary for our understanding of the functioning of these receptors and has been reviewed elsewhere (8). In brief, a molecule of the ligand (i.e. EGF) binds to a molecule of EGFR to form a stable 1:1 EGF: EGFR intermediate and dimerization of EGFR requires the binding of two such intermediates in a 2:2 EGFR:EGFR complex (7). This leads to the exposure of a critical dimerization loop that allows their juxtaposed intracellular portions to transphosphorylate each other on certain tyrosine residues. This dimerization loop sequence is conserved in the other HER family members and allows for transactivation of the various family members in all possible combinations of homo- and heterodimerization. Our improved understanding of the ligand-induced receptor activation and dimerization may lead to novel therapeutic strategies in the treatment of dysregulated HER family signaling.

3. HETEROLOGOUS TRANSACTIVATION

The role of HER family receptor tyrosine kinases in signaling is traditionally viewed as being exclusively at the level of the membrane, whereby the receptor transfers the signal represented by ligand binding from the external cell surface, across the membrane, to within the cells. Ligand binding induces receptor dimerization and autophosphorylation, association of a variety of signaling molecules and adaptor molecules and the tyrosine phosphorylation of cellular substrates by the receptor or associated kinases to trigger intracellular kinase/phosphorylation cascades. This ultimately leads to translocation of the kinases from the cytoplasm to the nucleus/nuclear envelope. Subsequent phosphorylation and activation of nuclear transcription factors enables the response to the initial signal to be effected at the level of gene expression (reviewed in 9). However, there is mounting evidence that the HER family receptors propagate not only signals initiated by their own ligands but also act as a point of integration for signals and cross-talk with various heterologous receptors. These trans-regulatory

interactions can be mediated through increased receptor ligand availability, direct phosphorylation of HERs by protein tyrosine kinases, and via novel heterodimerization partners and transactivation. The net result of these alternative activation strategies is enhanced HER signaling to multiple cell regulatory pathways.

The most extensively studied mechanism of HER family transactivation involves activation of G protein coupled receptors (GPRs). GPRs that are activated by ligands like lysophosphatidic acid (LPA), carbachol and thrombin can in turn activate either matrix metalloproteases (MMPs), which cleave EGF-like ligands thus freeing them for receptor activation, or cytoplasmic kinases such as Src and Jak2, which directly phosphorylate and activate EGFR. Another cytokine, interleukin-6, elevates tyrosine phosphorylation of ErbB2 by increasing its intrinsic catalytic activity. Signaling events of other classes of receptors can also indirectly increase or decrease receptor phosphorylation through activation of additional kinases or phosphatases and thus influence HER-mediated cell signaling (9). These interconnections to other signaling modules help to integrate and coordinate cellular responses to extracellular stimuli.

EGFR transactivation can also be mediated by prostaglandin (PG) E_2 (10). This novel activation pathway seems to be similar to that described for GPRs. PGs activate Src kinase that in turn activates MMPs which now release a tethered EGFR ligand, TGFα from the cell membrane, thus initiating ligand-mediated activation of EGFR (6). This new data may complete a positive feedback loop for HER family-mediated cellular growth regulation. COX2 converts arachidonic acid to prostaglandins and a growing body of evidence indicates that COX2-derived prostaglandins can promote angiogenesis and the invasiveness of colorectal and other types of cancers. A number of studies have indicated that the HER and COX2 pathways may be interconnected. For example, studies have shown that HER2 overexpression is associated with COX2 overexpression in breast, colon, prostate and pancreatic cancer (11). Furthermore, suppression of COX2 results in decreased HER2 tyrosine-kinase activity, while activation of the HER2/HER3 signaling pathway has been shown to be associated with expression of COX2. Taken together, these data provide a basis for investigating the combination of HER family inhibitors with COX2 inhibitors in the clinical setting. The selective COX2 inhibitor celecoxib was recently reported to decrease mammary tumor incidence and PGE_2 levels in mouse mammary tumor virus/HER2 transgenic mice (12). These new reports offer hope that selective targets could be used for therapy in the clinics.

Direct association and activation of EGFR and HER2 by cytokine receptors for growth hormone and interleukin 6, respectively has been demonstrated (8). Further, new reports indicate that EGFR is involved in

downstream signaling cascade initiated by the protease urokinase plasminogen activator (uPA), its cell surface receptor (uPAR), and integrins. uPA/uPAR overexpression has been implicated in progression and metastasis of a variety of tumors and is a predictor of poor prognosis (13). Overexpression of uPAR in Hep3 human carcinoma cells stimulates the $\alpha_5\beta_1$ integrin complex to activate EGFR upon binding to fibronectin (14) via focal adhesion kinase. The fibronectin-stimulated EGFR activity was independent of EGFR overexpression or the release of EGF-like ligands, but was rather dependent upon overexpression of uPAR and a functional uPA/uPAR/integrin complex. Thus overexpression of the receptor alone may not account for all aberrant HER family receptor activation. Rather, screening for the expression and activity of HER family transactivation partners in developing malignancies may help in the use of specific, targeted therapies. It was recently reported that dual inhibition of focal adhesion kinase and EGFR signaling cooperatively enhance apoptosis in breast cancer cells (15). The emerging central role of HER family kinases as integrators of diverse signals stresses the importance of these receptors as therapeutic targets.

Delineation of cytokine signaling pathways that control cellular growth, differentiation, survival and development has defned a novel class of proteins known as STATs that regulate these processes by modulating the expression of specifc target genes. STAT proteins are activated by cytokine engagement of cognate cell surface receptors and induce the expression of ligand-dependent genetic programs that determine the biological response to the stimulus. Although originally discovered as effectors of normal cytokine signaling, subsequent studies have demonstrated the participation of STATs in signaling by polypeptide growth factors and oncoproteins. Signifcantly, constitutive Stat3 activation in human breast cancer cells correlates with elevated EGF receptor and c-Src expression or activity (16). Using specifc TK-selective inhibitors, inhibition of signaling downstream of Src or JAKs was shown to abrogate constitutive Stat3 DNA-binding, inhibit cell proliferation and induce apoptosis in model human breast carcinoma cell lines (17,18). Since EGF can `super-activate' STATs in human breast cancer cells, it is possible that activated STATs participate in cooperative oncogenic signaling by EGF receptor and c-Src as a result of the aberrant expression of EGF-related ligands within the mammary gland microenvironment during breast cancer progression. Thus, better understanding of the mechanisms underlying aberrant STAT signaling during oncogensis may lead to the development of novel cancer therapies based on interrupting key steps in this pathway.

4. NUCLEAR FUNCTIONS FOR EGFR FAMILY MEMBERS

Although many of the changes that are elicited by signaling cascades occur in the cytosol, it becomes increasingly clear that growth-factor-receptor signaling also greatly affects nuclear events such as mitogenesis and changes in transcription. These signals were thought to be transmitted through multistep cascades, such as ERK translocation into the nucleus. However, a new mode of growth factor signalling to the nucleus was discerned when it was reported that EGFR activates STAT proteins. Excitingly, recent reports have opened up the possibility of a third mode of transcription activation, one that requires 'zero transfers' of information between the plasma membrane and the nucleus — direct nuclear translocation of full-length growth-factor receptors or fragments of them (19). Although nuclear localization of EGFR had been noted in previous publications, Lin et al. (19) reported that an EGFR receptor variant could be internalized and transported to the nucleus. Interestingly, this accumulation also required both ligand and full-length, membrane-integral EGFR (20). Both intracellular and extracellular domains of EGFR appear to move to the nucleus in a ligand-bound form and the proposed mechanisms remain speculative and thus need to be demonstrated.It has also been proposed that EGFR may transport STAT-1 from the cytosol into the nucleus (21) after tyrosine-phosphorylating it, which could then carry out its functions as a transcription factor.

ErbB-4, the most recently identified HER family member, was demonstrated to be proteolytically processed. Ectodomain cleavage involves TACE (a metalloprotease), while intramembrane proteolysis is affected by γ-secretase (PS-1). Processing by either route produces the cytosolic ErbB-4 fragment (s80), which translocates to the nucleus (22). This carboxyl-terminal HER4 fragment then associates with the WW-domain-containing transcriptional regulatory protein YAP (Yes-associated protein) and acts as a co-transcriptional activator (23). Thus, in addition to initiating numerous cytoplasmic signaling cascades upon activation, HER family members may directly influence transcriptional activity and nuclear function via translocation to the nuclear compartment.

5. HER FAMILY RECEPTORS AS TARGETS FOR CANCER THERAPEUTICS

HER family receptors and their ligands are frequently dysregulated in a number of tumor types and therefore might play an important role in the

pathogenesis of these diseases. This clearly suggests that ErbB receptors and their cognate ligands represent suitable targets for experimental therapeutic approaches in human tumors. Novel agents that modulate signaling through HER family receptors have recently emerged as promising therapies for primary or adjuvant cancer treatment. These new agents are the subjects of several recent reviews (24, 26). A number of strategies have been developed that target various components of the HER-kinase axis. These therapies may be divided into two basic strategies: (1) antibody-based inhibition of HER-kinase receptors, and (2) small-molecule inhibitors of the tyrosine kinase activity of HER-family receptors. Anti-receptor antibodies including C225 (Erbitux, against EGFR) and 4D5 (Trastuzumab or Herceptin, against HER2) bind to the receptor extracellular domain and induce internalization and degradation, thus effectively blocking receptor activation of subsequent cellular signaling cascades. Small molecule inhibitors interact with the extracellular domain and effectively block ligand binding, or act against the cytoplasmic tyrosine kinase domain and inhibit receptor tyrosine phosphorylation and cytoplasmic signaling. Other approaches include toxins conjugated to anti-receptor antibodies or receptor ligands, antisense therapies, and directed transcriptional repression to down regulate receptor or ligand expression. Most of these agents target EGFR or HER2, since these receptors are most often disregulated in human cancers.

Research on HER family inhibitors is rapidly evolving, with many new compounds in preclinical and clinical development. Several monoclonal antibodies (mAbs) targeted toward the EGFR extracellular region have been produced with the most recent ones being EGFR (EMD 55900) (27) and HER2 (2C4) (28) monoclonal antibodies.

As tyrosine kinase activity is required for EGFR-mediated tumorigenicity, therapies that ablate this function are currently being tested in clinical trials. Mutations in the EGFR ATP-binding site were shown to eliminate receptor kinase activity and prevent cellular transformation. Thus, small molecule tyrosine kinase inhibitors (TKIs) that competitively block ATP binding were designed as potential anticancer agents. Importantly, since these agents target an intracellular region of the EGFR, they could potentially inhibit the highly tumorigenic EGFR mutant vIII, which is a truncated receptor frequently found in breast cancer and may be inaccessible to mAbs. Quinazoline compounds represent a class of competitive inhibitors of the ATP-binding site that are orally active, potent, and selective tyrosine kinase inhibitors. Among the most widely examined thus far are the EGFR-specific ZD1839 and OSI-774 (29), both EGFR and HER2 (PKI-166 (30) and GW572016 (31)) and an inhibitor of all four Her family receptors (called a pan-Her inhibitor), CI-1033 (32). Of the HER family-directed therapies, ZD1839 (IRESSA), a substituted aniloquinazoline, has progressed the

furthest in clinical development. IRESSA is effective against numerous tumor types in preclinical testing (33,34). This EGFR tyrosine kinase inhibitor has shown consistent and clinically meaningful disease stabilization and a low frequency of regression across a variety of tumor types, with manageable side effects (35-37). ZD1839 recently received FDA approval for use as a third line therapy in treating non-small cell lung cancer (38). As clinical experience with these and other new inhibitors increases, the ability to direct therapies towards individuals with specific HER family and genetic alterations may be possible.

Evidence that HER2 overexpression correlates with poor clinical outcome, the existence of cross-talk between the HER2 and ER signalling pathways in breast cancer, and the lack of benefit achieved with hormonal therapy in patients with ER-positive/HER2-positive disease, and hence the fact that these patients are receiving sub-optimal treatment, suggests that combining treatments that target these different pathways may provide additional clinical benefits for patients with breast cancer. Twenty percent to 30% of human breast cancers overexpress ErbB-2, usually as a result of gene amplification. ErbB-2 expression is more common in ER^- and PgR^- breast cancers, and these cancers naturally exhibit endocrine therapy resistance because of the absence of the relevant target. Indeed, ErbB-2-activated mitogen-activated protein kinase (MAPK) signaling may be directly responsible for ER downregulation (39).

An example of rationale combinatorial chemotherapy is choice of inhibitors of growth factor signaling and angiogenesis for dual targeted therapy. Preliminary translational laboratory studies provided molecular data showing a clear link between HER2 overexpression and VEGF production in human breast cancer cells. Overexpression of HER2 in human tumor cells is closely associated with increased angiogenesis and expression of vascular endothelial growth factor (VEGF). This effect on VEGF expression may be mediated via upregulation of hypoxia-inducible factor 1 alpha or activation of p21-activated kinase (Pak), a transcriptional activator and intracellular signaling molecule, respectively, that help control VEGF gene expression (40,41). Indeed, when the VEGF pathway is inhibited, tumor growth is suppressed. The anti-HER2 blocking antibody trastuzumab has been shown to inhibit tumor cell growth and VEGF expression. Cancer cell invasiveness can be promoted, even in the absence of HER2 overexpression, by transregulation of HER2 by heregulins that bind to HER3 and HER4. Accordingly, heregulin beta1 regulates the expression and secretion of VEGF in breast cancer cells, and trastuzumab inhibits heregulin-mediated angiogenesis both in vitro and in vivo (41, 42). The strategy of dual inhibition has also proven effective with antibodies against EGFR and

VEGF in pancreatic cancer (43). Thus, potential upregulation of VEGF in cancer epithelial cells likely supports angiogenesis, sustaining and promoting survival and metastasis of tumor cells.

A variety of novel studies have elaborated on the complexity of ErbB family proteins and open up new windows for therapeutic intervention. Protein core of decorin, a prototype member of an expanding family of small leucine-rich proteoglycans, binds to a discrete region of the EGFR, partially overlapping with but distinct from the EGF-binding epitope. Decorin interacts with the EGFR in a protracted way, leading to a sustained down-regulation of EGFR kinase activity (44). This antagonist to EGFR signaling may be a key negative regulator of tumor growth. Future investigations may lead to the generation of protein mimetics that could antagonize EGFR activity in a variety of tumors in which EGFR is overexpressed. Also, a recent report of down regulation of EGFR-mediated growth-promoting signals by treatment with 1,25-dihydroxyvitamin D-3 (45) opens up new possibilities for EGFR regulation.

Recently, the histone deacetylase inhibitors sodium butyrate and trichostatin A were identified as potent and relatively specific ErbB2 promoter-inhibiting agents (46). This finding indicates that human breast cancers with ErbB2 amplification and overexpression represent unusually sensitive clinical targets for HDAC inhibitor therapy. HER2/*neu* overexpression could also be repressed by attenuating the promoter activity of the HER2/*neu* gene by potent transcriptional regulators like the adenovirus type 5 *E1A* (47). Targeted disruption of transcriptional complexes essential for HER2 expression using short, cell-permeable peptides has also been demonstrated (48).

Finally, new insights into protein turnover and targeted degradation could lead to novel therapies. Csk homologous kinase (CHK) binds, via its SH2 domain, to Tyr1253 of the activated ErbB-2/neu and down-regulates the ErbB-2/neu-mediated activation of Src kinases, thereby inhibiting breast cancer cell growth. This data strongly suggest that CHK is a novel negative growth regulator in human breast cancer (49 and references therein). ERRP (EGFR-related protein), a recently identified negative regulator of EGFR modulates EGFR function in colorectal carcinogenesis and expression of EGFR was found to be inversely related to ERRP in representative samples of normal and neoplastic tissues (50). Re-expression of novel negative regulatory proteins or induced expression of high affinity inhibitory proteins may restore normal receptor homeostasis in a deregulated setting and serve as potential future therapies. Pharmacologic manipulation of ubiquitination and degradation via ubiquitin ligases such as CHIP (51) and NEDD4 (52) also provide new routes for stimulated downregulation of dysregulated HER family members. Evidence to date suggests that direct targeting of growth

factor receptors is a promising therapeutic strategy for breast cancers with abnormalities in these pathways. The challenge is to identify the patient population most likely to benefit from this biological therapy approach.

6. CONCLUSIONS

A large body of knowledge has been accumulating in recent years on the role of the EGF family of ligands and receptors in embryonic development, physiology and pathology and much progress has made in understanding the mechanism of EGFR activation upon ligand binding. The EGFR is a complex signaling system important in normal physiology and in the maintenance of the tumorigenic state. Recent research has strengthened the basis for an intimate role of HER family kinases in a variety of cancers. In addition to propagating cytoplasmic signaling initiated by HER family receptor ligands, HER family members can also propagate signals initiated by multiple other signaling pathways and may serve as central nodes in conveying extracellular signals. Attenuation of HER family signaling is a developing strategy for the management of human malignancies and is the subject of ongoing clinical trials and preclinical mechanistic investigations. Finally, since the life of a cell is controlled by more than one signaling network, resolution of interaction between EGFR proteins and G-protein coupled receptors, cytokine receptors, cell adhesion molecules and other networks and shedding light on the way the convergence of networks is integrated and translates into specific outputs could potentially lead to the development of more effective treatment strategies in aberrant pathological situations.

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