Review

Tyrosine kinase signalling in breast cancer ErbB family receptor tyrosine kinases

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Abstract

ERBB family receptor tyrosine kinases are overexpressed in a significant subset of breast cancers. One of these receptors, HER2/neu, or ErbB-2, is the target for a new rational therapeutic antibody, Herceptin. Other inhibitors that target this receptor, and another family member, the epidermal growth factor (EGF) receptor, are moving into clinical trials. Both of these receptors are sometimes overexpressed in breast cancer, and still subject to regulation by hormones and other physiological regulators. Optimal use of therapeutics targeting these receptors will require consideration of the several modes of regulation of these receptors and their interactions with steroid receptors.

Keywords: epidermal growth factor receptor, ErbB-2, ErbB-3, ErbB4, HER2/neu

Introduction

The close of the millenium is marked by unprecedented advances in our understanding of the mechanisms that underlie carcinogenesis. Each procarcinogenic pathway presents pharmacologists with an array of therapeutic targets. Most importantly, the first rational therapeutic agents targeted at oncogene products have now navigated the tortuous drug development pipeline. A monoclonal antibody, 'Herceptin', which binds to the receptor tyrosine kinase (RTK) Her-2/Neu/ErbB-2 is effective for treatment of a subset of patients with advanced breast cancer [1••,2••]. This antibody is at the vanguard of a host of therapeutic compounds that are under development to antagonize the activities of ErbB family receptors.

The development and application of clinical tools that intervene in ErbB-regulated processes will be most effective if close attention is paid to the biologic properties of these RTKs. Signaling through these receptors, even when they are overexpressed in tumors, is still subject to regulation. This means that there are potentially many points of vulnerability at which therapeutic interventions can be targeted. Furthermore, the ability of overexpressed ErbB family RTKs to alter responses to genotoxic drugs and to steroid hormones means that ErbB-targeted therapies will have complex interactions with existing breast cancer therapeutic agents.

Amplification of ErbB family receptors

The *ERBB* family consists of four closely related genes: *ERBB* (*HER*), *ERBB-2* (*HER-2*, *NEU*), *ERBB-3* (*HER-3*), and *ERBB-4* (*HER-4*). *ERBB* (*HER*) encodes the prototype EGF receptor, and the other genes encode structurally similar RTKs. *ERBB-2* is amplified (extensively reduplicated) in 15–30% of breast cancers [3••]. Amplification is more common in tumors from patients with lymph node metastases than in those from node-negative patients (for review [4•]). Amplification and/or overexpression of ErbB-2 occurs most often (up to 60%) in *in situ* carcinomas [4•,5], so ErbB-2 overexpression may be an early change. ErbB-2 overexpression in conjunction with amplification, and in another few percent of breast cancers without gene amplification, is further accentuated

EGF = epidermal growth factor; RTK = receptor tyrosine kinase; TGF = transforming growth factor.

by transcriptional upregulation of *ERBB*2, which is mediated by the transcription factor AP-2 [6].

The finding that a particular tumor type is associated with amplification of a particular gene suggests that the amplification incurs a selective advantage for the tumor. Amplicons may affect more than one gene, but *ERBB2* is a common element in 17q21 amplification. Overexpression of coamplified genes, including *GRB*7, may also contribute to selection for this genetic change [7,8].

EGF family receptors normally function in regulation of mammary growth and differentiation. Overexpression of ErbB-2 in tissue culture or in transgenic mouse mammary glands leads to transformation, tumorigenicity, and metastasis, which is unusual for RTKs that are overexpressed in the absence of ligands [9•,10]. These findings implicate ErbB-2 as a major player in initiation and/or progression of breast cancer. This conclusion has now been substantiated with the demonstrated efficacy of ErbB-2-targeted therapeutic antibodies.

Other ErbB family receptors apart from ErbB-2 are amplified or overexpressed in breast cancer. Amplification and overexpression of the EGF receptor is well documented, but there is a wide range in the reported frequencies of EGF receptor overexpression [3••,11•]. Overexpression of *ERBB3* has been reported in breast cancer [12,13••,14]. Thus, EGF receptor, ErbB-2, and ErbB-3 are positively regulated in breast cancer. In contrast, overexpression of ErbB-4 is uncommon in breast cancer, and ErbB4 expression may be suppressed in carcinoma [15,16]. This might indicate that ErbB4 is either unimportant, or actually antagonizes carcinogenesis. In fact, there is evidence that ErbB4 expression is associated with positive prognostic indicators in breast cancer [17,18].

Regulation of ErbB family receptors by peptide growth factors

EGF family receptors are directly regulated by binding of diverse polypeptide hormones that each contain a 6-kDa domain that is homologous to EGF (for review [19•,20•, 21•]). The ligands consist of amphiregulin, betacellulin, EGF, epiregulin, heparin-binding EGF-like growth factor, various forms of neuregulin (neuregulin-1, -2, -3, and -4), and transforming growth factor (TGF)-α. They have different abilities to bind to and activate the ErbB family receptors when expressed singly. For example, EGF binds to the EGF receptor, but not to ErbB2, ErbB3, or ErbB4, and neuregulin-1 binds to ErbB3 or ErbB4, but not to the EGF receptor [19•,20•]. EGF family receptors can also be activated indirectly by agonists that bind serpentine G protein-coupled receptors [22].

Ligand-dependent regulation of the ErbB family of receptors is expanded through the promiscuous formation of receptor heterodimers [19•,20•,23]. In cells that express EGF receptor and ErbB2, any of the EGF agonists will induce formation of EGF receptor–ErbB2 heterodimers, as well as EGF receptor–EGF receptor homodimers. This cross-activation extends to most of the receptor combinations, so that activation of one receptor will generally lead to some activation of other coexpressed ErbB family RTKs. Heteromerization can also enhance the affinity of ligand binding [24•,25]. Heteromerization with other ErbB family receptors is required for activation of ErbB-3, which is devoid of intrinsic catalytic activity [26].

ErbB-2 is an orphan receptor, because none of the soluble ligands bind to ErbB-2 that is expressed independently. ErbB-2 is strongly activated through interactions with other EGF family receptors [27••], however, and ligand-induced ErbB-2 heteromers are favored over other heteromers or homomers [28••]. Because ErbB-2 is jointly expressed with other ErbB family receptors, it can be thought of as a common subunit that expands the signaling repertoire of the other ErbB family receptors [20•].

Differential regulation of the receptors is important because each receptor has unique signaling properties [19•,20•,29]. ErbB-3 is an extreme case, because it has multiple phosphoinositide 3-kinase-binding sites, and couples strongly to this signaling molecule [30]. The response of cells depends on which of the receptors are activated. Depending on the specific cell context, activation of these receptors may promote proliferation, motility, differentiation, or even apoptosis [31–33]. On aggregate, these interactions may significantly add to, or even alter the response of cells to ligands [19•]. For example, ErbB2 greatly augments the amplitude and duration of mitogen-activated protein kinase activation by EGF or neuregulin [34••]. The differential activation of different receptors and receptor combinations, as well as the different signaling abilities of the four ErbB receptors, contributes to the extraordinary diversity of signals that can be regulated by the ligands. It also means that the response to a particular agonist is affected by the spectrum of agonistic hormones that regulate the system, because there may be inter-receptor competition for dimerization partners.

Activation of ErbB receptors induces dimerization and tyrosine phosphorylation. The activation-induced phosphopeptides recruit docking proteins that themselves convey the signal further. Although specific ErbB receptors have been traced to specific responses, less is known about how these responses correlate with recruitment of specific pathways and substrates. Once activated, signals are damped through ligand–receptor dissociation [25], through phosphorylation (eg by protein kinase C), and by receptor-mediated endocytosis, which can be followed by recycling to the cell surface, or proteolytic destruction of the receptor. The activities of tyrosine phosphatases,

including basal phosphatases and those that are recruited after ligand activation, will further influence the intensity and duration of signaling.

ErbB family receptors in mammary gland development

The frequent association of ErbB receptor signaling with breast cancer reflects the fact that the four ErbB receptors are involved in normal mammary gland development. The female mammary gland undergoes extensive postnatal development under the influence of systemic hormones. Peptide hormones including EGF family ligands, fibroblast growth factors [35], and insulin-like growth factor-I are thought to act under control of the systemic hormones as local mediators of mammary development. All four ErbB family receptors are expressed in the mammary gland of adult females, but EGF receptor and ErbB-2 are preferentially expressed in young females [36,37••,38••].

The first postnatal episode of mammary development occurs at puberty, and leads to elongation and branching of the mammary ducts to extend throughout the fatty mesenchyme. EGF receptor and ErbB-2 are present in stroma and epithelium, and are tyrosine-phosphorylated, which is indicative of signaling activity [37••,38••]. The mid-gestation lethality of disruptions in ErbB-2, ErbB-3, and ErbB-4 has hampered characterization of mammary functions of these receptors [39•–41•]. The EGF receptor is important at puberty, because expression of a dominant-negative EGF receptor impairs ductal morphogenesis [42••]. Surprisingly, reconstitution of stromal/epithelial tissue chimeras showed that EGF receptor is absolutely required in mesenchyme, but not in the epithelium [43••]. This does not rule out redundant functions for EGF receptor in the epithelium.

The second wave of activation (tyrosine phosphorylation) of ErbB family receptors occurs in pregnancy [37••,38••]. The activation occurs later than the major period of proliferation. Instead, ligand expression and receptor activation is most extensive late in pregnancy and after parturition. Dominant-negative ErbB-2 and ErbB-4 transgenes interfere with lobuloalveolar expansion and milk protein production early and late postpartum, respectively, which is consistent with the ability of neuregulin-activated ErbB-2 to drive mammary differentiation [36,44•,45••,46•].

At least five EGF family ligands are expressed at various phases of mammary development, where they may promote proliferation and/or differentiation [37••,38••]. Disruption of mouse amphiregulin, EGF, and TGF-α genes identified amphiregulin as a particularly important ligand for regulation of both ductal outgrowth and lactation. This is consistent with the high expression of amphiregulin in the mammary gland [47]. In both the amphiregulin knockout mice, and transgenic mice that express the ErbB-4 dominant-negative transgene, the lactational phenotype is associated with a failure to activate the important intracellular mediator of mammary development Stat5, an SH2 domain-containing transcription factor that regulates production of milk proteins [45••,48••].

The functions of ErbB receptors in the mammary gland may be connected with their roles in carcinogenesis. EGF agonists are mitogenic in the mammary fat pad of young females [49], and probably work by concomitant activation of EGF receptor and ErbB-2. The initial selection for overexpression of these receptors in cancer precursor cells might be based on mitogenic coupling of the receptors in ductal epithelial cells. The elongation of mammary ducts, which is impaired in amphiregulin-disrupted mice [48••], involves processes that mirror aspects of tumor invasion, including changes in intercellular adhesion, and motility. These changes, even divorced from mitogenic activity of the receptors, may be important in early stages of tumor progression. Interestingly, the proliferation and survival of cells in the mammary gland seems unaffected by disruption of amphiregulin. Similarly, defects in terminal differentiation and lactation of transgenic mice that express dominant-negative ErbB-2 and ErbB-4 occur after the major period of proliferation [44•,45••].

ErbB receptor activation and signaling heterogeneity

Activation of oncogenes such as *RAS* in human cancer is caused by structural mutations that strongly and constitutively activate Ras signaling. The tumor-associated changes in ErbB family receptors in breast cancer are generally changes in expression, however, so that normal modes of regulation are still in operation. Two tumors that overexpress an ErbB family receptor to the same degree may be influenced by the receptor in quantitatively or qualitatively different ways if other regulatory influences differ.

The fact that overexpressed ErbB receptors can still be regulated may facilitate drug development. Negative regulators of these receptors that may be worthy therapeutic targets or models include protein kinase C isoforms, tyrosine phosphatases, Cbl (which enhances EGF receptor turnover; alternative forms of erbB family receptors), and 'argos', a physiologic antagonist of the *Drosophila* EGF receptor [50•].

In tissue culture, ligand-independent activation of ErbB-2 is sufficient to transform cells even though overexpression of the EGF receptor has no effect [10]. Overexpression probably increases receptor phosphorylation and signaling by increasing the rate of inter-receptor collisions. Differences between transforming activities of ErbB-2 and the EGF receptor have been traced to specific portions of the intracellular domains, indicating that differential signaling qualities are important, and ErbB-2 may also be better

at ligand-independent dimerization [51,52]. Overexpression of wild-type ErbB-2 in the mammary glands of transgenic mice induces metastatic breast cancer [9•,53]. This animal model revealed that carcinogenesis initiated by overexpression of ErbB-2 is a multistep process. Tumorigenicity requires additional structural alterations of ErbB-2, mutations in other oncogenes, or excessive production of EGF agonists [54–56]. The structural changes are small in-frame deletions just outside of the ErbB-2 transmembrane domain. These deletions leave unpaired cysteine residues that cause constitutive dimerization and transformation mediated by disulfide bonds. Although such deletions have not been identified in human tumors, a splice variant may achieve the same result.

Alternative forms of ErbB family receptors

Variant forms of EGF receptor, ErbB-2, and ErbB-3 can be produced as a result of differential splicing or proteolytic processing. Oncogenic forms of the EGF receptor have been described, albeit seldomly in breast cancer (for review [57,58]). An alternative spliced form of ErbB-2 has also been described, in which the exon encoding protein sequences immediately preceding the transmembrane domain is skipped. The resulting polypeptide has an unpaired cysteine and, like the deletion-activated receptor in the mouse transgenic model, dimerizes constitutively and transforms cells [13••,59•]. Hence, this spliced variant mRNA may encode a subpopulation of ErbB-2 molecules that is primarily responsible for carcinogenesis.

Another set of variant molecules might be less active than the common receptor forms and dominantly inhibit signaling. Soluble extracellular domains of EGF receptor, ErbB-2, and ErbB-3 that are encoded by alternative spliced products may act as sinks or buffers that bind agonists [60,61]. They can be produced either through direct coding by spliced variant mRNAs, or through proteolytic processing of full-length receptors. One such molecule, 'herstatin', is encoded by a spliced isoform of ErbB-2, and disrupts ErbB-2 dimerization and signaling [62•].

Truncated receptors can also be produced through proteolytic cleavage of full-length molecules. Cleavage of the extracellular juxtamembrane domain would hypothetically yield a soluble ectodomain, which may function as an inhibitor, and concomitantly a truncated anchored receptor that would be constitutively active. Such an activated form of ErbB-2 is found in a subset of tumors that overexpress ErbB-2. It is apparently produced through cleavage by one or more metalloproteinases. This extracellularly truncated form is activated for signaling, and may be associated with lymph node metastasis [63•].

Other sources of signaling heterogeneity

The ligand-activated EGF receptor is downregulated through receptor-mediated endocytosis. ErbB-2, ErbB-3, and ErbB-4 show little ligand-dependent endocytosis compared with the EGF receptor [64•]. Once internalized, the EGF receptor may be degraded, or, alternatively, recycled to the cell surface. The strong downregulation of the EGF receptor occurs in part because it preferentially associates with the ring-finger protein Cbl, which in turn favors degradation [65••]. Stability and signaling of the EGF receptor may be further enhanced by overexpression of ErbB-2, providing another mechanism through which ErbB2 overexpression enhances signaling by the EGF receptor [66].

Autocrine and paracrine regulation of the receptors is probably of paramount importance in determining the amount of signal produced by overexpressed ErbB family receptors. Production of TGF-α, amphiregulin, and neuregulin have all been described in breast cancer (for review [21•]). The regulation of ErbB receptors on tumor cells may change during tumor progression, because the availability of paracrine ligands may change as tumors move out from the intraductal environment to invade locally and distantly.

Another consequence of hormonal stimulation is that it may qualititatively alter the signaling activity of the receptors. Different kinase–substrate pairs are juxtaposed in different receptor dimers, so that signaling by EGF receptor–ErbB2 heterodimers, for example, is different from the sum of signals emitted by the respective homomers. Differential positioning of receptors by different ligands may also influence signaling qualitatively [67•,68,69•].

ErbB receptors are sometimes jointly overexpressed in breast cancer, suggesting either that they cooperate in carcinogenesis, or that overexpression of one receptor upregulates expression of another. In transfection experiments, EGF receptor and ErbB2 synergize in cell transformation, which is consistent with the former model [24•]. Synergy may result from the ability of ErbB2 in heterodimers to stabilize ligand binding and impair EGF receptor turnover, as discussed above, to expand the repertoire of pathways activated, or to enhance the duration of signaling by the two receptors [19•,34••]. In *MMTV-NEU* transgenic mouse models, tumors express high levels of EGF receptor [70] or ErbB-3 [13••]. The latter apparently results from post-transcriptional upregulation of ErbB-3 by ErbB-2 [53].

Clinical impact

From the perspective of the clinician, the knowledge that ErbB family receptors are altered in breast cancer is most important if it has prognostic value, if it can be used to make treatment decisions, or if the receptors or ligands are good therapeutic targets. The most extensive clinical data are available for ErbB-2. Amplification or overexpression of ErbB-2 is associated with accelerated relapse and

mortality [3••] (for review [4•,71•]). Although ErbB-2 status is not currently in common use for breast cancer treatment decisions, it may be helpful in borderline situations. Because Herceptin is most likely to be valuable for patients with tumors that overexpress ErbB-2, such patients were selected for earlier clinical trials, and the drug is approved for use in the USA in this subset of patients.

Clinical tests that are based on fluorescent *in situ* hybridization or immunohistochemistry are typically used to identify ErbB-2 amplification or overexpression. As discussed above, signaling heterogeneity could significantly affect clinical changes associated with receptor overexpression and the response to therapeutic antagonists; whatever role an individual receptor plays in breast cancer, the clinical variables should correlate best with the signaling activity of the receptor, rather than with its abundance. The use of phosphorylation-sensitive antibodies that read out ErbB receptor phosphorylation at specific sites may be the broadest approach to contending with these competing forms of regulation, because the phosphorylation status of the receptors is a direct measure of their signaling activity [72,73]. Antibodies that recognize tyrosine phosphorylated (active) forms of ErbB-2 identify a subset of breast cancer patients with poor prognosis [74]. Better understanding and quantification of these variables should greatly augment the clinical utility of ErbB receptor status (for review [71•]).

The signaling diversity engendered by the different signaling activities of the four receptors means that activation of specific receptor combinations may be most strongly linked to tumor aggressiveness, whereas others may be associated with better prognosis. Thus, the most powerful clinical predictions may be obtained through quantification of different receptor activations, and the most powerful therapeutic strategies may work by targeting specific subsets of receptors. For example, the synergy of EGF receptor and ErbB2 in carcinogenesis has suggested the development of binary therapeutic agents targeting both receptors [75].

In both tissue culture and clinical studies, overexpression of ErbB receptors has been associated with resistance to specific chemotherapeutic agents [76]. Other studies have suggested that patients with ErbB-2-overexpressing tumors may benefit from high doses of chemotherapy. Finally, synergy has been observed between genotoxic drugs and EGF receptor or ErbB2 antagonists [77–79]. Because of discordances in the research literature, the extent and mechanisms of these phenomena are controversial, and the reader is referred to reviews of the subject [4•,71•,80•]. Nonetheless, the evidence clearly demonstrates that the deployment of ErbB-directed therapies can have significant impact on the response to other agents.

Changes in ErbB family receptors may also affect response to hormone therapy with estrogen antagonists. This may have major practical consequences as therapeutic agents targeting the ErbB receptors are produced, given the importance of hormone therapy in treating breast cancer. At least two molecular links have been established between estrogen receptor and ErbB receptors. Estrogen suppresses ErbB-2 transcription and expression [81•], and, conversely, the antagonist tamoxifen has the undesirable property of augmenting ErbB2 expression [82•]. ErbB receptors themselves regulate estrogen-signaling pathways, either by directly phosphorylating the estrogen receptor, or by activating mitogen-activated protein kinases, which in turn enhance estrogen receptor signaling [83•,84•].

Conclusion

The ErbB family of receptors are outstanding targets for breast cancer therapies (and diagnosis). Optimal clinical performance of ErbB-targeted reagents will best be achieved by harnessing knowledge about the biologic activities of these receptors in the mammary gland. Although the fundamentals of ligand regulation of these receptors have been established, there is still much to be learned. Work in *Drosophila melanogaster* suggests that fine tuning of expression and processing of ligands and receptors, along with the operation of natural antagonists, will be important for normal regulation [85•]. The association of specific signaling pathways with receptor-specific functions is not well understood. Finally, understanding the mechanism of action of Herceptin (for review [86•]) may lead to improved second-generation therapeutics.

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