Redundancy of Radioresistant Signaling Pathways Originating from Insulin-like Growth Factor I Receptor*

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The insulin-like growth factor I receptor (IGF-IR) has the ability to confer clonogenic radioresistance following ionizing irradiation. We attempted to determine the downstream pathways involved in IGF-IR-mediated radioresistance and used mouse embryo fibroblasts deficient in endogenous IGF-IR (R−) as recipients for a number of mutant IGF-IRs. Mutational analysis revealed that the tyrosine at residue 950 (Tyr-950) of IGF-IR, as well as the C-terminal domain, are required for radioresistance and that both domains must be mutated to abrogate the phenotype. Furthermore, the contribution of downstream pathways was analyzed by combining the use of wild-type or Tyr-950 and C-terminal mutants with specific inhibitors of phosphatidylinositol 3′-kinase (PI3-K) or mitogen-activated protein extracellular signal-regulated kinase (ERK) kinase (MEK). Radioresistance could be induced by IGF-IR as long as the ability of the receptor to stimulate the MEK/ERK pathway was retained. This was confirmed by the expression of constitutively active MEK in R− cells. The ability to stimulate the PI3-K pathway alone was not sufficient, but PI3-K activation coupled with MEK/ERK pathway-independent signals from the C terminus was able to induce radioresistance. Taken together, these results indicate that the IGF-IR-mediated radioresistant signaling mechanism progresses through redundant downstream pathways.

Intrinsic radiosensitivity is one of the critical factors that determines the probability of successful tumor cure or local control following radiotherapy (1, 2). Activation of oncogenes, including ras, and mutation of tumor suppressors such as p53 are well known to induce radioresistance (3–5), and the mechanisms by which this occurs have been investigated from a number of aspects, including cell cycle progression and signal transduction (6–8). It has been reported that phosphatidylinositol 3′-kinase (PI3-K), is important for mutant Ras-induced radioresistance, although both are known to convey potent survival signals (8). Some of the growth factors, which activate a variety of downstream pathways including Ras, also mediate cell survival functions through their cognate receptors (9). Of these, epidermal growth factor receptor (EGFR), which is often overexpressed in various tumor types, has been shown to induce radioresistance; specific antibodies for EGFR or expression of dominant negative EGFR significantly radiosensitizes tumor cells both in vitro and in vivo (10–12). It has been suggested that stimulation of survival signals such as the PI3-K and MAPK pathways following EGFR activation contributes to radioresistance (13, 14).

The insulin-like growth factor I receptor (IGF-IR) is a transmembrane tyrosine kinase, the amino acid sequence of which is highly homologous to that of the insulin receptor (IR) (15). It is a generally held view that IGF-IR activation plays a key role in cell growth, establishment, and maintenance of a transformed phenotype, cell survival, and differentiation (16–20). Elevated levels of IGF-IR have been observed in human tumors of breast (21), brain (22), and lung and colon (23) and, when observed, are associated with a poor prognosis (21). As IGF-IR was found to possess the ability to induce radioresistance (21, 24–26), directed study of this receptor is likely to shed light on the downstream pathways leading to this phenomenon. Comprehensive study from such a viewpoint has not been previously conducted, except that antisense targeting of IGF-IR reduces the activity of ataxia telangiectasia-mutated (ATM), a necessary factor for proper double strand break repair, resulting in enhanced radiosensitivity (27). Direct connection, however, between the IGF-IR pathway and ATM has not been established to date.

Two major pathways are thought to originate from IGF-IR, one through insulin receptor substrate-1 (IRS-1), which activates the PI3-K/Akt pathway, and the other through Shc, which activates the Ras/Raf/MEK/ERK pathway (28). These two substrates bind to the NPXY950 motif in the juxtamembrane domain, and Tyr-950 plays an important role in binding as revealed by a yeast two-hybrid assay (29, 30). The Raf/MEK/ERK pathway is also activated through 14-3-3 proteins, which bind to the C terminus of IGF-IR, a site not available on the IR (31–33). In addition to these main pathways, activation of c-Raf kinase by 14-3-3 proteins bound to the IGF-IR also results in its translocation to the mitochondria, where it exerts a survival effect with Nedd4 (28, 34). How these downstream pathways of IGF-IR influence radioresistance is not known.

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¶ The abbreviations used are: PI3-K, phosphatidylinositol 3′-kinase; MAPK, mitogen-activated protein kinase; EGFR, epidermal growth factor receptor; IGF-IR, insulin-like growth factor I receptor; IR, insulin receptor; ATM, ataxia telangiectasia-mutated; IRS-1, insulin receptor substrate-1; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase; WT, wild-type; HRP, horseradish peroxidase; HA, hemagglutinin; TBST, Tris-buffered saline plus Tween 20; PtdIns, phosphatidylinositol; Gy, gray.
In this study, we sought to determine the contributions of the different downstream pathways of IGF-IR to IGF-IR-mediated radioresistance. For this purpose, we used a series of mutant IGF-IRs, potentially relevant to PI3-K and MEK/ERK activation, expressed in R− cells deficient in endogenous IGF-IR (17).  

### EXPERIMENTAL PROCEDURES

**Materials**—Wortmannin, LY294002, and PD98059 were purchased from Sigma. Antibodies against IGF-IR α- and β-subunits, ERK2, goat IgG conjugated with horseradish peroxidase (HRP), rabbit IgG-HRP, and Protein A/G PLUS-agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibody (PY20) was purchased from Transduction Laboratory (Lexington, KY) and anti-phosphokinase B/AKT phosphoserine 473 antibody was purchased from Cell Signaling Technology (Danvers, MA). Anti-ACTIVE™ MAPK antibody was kindly provided by Dr. E. Nishida (Kyoto University, Kyoto, Japan).

**Plasmid Construction**—Wild-type, Y950F, Y1316F, and Δ1245 receptors were derived from human IGF-IR cDNA (15) as described previously (35, 36). For Y950F/1316F, the HindIII-BamHI fragment of pBluescript SK Y950F (35) was replaced by the HindIII-BamHI fragment of pBluescript SK Y950F/Y1316F (36) and was designated pBluescript SK Y950F/Y1316F. The Xhol-NotI fragment of pBPV IGF-IR (17), an expression plasmid for the wild-type (WT) receptor, was then replaced with the Xhol-NotI fragment of pBluescript SK Y950F/Y1316F, including the double mutation. For the Y950F/Δ1245 mutant, the HindIII-BamHI fragment of pBluescript SK Y950F was replaced by the corresponding fragment from pBluescript SK Δ1245 (36). Then, the Xhol-NotI fragment of pBPV IGF-IR was replaced by the Xhol-NotI fragment of the Y950F/Δ1245 cDNA in pBluescript SK. For the construction of IGF-IR truncated at residue 950, the Xhol-BamHI fragment of pBPV IGF-IR was transferred into the vector pEGFP-C1. The SacI-BamHI fragment of the IGF-IR cDNA in this vector was then replaced by the following oligodeoxynucleotides, 5′-ACTGAGAATTCC-3′ and 5′-TGACTCTTAAGCCTAG. The oligodeoxynucleotides were designed to terminate translation at residue 950 followed by the stop codon TGA, and they contained a BamHI restriction overhang for ligation to the BamHI site of the IGF-IR cDNA in pEGFP-C1 and an EcoRI restriction site for confirmation. The Xhol-BamHI fragment of pBluescript SK IGF-IR was replaced by the corresponding fragment of truncated IGF-IR cDNA in vector pEGFP-C1. The Xhol-NotI fragment of pBPV IGF-IR was then replaced with the corresponding fragment containing Δ950 in pBluescript SK.

**Cell Lines, Culture Conditions, and Transfections**—R− cells were obtained from mouse embryo fibroblasts possessing a null mutation of the IGF-IR gene (17). Plasmids containing wt or mutant IGF-IR cDNAs were stably transfected into R− cells with a pDVe6 plasmid carrying the puromycin resistance gene (37) by calcium phosphate precipitation. Cells were selected in 4 μg/ml puromycin, and the resultant clones were mixed and sorted as described previously (38). Mixed populations or clones were used in the present study. For transient expression of constitutively active MEK, cells, a plasmid containing LA-SDSE MAPK kinase cDNA (39) was transfected into R− cells, and the cells were prepared for Western blotting and a colony-forming assay 48 h after transfection. Mock-transfected R− cells were treated similarly as a control. All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO2 in Eagle's minimal essential medium containing 10% fetal bovine serum supplemented with 10% (v/v) fetal bovine serum. Exponentially growing cells were used for all experiments.

**Colony-forming Assay**—Radioresistance was determined by colony-forming assay as described previously (25). To assess the effect of exogenously added IGF-I or inhibitors of PI3-K or MEK, cells in plastic flasks grown for roughly 10 h were treated with inhibitors for 1 h and then γ-irradiated. Cells were transferred to a 37 °C incubator and rendered to form colonies. Surviving fraction was calculated based on the plating efficiency determined from the IGF-I- or inhibitor-treated cells. Cell survival was corrected using the equation $S = 1 - (1 - f)^{N}$, where $S$ is the single cell survival rate, $f$ is the measured surviving fraction, and $N$ is the multiplicity determined by plating the number of cells per microcolony at the time of irradiation. Multiplicity ranged from 1.1 to 1.2 for all cell lines under the described conditions.

**Western Blotting**—Cells were digested in a lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.1% SDS, 1 mM EDTA, 100 mM Na3VO4, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin). Equal amounts of lysates were separated in SDS-polyacrylamide gel (PAGE), and proteins were transferred to a nitrocellulose membrane in a Trans-glycine buffer containing 20% methanol. The membrane was blocked in 5% nonfat milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20). Filters were probed with primary antibodies against target proteins for 1 h at room temperature or overnight at 4 °C. Filters were washed three times in TBST, incubated with secondary antibodies conjugated with HRP in TBST for 1 h at room temperature, and then washed three times in TBST. Proteins were visualized using the ECL system. For the detection of activated proteins, cells were incubated in serum-free medium containing 1 μg/ml bovine serum albumin overnight. Serum-starved cells either treated or untreated with indicated concentrations of IGF-I or 10 min were processed as described above.

**IGF-I Binding Assay**—The number of IGF-I binding sites was determined in each cell line as described previously (38). Cells grown on six-well plate wells were washed with Hanks' balanced salt solution and incubated for 4 h at 4 °C in binding buffer (Eagle's minimal essential medium plus 25 mM Hepes, pH 7.4, and 1 μg/ml bovine serum albumin) containing 0.5 ng/ml [125I]-IGF-I and/or increasing concentrations of unlabeled IGF-I. After washing with cold Hanks' balanced salt solution, cells were lysed with 0.05% SDS, and cell-associated radioactivities were measured by an autowell γ-counter. Specific binding was expressed by subtracting nonspecific binding as determined in the presence of excess unlabeled IGF-I (200 ng/ml) relative to specific binding sites in cells incubated in buffer containing 0.5 ng/ml [125I]-IGF-I alone was determined in each cell line with values of WT 11 cells normalized to 1.0. Receptor number per cell and dissociation constants ($K_{d}$) were also estimated in some cell lines by Scatchard analysis as described previously (38).

**Phosphatidylinositol 3-Kinase Activity**—Activity of PI3-K was measured as described previously (40). Briefly, IGF-I-treated or untreated cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40, and phosphotyrosine-containing proteins were immunoprecipitated with anti-phosphotyrosine antibody (PY20) bound to Protein A/G-agarose. PI3-K activity in the immunoprecipitates was measured in a reaction mixture containing phosphatidylinositol and [γ-32P]ATP. After incubation, the reaction was stopped by the addition of a solution (chloroform:methanol:HCl = 2:1:0.1) and analyzed by thin layer chromatography.

**γ-Irradiation**—γ-Irradiation was performed using a 60Co therapeutic machine, RCR-120 (Toshiba, Tokyo, Japan), at a dose rate of 1.4-1.8 Gy/min.

**Statistical Analysis**—Statistical comparison of mean values was performed using the Student's t test or one-way analysis of variance followed by Fisher's protected least significant difference. Differences with a p value of <0.05 were considered statistically significant.

**RESULTS**

**Expression of Wild-type IGF-IR Confers Clonogenic Radioreistance in R− Cells**—We previously reported that introduction of IGF-IR into R− cells, which are deficient in endogenous IGF-IR, confers clonogenic radioresistance (25). To confirm this, we newly established several clones and their radiosensitivities were determined by colony-forming assay. Of these, mixed populations (WTmix), clones 9 and 11, which expressed similar levels of IGF-IR, exhibited a significant radioresistance (to a similar extent) compared with R− or R− (puro) cells expressing a marker gene alone (Fig. 1, A and B). Because the extent of radioresistance was relatively modest, we attempted to examine whether radioresistance was increased when cells were stimulated with exogenously added IGF-I, although
lyases were subjected to SDS-PAGE, and IGF-IR was detected as described under “Experimental Procedures.” The unprocessed form of the proreceptor was also detected with both antibodies. The antibody used to detect the IGF-IR β-subunit recognizes the C terminus of the subunit. β-Actin was used as a loading control. C, effect of exogenously added IGF-I on surviving fractions. WTmix and WT 11 (A11) cells were irradiated at a dose of 6 Gy in the presence or absence of exogenously added IGF-I in growth medium, and surviving fractions were determined. Lane 1, 0 ng/ml; lane 2, 10 ng/ml; lane 3, 20 ng/ml; lane 4, 50 ng/ml. Data represent the means ± S.D. of triplicate determinants. No significant differences were obtained versus cells irradiated in the absence of exogenously added IGF-I. Dose-survival curves of the cell lines in panels A and D are shown in panels B and E, respectively. Data shown are the means of at least two independent experiments.

**FIG. 1.** Expression of IGF-IR and dose-survival curves in cell lines expressing WT or mutant receptors lacking the tyrosine kinase domain. Figure shows Western blots for IGF-IR expression in R−, R− (puro clones), WT 9, WT 11, and WTmix cells (A), and in Δ950 clone 5 and Δ950 clone 7 cells (D, #5 and #7). Equal amounts of cell lysates were subjected to SDS-PAGE, and IGF-IR α- or β-subunits were detected as described under “Experimental Procedures.” The unprocessed form of the proroeptor was also detected with both antibodies. The antibody used to detect the IGF-IR β-subunit recognizes the C terminus of the subunit. β-Actin was used as a loading control. C, effect of exogenously added IGF-I on surviving fractions. WTmix and WT 11 (A11) cells were irradiated at a dose of 6 Gy in the presence or absence of exogenously added IGF-I in growth medium, and surviving fractions were determined. Lane 1, 0 ng/ml; lane 2, 10 ng/ml; lane 3, 20 ng/ml; lane 4, 50 ng/ml. Data represent the means ± S.D. of triplicate determinants. No significant differences were obtained versus cells irradiated in the absence of exogenously added IGF-I. Dose-survival curves of the cell lines in panels A and D are shown in panels B and E, respectively. Data shown are the means of at least two independent experiments.

**FIG. 2.** Schematic presentation of the WT and mutant IGF-IR β-subunits used in this study. For simplicity, only a single β-subunit is presented. TK, tyrosine kinase domain; Y, tyrosine; F, phenylalanine; S, serine. The number denotes amino acid number according to Ulrich et al. (15).

**Characteristics of Mutant Receptors—**We next attempted to determine which domains of the IGF-IR β-subunit are necessary for clonogenic radioresistance. For this purpose, we made various mutant receptors with specific mutations potentially relevant to activation of downstream pathways. For clarity, the mutant receptors used in this study are shown in Fig. 2. The tyrosine residue at position 950 is part of the NPXY motif, a major binding site for IRS-1 and Shc, which is conserved in IR (41) and interleukin 4 receptor (42). IRS-1 activates PI3-K, which phosphorylates phosphatidylinositol (PtdIns) phosphates, converting PtdIns 4,5-P2 to PtdIns 3,4,5-P3. This lipid activates phosphoinositol-dependent kinases 1 and 2, which in turn activate Akt (43). Shc strongly activates the Ras/Raf/MEK/ERK pathway (44, 45). Tyrosine 1316 is a constituent of the Y1316XXM motif, a binding site for the regulatory subunit p85 of PI3-K, and is able to stimulate its activity (46). This is also conserved in IR (47). Because each tyrosine, Tyr-950 and Tyr-1316, is reported to play a critical role in each binding function (29, 30, 46), these residues were mutated to phenylalanines to attenuate the relevant pathways. The C terminus of IGF-IR includes a quartet of serine residues 1280–1283, which is a binding site for 14-3-3 proteins (33) that in turn lead to activation of c-Raf and the MAPK pathway. c-Raf undergoes mitochondrial translocation and exerts a survival effect in cooperation with Nedd4 (34). We will refer to this pathway as 14-3-3/c-Raf hereafter to differentiate this c-Raf signaling event as separate from its activation of the MAPK pathway. To eliminate the binding site of the 14-3-3 proteins, the receptor was truncated at residue 1245. Double mutation at Tyr-950 and the C terminus was also introduced to exclude both signals. Mutant receptors were expressed in R− cells, and their expression levels were assayed by Western blotting using antibodies specific for the IGF-IR α- or β-subunit (Fig. 3A). Clones expressing levels of receptor almost similar to those of WT 9 or 11 were selected. To assess the number of mature cell-surface receptors, 125I-IGF-I-binding assay was also done (Fig. 3B). Although there were some variations in the number of IGF-I binding sites among cell lines, all the mutants possessed levels
Concentrations of unlabeled IGF-I was determined as described under "Experimental Procedures." B, IGF-I binding assay. Panel shows relative number of IGF-I binding sites per cell in each cell line. Cells were incubated in binding buffer containing 0.5 ng/ml 125I-IGF-I, and relative numbers of specific binding sites were determined. Results were presented with values of binding sites at least more than WT 11 cells. Specific binding ratios were plotted against unlabeled IGF-I concentrations.

To examine the functional properties of the Tyr-950 and the C terminus receptor mutants, we measured the activation of the PI3-K and MAPK pathways upon IGF-I stimulation (Fig. 4). Phosphorylation of IRS-1 and Akt was used as a marker of activation of the PI3-K pathway. The activation of both IRS-1 and Akt seemed to be somewhat mitigated in the Y950F, Y950F/Y1316F, and Y950F/Y1316F mutants compared with WT 11 cells. As an example, 125I-IGF-I binding competition in WT 11 and Y950F/Δ1245 clone 3 cells is shown in Fig. 3C, exhibiting similar displacement properties (IC50 = ~1 nM). Scatchard analysis revealed that receptor number per cell and dissociation constant (Kd) in WT 11 cells were 9 x 10^5 receptors and 0.6 nM, respectively. We could thus confirm that all cell lines express almost similar levels of high affinity surface receptors.

To analyze the functional properties of the Tyr-950 and the C terminus receptors, we measured the activation of the PI3-K and MAPK pathways upon IGF-I stimulation (Fig. 4). Phosphorylation of IRS-1 and Akt was used as a marker of activation of the PI3-K pathway. The activation of both IRS-1 and Akt seemed to be somewhat mitigated in the Y950F, Y950F/Y1316F, and Y950F/Δ1245 mutants compared with WT 11 after testing the dose dependence of the response (Fig. 4, A and B). The PI3-K assay was in agreement with the results regarding those of activation for IRS-1 and Akt (Fig. 4C). Taken together, we concluded that the PI3-K pathway is not abrogated, but inhibited in Y950F and Y950F/Δ1245 mutants. It is unlikely that Tyr-1316 contributes in a significant manner to the activation of the PI3-K pathway (Fig. 4A), which is consistent with both the results that the Δ1245 mutant had no significant effect on the activation of the PI3-K pathway (Fig. 4, B and C), and those from the studies of the IR C-terminal deletion mutants (48). The ability of the Y950F and Δ1245 mutants to activate the ERK pathway was intact, as determined by measurement of ERK-1 and -2 phosphorylation (Fig. 4, A and B). Double mutation of Tyr-950 and the C terminus was required to inhibit the ERK pathway (Fig. 4B), confirming previous reports (49).

Double Mutation at Tyr-950 and the C Terminus Is Required to Inactivate IGF-IR-mediated Radiosensitivity—The radiosensitivity of the various mutants were determined by colony-forming assays, and dose-survival curves were obtained as shown in Fig. 5A. Survival fractions after exposure to 6 Gy are also summarized in Fig. 5B. All the specific mutants except for Y950F/Δ1245 exhibited very similar radiosensitivity to WT cells, demonstrating that any single mutation does not influence the phenotype. Double mutation of Tyr-950 and the C-terminal domain was required to inactivate the phenotype. This suggests that signals from either of the two different sites, Tyr-950 or the C terminus, may be sufficient to induce clonogenic radiosensitivity in R- cells. Because Tyr-950 has the ability to stimulate both PI3-K and MAPK pathways and the C terminus has both MAPK and 14-3-3/Raf pathways, mutational analysis was thus still insufficient to clearly define the relative contributions of the three pathways.

Effects of Specific Inhibitors of PI3-K and MEK on Radiosensitivity in WT, Y950F, and C-terminal Mutants—We reasoned that it would be possible to analyze the relative contributions of the three pathways when Tyr-950 or C-terminal single mutants were combined with specific inhibitors of PI3-K or MEK. The resulting patterns of signal inhibition in WT 11 cells by each inhibitor are shown in Fig. 6A, and the survival fractions after a radiation exposure to 6 Gy of WT cells or cells expressing Y950F or Δ1245 receptors are shown in Fig. 6B. Wortmannin is a well known PI3-K inhibitor and has often been used in studies of radiosensitization. Previous reports have shown that concentrations of more than ~5 μM will significantly sensitize cells, and that significant sensitization is not observed at lesser concentrations (50, 51). This sensitization is a result of inhibition of ATM or DNA-dependent protein kinase, important factors in DNA double strand break repair, but not to inhibition of PI3-K itself. Specific inhibition for PI3-K is usually obtained at 0.1–1 μM (52). The survival rates of the cell lines used in this study were unaffected by 1 μM wortmannin (Fig. 6B), at which
concentration IGF-I-induced Akt activation is completely inhibited (Fig. 6A), but they were uniformly sensitized by 5 and 10 μM concentrations (data not shown). In addition, we tested another PI3-K inhibitor, LY294002, in a similar manner. Precise information was not available on the concentration at which ATM and DNA-dependent protein kinase inhibition occurred using this inhibitor. When 10 μM LY294002 was applied, a concentration frequently used to inhibit PI3-K (8, 20), IGF-I-induced Akt activation was significantly inhibited, but somewhat less so than after treatment with 1 μM wortmannin (Fig. 6A). The use of more than 10 μM LY294002, however, resulted in significant toxicity and a remarkable decrease in plating efficiency (data not shown). No effect was observed on the survival of the three cell lines (Fig. 6B), consistent with the results obtained after treatment with 1 μM wortmannin. The fact that the Δ1245 mutant was still resistant after treatment with PI3-K inhibitors was quite informative; cells displayed radioresistance even under conditions where the only known surviving pathway of IGF-IR was that of MEK/ERK. We further used an inhibitor of MEK, PD98059, to inhibit this pathway. Of interest, cells expressing WT receptors were not influenced, but both mutants were significantly radiosensitized upon treatment with 25 μM PD98059, a concentration that effected a nearly complete inhibition of IGF-I-induced ERK activation (Fig. 6, A and B). These results suggest that the ability of IGF-IR to activate the PI3-K pathway alone is insufficient to produce radioresistance, but radioresistance is achievable when PI3-K signals are combined with signals from the C terminus that are independent of the MEK/ERK pathway. The results of these two inhibitor experiments both point toward a strong contribution of the MEK/ERK pathway in the development of radioresistance. The results using inhibitors are summarized in Table I.

**Expression of Constitutively Active MEK Renders R− Cells Radioresistant**—To confirm the radioresistant effect of MAPK pathway signaling, constitutively active MEK was transiently expressed in R− cells (Fig. 6B). This resulted in significant toxicity and a remarkable decrease in plating efficiency (data not shown). No effect was observed on the survival of the three cell lines (Fig. 6B), consistent with the results obtained after treatment with 1 μM wortmannin. The fact that the Δ1245 mutant was still resistant after treatment with PI3-K inhibitors was quite informative; cells displayed radioresistance even under conditions where the only known surviving pathway of IGF-IR was that of MEK/ERK. We further used an inhibitor of MEK, PD98059, to inhibit this pathway. Of interest, cells expressing WT receptors were not influenced, but both mutants were significantly radiosensitized upon treatment with 25 μM PD98059, a concentration that effected a nearly complete inhibition of IGF-I-induced ERK activation (Fig. 6, A and B). These results suggest that the ability of IGF-IR to activate the PI3-K pathway alone is insufficient to produce radioresistance, but radioresistance is achievable when PI3-K signals are combined with signals from the C terminus that are independent of the MEK/ERK pathway. The results of these two inhibitor experiments both point toward a strong contribution of the MEK/ERK pathway in the development of radioresistance. The results using inhibitors are summarized in Table I.

**FIG. 6. Effect of specific inhibitors of PI3-K and MEK on clonogenic radioresistance in cells expressing WT or mutant receptors.** A, effect of inhibitors on IGF-I-induced activation of Akt or ERK in WT 11 (111) cells. Serum-deprived cells were incubated with inhibitors (Wort, 1 μM wortmannin; LY, 10 μM LY294002; PD, 25 μM PD98059) for 1 h and stimulated with 50 ng/ml IGF-I. Cells were lysed after 10 min, and phosphorylation of Akt or ERK was detected by Western blotting as described under “Experimental Procedures.” β-Actin and ERK-2 were used as loading controls. B, effect of inhibitors on clonogenic radioresistance. Appropriate numbers of cells grown in plastic flasks were treated with the same concentrations of inhibitors as described in panel A for 1 h and γ-irradiated at a dose of 6 Gy. Irradiated cells were transferred to an incubator and rendered to form colonies in the presence of inhibitors. Surviving fractions were calculated as described under “Experimental Procedures.” Data represent the means ± S.D. of three separate experiments. *p < 0.002; **p < 0.001 versus cells irradiated in the absence of inhibitors (analysis of variance).
Redundancy of IGF-IR-mediated Radioresistance

Table I
Summary of radiosensitivity of WT, Y950F, and Δ1245 cells after treatment with specific inhibitors of PI3-K or MEK

| Cell line | Wortmannin | LY294002 | PD98059 |
|-----------|------------|----------|---------|
| WT        | +          | +        | +       |
| Y950F     | (M + C)*  | (M + C)  | (P + C) |
| Δ1245     | +          | +        | -       |

*+, radiosensitivity is retained; −, radiosensitivity is lost.

Downstream pathways which can be stimulated through WT or mutant IGF-IRs in the presence of inhibitors: P, PI3-K; M, MEK/ERK; C, pathway from the C-terminus independent of the MEK/ERK pathway.

A

B

FIG. 7. Effect of constitutively active MEK expression in R- cells on clonogenic radiosensitivity. A, expression of constitutively active HA-tagged MEK in R- cells. R- cells were transfected with a plasmid containing constitutively active HA-MEK cDNA. Cell lysates were prepared 48 h after transfection and processed for Western blotting as described under “Experimental Procedures.” Proteins were probed with anti-HA or phosphorylated ERK antibodies. ERK-2 was used as a loading control. Preparations from mock-transfected cells without plasmids were also used as controls. B, effect of constitutively active MEK expression in R- cells on clonogenic radiosensitivity. Preparations from mock-transfected cells served as a loading control. Data represent the means ± S.D. of three separate experiments. *p < 0.05 versus untreated or mock-transfected cells (Student’s t test).

similar to those for R- (puro) cells (Fig. 7B). Cross-activation of IRS-1 or Akt was not detected in R- cells expressing constitutively active MEK (data not shown).

Discussion

Evidence is rapidly accumulating that IGF-IR is involved in cellular radioresistance. Mouse embryo fibroblasts exhibit a radioresistant phenotype when IGF-IR is overexpressed (21, 24, 25), and melanoma cells display an enhanced radiosensitivity after anti-sense targeting of IGF-IR (27). Furthermore, a specific inhibitor of the IGF-IR tyrosine kinase, tyrphostin AG 1024, significantly radiosensitized human breast cancer cells (26). The signaling mechanism whereby IGF-IR leads to radioresistance, however, has never been comprehensively pursued to date. We therefore wished to determine which of the downstream pathways of IGF-IR are related to the development of radioresistance.

As a measurement of radiosensitivity, clonogenic cell survival as determined by colony-forming assay was used during this study. It is a complex end point influenced by many factors such as cell death and/or growth, DNA repair, and the ability to escape growth arrest and re-enter the cell cycle. This in vitro assay has been used as a representative marker of radiosensitivity thought to reflect the reproductive integrity of tumor cells (1, 2, 54–56). It may be argued that clonogenic radioresistance induced by IGF-IR in this study is at best very modest, leading to no more than a 3-fold increase in survival fraction at 6 Gy. Although it is well established that activated Ras (6–8) or overexpression of EGFR (10–14) in tumor cells confers clonogenic radioresistance, its extent is similar to that obtained by IGF-IR as shown in our results. This level of difference, however, critically affects clinical radioreponse or tumor cure (1, 2) because radiotherapy is usually performed according to fractionated regimen, by repeating irradiation 20–30 times; the modest difference per irradiation is enormously enhanced at the end of the therapy (54).

Considering that exogenously introduced WT IGF-IR induces clonogenic radioresistance in R- cells, which are deficient in the endogenous IGF-IR, we attempted to use this system to determine which portions of the receptor structure are necessary for the origin of this signal. A Δ950 IGF-IR mutant that lacks the tyrosine kinase domain did not confer radioresistance, confirming that tyrosine kinase-dependent signal transduction is required for this activity. We therefore reasoned that the use of mutants with selective signaling defects could help to pinpoint which downstream pathways are involved in the development of radioresistance.

How is the IGF-IR activated under the present conditions? Ionizing radiation by itself activates receptor tyrosine kinases such as EGFR, which in turn activates its downstream pathways (13). Although such activation of IGF-IR was not apparent in WT 9 or 11 cells under the present conditions (data not shown), undetectable levels of activation could not be ruled out. Another factor that may affect activation of IGF-IR is relatively high levels of IGF-I (10–20 ng/ml) originally contained in the serum in growth medium (57). Unlike the case of stimulation of serum-deprived cells, activation of IGF-IR and its downstream pathways in cells chronically incubated with growth medium is very weak (25). Even under these conditions, IGF-IR-overexpressing cells can proliferate much faster than non-overexpressing cells by the continuous exposure to 10% serum medium (16, 17, 25). More pronounced effect is observed under anchorage-independent conditions (16, 17). It is thus possible that very low levels of IGF-IR activation through growth medium or irradiation may be enough to induce IGF-IR-mediated clonogenic radioresistance and easily reach to saturation. We inferred that retained functions of mutant IGF-IRs in growth medium following irradiation, which are barely detectable, could be qualitatively similar to those activated by IGF-I, which are easily visualized as shown in Fig. 4.

As described above, survival signals are transduced by the different regions of IGF-IR through specific docking proteins, and eventually converge to cause the activation of the PI3-K, MEK/ERK, and 14-3-3/c-Raf pathways. Mutational analysis revealed that Tyr-950 and the C terminus are required for conferral of the radioresistant phenotype, and that both domains must be mutated to inactivate it (Fig. 5). These patterns were reminiscent of those observed for IGF-IR-mediated differentiation of neuronal cells, where double mutation of Tyr-950 and the C terminus was similarly required to inactivate the function (20). This phenotype absolutely depends on MEK/ERK activity, and indeed cells expressing WT receptor lose differentiation ability when treated with the MEK inhibitor PD98059 (20). Although this similarity also implicated a contribution of the MEK/ERK pathway to clonogenic radioresistance, the finding that PD98059 did not radiosensitize cells expressing WT receptors seemed tentatively puzzling. However, further results helped explain this discrepancy by revealing the importance of redundancy in this system. Cells expressing Y950F or Δ1245 mutant receptors were effectively radiosensitized upon PD98059 treatment (Fig. 6B), whereas cells expressing either
WT or mutant receptors were unable to be sensitized by PI3-K inhibitor treatment, i.e. these results suggest that radiosensitivity may be obtained as long as the ability of the receptor to stimulate the MEK/ERK pathway is retained. This was confirmed by the demonstration of full radiosensitivity in R- cells transfected with constitutively active MEK (Fig. 7). Previous results confirmed that the MEK/ERK pathway is not involved in radioresistance (7, 58), mostly drawn from the simple finding that PD98059 treatment had no effect on radiosensitivity.

Here, a combination of mutational analysis with the use of specific inhibitors allowed us to reveal contributions to the pathway in the absence of redundancy. This study then serves as an important reminder that careful interpretation is of extreme importance when analyzing the findings from inhibitor studies. In addition, the PI3-K pathway alone is not sufficient to induce radioresistance, but full radioresistance can be achieved by a combination of PI3-K signals with those from the C terminus that are irrelevant to the MEK/ERK pathway. These non-MEK/ERK C-terminal signals are presumably mediated by the 14-3-3/c-Raf pathway. Activated c-Raf can migrate to mitochondria and exerts cell survival effects with Nedd4 through 14-3-3 proteins bound to the C terminus of IGF-IR (28, 34). The PI3-K pathway finally leads to Bad phosphorylation and induces binding with Bcl-x-L in mitochondria (28). Although it is still unclear exactly how cooperation of both pathways leads to clonogenic radioresistance, activation of the PI3-K or the 14-3-3/c-Raf pathway alone is unlikely to be sufficient at least in these cell lines. Similar combination of pathways is also required for cell survival of 32D cells expressing IGF-IR induced by interleukin 3 withdrawal (49). Gupta et al. (8) reported that Ras-induced clonogenic radioresistance is mediated exclusively by the PI3-K, and not the MEK/ERK, pathway. Because IGF-IR also activates Ras pathways, we cannot clearly explain this discrepancy. Considering that IGF-IR signaling and functions vary from one cell type to another, this variability in signaling may depend on the availability of substrates and transducing molecules in each cell type, as reported by Petley et al. (59).

ATM is known to be a sensor of DNA damage, especially as a result of ionizing radiation, and stimulates double strand break repair via its kinase activity, whereby it significantly contributes to clonogenic radioresistance (55). Recently, radiation-induced ATM activation was reported to be inhibited by antisense against IGF-IR (27), suggesting a possible connection between IGF-IR and ATM signaling. However, a direct link between these two molecules has not been established to date. Discovering a relationship between the MEK/ERK pathway or other ATM and IGF-IR pathway may be an interesting key to understanding IGF-IR-mediated clonogenic radioresistance.

The redundancy of the survival signals related to the development of clonogenic radioresistance revealed by this study may have clinical implications regarding the use of molecular targeting in radiotherapy. IGF-IR is overexpressed in several human tumors (21–23) and is associated with a very poor prognosis following radiotherapeutic treatment (21). To overcome these problems, the points of downstream convergence of the IGF-IR pathways known to be involved in the development of radioresistance, PI3-K and MAPK, may be concomitantly targeted with radiotherapy to increase the efficacy of treatment for these cases (8). However, it is possible that the IGF-IR tyrosine kinase itself, the origin of many features of this robust radioresistant mechanism, may be a more efficient target.

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