H-RAS V12–induced radioresistance in HCT116 colon carcinoma cells is heregulin dependent

Rubén W. Carón,[1,4] Adly Yacoub,[1] Xiaoyu Zhu,[1] Clint Mitchell,[1] Song Iy Han,[1] Takehiko Sasazuki,[3] Senji Shirasawa,[3] Michael P. Hagan,[1] Steven Grant,[2] and Paul Dent[1]

Departments of [1]Radiation Oncology and [2]Hematology/Oncology, Virginia Commonwealth University, Richmond, Virginia; [3]Department of Pathology, International Medical Center of Japan, Tokyo, Japan; and [4]Instituto de Medicina y Biología Experimental de Cuyo-CONICET, Mendoza, Argentina

Abstract

The abilities of mutated active K-RAS and H-RAS proteins, in an isogenic human carcinoma cell system, to modulate the activity of signaling pathways following exposure to ionizing radiation is unknown. Loss of K-RAS D13 expression in HCT116 colorectal carcinoma cells blunted basal extracellular signal-regulated kinase 1/2 (ERK1/2), AKT, and c-Jun NH_2-terminal kinase 1/2 activity. Deletion of the allele to express K-RAS D13 also enhanced expression of ERBB1, ERBB3, and heregulin but nearly abolished radiation-induced activation of all signaling pathways. Expression of H-RAS V12 in HCT116 cells lacking an activated RAS molecule (H-RAS V12 cells) restored basal ERK1/2 and AKT activity to that observed in parental cells but did not restore or alter basal c-*jun* NH_2-kinase 1/2 activity. In parental cells, radiation caused stronger ERK1/2 pathway activation compared with that of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which correlated with constitutive translocation of Raf-1 into the plasma membrane of parental cells. Inhibition of mitogen-activated protein kinase/ERK1/2, but not PI3K, radiosensitized parental cells. In H-RAS V12 cells, radiation caused stronger PI3K/AKT pathway activation compared with that of the ERK1/2 pathway, which correlated with H-RAS V12–dependent translocation of PI3K into the plasma membrane. Inhibition of PI3K, but not mitogen-activated protein kinase/ERK1/2, radiosensitized H-RAS V12 cells. Radiation-induced activation of the PI3K/AKT pathway in H-RAS V12 cells 2 to 24 hours after exposure was dependent on heregulin-stimulated ERBB3 association with membrane-localized PI3K. Neutralization of heregulin function abolished radiation-induced AKT activation and reverted the radiosensitivity of H-RAS V12 cells to those levels found in cells lacking expression of any active RAS protein. These findings show that H-RAS V12 and K-RAS D13 differentially regulate radiation-induced signaling pathway function. In HCT116 cells expressing H-RAS V12, PI3K-dependent radioresistance is mediated by both H-RAS-dependent translocation of PI3K into the plasma membrane and heregulin-induced activation of membrane-localized PI3K via ERBB3. [Mol Cancer Ther 2005;4(2): 243–55]

Introduction

Ionizing radiation is used as a primary treatment for many types of carcinoma. Although it has been appreciated for many years that radiation causes cell death, it has only recently become accepted that radiation has some potential to enhance proliferation in the surviving fraction of cells (1, 2). We and others have discovered that exposure of carcinoma cells to low radiation doses causes an initial early activation of growth factor receptors in the plasma membrane followed by secondary receptor activation that is dependent on autocrine growth factors (3, 4). Receptor activation enhances the activities of RAS family molecules that signal to cause activation of multiple intracellular signal transduction pathways. Secondary activation of intracellular signal transduction pathways by growth factors and radiation has been correlated to altered expression of cell cycle regulatory proteins and may under certain circumstances promote cell proliferation (5, 6).

Growth factors interact with plasma membrane receptors, which transduce signals through the membrane to its inner leaflet (7–14). Growth factor signals, via guanine nucleotide exchange factors, can increase the amount of GTP bound to membrane-associated GTP binding proteins, including RAS (15, 10). There are three widely recognized isoforms of RAS: Harvey RAS (H-RAS), Kirsten RAS (K-RAS), and neuroblastoma RAS (N-RAS; ref. 16). GTP-RAS can interact with multiple downstream effector molecules, including the Raf-1 protein kinase and the phosphatidylinositol 3-kinase (PI3K) lipid kinase. Receptor-stimulated guanine nucleotide exchange of ''RAS'' to the GTP-bound form permits Raf-1 and p110 PI3K to associate with ''RAS,'' resulting in kinase translocation to the plasma membrane environment where activation of these kinases, via complex mechanisms, takes place. RAS contains a GTPase activity

that converts bound GTP to GDP resulting in inactivation of the RAS molecule. PI3K enzymes are also translocated to the plasma membrane environment via the p85 SH2 domain interaction with phosphorylated tyrosine residues on adaptor proteins and growth factor receptors (e.g., GAB2, IRS-1, and ERBB3; ref. 17).

Mutation of RAS results in a loss of GTPase activity, generating a constitutively active RAS molecule that can lead to elevated activity within downstream signaling pathways. One third of human cancers have RAS mutations, primarily the K-RAS isoform that also leads to a radioprotected phenotype (15, 18). Of note is that some studies suggest that K-RAS and H-RAS have different but overlapping signaling specificities to downstream pathways as judged by *in vitro* cell-based studies and in animal knockout models: thus, mutant K-RAS is thought to preferentially activate the Raf-1/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, whereas mutant H-RAS is believed to preferentially activate the PI3K/AKT pathway (19–21). It has been argued that ERK1/2 and PI3K signaling downstream of K-RAS and H-RAS, respectively, can in turn control cell growth and cell survival following exposure to multiple growth factors [e.g., epidermal growth factor (EGF); refs. 4, 8, 9].

Loss of mutant K-RAS expression has been shown in HCT116 cells to abolish tumor formation in athymic mice and enhance radiosensitivity (22, 23). The findings presented in these studies were linked to reduced expression of the paracrine growth factor epiregulin. Repeated irradiation of tumor cells can also increase expression of transforming growth factor-α (TGF-α) and ERBB1, and radiation is known to promote the cleavage and release of TGF-α from tumor cells in an ERK1/2-dependent fashion (24–26). Increased proliferative rates and poor prognosis of carcinomas *in vivo* have also been correlated with increased expression of ERBB1 (27).

Recent studies in rodent fibroblasts have argued that H-RAS and K-RAS can differentially alter radiosensitivity, with mutated active K-RAS promoting radiation sensitization, which was linked to activation of the p38 mitogen-activated protein kinase pathway (28); however, the roles of different RAS isoforms and the signaling pathways used by radiation to alter radiosensitivity of isogenic human *carcinoma* cells are unknown. The studies in this article were undertaken to determine whether constitutively active mutant K-RAS or mutant H-RAS differentially alters the signaling properties and radiosensitivity of HCT116 colon carcinoma cells.

Materials and Methods

Materials

Anti-phospho-ERK1/2 (sc-7383), anti-total-ERK2 (sc-154), anti-β-actin (sc-8432) antibodies, and protein A/G–conjugated agarose (sc-2003) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho–c-Jun NH$_2$-terminal kinase 1/2 (JNK1/2) (9255), anti-phospho-Ser^{473}-AKT (9271), anti-phospho-Thr^{308}-AKT (9275), anti-

total-AKT (9272), anti-phospho-Ser^9-GSK3β (9336), anti-GSK3β (9332), anti-phospho-Ser^{241}-PDK1 (3061), anti-phospho-Tyr$^{373/376}$-PDK1 (3065), anti-total-PDK1 (3062), anti-phospho-Ser^{259}-RAF (9421), anti-phospho-Tyr^{1289} HER3/ERBB3 (4791), and anti-phospho-p38 (9211) antibodies were from Cell Signaling Technology (Beverly, MA). Antibodies to detect total RAS (OP24), H-RAS (OP23), and mutant K-RAS (OP38) and the neutralizing anti-heregulin antibody (PC185L) were from Oncogene Research Products (Cambridge, MA). Antibodies to detect ERBB1 (MS-610-P), ERBB2 (MS-267-P), ERBB3 (MS-313-P), and ERBB4 (MS-637-P) and for immunoprecipitation of ERBB3 (MS-262-P) were from Neomarkers Lab Vision Corp. (Fremont, CA). Antibodies anti-PI3K p85 (06-496) and anti-PI3K p110α (06-567) were from Upstate Biotechnology (Lake Placid, NY). Oligonucleotides for semiquantitative PCR were synthesized by the Virginia Commonwealth University Oligonucleotide Core Facility and were based on published sequences (sense K-RAS: GAGAGAGGCCTGCT-GAAAATG, antisense K-RAS: TTCTGAGGACCGACA-CACTTT, sense H-RAS: GCAGGCCCCTGAGGAGCGATG, antisense H-RAS: TCAGGGAGAGCACACACTTGCAGC, sense N-RAS: GGTTCTTGCTGGTGTGAAATG, antisense N-RAS: ACTTTAAAAGTAATCTTGTTACAT, sense ERBB1: CAGCGCTACCTTGTCATTCAG, antisense ERBB1: TCATACTATCCTCCGTGGTCA, sense ERBB2: CGCTTTGTGGTCATCCAGAATG, antisense ERBB2: TCGTGTTCACACTGGCACGTC, sense ERBB3: TGGCCCGAGACCCACCACGGTATCTG, antisense ERBB3: AGTTACGTTCTCTGGGCATTAGCCTT, sense ERBB4: AGTTTTCAAGGATGGCTCGAGACCCTC, antisense ERBB4: AGCTTACACCACAGTATTCCGGTGTCT, sense epiregulin: GCGCCCGCTCCCATCGCCG, antisense epiregulin: CAGAAATAATATGTGGAACC, sense EGF: TGCAACTGTGTTGTTGGCTACATC, antisense EGF: TGGTTGACCCCCATTCTTGAG, sense HB-EGF: ACAAG-GAGGAGCACGGGAAAAG, antisense HB-EGF: CGAT-GACCAGCAGACAGACAGATG, sense heregulin: CGTGGAATCAAACGAGATCATCAC, antisense heregulin: GGTTATGGTCAGCACTCTCTTCTGG, sense TGF-α: TCAGTTCTGCTTCCATGCAACC, antisense TGF-α: TTTCTGAGTGGCAGCAAGCG, sense β-actin: GAACCC-TAAGGCCAACCGTGAAAAGATGAC, antisense β-actin: TGATCTTCATGGTGCTAGGAGCCAGAGCAG). The mitogen-activated protein kinase/ERK1/2 (MEK1/2) inhibitor U0126, the selective inhibitor of farnesyltransferase FTI277, and the PI3K inhibitor LY294002 were from Calbiochem (San Diego, CA). Other reagents were as in refs. 19–31.

Generation of HCT116 Cell Lines without Mutant K-RAS and Expressing Mutant H-RAS

HCT116 mutant K-RAS-deleted cells were generated by homologous deletion of the mutant K-RAS allele as described (22, 32). A plasmid to express mutant active H-RAS (H-RAS V12) was kindly provided by the laboratory of Dr. M. Wigler (Cold Spring Harbor, NY). A plasmid to express dominant-negative AKT (AKT1-S473A/T308A mutant) was kindly provided by Dr. R.A. Roth (Stanford

University, Palo Alto, CA). HCT116 cell lines were transfected by electroporation at 600 V for 60 ms using a Multiporator Eppendorf (Hamburg, Germany) with control plasmids ("C2" cells) or plasmids to express H-RAS V12 ("C10" and "C3" cells) or dominant-negative AKT. Pools of transfected cells were obtained by puromycin (RAS) or neomycin (AKT) selection, and individual colonies were isolated and then characterized.

Culture of HCT116 Cell Lines

Asynchronous carcinoma cells were cultured in DMEM supplemented with 10% (v/v) FCS at 37°C in 95% (v/v) air/5% (v/v) CO_2. Cells were plated at a density 3×10^3 cells per cm^2 plate area, and all cells were plated from log-phase cultures. For radiation-induced activations of protein kinases, cells were cultured for 4 days in this medium and for 24 hours before irradiation in serum-free DMEM. For colony formation assays, cells were plated at low density (250–2,000 cells per dish) for 24 hours after plating and for 24 hours before irradiation in serum-free DMEM. Cells were irradiated (1–4 Gy) or as indicated in the text: medium was replaced with serum containing medium 24 hours after radiation exposure. Ten to 14 days after exposure, plates were washed in PBS, fixed with methanol, and stained with a filtered solution of crystal violet (5% w/v). After washing with tap water, the colonies were counted both manually (by eye) and digitally using a ColCount plate reader. Data are arithmetic means ± SE from both counting methods from multiple studies.

Exposure of Cells to Ionizing Radiation and Cell Homogenization

Cells were cultured as described above. Before irradiation (1 hour), cells were treated with vehicle (DMSO), U1026 (1 μmol/L), or LY294002 (1 μmol/L). Treatment was from a 100 mmol/L stock solution and the maximal concentration of vehicle in medium was 0.01% (v/v). Cells were irradiated using a ^{60}Co source at dose rate of 1.8 Gy/min. Cells were maintained at 37°C throughout the experiment, except during irradiation. Zero time is designated as the time point at which radiation exposure ceased. After radiation treatment, cells were incubated for specified times followed by aspiration of medium and immediately homogenized in 1 mL SDS-PAGE lysis buffer [5% (w/v) SDS, 40% (v/v) glycerol, 250 mmol/L Tris-HCl, 10% (v/v) 2-mercaptoethanol]. Homogenates were sonicated, boiled for 10 minutes, and stored frozen (−20°C) before use, and protein concentration was determined by Bradford assay (Coomassie Protein Assay kit, Pierce Biotechnology, Rockford, IL).

SDS-PAGE and Western Blotting

Depending on the protein to be studied, a volume of homogenate containing 10, 20, or 40 μg of total protein was loaded in 12% (w/v) acrylamide gels and subjected to SDS-PAGE. Gels were transferred to nitrocellulose and Western blotted using specific antibodies. Blots were developed using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA) and Kodak X-ray film or using an Odyssey IR Imaging System (LI-COR Biosciences, Lincoln, NE), which has 3-log detection sensitivity over conventional enhanced chemiluminescence immunoblotting. This machine uses dye-conjugated secondary antibodies that fluoresce in IR light as a detection system rather than an enzymatic chemiluminescent reaction. Secondary antibodies are available that fluoresce red or green, which are detected on two separate channels in/by the machine (Molecular Probes, Eugene, OR and Rockland ImmunoChem, Gilbertsville, PA). Thus, immunoblots to detect multiple proteins of different masses can be digitally scanned/probed on the same piece of nitrocellulose at the same time. Densitometric analysis for enhanced chemiluminescence immunoblots and reverse transcription-PCR (RT-PCR) analyses was done using a Fluorochem 8800 Image System and the respective software (Alpha Innotech Corp., San Leandro, CA), and band densities were normalized to that of β-actin in the same sample and expressed as percentages of the respective control in each experiment as indicated in the figure legends. Blots were digitally scanned using Adobe Photoshop 7.

PCR Analysis of mRNA Levels

Cells were plated in 10% FCS-DMEM, and 96 hours after plating, the medium was replaced by either serum containing medium or medium without serum. Twenty-four hours after the medium change, cells were homogenized in buffer containing guanidine isothiocyanate for the extraction of total RNA with a commercial kit (RNeasy) following the direction of the manufacturer (Qiagen, Valencia, CA). RNA yield and purity were determined following measurement of absorbance at 260 and 280 nm. The RT-PCR was done using a commercial kit (Qiagen One-Step RT-PCR) and 1 μg of RNA in a final volume of 25 μL and following the instructions of the manufacturer (Qiagen). To determine the expression of H-RAS, K-RAS, and N-RAS and β-actin, epiregulin, heregulin, TGF-α, HB-EGF, EGF, ERBB1, ERBB2, ERBB3, and ERBB4, the respective primers described in Materials at a final concentration of 0.6 μmol/L were used. Both reverse transcription and DNA synthesis were done in the same tube in a Peltier Thermal Cycler 200 (MJ Research, Inc., Boston, MA). The reverse transcriptase reaction was done at 50°C for 30 minutes followed for a step of activation of the DNA polymerase and inactivation of the reverse trancriptase at 95°C for 15 minutes and 25 cycles of denaturalization at 95°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute and 1 cycle of 10 minutes at 72°C. All PCR products were analyzed by gel electrophoresis through a 1% agarose gel containing ethidium bromide at 100 V for ~1 hour in Tris-borate-EDTA buffer. Densitometric analysis was done using a Fluorochem 8800 Image System and the respective software, and band densities were normalized to that of β-actin in the same sample and expressed as percentages of the respective control in each experiment as indicated in the figure legends.

Cell Death Assays: Wright Giemsa for Apoptosis

Cells were plated at 5×10^4 cells per well in 12-well plates and 24 hours later were serum starved. Twenty-four hours later, the plates were mock exposed or irradiated with 1 or 4 Gy and harvested 96 hours after

irradiation by trypsinization followed by centrifugation onto glass slides (cytospin) at 800 rpm for 10 minutes. The cells were fixed and stained with a commercial kit (Diff-Quik) following the instruction of the manufacturer (Dade Behring AG, Düdingen, Switzerland). Randomly selected fields of stained cells ($\sim$200 cells per field, $n = 5$ per slide) were counted for apoptotic nuclear morphology.

Membrane Preparation from HCT116 Cells

Cells were cultured as described above. Twenty-four hours after serum withdrawal/starvation, cells were scraped into 10 mL of 1 mmol/L $NaHCO_3$ (pH 7.4), 1 mmol/L sodium pyrophosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L EDTA, 1 mmol/L EGTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride and incubated for 1 hour at 4°C. Crude membrane preparations were prepared as described in refs. 33, 34 by sucrose density overlayer centrifugation.

Data Analysis

Comparison of the effects of treatments was done using one-way ANOVA and a two-tailed t test. Differences with $P < 0.05$ were considered statistically significant. Experiments, except where indicated, are the means $\pm$ SE of multiple individual points from multiple separate experiments.

Results

Generation and Characterization of Wild-type/Parental, Mutant K-RAS-Deleted, and H-RAS V12–Expressing HCT116 Cells

To examine the relative roles of mutant active K-RAS D13 and mutant active H-RAS V12 molecules in the responses of isogenic tumor cells to radiation, we obtained previously described HCT116 cell lines that had been genetically manipulated with the allele expressing K-RAS D13 deleted by homologous recombination while leaving the other allele of wild-type (WT) K-RAS intact (termed hereafter ''mutant K-RAS-deleted cells''; refs. 22, 32). HCT116 cell lines were stably transfected with either a vector control plasmid or a plasmid to express H-RAS V12. Colonies were initially analyzed/characterized and selected for further study based on total RAS protein expression (data not shown).

Expression of Mutant K-RAS and Mutant H-RAS Differentially Regulate the Expression of ERBB Family Receptors and Their Ligands

Compared with parental HCT116 cells and as would be predicted on losing function of one allele of a gene, HCT116 mutant K-RAS-deleted cells (clone C2) exhibited a $\sim$50% reduction in the levels of K-RAS mRNA and protein (Fig. 1A and B). Transfection of active H-RAS V12 into mutant K-RAS-deleted cells (clones C10 and C3) resulted in a $\sim$80% increase in H-RAS protein levels above WT cells and a restoration of H-RAS mRNA levels to near those found in WT cells (Fig. 1A and B). N-RAS mRNA was almost undetectable, and the N-RAS isoform was not reproducibly identified by protein immunoblotting in any HCT116 cell line (Fig. 1A; data not

shown). As judged by immunoblotting, all HCT116 cell lines expressed the growth factor receptors ERBB1, ERBB2, and ERBB3, but not ERBB4, with loss of the K-RAS D13 allele promoting increased expression of ERBB1 and ERBB3 (Fig. 1C; data not shown).

ERBB family receptor activity in tumor cells is frequently enhanced by the actions of paracrine ligands. Parental HCT116 cells expressed high levels of epiregulin with considerably lower expression levels of HB-EGF, heregulin, EGF, and TGF-α (Fig. 1D; data not shown). In HCT116 cells with their allele of mutant K-RAS-deleted (C2 cells), the expression of epiregulin declined to $\sim$25% of WT cells, whereas surprisingly that of heregulin increased by $\sim$1,400%. Expression of H-RAS V12 in mutant K-RAS-deleted cells (C10 cells) further decreased epiregulin levels without causing any additional enhancement of heregulin expression. In contrast, a ''knock-in'' transfection of K-RAS D13 into mutant K-RAS-deleted cells restored epiregulin expression (ref. 22; data not shown). Identical data were obtained using nontransfected K-RAS D13–deleted cells and in an additional HCT116 clone expressing H-RAS V12 (data not shown). Elevated heregulin mRNA levels were paralleled with similar increases in the expression of heregulin protein levels (Fig. 1E and F). Parallel experiments could not be done to examine epiregulin protein levels due to the lack of a commercially available antibody for this growth factor.

Differential Regulation of Radiation-Induced Signaling Pathway Activation by Mutant K-RAS and Mutant H-RAS

RAS molecules have been proposed to differentially regulate the activities of multiple downstream pathways comparing basal and growth factor–stimulated kinase activities in nonisogenic cell types, particularly K-RAS regulating the Raf-1/MEK/ERK1/2 pathway and H-RAS controlling the PI3K/AKT/GSK3 pathway. Radiation resistance downstream of mutant K-RAS proteins and mutant H-RAS proteins, also using nonisogenic cells, has generally been linked by several groups predominantly to activation of the PI3K pathway (e.g., refs. 35–37).

Loss of K-RAS D13 expression significantly reduced basal ERK1/2 and AKT activity in HCT116 cells by 28.3 $\pm$ 1.7% and 40.9 $\pm$ 1.9% ($P < 0.05 \pm$ SE, $n = 4$; Fig. 2A). Transfection of mutant K-RAS D13–deleted cells with H-RAS V12 restored basal ERK1/2 and AKT activity to those found in WT cells. Loss of K-RAS D13 expression reduced basal JNK1/2 activity by 26.3 $\pm$ 5.9% ($P < 0.05 \pm$ SE, $n = 4$), which was not restored by expression of H-RAS V12 (Fig. 2A). Identical data for ERK1/2, AKT, and JNK1/2 were obtained using nontransfected mutant K-RAS-deleted cells and in an additional HCT116 clone expressing mutant H-RAS V12 (data not shown). In contrast to recent studies in rodent fibroblasts transfected to express active H-RAS and K-RAS proteins (28), basal p38 activity was not detected in any of the HCT116 carcinoma cell lines examined (data not shown).

Radiation-Induced ERK1/2 Pathway Activation in Parental Cells Correlates with K-RAS D13 Expression and Radiation-Induced PI3K/AKT Pathway Activation in H-RAS V12 (C10) Cells Correlates with H-RAS V12 Expression

We next examined the modulation of signaling pathway activities over a 24-time course following a 1 Gy radiation exposure in our transfected HCT116 cell lines. In contrast to findings in rodent fibroblasts, p38 was not activated following a 1 Gy exposure in any HCT116 cell type examined (data not shown). In parental/WT HCT116 cells, JNK1/2 was activated in two phases, 5 to 30 minutes after exposure with a rebound back to basal levels within 1 to 6 hours (Fig. 2B and C). In contrast, inhibition of JNK1/2 activity was observed in the mutant K-RAS D13–deleted or H-RAS V12 cell lines.

ERK1/2 and AKT were also activated in multiple phases by radiation in HCT116 cell lines (Fig. 2D–G). In parental

Figure 1. Expression of H-RAS, K-RAS, and N-RAS in HCT116 cells transfected with empty vector or a mutant H-RAS plasmid. **A,** levels of mRNA for H-RAS, K-RAS, and N-RAS in HCT116 cell clones. mRNA levels of H-RAS, K-RAS, N-RAS, and β-actin were determined by RT-PCR using the specific primers as described in Materials and Methods. Densitometry values of each RAS isoform mRNA were normalized with respect to the total amount of β-actin mRNA in each sample and then expressed as percentages with the mRNA content for K-RAS in HCT116 (WT) cells being defined as a 100% value. *Columns,* mean of four independent experiments; *bars,* SE. *, $P < 0.05$ less than corresponding WT cell value. *Inset,* quantitative RT-PCR H-RAS, K-RAS, and N-RAS mRNA levels from a representative experiment showing data from WT, C2 (mutant K-RAS-deleted), C10 (mutant K-RAS-deleted expressing H-RAS V12), and C3 (mutant K-RAS-deleted expressing H-RAS V12). **B,** expression of K-RAS and H-RAS proteins determined by immunoblotting using RAS isoform-specific antibodies. Densitometry values were normalized with respect to the total amount of β-actin in each sample and then expressed as percentages with the value for K-RAS expression in WT cells being defined as a 100% value. *Columns,* mean of four independent experiments; *bars,* SE. *, $P < 0.05$ less than corresponding WT cell value; #, $P < 0.05$ greater than corresponding WT cell value. *Inset,* H-RAS and K-RAS protein levels from a representative experiment showing data from WT, C2, and C10. **C,** expression of ERBB1, ERBB2, and ERBB3 proteins determined by Western blot using receptor-specific antibodies. Densitometry values were normalized with respect to the total amount of β-actin in each sample and then expressed as percentages with the value for ERBB1 expression in WT cells being defined as a 100% value. *Columns,* mean of four independent experiments; *bars,* SE. #, $P < 0.05$ greater than corresponding WT cell value. *Inset,* ERBB1, ERBB2, and ERBB3 protein levels from a representative experiment showing data from WT, C2, and C10. **D,** levels of mRNA for ERBB family paracrine growth factors in WT, C2, or C10. Growth factor expression was determined by RT-PCR using specific primers as described in Materials and Methods. Densitometry values of the mRNAs for epiregulin (*EPI*) and heregulin (*HER*) were normalized with respect to the total amount of β-actin in each sample and then expressed as percentages with the value for epiregulin mRNA levels in WT cells being defined as a 100% value. *Columns,* mean of five experiments; *bars,* SE. *, $P < 0.05$ less than corresponding WT cell value; **, $P < 0.05$ less than mutant K-RAS deleted value; #, $P < 0.05$ greater than corresponding WT cell value. *Inset,* epiregulin and heregulin mRNA levels from a representative experiment. **E** and **F,** heregulin protein expression by Western blot (**E**) and heregulin expression by mRNA content (**F**) in WT, C2, and C10. Densitometry values were normalized with respect to the total amount of β-actin protein/mRNA in each sample and expressed as percentages of the protein or mRNA content in WT cells (defined as 100%). *Columns,* mean of five experiments; *bars,* SE. #, $P < 0.05$ greater than corresponding WT cell value. *Inset,* epiregulin and heregulin mRNA and protein levels from a representative experiment.

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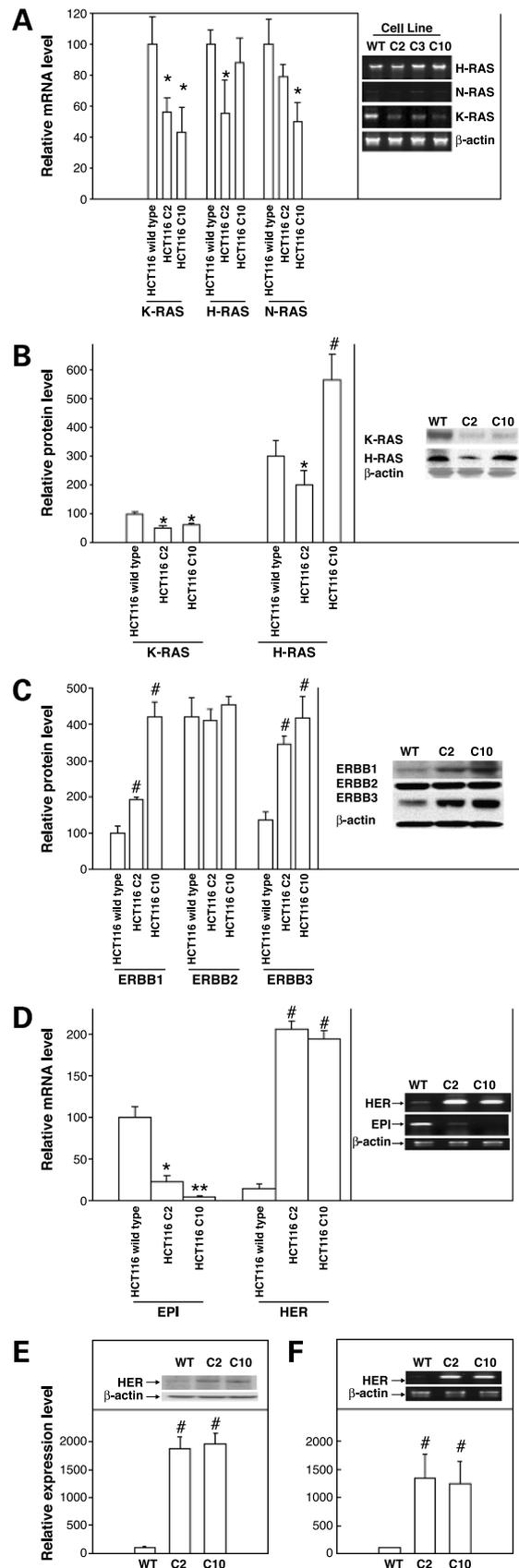
cells, ERK1/2 was activated in three phases following radiation exposure. ERK1/2 phosphorylation was enhanced by radiation rapidly (5–30 minutes), several hours (1–4 hours) after exposure, and many hours (6–24 hours) following exposure (Fig. 2D and E). Compared with WT cells, the activation of ERK1/2 was significantly reduced 5 to 30 minutes after exposure in mutant K-RAS-deleted (C2) cells and was abolished thereafter (30 minutes–24 hours). In cells expressing H-RAS V12 (C10 cells), the activation of ERK1/2 was enhanced to a greater extent than in WT cells shortly following exposure (5–30 minutes); however, ERK1/2 activation

was only partially restored several hours after exposure (1–4 hours). At later times (6–24 hours), ERK1/2 was not activated by radiation in H-RAS V12 cells.

In parental HCT116 cells, AKT was modestly activated shortly following exposure (5–30 minutes) and several hours (1–4 hours) after exposure but was not activated at later times (6–24 hours; Fig. 2F and G). Activation of AKT was abolished in mutant K-RAS-deleted cells. In cells expressing H-RAS V12 (C10), AKT was strongly activated, which occurred in three phases: 5 to 30 minutes, 1 to 4 hours, and 6 to 24 hours after exposure. The fold increase in AKT activation in cells expressing H-RAS V12 was significantly enhanced beyond the level observed for AKT activation in parental/WT cells. Similar data for AKT activation by radiation were obtained in one additional HCT116 cell clone expressing H-RAS V12 (data not shown).

The modulation of radiosensitivity by small molecule signaling pathway inhibitors after a 1 Gy exposure was determined in parental HCT116 cells. In general agreement with our findings and those published by Ries et al. (23), incubation of parental HCT116 cells with a MEK1/2 inhibitor (U0126, 1 μmol/L) significantly reduced survival in colony formation assays from 0.69 ± 0.08 to 0.43 ± 0.07 ($P < 0.05$, $\pm$ SE, $n = 3$). In contrast, an inhibitor of PI3K (LY294002, 1 μmol/L) had no significant effect on the survival of WT HCT116 cells. Additional studies then examined cell survival in mutant K-RAS D13–deleted cells and H-RAS V12 cells. In mutant K-RAS-deleted cells, inhibition of PI3K modestly enhanced radiosensitivity,
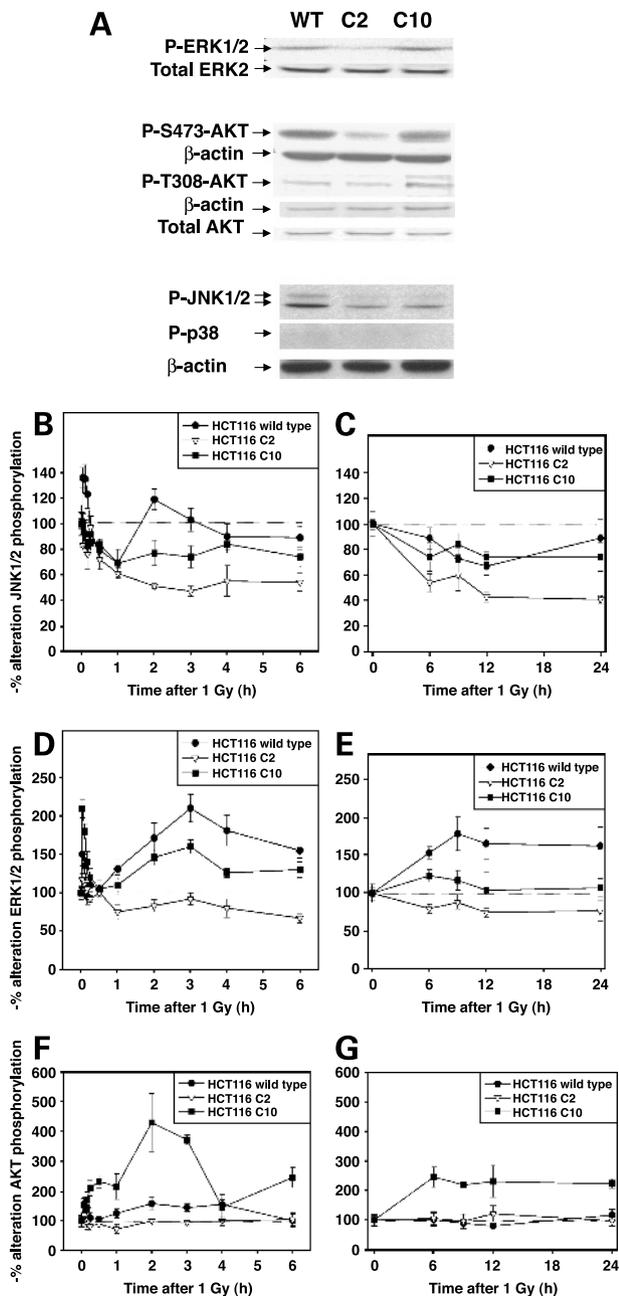
Figure 2. Phosphorylation of P-JNK1/2, P-ERK1/2, and P-AKT Ser[473] in HCT116 cells after a 1 Gy radiation exposure. **A,** phosphorylation of protein kinases was determined by immunoblotting using antibodies for the phosphorylated forms of ERK1/2, AKT Ser[473]/Thr[308], JNK1/2, and p38 in parental WT, C2, or C10. Total β-actin, ERK2, and AKT1/2 expression was blotted in the same membrane as a loading control. **B,** alteration of JNK1/2 phosphorylation 0–6 h after a 1 Gy exposure in WT, C2, or C10. Densitometry values were normalized with respect to total β-actin protein expression and are expressed as percentages of JNK1/2 phosphorylation in WT cells at $t = 0$. *Points,* mean of three experiments; *bars,* SE. **C,** alteration of JNK1/2 phosphorylation 6–24 h after a 1 Gy exposure in WT, C2, and C10. Densitometry values were normalized with respect to total β-actin protein expression and are expressed as percentages of JNK1/2 phosphorylation in WT cells at $t = 0$. *Points,* mean of three experiments; *bars,* SE. **D,** alteration of ERK1/2 phosphorylation 0–6 h after a 1 Gy exposure in WT, C2, or C10. Densitometry values were normalized with respect to total β-actin protein expression and are expressed as percentages of ERK1/2 phosphorylation in WT cells at $t = 0$. *Points,* mean of three experiments; *bars,* SE. **E,** alteration of ERK1/2 phosphorylation 6–24 h after a 1 Gy exposure in WT, C2, and C10. Densitometry values were normalized with respect to total β-actin protein expression and are expressed as percentages of ERK1/2 phosphorylation in WT cells at $t = 0$. *Points,* mean of three experiments; *bars,* SE. **F,** alteration of AKT Ser[473] phosphorylation 0–6 h after a 1 Gy exposure in WT, C2, and C10. Densitometry values were normalized with respect to total β-actin protein expression and expressed as percentages of AKT phosphorylation in WT cells at $t = 0$. *Points,* mean of four independent experiments; *bars,* SE. **G,** alteration of AKT Ser[473] phosphorylation 6–24 h after a 1 Gy exposure in WT, C2, and C10. Densitometry values were normalized with respect to total β-actin protein expression and expressed as percentages of AKT phosphorylation in WT cells at $t = 0$. *Points,* mean of four independent experiments; *bars,* SE.

reducing survival after a 1 Gy exposure from 0.48 ± 0.07 to 0.39 ± 0.06 ($P < 0.05$, ± SE, $n = 3$), whereas inhibition of MEK1/2 did not alter survival. In H-RAS V12 cells, inhibition of PI3K blocked AKT activation and caused a large reduction in colony formation from 0.89 ± 0.08 to 0.30 ± 0.07 ($P < 0.05$, ± SE, $n = 3$) after irradiation, whereas inhibition of MEK1/2 had no effect on cell survival.

In support of the overall concept that PI3K signaling generates a greater cytoprotective response against radiation toxicity than ERK1/2 signaling, deletion of mutant K-RAS D13 increased the radiosensitivity of HCT116 cells, whereas expression of H-RAS V12 significantly enhanced both plating efficiency and radioresistance of H-RAS V12 cells above that observed in parental cells (Fig. 3A and B).

AKT Activation Promotes Survival in Irradiated H-RAS V12 Cells

Several potential pathways exist downstream of PI3K that could mediate radiation resistance in H-RAS V12 cells, including the PDK1/AKT/GSK3 pathway that has been linked to PI3K-dependent protection from cytotoxic drug lethality.

Some investigators have argued that PI3K signaling may protect cells from ionizing radiation via AKT-independent pathways (38–40). Thus, we investigated whether inhibition of AKT function altered HCT116 cell survival after radiation exposure. Expression of dominant-negative AKT also radiosensitized H-RAS V12 cells (Fig. 3C). Cell survival after exposure to 1 and 4 Gy in H-RAS V12 cells expressing dominant-negative AKT (0.49 ± 0.06 and 0.13 ± 0.02, respectively) was similar to that in the vector control transfected mutant K-RAS-

deleted cells when exposed to 1 and 4 Gy (0.47 ± 0.05 and 0.10 ± 0.02), respectively ($P > 0.05$, ± SE, $n = 3$). These data suggest that part of the downstream radioprotective effect of H-RAS V12 expression in HCT116 cells was mediated by enhanced AKT signaling.
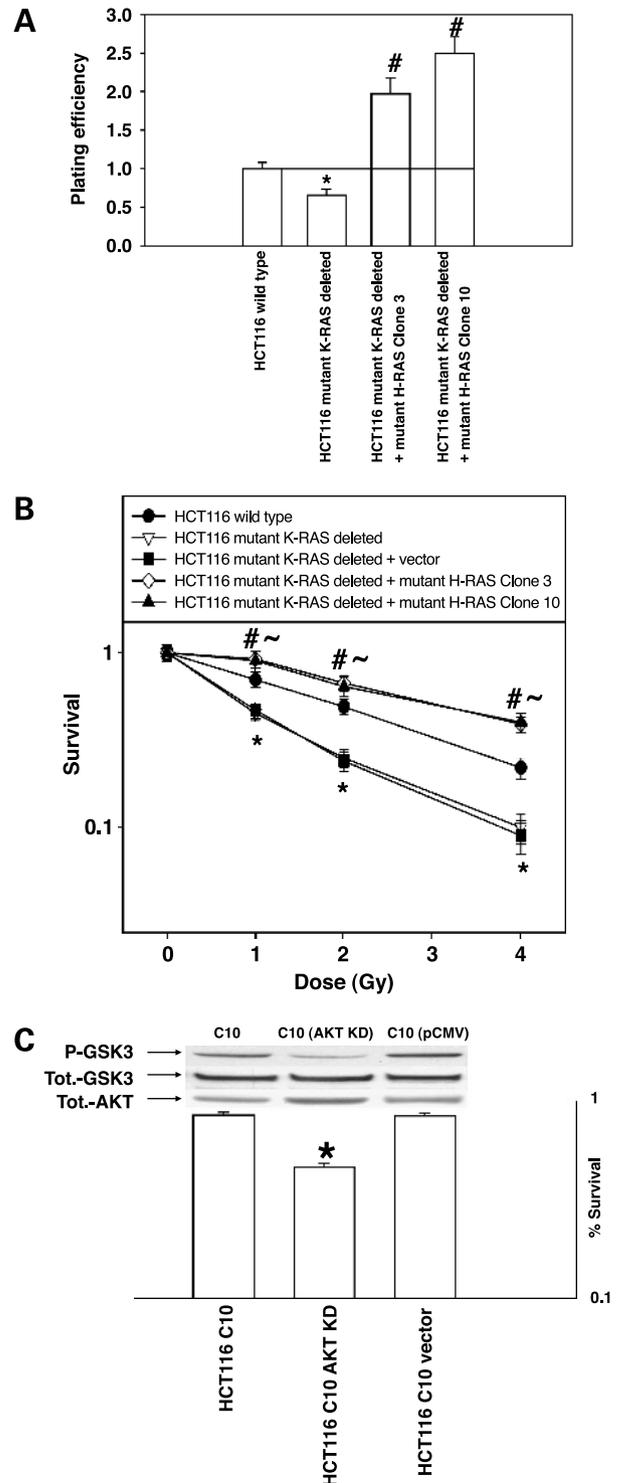
Figure 3. Expression of H-RAS V12 causes a greater radioprotective/survival effect than K-RAS D13 in HCT116 cells. **A,** plating efficiency (colonies formed per total number of cells plated) of WT, C2, or C10/C3 cells. Cells were plated as described in Materials and Methods, and the relative efficiency of cell plating (percentage colonies recovered per total number of cells plated) was calculated making the plating efficiency value for WT equal 1.00. *Columns,* means of six separate dishes from three separate experiments; *bars,* SE. *, $P < 0.05$ less than corresponding WT cell value; #, $P < 0.05$ greater than corresponding WT cell value. **B,** dose-response survival curve after exposure of HCT116 cell lines to ionizing radiation: WT cells, mutant K-RAS-deleted cells, and mutant K-RAS-deleted cells transfected with the empty vector (C2) or with the mutant H-RAS V12 plasmid (C3 and C10). Cells were serum starved for 24 h before exposure to the respective dose of radiation, medium containing serum was added 24 h after exposure, and 10–14 d later colonies were counted as indicated in Materials and Methods. Numbers of colonies were expressed as a fraction of the respective mock irradiated cells and plotted in a semilog graph. *Points,* means of six separate dishes from three separate experiments; *bars,* SE. *, $P < 0.05$ less than corresponding WT cell value; #, $P < 0.05$ greater than corresponding WT cell value; ~, $P < 0.001$ greater than mutant K-RAS-deleted cells value. **C,** HCT116 cells expressing H-RAS V12 (C10 cells) were transfected with vector control plasmid or plasmid to express dominant-negative AKT as described in Materials and Methods and in Fig. 5 legend. Pools of transfected cells were plated for colony formation assays and irradiated (1 Gy) as described in Materials and Methods. *Columns,* means of three separate dishes from three separate experiments; *bars,* SE. *, $P < 0.05$ less than corresponding C10 cell or C10 control plasmid cell value. *Inset,* expression of total AKT, total GSK3, and P-GSK3 S9 from a representative experiment ($n = 3$).

Heregulin and ERBB3 Signaling Is Essential for Prolonged Radiation-Induced AKT Activation in H-RAS V12 Cells

In prior studies using other carcinoma cell lines, we showed that the paracrine ligand TGF-α is processed and released into the growth medium several hours after exposure of cells to ionizing radiation. Medium containing released TGF-α could be transferred onto plates of unirradiated cancer cells to cause activation of ERBB1 and the ERK1/2 pathway (4, 25, 26). Heregulin, which is overexpressed in H-RAS V12 (C10) cells, has been linked by others to ERBB3-dependent activation of the PI3K/AKT pathway (17). Hence, we did additional studies in H-RAS V12 (C10) cells using heregulin neutralizing antibodies to determine whether the high levels of AKT activation we observed after radiation exposure in these cells were dependent on the actions of this paracrine ligand.

Addition of a heregulin neutralizing antibody, but not a control antibody, into H-RAS V12 cell growth medium *in situ* blocked radiation-induced activation of AKT 1 to 24 hours after exposure (Fig. 4A; data not shown). Parallel studies then determined whether radiation exposure caused HCT116 cells to release heregulin into their growth medium. H-RAS V12 cells were irradiated or mock exposed, and their growth medium was removed several hours after exposure. The isolated medium was incubated with control or neutralizing heregulin antibodies and then transferred onto plates of unirradiated H-RAS V12 cells to determine whether the medium contained any "factors" that could stimulate AKT activity. Medium from irradiated H-RAS V12 cells caused activation of AKT when transferred onto plates of unirradiated H-RAS V12 cells, which was abolished by the heregulin neutralizing antibody (Fig. 4B). Collectively, these data show that a significant portion of the total radiation-induced AKT activation in HCT116 H-RAS V12 (C10) cells is heregulin dependent.

As inhibition of either PI3K or AKT function radiosensitized H-RAS V12 (C10) cells, and because radiation-induced AKT activation was largely dependent on heregulin/PI3K signaling, we then investigated whether neutralization of heregulin function would also enhance H-RAS V12 cell radiosensitivity. As a single agent, incubation of H-RAS V12 (C10) cells with a heregulin neutralizing antibody reduced plating efficiency to 0.80 ± 0.07 of the value found in control IgG-treated cells. H-RAS V12 cells were also radiosensitized by a heregulin neutralizing antibody (Fig. 4C), reducing 1 Gy survival from 0.86 ± 0.06 to 0.51 ± 0.05 ($P < 0.05$, ± SE). Survival at 4 Gy was reduced from 0.19 ± 0.04 to 0.09 ± 0.03 ($P < 0.05$, ± SE). Vector control (C2) cell radiosensitivity/survival under the same treatment conditions following a 1 Gy exposure was 0.47 ± 0.05, which was not significantly modified by the inclusion of a heregulin neutralizing antibody in the growth medium (cf. to data presented in Fig. 3B). Of note, cell survival of H-RAS V12 cells expressing dominant-negative AKT also was not modified by the inclusion of a heregulin neutralizing antibody in the
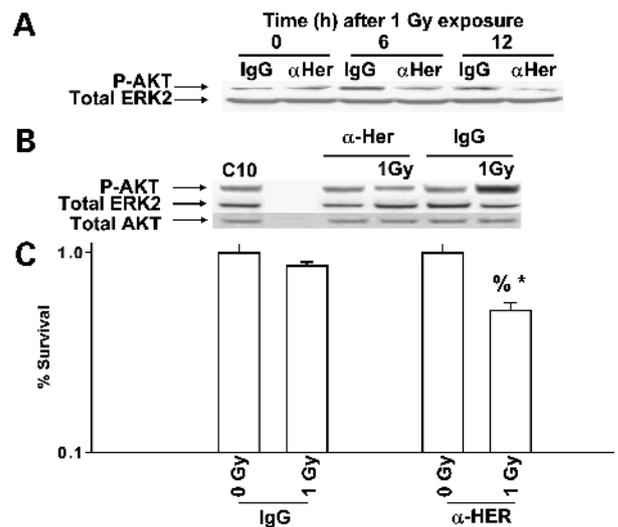
Figure 4. Neutralization of heregulin function in HCT116 H-RAS V12 (C10) cells inhibits radiation-induced AKT activation and causes H-RAS V12 cell radiosensitization. **A,** H-RAS V12 (C10) cells were serum starved for 24 h and 30 min before irradiation treated with an anti-heregulin antibody (αHer) 14 μg/mL (final in medium) or a nonspecific IgG of the same subtype. Cells were irradiated (1 Gy) or mock exposed, and homogenates were taken 0–24 h afterward to determine AKT Ser[473] phosphorylation and total ERK2 expression as described in Materials and Methods. Data are from 6 and 12 h after irradiation. Representative experiment ($n = 3$). **B,** H-RAS V12 cells (C10) were serum starved for 24 h and either mock exposed or irradiated (1 Gy). Six hours after irradiation, medium was removed from the irradiated plates and incubated for 1 h with anti-heregulin antibody or a nonspecific IgG of the same subtype. After 1 h, medium was added to unirradiated plates of H-RAS V12 (C10) cells. Ten minutes after medium addition, cells were lysed and prepared to determine AKT Ser[473] phosphorylation. Representative experiment ($n = 3$). **C,** effect of neutralizing antibody against heregulin on the survival of irradiated H-RAS V12 cells. Plated cells were serum starved for 24 h and treated with 14 μg/mL anti-heregulin antibody or an identical concentration of an unspecific IgG of the same isotype and subjected to a 1 Gy or a mock exposure as described in Materials and Methods. Colonies were counted 10–14 d later as described in Materials and Methods. Numbers of colonies were expressed as percentages of the respective mock exposed group. *Columns,* means of six separate dishes from three separate experiments; *bars,* SE. *, $P < 0.05$ less than corresponding unirradiated cell value; %, $P < 0.05$ less than corresponding WT cell value.

growth medium (data not shown). Thus, neutralization of heregulin function, which blocked radiation-induced AKT activation, also abolished the radioprotective properties of H-RAS V12 in HCT116 cells.

Heregulin Signaling Prevents Radiation-Induced Activation of the Intrinsic Caspase/Apoptosis Pathway
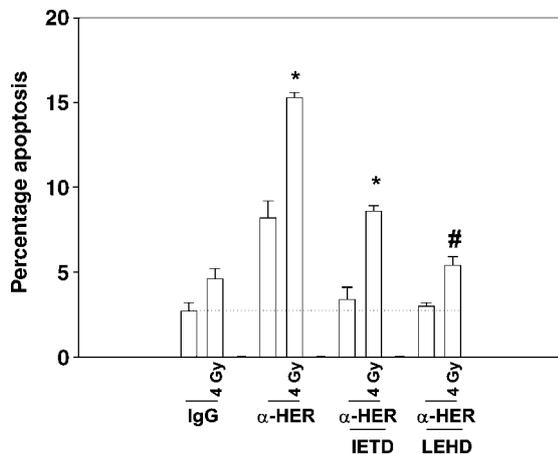
To further investigate the mechanisms by which heregulin enhanced radiosensitivity, we examined the induction of apoptosis 96 hours after exposure (Fig. 5). Inhibition of heregulin function over 96 h enhanced basal levels of apoptosis, in general agreement with the ability of the neutralizing antibody to reduce plating efficiency in colony formation assays. Also in agreement with data in Fig. 4, loss of heregulin function enhanced the lethality of radiation. Inhibition of either caspase-8 or caspase-9 function blunted the cytotoxic effect of the heregulin neutralizing antibody as a single agent; however, only inhibition of caspase-9

prevented the heregulin neutralizing antibody from enhancing radiosensitivity (Fig. 5). Collectively, these findings suggest that the heregulin neutralizing antibody enhances radiosensitivity by promoting mitochondrial dysfunction/activation of pro-caspase-9 and to a lesser extent pro-caspase-8.
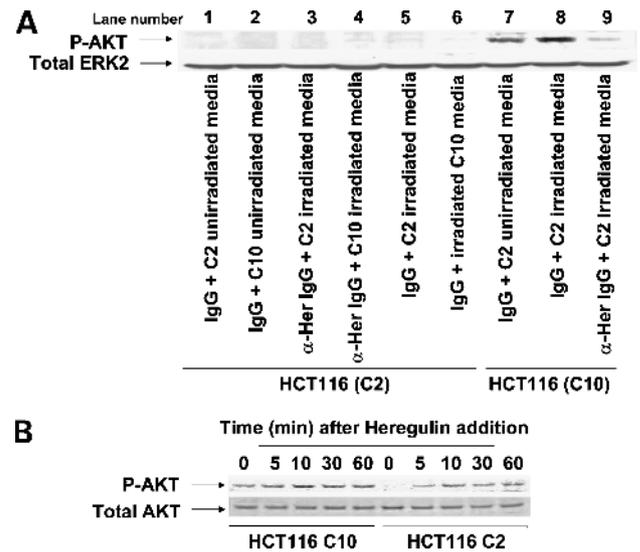
Culture Medium from Irradiated Mutant K-RAS D13– Deleted Cells Causes AKT Activation in Unirradiated H-RAS V12 Cells, Which Is Heregulin Dependent

Based the findings in Fig. 4, and our data showing that mutant K-RAS D13–deleted cells and H-RAS V12 cells express similarly high levels of heregulin and ERBB3 (Fig. 1C and D), it could have been *a priori* predicted that similar levels of radiation-induced AKT activation would be observed in both of these cell lines. However, radiation did not activate AKT in mutant K-RAS-deleted cells (Fig. 2E and F). One possible explanation for these findings is that for heregulin to activate ERBB3 and AKT it must first be released into the growth medium after irradiation as was observed in H-RAS V12 cells (Fig. 4A and B). Thus, it is possible that radiation will not stimulate heregulin release in mutant K-RAS-deleted cells.

To test whether mutant K-RAS-deleted cells release heregulin into their growth medium after irradiation, we irradiated mutant K-RAS-deleted cells and removed their growth medium several hours after exposure. Isolated medium was incubated with a control or a neutralizing anti-heregulin antibody and then tested for heregulin-dependent "AKT activating ability" in unirradiated H-RAS V12 cells. Medium from irradiated mutant K-RAS-deleted cells caused heregulin-

Figure 6. A neutralizing antibody against heregulin inhibits AKT activation in H-RAS V12 (C10) cells induced by medium from irradiated mutant K-RAS-deleted (C2) cells. **A,** mutant K-RAS deleted (C2) cells and H-RAS V12 (C10) cells were serum starved for 24 h and either mock exposed or irradiated (1 Gy). Six hours after exposure, medium was removed from the treated plates and incubated for 1 h with 14 μg/mL (final in medium) of anti-heregulin antibody or with an identical concentration of an unspecific IgG of the same subtype. After 1 h, medium was added to unirradiated plates of either mutant K-RAS-deleted cells (HCT116 C2) or H-RAS V12 (HCT116 C10) cells. Ten minutes after medium addition, cells were lysed and prepared to determine AKT Ser^{473} phosphorylation. *Lane 1,* C2 cells treated with control IgG + C2 unirradiated medium; *lane 2,* C2 cells treated with control IgG + C10 unirradiated medium; *lane 3,* C2 cells treated with α-Her IgG + C2 irradiated medium; *lane 4,* C2 cells treated with α-Her IgG + C10 irradiated medium; *lane 5,* C2 cells treated with control IgG + C2 irradiated medium; *lane 6,* C2 cells treated with control IgG + irradiated C10 medium; *lane 7,* C10 cells treated with control IgG + C2 unirradiated medium; *lane 8,* C10 cells treated with control IgG + C2 irradiated medium; *lane 9,* C10 cells treated with α-Her IgG + C2 irradiated medium. Representative experiment (*n* = 3). **B,** heregulin activates AKT in C2 and C10 cell lines. Mutant K-RAS-deleted (C2) cells and H-RAS V12 (C10) cells were serum starved for 24 h and either mock treated with vehicle (PBS) or treated with heregulin (10 ng/mL final). Cells were isolated at the indicated times after treatment. Cells were lysed and prepared to determine AKT Ser^{473} phosphorylation by immunoblotting. Representative experiment (*n* = 3).

Figure 5. Neutralization of heregulin function promotes radiation-induced apoptosis by caspase-8 – and caspase-9 – dependent mechanisms. H-RAS V12 (C10) cells were serum starved for 24 h and 30 min before irradiation treated with an anti-heregulin antibody (α-*Her*) 14 μg/mL (final in medium) or a nonspecific IgG of the same subtype. Cells were irradiated (1 Gy) or mock exposed and cells were isolated 0 – 96 h afterward to determine the percentage apoptosis 96 h after exposure in the presence or absence of vehicle (DMSO), the caspase-8 inhibitor *IETD* (50 μmol/L), or the caspase-9 inhibitor *LEHD* (50 μmol/L) as described in Materials and Methods. *Columns,* mean of individual triplicate samples from two experiments; *bars,* SE. *, $P < 0.05$ greater than corresponding vehicle-treated value; #, $P > 0.05$ for value compared with corresponding irradiated value.

dependent AKT activation in unirradiated H-RAS V12 cells (Fig. 6A: compare P-AKT intensity in *lanes 7–9*). Thus, mutant K-RAS-deleted cells are competent to release heregulin into their growth medium after irradiation, which can promote activation of AKT in H-RAS V12 cells.

Parallel studies also explored whether medium from irradiated H-RAS V12 cells could activate AKT in unirradiated mutant K-RAS-deleted cells (the reverse experiment to that described above). Medium from irradiated H-RAS V12 cells was unable to alter AKT phosphorylation when transferred onto plates of unirradiated mutant K-RAS-deleted cells (Fig. 6A: compare P-AKT levels in *lanes 2, 4,* and *6*). These findings imply that in mutant K-RAS-deleted cells a portion of the PI3K/AKT signaling pathway, downstream of heregulin and ERBB3, is either defective or present at lower levels compared with that in H-RAS V12 cells.

In contrast to our findings using medium from irradiated plates of HCT116 cells, exposure of mutant K-RAS-deleted (C2) cells and H-RAS V12 (C10) cells to the pure ligand heregulin (10 ng/mL) caused rapid AKT activation in both cell lines (Fig. 6B). The total amount of heregulin-induced AKT activation in mutant K-RAS deleted cells was less than that in H-RAS V12 cells, although as a *fold* increase, heregulin caused a greater activation of AKT in C2 cells than in C10 cells. These findings suggest that the lack of AKT activation in mutant K-RAS-deleted cells treated with medium from irradiated cells (Fig. 6A) may due to the relatively low concentration of heregulin released into the growth medium after irradiation.

H-RAS V12 (C10) Cells Constitutively Localize PI3K (p110 and p85) and AKT in Their Plasma Membranes: PI3K Localization Is H-RAS V12 Dependent

A possible explanation for our findings in Figs. 4 and 6 is that the high levels of radiation/heregulin-induced AKT activation in H-RAS V12 cells are dependent on constitutive translocation of PI3K into the plasma membrane environment in these cells (41). Plasma membrane–localized PI3K would be predicted to more readily interact with heregulin-stimulated ERBB3 and thus become more highly activated. In agreement with this possibility, high levels of PI3K (p85 and p110 subunits) were detected in unstimulated plasma membranes from H-RAS V12 cells, with much lower amounts of these proteins detected in membranes from both WT and mutant K-RAS-deleted cells (Fig. 7A). Radiation promoted a heregulin-dependent association of PI3K p110 and p85 with ERBB3 in H-RAS V12 cells within 1 hour of exposure (Fig. 7B). These findings correlated with radiation-induced, heregulin-dependent tyrosine phosphorylation of ERBB3 (Fig. 7B).

To determine whether translocation of PI3K into the plasma membrane environment was H-RAS V12 dependent, H-RAS V12 (C10) cells were treated with a farnesyltransferase inhibitor. Incubation of H-RAS V12 cells with a farnesyltransferase inhibitor (FTI277, 2 μmol/L for 24 hours) almost abolished the detection of H-RAS and PI3K (p110 and p85) in isolated plasma membranes (Fig. 7C). In contrast, treatment with an anti-heregulin neutralizing antibody altered neither H-RAS nor PI3K p110 membrane localization (Fig. 7C). FTI277 treatment did not alter the total amount of membrane-associated AKT but reduced AKT phosphorylation and AKT activation after irradiation as shown previously by others (refs. 42, 43; data not shown). In agreement with data presented in refs. 42, 43, FTI277 also radiosensitized H-RAS V12 (C10) cells (data not shown).

Discussion

Several studies have linked mutated active forms of K-RAS and H-RAS to enhanced radiation resistance in a variety of unrelated human tumor cell types or in rodent fibroblasts. The present studies were initiated to examine the radioprotective properties of mutant active K-RAS and mutant active H-RAS in an isogenic epithelial carcinoma cell
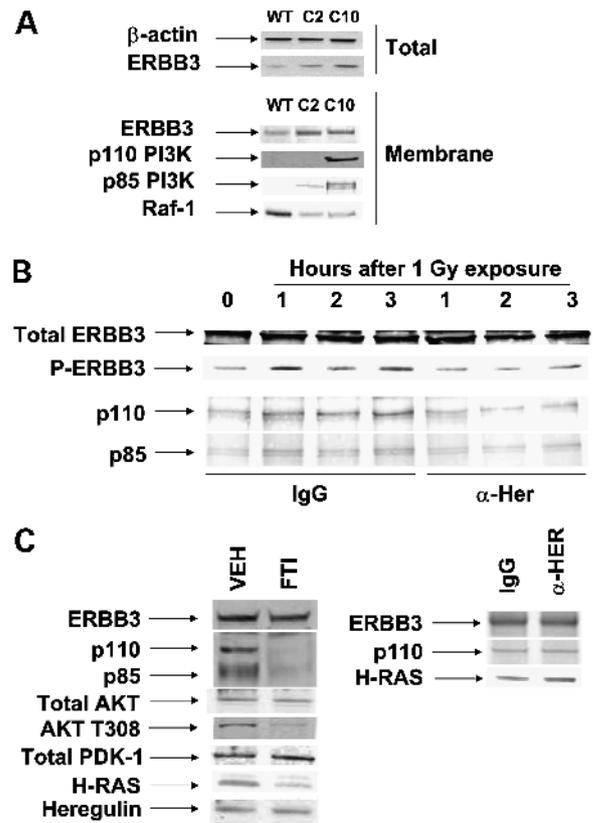
Figure 7. AKT activation and radiation resistance is dependent on heregulin-stimulated association of membrane-associated PI3K with ERBB3. **A,** WT, C2, and C10 cells were plated in parallel and serum starved for 24 h. Cells were then either lysed to determine total ERBB3 and β-actin expression or lysed with hypotonic buffer before preparation of plasma membranes as described in Materials and Methods. Equal amounts of membrane protein were loaded onto SDS-PAGE and immunoblotting done against the indicated membrane-associated proteins. Representative experiment (*n* = 4). **B,** H-RAS V12 (C10) cells were serum starved for 24 h and treated with 14 μg/mL (final in medium) of anti-heregulin antibody or an identical concentration of an unspecific IgG of the same subtype. Cells were subjected to 1 Gy irradiation or mock exposed and cells were isolated 1–3 h after exposure. ERBB3 was immunoprecipitated from the cell lysates as described (38), and the immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting to determine the levels of ERBB3 (protein loading control), ERBB3 tyrosine phosphorylation, and presence/association of PI3K p85 and PI3K p110. Representative experiment (*n* = 3). **C,** H-RAS V12 (C10) cells were serum starved for 24 h in the presence of a farnesyltransferase inhibitor (*FTI*; 2 μmol/L FTI277). In parallel, serum-staved cells that were vehicle treated were exposed to a neutralizing anti-heregulin antibody or control antibody for 3 h. Cells under all treatment conditions were then lysed with hypotonic buffer before preparation of membranes as described in Materials and Methods. Equal amounts of membrane protein were loaded onto SDS-PAGE. Representative experiment (*n* = 3).

system. To this end, we obtained HCT116 WT and HCT116 mutant K-RAS-deleted cells and stably transfected the mutant K-RAS-deleted cells with a plasmid to express H-RAS V12.

Loss of mutant active K-RAS D13 expression in HCT116 cells reduced basal activity of ERK1/2, AKT, and JNK1/2 as well as almost abolishing radiation-induced activation of ERK1/2, AKT, and JNK1/2. Introduction of mutant H-RAS

V12 into these cells restored basal ERK1/2 and AKT activity to those levels found in WT cells. However, H-RAS V12 did not modify the basal or radiation-stimulated activity of JNK1/2. In contrast to studies in rodent fibroblasts transfected to express activated K-RAS and H-RAS proteins, basal or stimulated p38 activity was not detected in HCT116 cells. These findings argue that in HCT116 carcinoma cells mutant active H-RAS V12 preferentially regulates the PI3K/AKT pathway compared with the ERK1/2 pathway.

The differential activation of the ERK1/2 and AKT pathways by radiation in cells expressing mutant active K-RAS D13 or mutant active H-RAS V12 was also examined with respect to the duration and amplitude of pathway activation. In WT cells, ERK1/2 was activated in three phases. However, in H-RAS V12 cells, the overall activation of ERK1/2 was lower than that in WT cells and the final phase of ERK1/2 activation was absent: in part, this may be due to lower levels of membrane-associated Raf-1 under basal conditions. AKT was activated in two waves in WT cells and in three waves in H-RAS V12 cells: all of the second and third phases of AKT activation in H-RAS V12 cells were abolished by an anti-heregulin neutralizing antibody. As heregulin binds to only ERBB3 and ERBB4, and as HCT116 cells did not express detectable amounts of ERBB4, these findings imply that heregulin/ERBB3 signaling is an essential upstream activator of AKT in irradiated H-RAS V12 cells.

In one respect, the data in mutant K-RAS-deleted (C2) cells were somewhat surprising based on our parallel findings examining the expression of receptor tyrosine kinases and their ligands, proteins that are known upstream transducers of basal ERK1/2 and AKT pathway activity and radiation-induced signaling by these pathways. Although mutant K-RAS-deleted cells expressed significantly less epiregulin than WT cells, they also displayed elevated levels of the ligand heregulin and of the receptor for heregulin, ERBB3. Enhanced heregulin and ERBB3 expression would be predicted to significantly increase basal AKT activity and radiation-induced activation of AKT in both mutant K-RAS-deleted and H-RAS V12 cells; however, this observation was only evident in H-RAS V12 cells.

One possible explanation of our findings is that for radiation to cause a measurable large amount of AKT activation in HCT116 carcinoma cells expressing high levels of ERBB3 and heregulin requires, additionally, the expression of an active mutant form of RAS in the plasma membrane. In other systems, H-RAS V12 has been reported to preferentially activate the PI3K/AKT pathway partly by directly binding to the p110 subunit of PI3K (e.g., ref. 41), and it could be hypothesized in our system that H-RAS V12 acted as a binding protein to localize PI3K p110 in the plasma membrane. Plasma membrane localization of PI3K and Raf-1 via RAS proteins has been shown by others to cause partial enzymatic activation (e.g., refs. 33, 41). We determined that a farnesyltransferase inhibitor abolished membrane association of H-RAS and PI3K in H-RAS V12 cells, which also abolished radiation-induced AKT activation. Furthermore, and in contrast to H-RAS V12, K-RAS D13 did not promote PI3K localization in the plasma membrane of WT HCT116 cells. Collectively, these findings show that activated forms of H-RAS and K-RAS differentially act to membrane localize PI3K in HCT116 carcinoma cells.

Once localized in the plasma membrane environment via H-RAS V12, additional studies showed that heregulin, via ERBB3, played an essential role in radiation-stimulated PI3K/AKT signaling, which in turn was required for H-RAS V12–stimulated radioresistance. Thus, our data suggest that H-RAS V12, acting solely as a docking protein to translocate PI3K to the plasma membrane, cannot by itself protect HCT116 cells from the toxic effects of ionizing radiation. Instead, our findings argue that H-RAS V12 acts to facilitate radiation-induced activation of the protective AKT pathway and has to cooperate with additional signaling modules, such as heregulin/ERBB3, to promote cell survival. Hence, the lack of radiation-induced AKT activation in mutant K-RAS-deleted cells can in part be attributed to a lack of membrane-associated PI3K in these cells.

In further support of the concept that paracrine ligand signaling plays a protective role, medium from irradiated mutant K-RAS-deleted cells and H-RAS V12 cells promoted heregulin-dependent AKT activation when transferred onto unirradiated H-RAS V12 cells, whereas in mutant K-RAS deleted cells, where PI3K is not constitutively present in the plasma membrane, little or no AKT activation was observed following medium transfer. However, treatment of both cell lines with a saturating amount of heregulin caused AKT activation, which suggests that the lack of AKT activation in mutant K-RAS-deleted cells in the medium transfer experiments may have been due to the relatively low amounts of heregulin secreted into the growth medium after irradiation. Collectively, because (*a*) mutant K-RAS-deleted cells and H-RAS V12 cells expressed comparable levels of ERBB3 and heregulin, (*b*) medium transfer from mutant K-RAS-deleted cells and H-RAS V12 cells caused similar amounts of AKT activation in H-RAS V12 cells, and (*c*) only mutant H-RAS V12 cells contained membrane-associated PI3K, our findings strongly argue that H-RAS V12 cooperates with heregulin and ERBB3 to promote radiation-induced activation of the radioprotective PI3K/AKT signaling pathway.

Ries et al. showed that inhibitors of MEK1/2 radiosensitized WT HCT116 cells but not mutant K-RAS-deleted cells (23). In agreement with these findings, we observed that U0126 enhanced the toxicity of radiation in WT but not mutant K-RAS-deleted HCT116 cells. In H-RAS V12 cells, despite H-RAS V12 returning basal ERK1/2 activity and partially restoring radiation-induced ERK1/2 activation to those levels found in WT cells, U0126 did not significantly enhance radiosensitivity. Basal AKT activity in H-RAS V12 cells was also similar to that found in WT cells, although radiation-induced AKT activation was significantly greater and more prolonged in H-RAS V12 cells than in WT cells. Inhibition of PI3K and AKT in H-RAS V12 cells, but not in

WT cells, enhanced radiosensitivity. Thus, a more pronounced activation of AKT in H-RAS V12 cells generated a greater overall radioprotective response than a similar amount and duration of ERK1/2 activation in WT cells. These findings in isogenic cells strongly support the concept that PI3K/AKT signaling has a greater radioprotective effect than MEK1/2-ERK1/2 signaling. In addition, our findings also suggest that the basal activity of ERK1/2 and AKT correlate poorly both with plating efficiency and with any *a priori* predicted impact of kinase inhibitors on cellular radiosensitivity.

In conclusion, studies in nonisogenic cell types have argued that activated mutant forms of H-RAS, N-RAS, and K-RAS protect cells after radiation exposure via activation of the PI3K/AKT pathway (35–37, 42, 43). Other studies have linked ERK1/2 signaling to both enhanced survival and sensitivity to radiation in a cell type–dependent manner that was unrelated to the expression of mutant active RAS molecules (4, 44). The present studies show that in isogenic HCT116 carcinoma cells mutant active K-RAS D13 promotes cell survival after radiation exposure predominantly via the ERK1/2 pathway. In contrast, mutant active H-RAS V12 enhances HCT116 cell survival more potently than mutant K-RAS D13 and does so by suppressing cell killing via the PI3K/AKT pathway. Although expression of H-RAS V12 strongly promotes a radioresistant phenotype, without the costimulatory actions of heregulin and ERBB3, the H-RAS V12 oncogene cannot enhance radioresistance in HCT116 colorectal tumor cells.

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