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ROLES OF ERBB FAMILY RECEPTOR TYROSINE KINASES, AND DOWNSTREAM SIGNALING PATHWAYS, IN THE CONTROL OF CELL GROWTH AND SURVIVAL

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1. ABSTRACT

Within the last 20 years, multiple novel intracellular signal transduction pathways, downstream of plasma membrane receptors, have been discovered. These pathways have been linked to the regulation of diverse cellular events such as proliferation, senescence, differentiation and apoptosis. This review will focus upon the roles of signaling by the ErbB receptor tyrosine kinase family (ErbB1-4) in the survival of cells in response to cytotoxic stresses. In addition, plasma membrane-to-nucleus signaling pathways downstream of these receptors, such as mitogen activated protein kinase (MAPK) and phosphatidyl inositol 3-kinase (PI3K), in the control of cell survival will be discussed. Recent evidence suggests that signaling by the MAPK and PI3K pathways can both enhance proliferation as well as protect cells from apoptosis. We describe potential mechanisms by which modulation of pathway activities following inhibition of ErbB receptor function may alter the sensitivity of cells to toxic insults, leading to increased apoptosis and loss of clonogenic survival.

2. THE Erb B FAMILY OF RECEPTOR TYROSINE KINASES

The ErbB family of receptor tyrosine kinases comprise ErbB1-ErbB4. ErbB1 is more commonly known as the epidermal growth factor (EGF) receptor and these molecules are also referred to as the EGFR and HER2-HER4 (1). ErbB1 and its autocrine ligands epidermal growth factor and transforming growth factor alpha (TGFα) were described over 20 years ago (1-3). The EGF receptor was found to have a tyrosine kinase within its intracellular domain whose activity was stimulated upon ligand binding (4,5). Further studies showed that the EGF receptor had homology with the v-Erb-B oncogene and that the EGF receptor was frequently over-expressed in a wide range of carcinomas (6,7). Another oncogenic form of the EGF receptor, EGFR VIII, has been described in a variety of tumor cell types: EGFR VIII lacks the ligand binding portion of the EGF receptor and is believed to have significant basal tyrosine kinase activity (8). Several other truncated forms of the EGF receptor are also known to exist that appear to play a role in tumorigenic processes (9) and ErbB1 truncated forms are also over-expressed in many types of tumor cell (10). These findings strongly argue that signaling by ErbB1 plays a role in tumor cell growth.

ErbB1 was shown, upon ligand binding, to homo- and heterodimerize with other ErbB family molecules and for the tyrosine kinase domain of each ErbB1 molecule to trans-phosphorylate its partner (11): thus ErbB1 can mediate the activation of ErbB1 as well as ErbB2-4. ErbB2, also called HER2/neu, was the second proto-oncogene of the ErbB family to be discovered, and like ErbB1, contains a tyrosine kinase motif within its intracellular domain (12). Currently, no ligand that binds to ErbB2 has been described and it is believed that this molecule has enhanced basal tyrosine activity compared to

ErbB1. ErbB2 is thought to play a facilitatory role in the activation of all ErbB family members via heterodimerization (13-15). ErbB2 is also over-expressed in solid tumors (15-25%), including mammary carcinoma, and is believed, together with ErbB1, to play a protective role against cytotoxic insults (16,17).

In contrast to ErbB1 and ErbB2, ErbB3 does not appear to have an active tyrosine kinase domain within the molecule due to an asparagine for aspartic acid substitution in the catalytic site (18). Unlike ErbB1 and ErbB2, Erb3 is capable of binding to ligands of the NDF/heregulin family but does not bind to ligands of the EGF / TGF alpha family (19). Thus signaling by ErbB3 has to be mediated in the context of interactions with heterodimeric ErbB complexes using ErbB receptors that contain an active tyrosine kinase domain to mediate signals (20). In a similar manner to ErbB3, ErbB4 also can bind ligands of the NDF/heregulin family (21). However, the kinase domain of ErbB4 is functional and it has been proposed that ErbB4 can play roles in pathological processes including cancer and heart disease (22). Similar to the truncations observed in ErbB1 during transformation, naturally occurring variants of ErbB4 have also been shown to exist, although their roles in the process of cellular transformation are less clear at present (23). Downstream of the ErbB family of receptors are intracellular signal transduction cascades that mediate receptor signaling into the cell. In this review, we will focus primarily on the "classical" mitogen activated protein kinase pathway and the phosphatidyl-inositol 3-kinase pathways.

3. THE MITOGEN ACTIVATED PROTEIN KINASE (MAPK) AND PHOSPHATIDYL INOSITOL 3-KINASE (PI3K) PATHWAYS

"MAPK" was first reported in 1986 (24). This protein kinase was originally described as a 42-kDa insulin-stimulated protein kinase activity whose tyrosine phosphorylation increased after insulin exposure, and which phosphorylated the cytoskeletal protein MAP-2. Contemporaneous studies identified an additional 44-kDa isoform of MAPK, ERK1 (Extracellular signal Regulated Kinase) (25). Since many growth factors and mitogens could activate MAPK, the acronym for this enzyme has subsequently been considered to denote Mitogen-Activated Protein (MAP) kinase. Additional studies demonstrated that the p42/p44 MAPKs regulated another protein kinase activity ($p90^{rsk}$) (26), and that they were themselves regulated by protein kinase activities designated MKK1/2 (MAPK kinase) or MEK1/2 (27-30). MKK1/2 were also regulated by reversible phosphorylation. The protein kinase responsible for catalyzing MKK1/2 activation was the proto-oncogene Raf-1 (31,32). It has been suggested that other enzymes at the level of MKK1/2 can phosphorylate and activate p42/44 MAPK e.g. RIP2 (33) that plays a role in TNF alpha-induced, but not EGF-induced, MAPK activation.

Raf-1 is a member of a family of serine-threonine protein kinases termed Raf-1, B-Raf, and A-Raf (34,35). All "Raf" family members can phosphorylate and activate MKK1/2, although the relative ability of each member to catalyze this reaction varies (B-Raf > Raf-1 > A-Raf) (36,37). Raf kinases thus act at the level of a MAPK kinase kinase (MAPKKK). Several studies demonstrated that the NH_2 domain of Raf-1 could reversibly interact with Ras in the plasma membrane and that the ability of Raf-1 to associate with Ras was dependent upon the Ras molecule being in the GTP-bound state (38,39). Other findings proved that the ability of Raf-1 to be activated depended upon Raf-1 translocation to the plasma membrane (40,41).

The regulation of Raf-1 activity appears to be very complex, with several mechanisms co-ordinately regulating activity when in the plasma membrane environment. Stokoe and McCormick have demonstrated that association of Raf-1 with Ras is sufficient for partial stimulation of Raf-1 activity (42). The binding of 14-3-3 proteins to phospho-serine residues (S259, S621) in Raf-1 have been suggested to play a role in Raf-1 activation (43-46). Others have argued that 14-3-3 proteins binding to these sites inhibit Raf-1 activation (47). Phosphorylation of S338 by PAK enzymes has more recently been shown to play a role in the activation process (48). Other investigators have suggested that another lipid second messenger, ceramide, may also be able to play a role in Raf-1 activation (49,50). Data from several laboratories has suggested that protein serine/threonine and tyrosine phosphorylations play a role increasing Raf-1 activity when in the plasma membrane environment (51-53). Other studies have also suggested that PKC (protein kinase C) isoforms can directly regulate Raf-1 activity (54,55). In contrast to data from earlier studies, phosphorylation of Raf-1 at S259 by Akt has been shown to inhibit Raf-1 activity and its activation by upstream stimuli (46,56,57). At the same time that Raf-1 was shown to associate with Ras, it was found that growth factors, via their plasma membrane receptors, stimulate GTP for GDP exchange in Ras using guanine nucleotide exchange factors (58,59). Thus a signaling pathway (often termed the "classical" MAPK pathway) was delineated from plasma membrane growth factor receptors, through guanine nucleotide exchange factors and the Ras proto-oncogene, to Raf-1/MKK/MAPK/$p90^{rsk}$.

Inositol phospholipids were first argued to be important second messenger signaling molecules in the 1980's (60). Phospholipase C γ, when activated by mitogens such as EGF and TGF alpha, cleaved inositol phospholipids into diacylglycerol and IP_3, with the release of IP_3 into the cytoplasm (61). IP_3 interacts with a receptor in the endoplasmic reticulum leading to Ca^{2+} release into the cytosol and Ca^{2+}, together with diacylglycerol, can cause activation of PKC isoforms (62,63).

PI3K enzymes consisit of two subunits, a catalytic p110 subunit and a regulatory and localizing subunit, p85: several different classes of PI3K enzymes exist (64,65). The p85 subunit of PI3K enzymes contains a phospho-tyrosine (SH2) binding domain (66). The major catalytic function of the phosphatidyl inositol 3 kinase enzymes is in the p110 subunit that acts to phosphorylate inositol phospholipids (PIP2: phosphatidyl inositol 4,5 bis

phosphate), in the plasma membrane, at the 3 position within the inositol sugar ring. The activation of PI3K enzymes is complicated and appears to have some degree of agonist specificity. Mitogens such as TGF alpha and heregulin stimulate tyrosine phosphorylation of ErbB family receptors, providing acceptor sites for the SH2 domain of p85 (67,68). Binding of p85 to active ErbB receptors (predominantly ErbB3) results in p110 PI3K activation. Other studies have suggested in cells expressing mutant oncogenic Ras or which are stimulated by mitogens that utilize serpentine receptors, that the p110 subunit of PI3K can directly bind to Ras-GTP, leading to catalytic activation of the kinase (69-71).

When other positions within the inositol ring are phosphorylated by additional PI-kinases (e.g. PI 4-kinase, PI 5-kinase), the inositol 3, 4, 5 trisphosphate molecule becomes an acceptor site in the plasma membrane for molecules that contain a plecstrin binding domain (PH domain), in particular, the protein kinases PDK1 and Akt (also called protein kinase B, PKB) (72). Of note, PDK1 can also be regulated by protein phosphorylation (73). The phosphorylation of the inositol sugar ring can be reversed by the tumor suppressor lipid phosphatase PTEN (phosphatase and tensin homologue on chromosome ten) (74,75). Loss of PTEN expression is frequently found in some tumor cell types e.g. glioblastoma multiforme (76) resulting in an apparent constitutive activation of PDK1 and Akt (56,57,77).

Signaling by PDK1 to Akt and by PDK1 and Akt downstream to other protein kinases such as PKC isoforms, GSK3, mTOR, p70 ^{S6K} and p90 ^{S6K}, has been shown to play a key role in mitogenic and metabolic responses of cells as well as protection of cells from noxious stresses (78-82). As with the previously discussed "Raf" molecules, the regulation of Akt appears to be very complex, with multiple phosphorylation sites playing various roles in the activation process (83). Indeed, evidence is now emerging that in addition to PDK1, other protein kinases including p38 MAP kinase (suggested to be "PDK2") and the proto-oncogene c-Src can phosphorylate Akt on multiple PDK1-independent sites resulting in modified Akt activity (84,85).

4. INHIBITORS OF ErbB RECEPTORS CAN MODIFY THE GROWTH AND SURVIVAL OF NORMAL AND TUMOR CELLS

Signaling by the ErbB family of receptors is, in general, thought to be pro-proliferative and cytoprotective (86). In some cell types, however, EGF and EGF receptor signaling is known to promote growth arrest and apoptosis (e.g. 87,88). Because both receptor expression as well as autocrine growth factor levels are often increased in carcinoma cells compared to normal tissue, many laboratories have studied signaling by the ErbB family in tumor cell growth and survival control. Thus it has been discovered that when signaling from ErbB family receptors is blocked, either by use of inhibitory antibodies (e.g. C225; 4D5 Herceptin; monoclonal antibody 806), small molecular weight inhibitors of receptor tyrosine kinases (e.g. PD183805 (also called CI1033); PKI166; AG1478; PD153035; ZD1839; PD169414; OSI774; AG825; AG879), dominant negative truncated receptors (e.g. dominant negative EGFR-CD533; dominant negative ErbB2) or antisense approaches (antisense EGFR), that tumor cell growth can be reduced and the sensitivity of these cells to being killed by noxious stresses increased (89-104).

The antibodies C225 and 4D5 herceptin bind to the extracellular portions of ErbB1 and ErbB2, respectively (105,106). In the instance of ErbB1, C225 appears to bind to the portion of the molecule that associates with growth factor ligands such as EGF and TGF alpha (105). Thus the ability of growth factors, in the presence of receptor bound C225, to stimulate ErbB1 receptor function is abolished. The anti-proliferative and anti-survival mechanisms of action of herceptin appear to be more complex, in as much as while herceptin binds to ErbB2, this receptor has no known ligand. Instead, it appears that Herceptin acts by causing the internalization and degradation of ErbB2, as well as by blocking ErbB2 heterodimerization with other ErbB family members (107). Both C225 and Herceptin have been shown to individually kill cells, and to interact in a synergistic fashion in combination with standard therapeutic regimens such as ionizing radiation, cisplatin and taxol to reduce tumor cell survival both in vitro and in vivo (108-111). Both C225 and Herceptin are currently in phase III trials and it is very likely that both agents will become standard tools in the treatment of epithelial cell cancers. More recent studies have also used monoclonal antibodies to target truncated forms of ErbB1 e.g. EGFR VIII (91,112). In these studies, a novel monoclonal antibody, 806, was found to potently inhibit truncated forms of ErbB1 and more weakly inhibit full length receptor (112). The inhibition of receptor function correlated with reduced tumor cell growth in vitro and in vivo. Of note, however, it is presently unclear whether all of the anti-tumor effects of anti-ErbB receptor antibodies are mediated solely via receptor inhibition or by a combination of receptor inhibition and enhanced immunological reactivity in vivo due to the Fc portion of the antibody (Figure 1).

Small molecule inhibitors of the tyrosine kinase domains of the ErbB family of receptors have also been used with some success in blocking tumor cell growth and survival both in vitro and in vivo. The inhibitors AG1478, ZD1839 ("Iressa"), PD153035 (also called AG1517), PKI166, OSI774, CI1033 (PD183805) and PD169414 (an irreversible inhibitor), all bind to the catalytic kinase domain of ErbB1 and inhibit tyrosine kinase activity (92-100,113-115). Some studies have suggested that CI1033 binds to, and inhibits, all ErbB kinase domains. Inhibition of ErbB1 kinase activity not only blocks phosphorylation of ErbB1 itself in response to the growth factors which it binds, but also inhibits the trans-phosphorylation of other ErbB family members by ErbB1. In addition to inhibiting ErbB1, the tyrphostin AG1478 has also been shown to inhibit ErbB4 (116). The tyrphostin inhibitors AG825 and AG879 are ErbB2 inhibitors with an apparently weaker kinase specificity than AG1478 for ErbB1/4, in that they

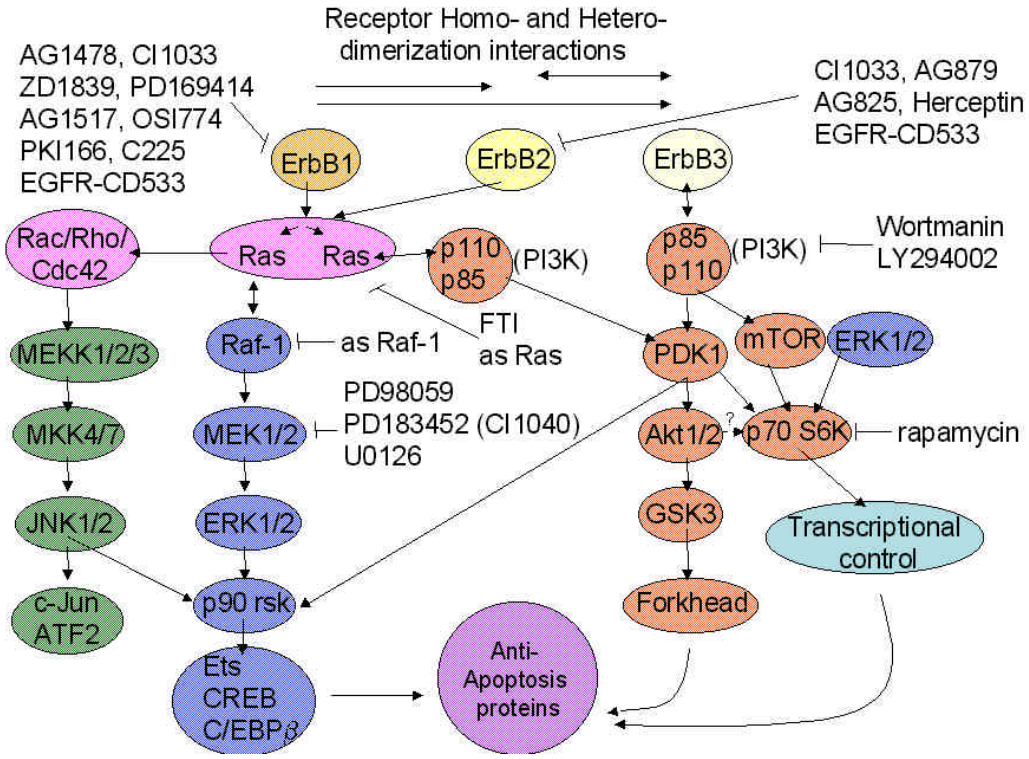

Figure 1. Signaling by ErbB receptors to downstream signaling pathways and the inhibitors of the receptors and downstream signaling pathways. ErbB receptors homo- and hetero-dimerize with each other to initiate signaling processes. Activation of small molecular weight GTP binding proteins (Ras, Rac etc.) leads to activation of cytoprotective modules such as the MAPK and PI3K pathways. Signaling pathways control, via transcription factors, the expression of proteins that control cell cycle progression and protection from noxious stresses. Inhibitors with varying degrees of specificity, as shown in the Figure, have been developed to block signaling by ErbB1, ErbB2, Ras, Raf-1, p110 (PI3K), MEK1/2, p70 S6K.

can also inhibit Trk receptors (99,100). Thus, AG825 / AG879, together with AG1478, have the potential to not only impact on EGF/TGF alpha signaling through ErbB1, but also neuregulin/heregulin signaling through ErbB4 and ErbB3 (117). Small molecular weight ErbB inhibitors are currently in clinical trials, both as stand-alone agents and in combination with ionizing radiation and other standard chemotherapeutic agents (e.g. 118-121) (Figure 1).

In addition to use of antibodies and small molecular weight inhibitors, the ErbB family of receptors have also been inhibited by the use of dominant negative and antisense approaches. In particular, expression of truncated forms of ErbB1 (EGFR-CD533), ErbB2 and ErbB3 in a variety of cell types has been shown to reduce proliferation and survival of both normal and tumor cells in vitro and in vivo (101, 122-127). The dominant negative approaches are believed to act by blocking homo- and heterodimerization of ErbB family members, reducing receptor transphosphorylation and thus downstream signaling by the receptors. Initial studies demonstrated that radiation could activate the EGFR (122,123) and subsequent investigations using dominant negative EGFR-CD533 demonstrated that it could block radiation- and bile acid-induced phosphorylation of the EGFR (124-127). In both mammary carcinoma and glioblastoma cells expression of EGFR-CD533, by use of a recombinant adenovirus injected into the tumor, was then shown to enhance radiosensitivity both in vitro and in vivo (101, 124-126). Collectively, these findings demonstrate that the EGFR is a key cyto-protective molecule whose activity is increased in response to radiation exposure and that a recombinant adenovirus to express dominant negative molecules such as EGFR-CD533 has the potential to be used clinically.

5. PATHWAYS DOWNSTREAM OF ErbB FAMILY RECEPTORS CAN MEDIATE SURVIVAL SIGNALING

Signaling by the ErbB family of receptors in response to growth factors is believed to play an important anti-apoptotic role in both normal and tumor cells. Downstream of the receptors are signaling modules each of which, in a variety of cell types, has been shown to be an anti-apoptotic effector pathway. Previously in this review, the PI3K and MAPK pathways were discussed. However, it should be noted that other pathways downstream of ErbB signaling including JAK/STAT molecules (128,129) and the c-Jun NH_2-terminal kinase pathway (130) are also known to mediate ErbB receptor anti-apoptosis signaling in a cell type and toxic-stress specific manner (Figure 2).

Many extracellular stresses, in a growth factor/ ligand-independent manner, can activate the ErbB family of

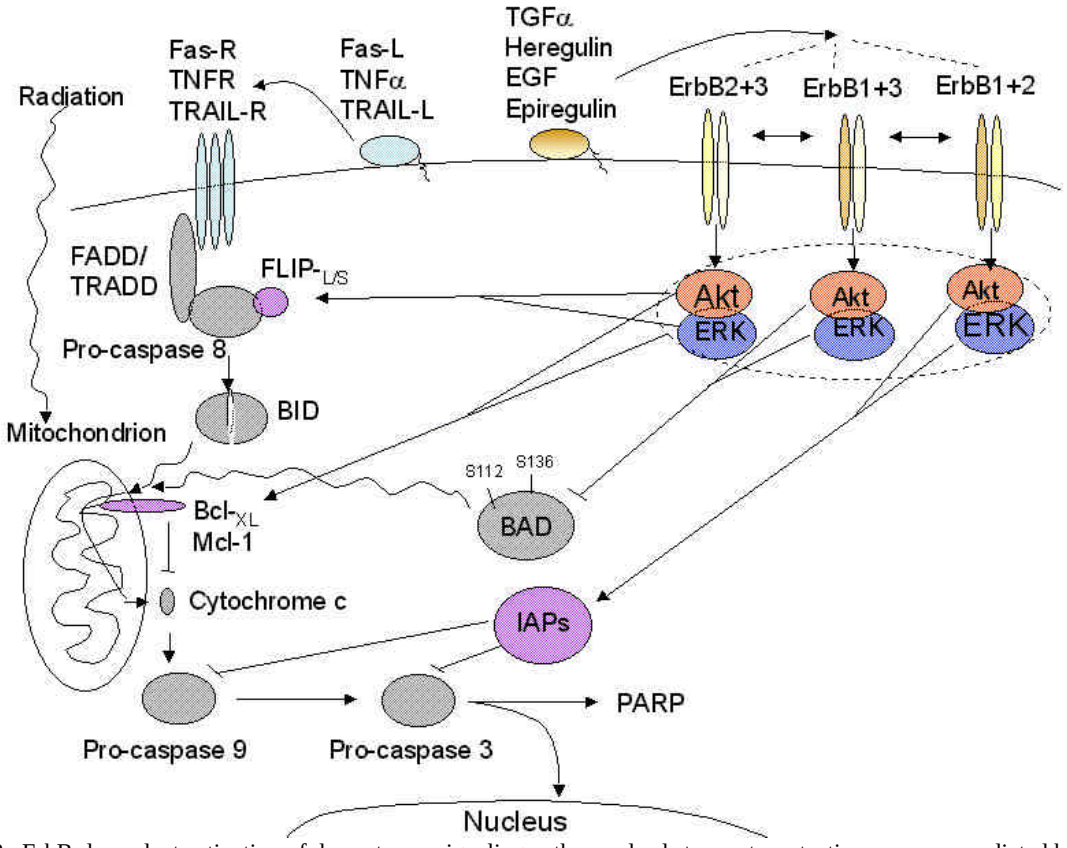

Figure 2. ErbB-dependent activation of downstream signaling pathways leads to a cytoprotective response mediated by multiple anti-apoptotic proteins: control of apoptosis by caspase enzymes. ErbB family receptors and associated autocrine ligands initiate signaling through downstream modules such as the MAPK/ERK and PI3K pathways. Inhibition of MAPK/ERK and PI3K signaling can reduce expression of anti-apoptotic mitochondrial proteins Bcl-XL and Mcl-1 and caspase inhibitor proteins such as FLIP isoforms and XIAP. Loss of ERK/MAPK and PI3K signaling can reduce phosphorylation of BAD at S112 and S136, respectively, leading to functional activation of the pro-apoptotic BAD molecule. Thus loss of MAPK/ERK and PI3K signaling will promote enhanced basal levels of apoptosis and will potentiate cell killing induced by death receptors and agents that disrupt mitochondrial function.

receptors, including ionizing and UV radiation, cytotoxic drugs and bile acids. In addition to causing ligand independent activation of ErbB receptors, ionizing radiation and other stresses can also cause the synthesis and release of autocrine growth factors such as TGF alpha from tumor cells that can re-energize the ErbB receptor system hours after the initial exposure to the stress (131-134). Depending upon the milieu of ErbB receptor expression, these receptor activation(s) will result in the activation of multiple downstream pathways such as PI3K and MAPK. That stresses can also cause the transient activation of other receptor molecules e.g. TNF alpha / FAS / TRAIL receptors, and the fact that TNF alpha / FAS / TRAIL signaling towards death can be promoted by inhibition of ErbB receptors provides further evidence of the complexity of responses emanating when ErbB receptor family function is altered (135).

The anti-apoptotic role of the PI3K / Akt pathway has been well documented by many investigators in response to numerous noxious stimuli, and in some cell types, the anti-apoptotic effects of ErbB receptor signaling have been attributed to activation of the PI3K / Akt pathway (136,137). ErbB signaling to PI3K / Akt has been proposed to enhance the expression of the mitochondrial anti-apoptosis proteins Bcl-$_{XL}$, Mcl-1 and caspase inhibitor proteins such as c-FLIP isoforms (138-140). Enhanced expression of Bcl-$_{XL}$ and Mcl-1 will protect cells from apoptosis via the intrinsic / mitochondrial pathway whereas expression of c-FLIP isoforms will block killing from the extrinsic pathway via death receptors (141). In addition, Akt has also been shown to phosphorylate BAD and human pro-caspase 9, thereby rendering these proteins inactive in apoptotic processes (142,143). Inhibitors of ErbB signaling have been shown to decrease the activity of the PI3K / Akt pathway in a variety of cell types and to increase the sensitivity of cells to a wide range of toxic stresses including cytotoxic drugs and radiation (144). Activation of Akt has also been able to protect cells from death in the presence of ErbB receptor inhibition (145). These findings strongly argue that PI3K / Akt signaling is a key cyto-protective response in many cell types downstream of ErbB family receptors (Figure 2, and legend).

Signaling by the MAP kinase pathway downstream of ErbB receptors also protects from various noxious stresses. Signaling from ErbB receptors through the MAPK pathway can also lead to increased expression of Bcl-$_{XL}$, Mcl-1 and c-FLIP isoforms (146-148). In addition, the downstream effector of the ERK1/2 enzymes, p90 ^{rsk}, phosphorylates the transcription factor CREB which can activate the promoters of several anti-apoptotic proteins (e.g. 149,150). Of note, p90 ^{rsk} also needs PDK1 phosphorylation to be catalytically active (64). In some cell systems, MAPK signaling appears to block apoptosis at levels above the mitochondrion / cytochrome c whereas in others it blunts the actions of caspases downstream of cytochrome c release (e.g. 151,152).

The cytotoxic effects of drugs, as well as radiation, can be magnified by inhibition of ErbB receptors that is paralleled by a reduced ability of cells to activate the MAPK pathway (153-155). For example, expression of dominant negative EGFR-CD533 radiosensitized MDA-MB-231 mammary carcinoma cells that was dependent upon, at least in part, inhibition of radiation-induced MAPK signaling (101,124,125). Expression of this dominant negative ErbB1 molecule also could radiosensitize glioblastoma cells that correlated with both reduced MAPK activity and -induced activation (126). The proto-oncogene Ras, downstream of ErbB receptors, can activate both the PI3K and the MAPK pathways. In certain tumor cell types, the impact of enhanced Ras signaling on tumor cell survival is mediated via the PI3K pathway (156). However in other cell types with mutant Ras, protection appears to be mediated via either the MAPK pathway (101,157) or NFκB (158,159). Of particular note are the findings in many cell types that inhibitors of the MAPK pathway do not significantly alter cell survival in response to toxic stresses, and in some cases act to protect cells from stress-induced cell death (e.g. 156,160). This may be due to the ability of MEK1/2/5 inhibitors to cause profound growth arrest in certain cell types. In contrast, signaling by the PI3K pathway appears to be cytoprotective in virtually all cell systems. Thus it is possible in the future that combined inhibition of ErbB receptors and the MAPK pathway / PI3K pathway could be employed to inhibit multiple cytoprotective pathways in tumor cells (Figure 2).

6. THE REGULATION OF TWO PUTATIVE CASPASE CASCADES (IN A SIMPLIFIED MANNER) (see Figures 1 and 2).

As noted in previous sections, ErbB signaling, via MAPK and PI3K, can alter cell survival and apoptosis. The following section provides a brief explanatory background to Figures 1 and 2.

1. Apoptotic stimuli, such as ionizing radiation, can promote a reversal in the mitochondrial membrane potential ($\Delta\Psi$m) and cause the release of cytochrome c into the cytosol. Of note, however, several groups have recently shown that cytochrome c can also be released without loss of $\Delta\Psi$m. Cytosolic cytochrome c binds to the adapter protein *apo*ptosis *a*ctivating *f*actor-1 (Apaf-1). In the presence of dATP the Apaf-1-cytochrome c complex binds to pro-caspase-9 leading to activation of the caspase 9 molecule. Active caspase 9 can cleave and activate the effector pro-caspase 3, as well as other effector pro-caspases such as caspases 6 and 7. Active caspases 3/6/7 can then degrade various cellular proteins, ultimately leading to apoptosis.

2. Exposure of cells to the pro-apoptotic cytokine Fas-L activates its receptor Fas-R, and the associated cytosolic death domain adapter protein FADD; forming a DISC (*d*eath-*i*nducing *s*ignaling *c*omplex). FADD interacts with pro-caspase 8 causing its cleavage. Cleaved, active caspase 8 is released from the DISC and in turn cleaves/activates the effector pro-casapse 3. A similar mechanism exists for TNFα signaling through the TNFα receptor I and its death domain, to TRADD, FADD and pro-caspase 8. The cell types we propose to examine in our studies express both TNFα I receptors and Fas-R. Of note, we know that radiation can activate the TNFα receptor and inhibition of MAPK and PI3K signaling can potentiate FAS-R signaling to cause cell death.

Of note, however and unfortunately, there is also a third further more complicated possibility. For example, the cytochrome c / caspase 9 pathway can play a role in activating caspase 8 via caspase 3. (Caspase 9 cleaves Caspase 3 which can cleave Caspase 8). Similarly, in many cell types the death receptor/caspase 8 pathway can cleave BID, thereby releasing cytochrome c from the mitochondria, activating caspase 9 followed by caspase 3, and then active caspase 3 can play a role in the further activation of caspase 8. In these cells, caspase 8 does not initially act to cleave pro-caspase 3. Thus it is possible for either caspase 8 or caspase 9 to play a role in an "amplification loop" for further activation of either caspase 9 or caspase 8, respectively.

The ability of a cell to undergo apoptosis is also impacted by expression of intracellular apoptosis caspase inhibitors, Inhibitor of Apoptosis (IAPs). IAP proteins can bind to domain proteins such as FADD and pro-caspase 3, and inhibit their function. Another protein shown to play a role in inhibiting FADD and pro-caspase 8 function are c-FLIP$_S$ and c-FLIP$_L$; c-FLIP isoforms can inactivate the "DISC" by blocking recruitment of pro-caspase 8. Of further note, c-FLIP expression can be regulated by Akt and MAPK signaling.

Alternatively, high expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xl may stabilize the mitochondrial membrane potential, reducing the likelihood of cytochrome c release / caspase activation. Phosphorylation of Bcl-2 can modulate its anti-apoptotic function in a stimulus-specific manner; recently, the JNK was proposed to phosphorylate and inactivate Bcl-2 after exposure of cells to taxanes. Phosphorylation of the pro-apoptotic protein BAD, potentially by MAPK and Akt signaling, can also reduce its ability to modify the mitochondrial membrane potential and cause apoptosis. Recently, it was demonstrated that MAPK signaling could reduce caspase 9 activation in the presence of cytosolic cytochrome c. This may be by direct phosphorylation of Caspase 9, since it is already known that human caspase 9

can be phosphorylated by Akt. This demonstrates that MAPK and Akt signaling can impact on caspase activation downstream of the mitochondria. In addition, others have shown that TNFα and Fas-L induced apoptosis can be potentiated by MAPK inhibition and blunted by MAPK and Akt activation. These data suggest that MAPK and PI3K signaling may therefore be able to attenuate or oppose caspase activation.

7. CONCLUSIONS

Drug companies and many research groups have utilized inhibitors of ErbB family receptors to investigate signaling by EGF, TGF alpha and the neuregulin / heregulin families of growth factors. In these studies it was discovered that inhibition of ErbB receptor function by multiple mechanisms results in a blocking of ligand-dependent receptor activation which resulted in reduced proliferation of agonist-treated cells. This correlated with reduced activation of multiple downstream signal transduction pathways. Subsequently, it was discovered that cytotoxic stresses could cause ligand-dependent and ligand-independent activation of ErbB family receptors. Use of ErbB receptor inhibitors demonstrated that the toxicity of stresses are magnified when the functional activation of the receptors is inhibited. Similarly, inhibition of downstream protective pathways such as the PI3K and MAPK pathways was shown to mediate the protective effects of ErbB receptor signaling. Clinical trials are currently in progress using ErbB, PI3K and MAPK inhibitors as agents alone or in combination with cytotoxic drugs or radiation. It will still be several years, however, before full knowledge of the impact of inhibiting ErbB receptor function has on the survival of patients treated in combination with standard therapeutic regimens.

Apoptosis is a physiologic cell suicide program that is mediated by regulated signaling molecules *via* specific limited proteolysis. The caspases are aspartate-specific cysteine proteases which play a key role in apoptotic cell death. These enzymes are synthesized as inactive zymogens which are activated by apoptotic signals, such as proteolytic cleavage or binding to a co-factor. This leads to the cleavage of chromosomal DNA as well as key cellular proteins such as PARP. The cytoplasm and nucleus shrink, the plasma membrane blebs, chromatin condenses and DNA fragments.

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