Review

Tyrosine kinase signalling in breast cancer
Epidermal growth factor receptor and c-Src interactions in breast cancer
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Abstract

Both the non-receptor tyrosine kinase, c-Src, and members of the epidermal growth factor (EGF) receptor family are overexpressed in high percentages of human breast cancers. Because these molecules are plasma membrane-associated and involved in mitogenesis, it has been speculated that they function in concert with one another to promote breast cancer development and progression. Evidence to date supports a model wherein c-Src potentiates the survival, proliferation and tumorigenesis of EGF receptor family members, in part by associating with them. Phosphorylation of the EGF receptor by c-SRC is also critical for mitogenic signaling initiated by the EGF receptor itself, as well as by several G-protein coupled receptors (GPCRs), a cytokine receptor, and the estrogen receptor. Thus, c-Src appears to have pleiotropic effects on cancer cells by modulating the action of multiple growth-promoting receptors.

Keywords: c-Src, epidermal growth factor receptor, human epidermal growth factor receptor 2/neu, signal transducers and activators of transcription, tyrosine phosphorylation

Introduction

Recent evidence has implicated an involvement of tyrosine kinases in human breast cancer development. Two families in particular have been examined, namely, the human epidermal growth factor and the Src families of tyrosine kinases.

Human epidermal growth factor receptor (HER1) in human breast cancer

The human epidermal growth factor receptor (HER1) is the prototype of a family that consists of four known members (EGF receptor/HER1, neu/herB2/HER2, erbB3/HER3, and erbB4/HER4). These receptor tyrosine kinases are characterized by an extracellular ligand-binding domain, an internal kinase domain, and a carboxyl-terminal domain that contains multiple tyrosine residues. Upon binding of EGF, HER1 dimerizes and becomes phosphorylated on these carboxyl-terminal tyrosyl residues, which in turn act as docking sites for multiple signaling proteins that contain SH2 domains. HER1 plays a variety of roles in normal development, and is found in ductal epithelial cells of normal breast tissue [1**].

The link between HER1 and human cancer initially came from studies by Velu et al [2], who demonstrated that cells that overexpress HER1 become transformed when they are grown in the continuous presence of EGF. HER1 is overexpressed in a variety of human cancers, including...
benign skin hyperplasia, glioblastoma and cancers of the breast, prostate, ovary, liver, bladder, esophagus, larynx, stomach, colon, and lung [3\textsuperscript{,}]. Approximately 30\% of human breast tumors overexpress HER1, and this overexpression is correlated with a loss of estrogen responsiveness and a poorer prognosis [4,5]. Much evidence suggests that HER1 is involved in later stages of human breast cancer and may play a role in the metastatic process [6].

Human epidermal growth factor receptor 2 (HER2) in breast cancer

Among the HER family members, HER2 is most closely related to HER1 [7] and has been found to be amplified in 10–35\% of human breast carcinomas, an event that portends a poor disease prognosis [8\textsuperscript{,},9\textsuperscript{,}]. Overexpression of HER2 occurs more frequently in the early stages of breast cancer, and is therefore thought to be involved in tumor initiation and early stages of progression [10\textsuperscript{,}]. The involvement of HER2 in human breast cancer is further supported by the success of recent immunotherapy trials, which targeted the receptor in conjunction with current chemotherapeutic protocols [11].

c-Src in breast cancer

c-Src, a nonreceptor tyrosine kinase that is localized to intracellular membranes of the cell, has also been found to be overexpressed or highly activated in a number of human neoplasms, including carcinomas of the breast, lung, colon, esophagus, skin, parotid, cervix, and gastric tissues, as well as in neuroblastomas and myeloproliferative disorders. In several studies that together examined over 125 human breast tumor specimens and cell lines [12,13,14\textsuperscript{,}], more than 70\% of the samples contained levels of c-Src tyrosine kinase activity that were two-fold to 50-fold greater than those found in normal breast epithelium or immortalized mammary epithelial cells. This elevated activity could be accounted for solely by an increase in c-Src protein levels, and did not reflect an increase in specific activity of the enzyme [14\textsuperscript{,}]. Such striking increases in c-Src protein levels in a surprisingly high percentage of human breast neoplasms provide correlative evidence that c-Src is involved in some facet of breast cancer development.

Unlike the EGF receptor, overexpression of c-Src alone is insufficient to transform murine fibroblasts in culture or to sustain tumor growth in intact animals [15,16,17\textsuperscript{,}]. However, expression of dominant interfering forms of the pair [17\textsuperscript{,}]. This enhanced oncogenesis correlated with the EGFr-dependent physical association between c-Src and HER1, increased phosphorylation of the HER1 substrate, Shc and phospholipase C\gamma, and the phosphorylation of two novel tyrosyl residues on the receptor, which have been identified by phosphotryptic mapping to be Tyr 845 and Tyr 1101 [17\textsuperscript{,}•,24\textsuperscript{,}]. Stover et al [25\textsuperscript{,}] showed that activated Src can phosphorylate HER1 at Tyr 891 and Tyr 920 in vitro, and that these sites can mediate binding of the SH2 domains of phosphatidyl inositol-3 kinase (PI-3K) and Src itself. These same sites have been

Structure of c-Src. C-Src is the prototype of a large family of cytoplasmic tyrosine kinases that associate with cellular membranes through lipid modifications at their amino-termini. As a linear molecule, the relationship between the various domains can be seen: an amino-terminal membrane-association domain that contains the site of myristylation; a unique domain that exhibits the widest sequence divergence among family members of any of the domains; an SH3 domain that binds polyproline motifs on target molecules; a SH2 domain that binds phosphotyrosine residues on target molecules; a SH2/kinase linker; the catalytic domain; and the negative regulatory domain that contains the predominant site of tyrosine phosphorylation on the inactive molecule (Y527 in chicken, Y530 in human). Mutations that abrogate myristylation, the SH2 domain, and the catalytic activity were shown to reduce EGF-stimulated DNA synthesis in C3H10T\textsuperscript{½} murine fibroblasts, providing evidence for the involvement of c-Src in mitogenic pathways [18\textsuperscript{,}].

Synergism between c-Src and human epidermal growth factor receptor 1 in oncogenesis

The fact that c-Src and HER1 are co-overexpressed in many of the same tumor types suggests that these two kinases may participate in regulating the genesis and/or progression of human cancers. In a direct test to resolve this question, dual overexpression of both c-Src and HER1 in C3H10T\textsuperscript{½} mouse fibroblasts was found to lead to synergistic increases in EGF-induced DNA synthesis, soft agar colony formation, and tumor formation in nude mice, when compared with cells that express only one of the pair [17\textsuperscript{•}]. This enhanced oncogenesis correlated with the EGFr-dependent physical association between c-Src and HER1, increased phosphorylation of the HER1 substrate, Shc and phospholipase C\gamma, and the phosphorylation of two novel tyrosyl residues on the receptor, which have been identified by phosphotryptic mapping to be Tyr 845 and Tyr 1101 [17\textsuperscript{•},24\textsuperscript{,}]. Stover et al [25\textsuperscript{,}] showed that activated Src can phosphorylate HER1 at Tyr 891 and Tyr 920 in vitro, and that these sites can mediate binding of the SH2 domains of phosphatidyl inositol-3 kinase (PI-3K) and Src itself. These same sites have been
Recently, it was shown \[32••\] that phosphorylation of Tyr

data not provided

predicted to mean either that the site was not phosphory-
receptor, as well as activation of its downstream effectors Shc and MAPK, and that this activation is dependent on c-Src kinase activity [34•,35,36]. In addition, stimulation of the growth hormone cytokine receptor has been found to induce EGF receptor phosphorylation via janus kinase 2 [37]. Recent work in our laboratory (Biscardi et al, unpublished data) has demonstrated that treatment of 10T½ cells with different GPCR ligands (thrombin, endothelin, and LPA) or with growth hormone induces increases in overall tyrosine phosphorylation of HER1, as well as in Tyr 845 phosphorylation. Interestingly, the kinase activity of c-Src is also required for phosphorylation of Tyr 845 via these alternate receptors, and mitogenesis is dependent on the phosphorylation of Tyr 845. 10T½ cells that express the Tyr845Phe variant of HER1 are impaired in their ability to synthesize DNA in response to these stimuli. However, the weakly mitogenic effects of isoproterenol, which signals through a Gαα coupled pathway, are not affected by the Tyr845Phe mutation, indicating that this mutation does not act as a general inhibitor of mitogenesis.

Estrogen receptor, c-Src, and human epidermal growth factor receptor 1

Accumulating evidence also points to an intricate network of cross-talk between the estrogen receptor and HER1. Early work by Ignar-Trowbridge et al [38,39] demonstrated that EGF can transcriptionally activate genes that contain estrogen response elements. More recently, Migliaccio et al [40,41] showed that estrogen is able to activate many of the effectors classically thought to be linked to the EGF receptor signaling pathway, including c-Src, Ras, and MAPK. These researchers have also shown that estrogen requires c-Src kinase activity in order to trigger its mitogenic effects [42•]. To further investigate this phenomenon, our laboratory has examined the effects of the Tyr845Phe mutation on estrogen-dependent DNA synthesis in the estrogen-responsive MCF7 breast cancer cell line. As was the case for the GPCR and cytokine receptor coupled agonists, estrogen-stimulated DNA synthesis was decreased to basal levels as a result of expression of the Y845F mutant (Biscardi et al, unpublished data). Taken together, these findings suggest the possibility that the EGF receptor plays an important, perhaps widespread, role in mediating the cell’s response to an array of external signals and that the c-Src mediated phosphorylation of EGF receptor Tyr 845 appears to be a critical event in this process (Fig. 3).

Human epidermal growth factor receptor 2 and c-Src in breast cancer

Evidence supporting bidirectional interactions between c-Src and the EGF receptor raises the question of whether c-Src interacts in a similar manner with other HER family members. Some indications that HER2 and c-Src can physically and/or functionally interact have emerged over recent years, but little is currently known about the relationship between c-Src and either HER3 or HER4. In vitro studies have also demonstrated that HER2/neu can associate with the SH2 domain of c-Src in a tyrosine phosphorylation-dependent manner [43,44••], and in vivo coassociation between HER2/neu and c-Src has been detected in murine mammary tumors, human breast cancer cell lines, and human tumor tissues [44••] (Belsches-Jablonski AP et al, unpublished data). Furthermore, transgenic murine tumor tissues or human mammary epithelial cell lines expressing mutationally activated Neu exhibit a correlative increase in c-Src activity [44••,45]. These results suggest that c-Src may be downstream of HER2 signaling. It has also been demonstrated in vitro [25], however, that c-Src is able to phosphorylate HER2 at nonautophosphorylation sites. The identity of these sites, their existence in intact cells, and their functional significance have not yet been determined. Nevertheless, the currently available information suggests that HER2 and c-Src are able to interact physically and that bidirectional signaling may be a mechanism of interaction between these two tyrosine kinases, as it is for HER1 and c-Src.

The functional consequences of coassociation between HER2 and c-Src remain unclear. In fact, available evidence suggests that the HER2–c-Src interaction may affect different parameters of oncogenesis to different extents and perhaps by different mechanisms than the HER1–c-Src association. Results from recent studies of MCF10A cells that ectopically express mutationally activated rat p185neu [45], and a panel of 13 human breast cancer cell lines and 13 human mammary tumor samples (Belsches-Jablonski AP et al, unpublished data) suggest that the HER2–c-Src complex may play an important role in heregulin-stimulated anchorage-independent growth and antiapoptotic or survival mechanisms, but have less of an effect on anchorage-dependent growth. In contrast, the HER1–c-Src complex has been found to have striking effects on anchorage-dependent growth and on anchorage-independent growth, but its role in survival signaling is unclear.

Whether c-Src mediates the phosphorylation of the Tyr 845 homolog in HER2 (Tyr 877) as it does in HER1 is not known. The comparable site in the activated, rat p185neu protein (Tyr 882) is an autophosphorylation site, and mutation of this residue reduces the intrinsic kinase activity of the protein and its transforming potential [46•]. These findings suggest that the Tyr 845 homolog in HER2 homolog in HER2 (Tyr 877) as it does in HER1, and raise the possibility that the mechanism by which c-Src interacts with HER2 may be distinct from that by which c-Src interacts with HER1.

Signals activated by c-Src/human epidermal growth factor receptor 1 interactions

Although mutation of Tyr 845 has profound effects on the cell’s ability to respond mitogenically to EGF, many of the
downstream targets of HER1 are unaffected. The Y845F mutant HER1 kinase activity appears to be unchanged, as does its ability to associate with c-Src. Moreover, the phosphorylation and/or activation of a number of HER1 effectors, including Shc, MAPK, signal transducer and activator of transcription (STAT)3, and phospholipase Cγ [32••] (Tice and Biscardi, unpublished data) are likewise unaffected. However, recent evidence, produced in a collaborative effort between our laboratory and that of Silva (unpublished data), suggests that STAT5b might be a physiologically relevant downstream effector of Tyr845.

The STATs are a family of transcription factors that are activated at the plasma membrane by tyrosine phosphorylation in response to signals from cytokine and growth factor receptors [47•,48]. Tyrosine phosphorylation results in STAT dimerization, nuclear translocation, and binding of STAT dimers to consensus elements upstream of regulated genes.

Increasing evidence indicates that STAT proteins are involved in the process of oncogenesis [49•,50]. Two laboratories have shown that STAT3 is required for v-Src transformation [51••,52••], whereas deGroot et al [53] demonstrated that active STAT5 is necessary for the soft agar growth of BCR-Abl transformed leukemia cells. Recent studies [54••] have also indicated a direct role for c-Src in the activation of STAT proteins. For example, c-Src was shown to mediate the EGF stimulation of STATs 1, 3, 5a, and 5b in NIH3T3 cells engineered to overexpress HER1, as well as in A431 cells, which endogenously express high levels of HER1. In contrast, another group [55] has recently described a role for c-Src in the tyrosine phosphorylation (but not the transcriptional activation) of STAT5a and STAT5b in a COS cell transfection model.

Our recent studies (Silva et al, unpublished data) indicate a role for the STAT proteins in signaling pathways that are activated in 10T1½ and breast cancer cells co-overexpressing c-Src and EGF receptor. We have shown that c-Src tyrosine kinase activity is required for maximal transcriptional activation of STAT5b by EGF, and that phos-
phorylation of Tyr 845 is required for both the EGF-induced association between STAT5b and HER1 as well as tyrosine phosphorylation of STAT5b. These studies suggest a model whereby HER1 and c-Src overexpression and EGF stimulation lead to the phosphorylation of Tyr845 and the recruitment and activation of STAT5b.

A number of other signaling molecules have also been linked to EGF-induced mitogenesis in various cell systems, and should be considered as additional candidates for downstream effectors of Tyr845. These signaling molecules include PI-3K, big MAPK [BMK1 or extracellular-signal-regulated kinase (ERK)5], and the transcription factor Myc. After growth factor activation, PI-3K interacts, through its SH2 domain, with tyrosine phosphorylated growth factor receptors, resulting in an increase in PI-3K activity. Studies using specific antibodies to PI-3K [56] demonstrated that its catalytic activity is required for EGF (and platelet-derived growth factor)-induced mitogenesis. Although PI-3K associates with the EGF receptor, the binding site has not been characterized and thus it is interesting to speculate that this function may be fulfilled by Tyr845. ERK5, a member of the MAPK family, was first shown to be activated in response to oxidative stress, hyperosmolality, and serum. Recent studies [57••,58] have shown that this kinase is also activated in response to EGF and nerve growth factor. Furthermore, dominant-negative ERK5 blocks EGF-induced cell proliferation in a breast epithelial cell line by preventing cells from entering the S phase [57••]. Studies in mouse fibroblasts have shown that c-Src kinase is required for ERK5 activation in response to hydrogen peroxide. Although a role of c-Src in EGF activation of ERK5 has not yet been demonstrated, these studies provide the background for a potential role of ERK5 in the c-Src-mediated activation of HER1. One substrate of ERK5 is the early response gene c-myc [59]. C-myc encodes a nuclear phosphoprotein, which, in combination with Max, activates gene transcription. C-Myc expression correlates with the proliferative state [60], and has been shown to rescue platelet-derived growth factor signaling that is blocked by kinase-inactive c-Src [61]. Together these findings link c-Src and EGF with PI-3K, ERK5, and c-Myc, and are suggestive of a potential role for one or more of these molecules to function as downstream effectors of phosphorylated Tyr845.

Conclusion

Substantial evidence is accumulating to indicate functional synergism between the nonreceptor tyrosine kinase c-Src and members of the EGF receptor family in promoting breast cancer progression. Members of both families are overexpressed in approximately 70% or more of human breast cancers, and the human EGF receptor (HER1) and HER2/neu portend a poor prognosis for the disease.

It has been demonstrated that c-Src, which is nontransforming when overexpressed alone, can potentiate the tumorigenic capacity of overexpressed HER1. Recently, one mechanism by which c-Src synergizes with the HER1 has been uncovered. This mechanism involves the EGF-dependent association of c-Src with HER1 and phosphorylation of the receptor by c-Src on residues Tyr 845 and Tyr 1101. The functional consequences of Tyr 1101 phosphorylation are unknown, but phosphorylation of Tyr 845 is required for EGF-induced DNA synthesis and activation of members of the STAT family of transcription factors, particularly STAT5b, but not activation of Shc or MAPK. Whether the STATs are the predominant mediators of Tyr 845-dependent mitogenesis or whether there are other mitogenic signaling pathways that emanate from phosphorylated Tyr 845 remains to be determined. Surprisingly, Tyr 845 phosphorylation has also been found to be an intermediate in mitogenic signaling from a variety of GPCR, as well as from certain cytokine receptors and the estrogen receptor. Thus, HER1, and specifically phosphorylation of Tyr 845 by c-Src, appears to play an important, perhaps widespread role in mediating cell responses to an array of external signals.

c-Src also complexes with another member of the EGF receptor family, namely HER-2 or erbB-2/neu. This association is independent of extracellular ligand and appears to contribute more to cell survival and anchorage-independently grown breast cancer cells than to anchorage-dependent growth or migration. The mechanism of c-Src interaction with HER2 and how this interaction may transmit survival or anchorage-independent growth signals is not known, but it is speculated to be different than the interaction between c-Src and HER1.

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