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REVIEW

Stress and Radiation-Induced Activation of Multiple Intracellular Signaling Pathways[1]

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Exposure of cells to a variety of stresses induces compensatory activations of multiple intracellular signaling pathways. These activations can play critical roles in controlling cell survival and repopulation effects in a stress-specific and cell type-dependent manner. Some stress-induced signaling pathways are those normally activated by mitogens such as the EGFR/RAS/PI3K-MAPK pathway. Other pathways activated by stresses such as ionizing radiation include those downstream of death receptors, including pro-caspases and the transcription factor NFKB. This review will attempt to describe some of the complex network of signals induced by ionizing radiation and other cellular stresses in animal cells, with particular attention to signaling by growth factor and death receptors. This includes radiation-induced signaling via the EGFR and IGFI-R to the PI3K, MAPK, JNK, and p38 pathways as well as FAS-R and TNF-R signaling to pro-caspases and NFKB. The roles of autocrine ligands in the responses of cells and bystander cells to radiation and cellular stresses will also be discussed. Based on the data currently available, it appears that radiation can simultaneously activate multiple signaling pathways in cells. Reactive oxygen and nitrogen species may play an important role in this process by inhibiting protein tyrosine phosphatase activity. The ability of radiation to activate signaling pathways may depend on the expression of growth factor receptors, autocrine factors, RAS mutation, and PTEN expression. In other words, just because pathway X is activated by radiation in one cell type does not mean that pathway X will be activated in a different cell type. Radiation-induced signaling through growth factor receptors such as the EGFR may provide radioprotective signals through multiple downstream pathways. In some cell types, enhanced basal signaling by proto-oncogenes such as *RAS* may provide a radioprotective signal. In many cell types, this may be through PI3K, in others potentially by NFKB or MAPK. Receptor signaling is often dependent on autocrine factors, and synthesis of autocrine factors will have an impact on the amount of radiation-induced pathway activity. For example, cells expressing TGFα and HB-EGF will generate protection primarily through EGFR. Heregulin and neuregulins will generate protective signals through ERBB4/ERBB3. The impact on radiation-induced signaling of other autocrine and paracrine ligands such as TGFβ and interleukin 6 is likely to be as complicated as described above for the ERBB receptors.

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SIGNALING: CELLULAR RESPONSES TO STIMULI

Cell-to-cell communication, and how a cell translates such signals into metabolic, proliferative or death responses, has become a central area of study in many laboratories. Thus understanding how plasma membrane receptors, frequently through transducers such as GTP binding proteins, regulate signal transduction pathways has been the focus of intense study.

A historical overview of our understanding of signal transduction processes demonstrates the relative explosion of novel information that has occurred within the last 15 years. Initial studies in the 1930s and 1940s by Drs. Carl and Gertrude Cori argued that glycogen metabolism was a regulated process (*1, 2*), and further studies in the laboratories of Sutherland (*3*), Krebs and Fisher (*4, 5*), Leloir (*6*) and Larner (*7*) determined that protein phosphorylation played a key role in the control of glycogen metabolism and that second-messenger molecules such as cyclic AMP were important mediators of the action of epinephrine. By the late 1960s, the activities of five proteins were thought to be regulated by reversible protein phosphorylation (*8*).

Studies in the 1970s demonstrated that Ca^{2+} ions were second messengers and that epinephrine signaling was transduced into the cytoplasm by GTP binding proteins (*9–11*). The early 1980s saw the discovery of inositol lipid

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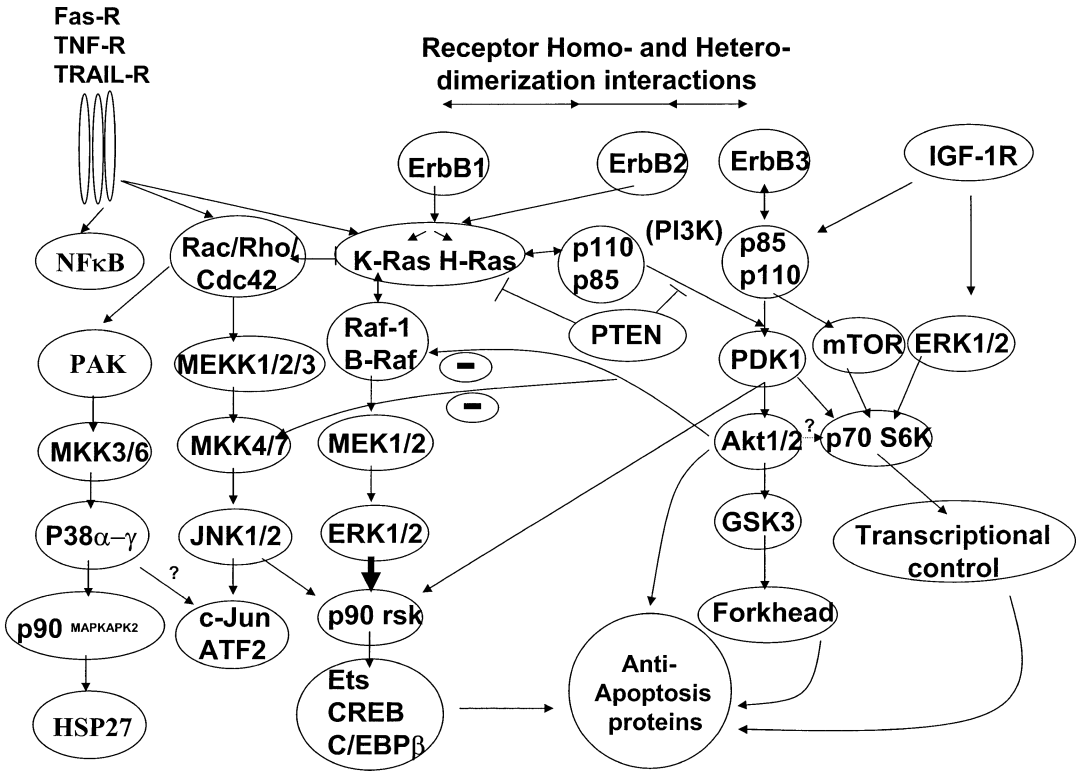

FIG. 1. Some of the characterized signal transduction pathways in mammalian cells. Growth factor receptors e.g. the ErbB family, can signal down through GTP binding proteins into multiple intracellular signal transduction pathways. Predominant among these pathways are the MAP kinase superfamily of cascades (ERK, JNK, p38) as well as the PI3K pathway.

second messengers (*12*, *13*) and the demonstration that tyrosine phosphorylation appeared to play a major role in many signaling processes, including those of insulin and the newly described oncogenes such as Src (*14*, *15*). The initial discovery of what are now often termed the mitogen-activated protein kinase (MAPK) pathways was made in the mid-1980s (Fig. 1).

THE MITOGEN ACTIVATED PROTEIN KINASE (MAPK) PATHWAY

''MAPK'' was first reported in 1986 (*16*). This protein kinase was originally described as a 42-kDa insulin-stimulated protein kinase activity whose tyrosine phosphorylation increased after insulin exposure and that phosphorylated the cytoskeletal protein MAP2. Contemporaneous studies identified an additional 44-kDa isoform of MAPK, ERK1 (extracellular signal regulated kinase; see Table 1 for a guide to the nomenclature used in this review) (*17*). 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 ($p90^{rsk}$) (*18*), and that they were themselves regulated by protein kinase activities designated MKK1/2 (MAPK kinase) or MEK1/2 (*19–22*). MKK1/2 were also regulated by reversible phosphorylation. The protein kinase responsible for catalyzing MKK1/2 activation was the proto-oncogene *RAF1* (*23*, *24*). It has been suggested that other enzymes at the level of MKK1/2 can phosphorylate and activate p42/44 MAPK, e.g. RIP2 (*25*), which plays a role in TNFα-induced, but not EGF-induced, MAPK activation (Fig. 1).

Raf-1 is a member of a family of serine-threonine protein kinases termed Raf-1, B-Raf, and A-Raf. 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) (*26*, *27*). Thus the ''Raf'' kinases act at the level of a MAPK kinase kinase (MAPKKK). Of note, we have found in A431 squamous carcinoma cells exposed to doses of less than 2 Gy that ionizing radiation activates Raf-1 but not B-Raf (*28*). 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 (*29*, *30*). Other findings proved that the ability of Raf-1 to be activated depended upon Raf-1 translocation to the plasma membrane (*31*, *32*) (Fig. 1).

In addition to the role Raf-1 plays in the activation of the MAPK pathway, it is important to note that Raf-1 may act upon substrates other than MEK1/2, such as the myosin phosphatase binding protein (*33*). Raf-1 has been proposed to act as an inhibitor of apoptosis signaling kinase 1

TABLE 1
Aliases and Official Names for Proteins and Genes Discussed in this Review

Alias(es) used	Official name
Akt, PKB	AKT1
A-Raf	ARAF
ASK1	MAP3K5
Bak	BAK
Bax	BAX
Bcl_{-XL}	BCL2L1
Bid	BID
B-Raf	BRAF
Cdc42	CDC42
c-FLIP	CFLAR
c-Jun	JUN
c-Myc	MYC
EGFR	ERBB1
ErbB	ERBB
ErbB3	ERBB3
ERK1	MAPK3
ERK2	MAPK1
Fas-L	TNFSF6
FAS-R	TNFRSF6
GSK3	GSK3A and GSK3B
HB-EGF	Heparin binding-EGF
HER1–4	ERBB1–4
H-Ras	HRAS
HSP27	HSLP1
cIAP	BIRC1–4
IGFI-R	IGF1R
JNK	MAPK8
JNK1	MAPK8
JNK2	MAPK9
JNK3	MAPK10
K-Ras	KRAS
MAPKKK	MAP3K family
Mcl-1	MCL1
MEK1	MAP2K1
MEK2	MAP2K2
MKK1	MAP2K1
MKK2	MAP2K2
MKK7	MAP2K7
MEKK1–4	MAP3K1–4
mTOR	FRAP1
NDF	NRG1
NFkB, NFκB	NFKB
p110	PIK3C2A/B
p38 (and isoforms)	p38β = MAPK11, p38γ = MAPK12, p38δ = MAPK13
p42	MAPK1
p44	MAPK3
$p70^{S6K}$	RPS6KB2
p85	PIK3R1/2
$p90^{rsk}$	RPS6KA1-3
PAR4	PAWR
PI4	PIK4CA
PI5	PIK5CA
PKC	PRKC (family)
PKCζ	PRKCZ
Rac1	RAC1
Raf-1	RAF1
Rho	RHO
RIP2	RIPK2
SAPK	MAPK
SEK1, MKK4	MAP2K4
Smac/DIABLO	second mitochondrial activator of caspases/direct IAP binding protein with low pI
Src, c-Src	SRC
TAK-1	NR2C2
TGFα	TGFA
TGFβ	TGFB
TNF-R	TNFR (superfamily)
Tpl-2	MAP3K8
uPA	PLAUR
v-Erb-B	ERBB (family)

(ASK1) by binding to ASK1: The inhibitory actions of Raf-1 were reported to be independent of Raf-1 protein kinase activity (*34*).

The regulation of Raf-1 activity appears to be very complex, with several mechanisms coordinately regulating activity 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 (*35*). 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 (*36–39*). Others have argued that 14-3-3 proteins binding to these sites inhibit Raf-1 activation (*40*). Phosphorylation of S338 by PAK enzymes has more recently been shown to play a role in the activation process (*41*). Other investigators have suggested that another lipid second messenger, ceramide, may be able to play a role in Raf-1 activation (*42, 43*). Data from several laboratories have suggested that protein serine/threonine and tyrosine phosphorylation play a role increasing Raf-1 activity in the plasma membrane environment (*44–46*). Other studies have suggested that PKC (protein kinase C; now known as PRKC) isoforms can directly regulate Raf-1 activity (*47, 48*). 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 (*39, 49, 50*). At the same time that Raf-1 was shown to associate with Ras, it was found that growth factors, through their plasma membrane receptors, stimulate GTP for GDP exchange in Ras using guanine nucleotide exchange factors (*51, 52*). Thus a signaling pathway (often termed the ''classical'' MAPK pathway) was delineated from plasma membrane growth factor receptors, through the guanine nucleotide exchange factors and Ras, to Raf-1/MKK/MAPK/$p90^{rsk}$ (Fig. 1).

THE PHOSPHATIDYL INOSITOL 3-KINASE (PI3K) PATHWAY

Inositol phospholipids were first proposed as important second messenger signaling molecules in the 1980 (*53*). Phospholipase C γ, when activated by mitogens such as

EGF and TGFα, cleaved inositol phospholipids into diacylglycerol and IP_3, with the release of IP_3 into the cytoplasm (*54*). IP_3 interacts with a receptor in the endoplasmic reticulum, leading to release of Ca^{2+} into the cytosol, and Ca^{2+}, together with diacylglycerol, can cause activation of PKC isoforms (*55, 56*).

PI3K enzymes consist of two subunits, a catalytic p110 subunit and a regulatory and localizing subunit, p85; several different classes of PI3K enzymes exist (*57, 58*). The p85 subunit of PI3K enzymes contains a phospho-tyrosine (SH2) binding domain (*59*). 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 TGFα and heregulin stimulate tyrosine phosphorylation of ErbB family receptors, providing acceptor sites for the SH2 domain of p85 (*60, 61*). 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 bind directly to Ras-GTP, leading to catalytic activation of the kinase (*62–64*) (Fig. 1).

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) (*65*). Of note, PDK1 can also be regulated by protein phosphorylation (*66*). PDK1 is proposed to phosphorylate and activate Akt, as well as to play a faciliatory role in the activation of other protein kinases such as $p90^{rsk}$. The PI3K-dependent phosphorylation of the inositol sugar ring can be reversed by the tumor suppressor lipid phosphatase PTEN (phosphatase and tensin homologue on chromosome ten) (*67, 68*). Loss of PTEN expression is found frequently in some tumor cell types, e.g. glioblastoma multiforme (*76*), resulting in an apparent constitutive activation of PDK1 and Akt (*49, 50, 70*) (Fig. 1).

Signaling by PDK1 to Akt and by PDK1 and Akt downstream to other protein kinases such as PKC isoforms, GSK3, mTOR, $p70^{S6K}$ and $p90^{rsk}$, has been shown to play a key role in mitogenic and metabolic responses of cells as well as in the protection of cells from noxious stresses (*71–75*). 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 (*76*). Indeed, evidence is now emerging that in addition to PDK1, other protein kinases including p38 MAPK and the integrin-linked kinase (suggested to be ''PDK2''-like enzymes) and the proto-oncogene c-Src can phosphorylate Akt on multiple PDK1-independent sites resulting in modified Akt activity (e.g. *77, 78*).

THE c-JUN NH_2-TERMINAL KINASE (JNK) PATHWAY

The c-Jun NH_2 terminal kinase (JNK) pathway was discovered and described in the early to mid-1990s (*79, 80*). JNK1/2 were initially described biochemically to be a stress-induced protein kinase activity that phosphorylated the NH_2-terminus of the transcription factor c-Jun; hence the pathway is often called the stress-activated protein kinase (SAPK) pathway. Multiple stresses increase JNK1/2 (and the subsequently discovered JNK3) activity including UV and γ radiation, cytotoxic drugs, and reactive oxygen species (H_2O_2). Phosphorylation of the NH_2-terminal sites Ser63 and Ser73 in c-Jun increases its ability to trans-activate AP-1 enhancer elements in the promoters of many genes (*81, 82*). It has been suggested recently that JNK can phosphorylate the NH_2-terminus of c-Myc, potentially playing a role in both proliferative and apoptotic signaling (*83*). In a similar manner to the previously described MAPK pathway, JNK1/2 activities were regulated by dual threonine and tyrosine phosphorylation, which were found to be catalyzed by a protein kinase analogous to MKK1/2, termed stress-activated extracellular regulated kinase 1 (SEK1), also called MKK4 (*84*). An additional isoform of MKK4, termed MKK7, was subsequently discovered (*85*). As in the case of MKK1/2, MKK4/7 are regulated by dual serine phosphorylation. In contrast to the MAPK pathway, which appears to use primarily the three protein kinases of the Raf family to activate MKK1/2, at least 10 protein kinases are known to phosphorylate and activate MKK4/7, including MKKK1-4, TAK-1 and Tpl-2 (*86*). The agonist and cell type specificity of each JNK pathway MAPKKK enzyme in the activation of this pathway is currently under intense investigation (Fig. 1).

Upstream of the MAPKKK enzymes are another layer of JNK pathway protein kinases, e.g. Ste20 homologues and low-molecular-weight GTP-binding proteins of the Rho family, in particular Cdc42 and Rac1 (Fig. 1) (*87*). It is not clear how growth factor receptors, e.g. EGF receptor, activate the Rho family of low-molecular-weight GTP-binding proteins; one proposed mechanism suggests activation by the *Ras* proto-oncogene, whereas other proposed mechanisms suggest activation by PI3K and/or protein kinase C isoforms (*88, 89*). In addition, other groups have shown that agonists acting through the tumor necrosis factor alpha (TNFα) receptor, through sphingomyelinase enzymes generating the lipid second-messenger ceramide, can activate the JNK pathway by mechanism(s) that may act through Rho family GTPases (*90*). Definitive answers to all of these questions await further investigation. In the following sections, potential roles in the control of growth, proliferation, cell survival and DNA repair for the JNK and MAPK pathways are examined.

THE p38 MAPK PATHWAY

The p38 MAPK pathway was originally described as a mammalian homologue to a yeast osmolarity sensing pathway (*91*). It was soon discovered that many cellular stresses activated the p38 MAPK pathway, in a manner not dissimilar to that described for the JNK pathway (*92*). Rho family GTPases appear to play an important role as upstream activators of the p38 MAPK pathway. Through several MAPKKK enzymes, e.g. the PAK family (*93*), they regulate the MAPKK enzymes MKK3 and MKK6 (*94*). At least four isoforms of p38 MAPK exist, termed p38 α, β, γ and δ (*95*). There are several protein kinases downstream of the p38 MAPK enzymes, including $p90^{MAPKAPK2}$ (*96*) and MSK1/2 (*97*). $p90^{MAPKAPK2}$ phosphorylates and activates HSP27, while MSK1/2 can phosphorylate and activate transcription factors such as CREB (*98, 99*) (Fig. 1).

The role of p38 MAPK signaling in cellular responses is diverse, depending on the cell type and stimulus. For example, p38 MAPK signaling has been shown to promote cell death as well as to enhance cell growth and survival (*100–102*). The ability of ionizing radiation to regulate p38 MAPK activity appears to be highly variable, with different groups reporting either no activation (*103*), weak activation (*104*) or strong activation (*105*). This is in contrast to the classical MAPK and JNK pathways, where radiation-induced activation has been observed by many groups in diverse cell types. In studies where p38 MAPK activation has been observed after irradiation, the p38γ isoform has been proposed to signal G_2/M-phase arrest (*106*). In these studies p38γ signaling was dependent on expression of a functional ATM protein. These findings suggest that specific inhibitors of p38γ may have therapeutic benefit.

THE ErbB FAMILY OF RECEPTOR TYROSINE KINASES

The ErbB family of receptor tyrosine kinases comprises ErbB1–ErbB4 (also called HER1–4). 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 (*107*). ErbB1 and its autocrine ligands epidermal growth factor and TGFα were described over 20 years ago (*107–109*). The EGF receptor was found to have a tyrosine kinase within its intracellular domain whose activity was stimulated upon ligand binding (*110, 111*). Further studies showed that the EGF receptor had homology with the v-Erb-B oncogene and that the EGF receptor was frequently overexpressed in a wide range of carcinomas (*112, 113*). 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 (*114*). Several other truncated forms of the EGF receptor are known to exist that appear to play a role in tumorigenic processes (*115*), and ErbB1 truncated forms are overexpressed in many types of tumor cells (*116*). These findings strongly argue that signaling by ErbB1 plays a role in tumor cell growth (Fig. 1).

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 (*117*). 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*, it contains a tyrosine kinase motif within its intracellular domain (*118*). 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 (*119–121*). ErbB2 is overexpressed in solid tumors (~15–25%), including mammary carcinoma, and is believed, together with ErbB1, to play a protective role against cytotoxic insults (*122, 123*) (Figs. 1–3).

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 (*124*). Unlike ErbB1 and ErbB2, ErbB3 is capable of binding to ligands of the NDF/heregulin family but does not bind to ligands of the EGF/TGFα family (*125*). 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 (*126*). In a similar manner to ErbB3, ErbB4 also can bind ligands of the NDF/heregulin family (*127*). 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 (*128*). Similar to the truncations observed in ErbB1 during transformation, naturally occurring variants of ErbB4 have been shown to exist, although their roles in the process of cellular transformation are less clear at present (*129, 130*).

IONIZING RADIATION ACTIVATES ErbB RECEPTORS

Several groups have shown that the epidermal growth factor receptor (EGFR, also called ErbB1 and HER1) is activated in response to irradiation of various carcinoma cell types (*28, 131–133*). The threshold dose at which radiation could induce Ca^{2+} oscillations and ErbB1 phosphorylation in MCF7, A431 and MDA-MB-231 carcinoma cells appeared to be ~0.5 Gy (*28, 132*). This may explain in part why some cell types irradiated with doses of 0.1–0.4 Gy that do not activate the ErbB receptor system exhibit hypersensitivity to radiation (*256*). Radiation exposure in the range of 1–2 Gy, through activation of the EGFR, can activate the MAPK pathway to a level similar to that observed by physiological, growth-stimulatory, EGF concentrations (~0.1 n*M*) (*28, 131–134*).

The proliferation of many carcinoma cells *in vitro* and *in vivo* is regulated in part by the synthesis and autocrine action of growth factors such as transforming growth factor α (TGFα) (*135*). Irradiation of tumor cells can increase expression of TGFα and activate the EGFR; this has been proposed as one mechanism by which radiation can increase the proliferation rate of surviving cells (*136, 137*). Increased proliferation rates and poor prognosis of carcinomas *in vivo* have been correlated with increased expression of the EGFR (*138*). These findings argue that radiation may have a self-limiting effect on its toxicity through increased activity of EGFR and associated downstream signaling modules.

The actions of ErbB receptor autocrine ligands have been shown to play important roles in the activation of receptors after radiation exposure. TGFα has been shown to mediate secondary activation of ErbB1 and the downstream MAPK and JNK pathways after irradiation in several carcinoma cell lines (*139, 140*). Radiation caused cleavage of pro-TGFα in the plasma membrane, which led to its release into the growth medium. Increasing the radiation dose from 2 Gy to 10 Gy enhanced both the secondary activation of ErbB1 and the secondary activation of the MAPK and JNK pathways, suggesting that radiation can promote a dose-dependent increase in the cleavage of pro-TGFα that reaches a plateau at ~10 Gy. It should be noted that in contrast to the secondary activation, primary activation of the receptor and signaling pathways appeared to reach a maximum between 2–3 Gy. In addition, signaling by Ras, MAPK and TP53, the activities of which can be increased after radiation exposure, has been shown in a variety of cell systems to increase the expression of HB-EGF and epiregulin (*141*). These findings argue that the activation of ErbB family receptors by radiation will be influenced by both the Ras and TP53 status (mutant or wild-type) of a given tumor cell. Thus, for example, in cells expressing a mutant K-Ras protein such as HCT116, loss of mutant Ras function lowers basal MAPK activity and reduces epiregulin expression (*142*). This in turn correlates with reduced basal and radiation-induced MAPK activation (Fig. 1; Dent, unpublished results).

More recent findings have shown that radiation can activate other ErbB family members including ErbB2, ErbB3 and ErbB4 (*143, 144*). In these studies, radiation-induced activation of ErbB2 did not appear to depend on ErbB1, suggesting that radiation causes an indiscriminate activation of multiple plasma membrane receptor tyrosine kinases. In addition to previous studies showing that ErbB1 generated an anti-apoptosis signal, more recent findings have demonstrated that radiation-induced ErbB2 activation generates a strong anti-apoptosis signal mediated by PI3K (*144*).

INHIBITORS OF ErbB AND OTHER GROWTH FACTOR RECEPTORS CAN MODIFY THE GROWTH AND SURVIVAL OF NORMAL AND TUMOR CELLS

Signaling by the ErbB family of receptors is generally thought to be pro-proliferative and cytoprotective (*145, 146*). In some cell types, however, EGF and EGF receptor signaling is known to promote growth arrest and apoptosis (e.g. *147, 148*). Because both receptor expression and 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), low-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 that the sensitivity of these cells to being killed by noxious stresses increased (*149–164*) (see Fig. 2).

The antibodies C225 and 4D5 herceptin bind to the extracellular portions of ErbB1 and ErbB2, respectively (*165, 166*). In the case of ErbB1, C225 appears to bind to the portion of the molecule that associates with growth factor ligands such as EGF and TGFα (*167*). Thus the ability of growth factors, in the presence of receptor-bound C225, to stimulate ErbB1 receptor function is abolished (Figs. 1 and 2). C225 does not block the primary activation of the receptor or MAPK after irradiation, in general agreement with the ligand-independent nature of this process. The anti-proliferative and anti-survival mechanisms of action of herceptin appear to be more complex, inasmuch 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 (*168*). Both C225 and herceptin have been shown individually to 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* (*169–172*). Both C225 and herceptin are currently in phase III trials, and it is likely, despite setbacks for C225 in FDA approval, that both agents will become standard tools in the treatment of epithelial cell cancers. More recent studies have used monoclonal antibodies to target truncated forms of ErbB1, e.g. EGFR VIII (*151, 173*). In these studies, a novel monoclonal antibody, 806, was found to potently inhibit truncated forms of ErbB1 and inhibit full-length receptor more weakly (*173*). 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 through receptor inhibition or by a combination of receptor inhibition and enhanced immunological reactivity *in vivo* due to the Fc portion of the antibody (Fig. 2).

Small molecule inhibitors of the tyrosine kinase domains of the ErbB family of receptors have been used with some

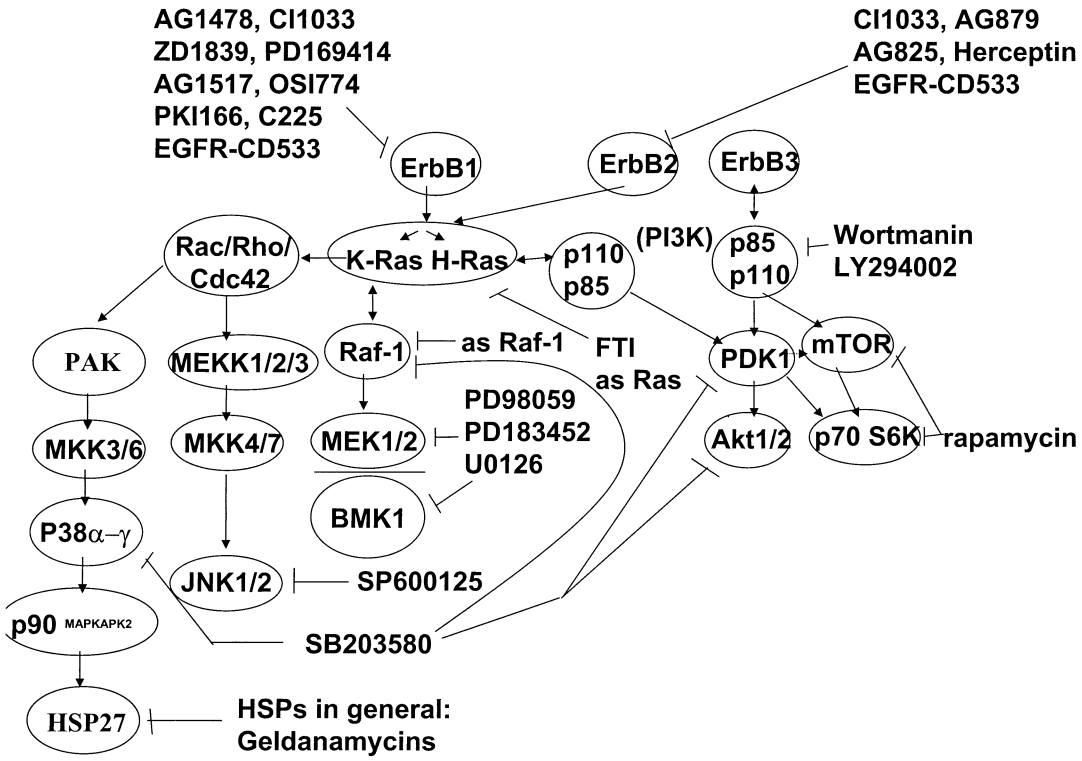

FIG. 2. Inhibitors of growth factor receptors and intracellular signal transduction pathways. Because growth factor receptors, Ras proteins and downstream pathways are often activated in tumor cells, and are often activated by radiation; inhibitors have been developed to block the function of these molecules, thereby slowing cell growth and promoting cell death responses after radiation exposure. Multiple inhibitors for the ErbB family receptors have been developed. Inhibitors of Ras farnesylation (and geranylgeranylation) are in clinical trials, as are inhibitors of the MAPK/ERK pathway. It should be noted that MEK1/2 inhibitors are capable of inhibiting the ''Big'' MAP kinase pathway by blocking activation of MEK5. PI3K inhibitors have been tested *in vivo*, but difficulties have emerged with systemic toxicity to these reagents. Geldanamycins are a class of agents that block the function of heat-shock protein 90 and down-regulate protein expression of proteins that bind HSP90 including Raf family members, ErbB2 and Akt.

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 (*152–160, 174–176*) (Fig. 2). 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 been shown to inhibit ErbB4 (*143*). The tyrphostin inhibitors AG825 and AG879 are ErbB2 inhibitors with apparently weaker kinase specificity than AG1478 for ErbB1/4, and they can inhibit Trk receptors (*159, 160*) (Fig. 2). Thus AG825/AG879, together with AG1478, has the potential to have an impact not only on EGF/TGFα signaling through ErbB1, but also on neuregulin/heregulin signaling through ErbB4 and ErbB3 (*177*). Low-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. *178–181*).

In addition to the use of antibodies and low-molecular-weight inhibitors, the ErbB family of receptors has 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* (*28, 161, 182–186*). 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 (*28, 182*) and subsequent investigations using dominant negative EGFR-CD533 demonstrated that it could block radiation-induced phosphorylation of the EGFR (183–186). 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* (*161, 183–186*) (Fig. 2). Collectively, these findings demonstrate that

the EGFR is a key cytoprotective 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 (Fig. 2).

THE ROLE OF OTHER GROWTH FACTOR RECEPTORS IN RADIATION RESPONSES

In addition to the ErbB family, other growth factor and cytokine receptors are believed to play an important role in cellular radiation responses. Cytokines such as TNFα, IL6, urokinase-type plasminogen activator (uPA), and TGFβ have all been proposed to control cell survival responses after irradiation (*187–190*).

Radiation has been shown to cause rapid activation of the TNFα receptor and, in addition, radiation-stimulated signaling modules such as the classical MAPK and p38 pathways are known to enhance the synthesis of TNFα ligand (*191*). TNFα signaling after irradiation may lead to the activation of both pro-caspase enzymes as well as the cytoprotective transcription factor nuclear factor kappa B (NFKB) (*192*). Thus the cellular outcome of radiation-induced TNFα receptor signaling will be a complex summation of opposing cellular signals.

IL6 is a cytokine that is proposed to regulate immune cell function as well as the ability of epithelial cells to proliferate (*188*). Several groups have shown that IL6 can generate anti-apoptosis signals in cells that are protective against the toxic effects of ionizing radiation (*193*). In some cell types, the protective effect of IL6 has been proposed to be mediated by the PI3K pathway (*194*), and in others the radiation-induced expression of IL6 is dependent upon prior activation of NFKB (*195*).

In nontransformed cells, TGFβ can cause growth arrest and differentiation (*196*). In tumor cells, TGFβ has been shown to cause either cytoprotection or apoptosis in a cell type-dependent manner (*197*). In some cells, TGFβ appears to confer a protective effect through MAPK signaling and potentially the expression of molecules such as HB-EGF and Bcl-$_{XL}$ (now known as BCL2L1) (*198–200*). In contrast, TGFβ in other cell types appears to protect cells in a Ras- and MAPK-independent manner that is dependent on PI3K signaling (*201*). Collectively, the findings described in this section argue that multiple cytokines, in addition to those for the ErbB family, play a role in the radiation responses of both nontransformed as well as tumor cells.

PATHWAYS DOWNSTREAM OF ErbB FAMILY AND OTHER GROWTH FACTOR RECEPTORS CAN MEDIATE SURVIVAL SIGNALING

Signaling by the ErbB family of receptors in response to growth factors is believed to play an important anti-apoptosis 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-apoptosis effector pathway. The PI3K and MAPK pathways were discussed earlier in this review. However, it should be noted that other pathways and molecules downstream of ErbB signaling including K-/H-Ras molecules, JAK/STAT molecules (*202*), and the c-Jun NH_2-terminal kinase pathway (*203*) are known to mediate ErbB receptor anti-apoptosis signaling in a cell type- and toxic stress-specific manner. The proto-oncogene *Ras* is a key effector downstream of plasma membrane receptors in the response of cells after radiation exposure, and farnesyltransferase inhibitors are under clinical investigation. This review is focusing primarily on the PI3K and MAPK pathways.

A simplified diagram showing possible interactions between signaling pathways and the apoptotic machinery of cells is shown in Fig. 3. Extensive efforts are under way to elucidate mechanisms governing apoptosis, a genetically regulated process of cell suicide that is particularly common in hematopoietic cells but is exhibited to a lesser extent by epithelial tumor cells (*204, 205*). Apoptosis occurs after activation of effector caspases (e.g. caspases 3, 6, 7) which can be triggered by either the extrinsic or intrinsic pathways (Fig. 3). The extrinsic pathway is characteristically initiated by ligation of the Fas ligand with its receptor, leading to formation of the death-inducing signaling complex (DISC), which permits the Fas-associated death domain (FADD) to cleave and activate procaspase 8 (*206*). Activated caspase 8 can activate effector caspases such as procaspase 3 or can initiate mitochondrial injury through Bid (*207*). The intrinsic or mitochondrial pathway becomes engaged after mitochondrial injury (e.g., loss of mitochondrial membrane potential; $\Delta\Psi$ m and/or release of pro-apoptosis proteins such as cytochrome c and Smac/DIABLO) (*208*). Cytochrome c, in association with dATP, promotes the caspase 9-mediated activation of procaspase 3 (*209*) (Fig. 3). Considerable cross-talk exists between the intrinsic and extrinsic pathways. For example, while caspase 8 directly activates caspase 3, it can cleave and activate the pro-apoptosis BH3-only domain Bcl-2 family member Bid, which then triggers cytochrome c release and results in further procaspase 3 activation (*210*). A large and expanding group of pro- and anti-apoptosis Bcl-2 family proteins has been described, which may act by modulating Bax/Bak interactions and mitochondrial pore function (*211*), or, in the case of IAP proteins, by directly inhibiting caspase activation (*212*). The relevance of apoptosis for tumor cell biology is underscored by accumulating evidence that diverse signaling pathways regulate cell survival and response to ionizing radiation and chemotherapy by modulating the apoptotic threshold (Fig. 3).

As noted above, many extracellular stresses, including ionizing and UV radiation and cytotoxic drugs, can activate the ErbB family of receptors, in a growth factor/ligand-independent manner. In addition to causing ligand-independent activation of ErbB receptors, ionizing radiation and other stresses can also cause the synthesis and release from

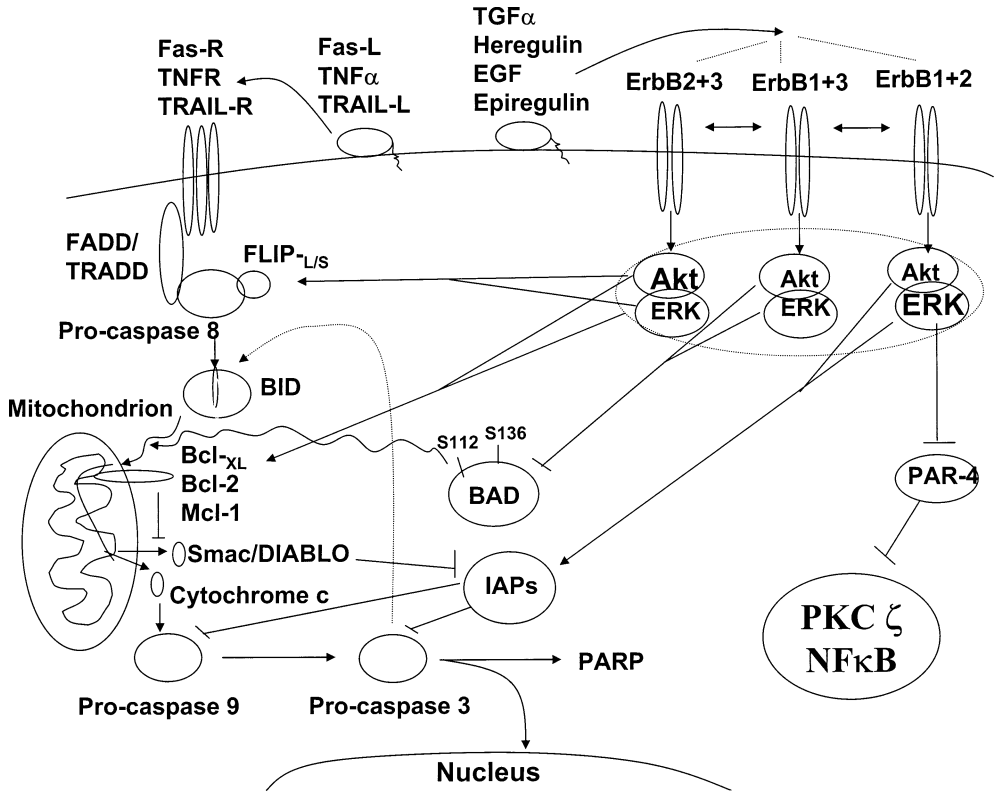

FIG. 3. Apoptotic response pathways and potential areas of control and overlap with kinase signal transduction pathways. Apoptosis occurs after activation of effector caspases (e.g. caspases 3, 6, 7), which can be triggered by either the extrinsic or intrinsic pathways. The extrinsic pathway is characteristically initiated by ligation of the Fas ligand with its receptor, leading to formation of the death-inducing signaling complex (DISC), which permits the Fas-associated death domain (FADD) to cleave and activate procaspase 8. Activated caspase 8 can activate effector caspases such as procaspase 3 or initiate mitochondrial injury through Bid. The intrinsic or mitochondrial pathway becomes engaged after mitochondrial injury (e.g. loss of mitochondrial membrane potential, $\Delta\Psi_m$, and/or release of pro-apoptosis proteins such as cytochrome c). Cytochrome c, in association with dATP, promotes the caspase 9-mediated activation of procaspase 3. Considerable cross-talk exists between the intrinsic and extrinsic pathways. For example, while caspase 8 directly activates caspase 3, it also cleaves and activates the pro-apoptosis BH3-only domain Bcl-2 family member Bid, which then triggers cytochrome c release and results in further procaspase 3 activation. A large and expanding group of pro- and anti-apoptosis Bcl-2 family proteins has been described that may act by modulating Bax/Bak interactions and mitochondrial pore function, or, in the case of IAP proteins, by directly inhibiting caspase activation. The relevance of apoptosis for tumor cell biology is underscored by accumulating evidence that diverse signaling pathways downstream of ErbB receptors (PI3K/Akt; MAPK) regulate cell survival and response to ionizing radiation and chemotherapy by modulating the apoptotic threshold.

tumor cells of autocrine growth factors such as TGFα that can re-energize the ErbB receptor system hours after the initial exposure to the stress (*139, 140, 213, 214*). Depending upon the milieu of ErbB receptor expression, this receptor activation(s) will result in the activation of multiple downstream pathways such as K-/H-Ras, PI3K, MAPK and NFKB. That stresses can cause the transient activation of other receptor molecules e.g. TNFα receptors, and the fact that TNFα signaling toward 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 (*215*).

The anti-apoptosis 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-apoptosis effects of ErbB receptor signaling have been attributed to activation of the PI3K/Akt pathway (*216, 217*). 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 (*218–220*). 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 through death receptors (*221*). In addition, Akt has been shown to phosphorylate BAD and human pro-caspase 9, thereby rendering these proteins inactive in the processes that lead to apoptosis (*222, 223*). 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 (*224*). Activation

of Akt was shown to protect cells from death in the presence of ErbB receptor inhibition (*225*). These findings strongly argue that PI3K/Akt signaling is a key cytoprotective response in many cell types downstream of ErbB family receptors.

Data from several groups has argued that the PI3K pathway is a key radioprotective pathway downstream of plasma membrane receptors. Inhibition of p110 PI3K function by use of the inhibitors LY294002 and wortmannin radiosensitizes tumor cells expressing mutant Ras molecules or wild-type Ras molecules that are constitutively active (*226–228*). It is possible that these inhibitors may exert a small portion of their radiosensitizing properties by inhibiting ATM, ATR and DNA-PK. Expression of a constitutively active p110 PI3K molecule was able to partially recapitulate the expression of mutant H-Ras. In these cell lines and culture conditions, inhibition of the MAPK pathway did not appear to alter the radiosensitivity of cells.

Signaling by the MAP kinase pathway downstream of ErbB receptors also protects cells from various noxious stresses. In part, the abilities of MEK1/2 inhibitors to enhance cell killing by radiation was linked to a derangement of radiation-induced G_2/M-phase growth arrest and enhanced apoptosis (*165, 229*). However, activation of the MAPK pathway after irradiation has been found to promote radiosensitivity in some cell types by abrogating the G_2/M-phase checkpoint (*230, 231*). The dual nature of MAPK signaling in the control of cell survival has been observed for other DNA-damaging agents such as adriamycin and UV radiation (*232–234*).

An excellent example of this dual nature of MAPK signaling is displayed by DU145 human prostate cancer cells. These cells secrete the EGFR ligand TGFα, which confers autocrine growth through MAPK signaling. Ionizing radiation markedly increases the release of TGFα, providing a growth stimulus that is at odds with cellular repair mechanisms. If EGFR-MAPK signaling is *transiently* blocked by either the tyrphostin AG1478 or a MEK1/2 inhibitor prior to irradiation, then growth of DU145 cells is retarded and cell killing is decreased. Moreover, if the EGFR is strongly activated by EGF or TGFα immediately after irradiation, then cell killing is increased, as would be expected. On the other hand, we reported previously that after irradiation, *prolonged* inhibition of MAPK can increase apoptosis and reduce clonogenic survival (*140, 238*). Therefore, the interruption of MAPK signaling can either enhance or degrade survival of DU145 cells depending on its timing and duration [*238* and unpublished data (MPH)].

Signaling from ErbB receptors through the MAPK pathway can lead to increased expression of Bcl-$_{XL}$, Mcl-1 and c-FLIP isoforms (*206, 235–237*). Radiation-induced MAPK activation has been linked to increased expression of the DNA repair proteins ERCC1 and XRCC1 (*140, 238*). Inhibition of ERCC1 and XRCC1 expression by a MEK1/2 inhibitor correlated with decreased DNA repair, increased micronucleus formation, and reduced clonogenic survival. In addition, the downstream effector of the ERK1/2 enzymes, p90^{rsk}, phosphorylates, with assistance from MSK1, the transcription factor CREB, which can activate the promoters of several anti-apoptosis proteins (e.g. *239, 240*). Of note, p90^{rsk} also needs PDK1 phosphorylation to be catalytically active, although this site may be constitutively phosphorylated in many cells (*57*). 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. *206, 241*).

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 and PI3K pathways. For example, expression of dominant negative EGFR-CD533 enhanced apoptosis and radiosensitized MDA-MB-231 mammary carcinoma cells; there were dependent, at least in part, upon inhibition of radiation-induced MAPK signaling. Expression of this dominant negative ErbB1 molecule could radiosensitize glioblastoma cells; this correlated with both reduced basal MAPK activity and radiation-induced activation. More recent findings have linked radiation-induced ErbB2 activation to a more potent anti-apoptosis signal (*242, 243*).

The transcription factor NFKB has been shown to be downstream of ErbB and TNFα receptors and was proposed to act as a radioprotector (*244*). However, other studies have argued against NFKB as a direct radioprotective factor (*245*). Many studies have suggested that NFKB signaling is regulated by the PI3K pathway whereas others have suggested MAPK signaling can regulate this transcription factor through autocrine mechanisms (*246–248*). MAPK signaling has the potential to inhibit expression of the protein PAR4 that is potentially downstream of mutant Ras molecules (*249, 250*). PAR4 is a protein inhibitor of PKCζ and NFKB function (*251–255*). More recently, PAR4 has been shown to radiosensitize prostate tumor cells (*254*). This may be due in part to enhanced signaling from death receptors (*255*). Thus PAR4 may be a link between MAPK signaling, NFKB function and radiosensitivity. Hence, in a cell type-dependent manner, PI3K, NFKB or MAPK signaling, downstream of receptors and Ras molecules, or a combination of these signals, may play a radioprotective role.

CONCLUSIONS

Ionizing radiation can activate multiple signaling pathways in cells. The ability of radiation to activate pathways *may* depend on the expression of growth factor receptors, autocrine factors, or Ras mutation. In other words, just because pathway X is activated by radiation in one cell type does not mean that pathway X will be activated in a different cell type.

In some cell types, enhanced basal signaling by oncogenes such as *Ras* may provide a radioprotective signal. In

many cell types, this may be by PI3K, in others potentially by NFKB or MAPK. Radiation-induced signaling through growth factor receptors such as the EGF receptor may provide radioprotective signals through multiple downstream pathways. Receptor signaling is often dependent on autocrine factors.

Synthesis of autocrine factors will have an impact on the amount of radiation-induced pathway activity: For example, cells expressing TGFα and HB-EGF will generate protection primarily through EGFR, and in a secondary manner through ErbB2/3/4. Cells expressing epiregulin will generate protection through EGFR/ErbB4 and in a secondary manner through ErbB2/3. Heregulin and neuregulins will generate protective signals through ErbB4/3 and with ErbB2 as a secondary effector. The impact of other ligands on radiation-induced signaling is likely to be as complicated as described above for the ErbB receptors.

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 through the PI3K pathway. However, in other cell types with mutant Ras, protection appears to be mediated through either the MAPK pathway or NFKB. 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. 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 that in the future combined inhibition of ErbB receptors and the MAPK pathway/PI3K pathway could be employed to inhibit multiple cytoprotective pathways in tumor cells.

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