Extremely Low-Dose Ionizing Radiation Causes Activation of Mitogen-activated Protein Kinase Pathway and Enhances Proliferation of Normal Human Diploid Cells

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ABSTRACT

We demonstrated here that X-ray irradiation at very low doses of between 2 and 5 Gy stimulated proliferation of normal human diploid cells and human tumor cells. Higher doses of irradiation at >1 Gy accumulated p53 protein and induced phosphorylation of extracellular signal-regulated kinase (ERK) 1/2. Phosphorylation of ERK1/2 decreased with dose down to 50 cGy, however, doses of between 5 cGy and 2 cGy phosphorylated ERK1/2 as efficiently as higher doses of X-rays, whereas the p53 protein level was not changed by doses <50 cGy. We found that mitogen-activated protein kinase (MEK) 1 was phosphorylated with both 2 cGy and 6 Gy of X-rays, and that activated ERK1/2 augmented phosphorylation of Elk-1 protein. The specific epidermal growth factor receptor tyrosine kinase inhibitor, AG1478, decreased phosphorylation of the ERK1/2 proteins induced by 2 cGy or 6 Gy of X-rays, and similar suppressive effect was observed with MEK inhibitor, PD98059. Suppression of ERK1/2 phosphorylation with these inhibitors alleviated enhanced proliferation of normal human cells by low-dose irradiation. Furthermore, overexpression of ERK2 in NCI-H1299 human lung carcinoma cells potentiated enhanced proliferation, whereas down-regulation of ERK2 using the antisense ERK2 gene abrogated the stimulatory effect of low-dose irradiation. These results indicate that a limited range of low-dose ionizing radiation differentially activates ERK1/2 kinases via activation of epidermal growth factor receptor and MEK, which causes enhanced proliferation of cells receiving very low doses of ionizing radiation.

INTRODUCTION

Several reports have indicated that extremely low doses of ionizing radiation cause an unpredicted response in cells (1–4). For example, a low dose of ionizing radiation, such as 2 cGy of X-rays, alleviates the lethal and mutagenic effects of subsequently higher doses of radiation (5–10). Induction of gene transcriptions or proteins has been found after low-dose irradiation (2, 11), indicating that the induction of gene transcription through the activation of signal transduction may be involved in the low-dose effects. Because doses of >10 cGy do not cause the effects (2, 9, 12), only a limited range of low doses may induce the differential stimulation of certain signal transduction pathways. To date, most signal transduction studies have used doses of ionizing radiation >1 Gy, therefore, very little is known about the effect of low-dose ionizing radiation on the activation of signal transduction pathways.

Ionizing radiation induces DNA double-strand breaks in the nucleus. In addition, it causes lipid peroxidation, ceramide generation, and protein oxidation in the membrane, cytoplasm, and nucleus (13). Therefore, it is very likely that ionizing radiation stimulates multiple signal transduction pathways simultaneously. One such pathway originates in the nucleus and transduces signal to p53 protein. p53 protein is a tumor suppressor gene product of which the function is involved in cell cycle arrest, apoptosis, DNA repair, and senescence (14–18). p53 protein has been shown to accumulate after ionizing radiation and to act as a transcription regulator (19–22). Several studies have suggested that DNA strand breaks cause accumulation and activation of p53 protein (23, 24). Furthermore, studies have shown that phosphorylation plays a crucial role in the regulation of the p53 accumulation and activity (25–30), and both ataxia telangiectasia-mutated and CHK2/Cds1 proteins, which are protein kinases, phosphorylate p53 protein at serine 15 and 20, resulting in an accumulation of p53 protein by the inhibition of the interaction between p53 and MDM2 proteins (31–36). Thus, DNA strand breaks caused by higher doses of radiation result in activation of the nuclear signal transduction, which is indispensable to the p53 response to ionizing irradiation. Once p53 is activated, it induces a variety of genes of which the products are involved in the induction of apoptosis, cell cycle arrest, and premature senescence. Recently, 0.5 Gy of γ-rays was reported to accumulate p53 protein in human skin diploid fibroblasts (37). It should therefore be examined whether a very low dose of ionizing radiation can activate p53 function to induce low-dose effects.

Another signal transduction pathway stimulated by ionizing irradiation is mediated by MAPKs. There are several members of MAPK including ERK1/2, JNK1/2, and p38-MAPK (38–41). Several studies have reported that high-dose ionizing radiation activates ERK1/2 through the stimulation of tyrosine kinase associated with the membrane (42–46), which results in the activation of several transcription factors regulating genes involved in cell growth. Thus, MAPK mediates the transduction of the signal from the membrane to the nucleus, and it may alleviate the lethal effects of radiation. Recently, a study reported that exposure to 1 Gy causes greater alterations in the activities of ERK1/2 than that to 6 Gy (47), however, it is not known whether very low doses of ionizing radiation cause activation of MAPK.

Although MAPK and p53 are activated by ionizing radiation, these pathways mediate pleiotropic response in cells. For example, p53, JNK1/2, and p38 have been shown to induce cell cycle arrest or apoptosis, whereas ERK1/2 promote cell growth (48, 49). These results suggest that the pathways activated differently in response to different doses of ionizing radiation cause differential effects.

In the present study, we examined the effects of low doses of ionizing radiation on cell proliferation of normal human diploid cells and determined whether MAPKs and p53 are activated in a dose-dependent manner at doses between 1 cGy and 6 Gy. The results demonstrated that irradiation of cells with a limited range of low-dose radiation between 2 cGy and 5 cGy stimulated cell proliferation, whereas doses >1 Gy showed lethal effect on cells. Differential activation of ERK1/2 via activation of EGFR and MEK could explain the enhanced cell proliferation of cells receiving low-dose irradiation.

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3 The abbreviations used are: MAPK, mitogen-activated protein kinase; ATF, activating transcription factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; PA, ponasterone A; JNK, c-Jun N-terminal kinase.
MATERIALS AND METHODS

Antibodies. The antibodies used in this study were anti-p53 monoclonal antibody and anti p21\(^{WAF/CIP}\) monoclonal antibody (Lab Vision Corp.) and anti-phospho-ERK1/2 antibody, anti-ERK1/2 antibody, anti-phospho-JNK1/2 antibody, anti-JNK1/2 antibody, anti-phospho-p38 antibody, anti-p38 antibody, anti-phospho-MEK antibody, anti-MEK antibody, anti-phospho-Erk1 antibody, anti-Erk1 antibody, and antiphosphorylated p53 (New England Biolabs).

Cell Cultures and Reagents. Normal human diploid (HE49) cells were cultured in Eagle’s MEM supplemented with 10% fetal bovine serum (Trace Bioscience Pty Ltd, ACN, Australia) as described previously (50). Cells seeded in T25 flasks (25 cm²) were subcultured every 3–4 days to maintain exponential growth. The specific MEK1 inhibitor PD98059 and EGFR inhibitor AG1478 were obtained from Calbiochem (San Diego, CA).

Constitution of the Sense- and Antisense-ERK2-expressing Cells. NCI-H1299 cells were obtained from American Type Culture Collection. H1299 cells were transfected with\(\Phi\)VgRXX plasmid to establish 99V9 cells, which express VgECR and RXR proteins. The 99V9 cells were transfected again with pIND-ERK2 or pIND-ASERK2, which contain the human ERK2 gene in a sense or antisense orientation, respectively. The pIND/GS H-Z11695 plasmid was obtained from Invitrogen, and the pIND-ERK2 and pIND-ASERK2 plasmids were constructed by cutting the pIND/GS H-Z11695 plasmid by Pme I and religated in a sense or antisense orientation.

Irradiation and Cell Survival. Exponentially growing cells were washed twice with PBS and exposed to a germicidal UV lamp (Model GL-10; Toshiba, Tokyo, Japan) at a dose rate of 0.45 J/m². For X-irradiation, exponentially growing cells were irradiated with 6 Gy of X-rays from an X-ray generator at 150 kVp and 5 mm with a 0.1-mm copper filter. The dose rate for X irradiation was 0.4 Gy/min and 0.1 Gy/min. After irradiation, the cells were trypsinized, and the number of cells was determined. Cell survival was determined as the plating efficiency by inoculating an appropriate amount of cells into 100-mm dishes. The cells were incubated at 37°C for 10 days, fixed with methanol, and then stained with Giemsa stain.

Western Blot Analysis. Cells were lysed in radioimmunoprecipitation assay buffer [50 mm Tris-HCl (pH 7.2), 150 mm NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS] containing 1 mm 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride as described previously (51). The cell lysate was cleared by centrifugation at 15,000 rpm for 10 min at 4°C, and the supernatant was used as total cellular protein. The protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). The protein samples (16 μg) were electrophoresed on a SDS-polyacrylamide gel. The proteins were electrophoretically transferred to polyvinylidene difluoride membrane in transfer buffer (100 mm Tris, 192 mm glycine). After an overnight incubation with blocking solution (10% skim milk), the membrane was incubated with primary antibodies. It was then incubated with a biotinylated secondary antibody and horseradish peroxidase conjugated streptavidin. The bands were visualized after reaction of 3,3′-diaminobenzidine solution and 2% hydrogen peroxide.

Assay for ERK1/2 Activity. To prepare total cell extracts, cells were lysed in lysis buffer [20 mm Tris-HCl (pH 7.5), 150 mm NaCl, 1 mm EDTA, 1 mm EGTA, 1% Triton X-100, 2.5 mm sodium pyrophosphate, 1 mm \(\beta\)-glycerophosphate, 1 mm Na\(_3\)VO\(_4\), 1 μg/ml leupeptin, 1 mm APMSF]. Total protein extracts (200 μg) were mixed with anti-phospho-ERK1/2 in 500 μl of lysis buffer overnight at 4°C. The phosphorylated ERK1/2 proteins were immunoprecipitated by adding protein A-Sepharose beads. The immune complexes were washed twice with lysis buffer and assayed for MAPK activity using a MAPK assay kit (New England Biolabs, Inc.). Briefly, the immunoprecipitates were washed once with kinase buffer (25 mm Tris-HCl, pH 7.5, 5 mm \(\beta\)-Glycerophosphate, 2 mm DTT, 0.1 mm Na\(_3\)VO\(_4\), 10 mm MgCl\(_2\)l), and were resuspended in 50 μl of kinase buffer containing 1 μl of 10 mm ATP and 2 μg of Elk1-GST fusion protein as a phosphorylation substrate. The reaction mixtures were then incubated for 30 min at 30°C with constant agitation. The reaction was terminated by adding Laemmli’s sample buffer [62.5 mm Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% \(\beta\)-mercaptoethanol]. The samples were analyzed by Western blot analysis using anti-phospho-Erk1 antibody.

RESULTS

Effect of Ionizing Radiation on Cell Survival and Cell Growth. Lethal effects of X-rays on normal human diploid cells were examined in Fig. 1. Cell killing was detected with doses >1 Gy, and cell survival was decreased in a dose-dependent manner. The effects of low-dose X-rays on cell proliferation were also investigated. Exponentially growing cells were irradiated and counted the number of cells to determine the initial cell number. The rest of the cells were incubated for 24 h before counting cell number. As shown in Fig. 2, cell proliferation was significantly enhanced by low-dose irradiation at doses between 0.02 and 0.05 Gy (\(P < 0.01\) and \(P < 0.05\), respectively).

Activation of MAPks in Normal Human Diploid Cells. Normal human diploid cells used in this study express constitutive levels of all three of the MAPks (Fig. 3). In the control cells, we detected constitutive phosphorylation of both ERK1 and ERK2 but little or no phosphorylation of JNK1 and JNK2 or p38. To determine the activation of the MAPK families, normal human diploid cells were irradiated with 4 Gy of X-rays and incubated for 5 h (Fig. 3). The amount of phosphorylated ERK1/2 increased 3–4 fold compared with the control level; however, no phosphorylation of JNK1/2 and p38 or of c-Jun and ATF-2 was observed for up to 12 h after X-irradiation. In contrast, cells exposed to 10 J/m² of UV showed phosphorylation of not only ERK1 and ERK2 but also JNK1/2 and p38 (Fig. 3). The activation of JNK1/2 and p38 was confirmed by the phosphorylation of the downstream effectors, c-Jun and ATF-2 transcription factors.

Dose-dependent Activation of ERK1/2 and p53. Dose-dependent activation of ERK1/2 and p53 protein is shown in Fig. 4. We found the dose-dependent increase in the phosphorylation of ERK1/2 at doses >2 Gy. The levels decreased with dose down to 1 Gy but increased again at doses between 0.02 Gy and 0.1 Gy. The increases
were comparable with those observed at ≥4 Gy. In contrast, p53 accumulation was not detected at doses <0.5 Gy, and between 1 and 6 Gy the level of p53 protein increased in a dose-dependent manner. Phosphorylation of p53 protein at serine 15 was detected at 0.1 Gy, and its level gradually increased as the X-ray dose increased. There was a slight increase of p21WAF1/CIP1 at 0.1 Gy and a dose-dependent increase was observed at ≥1.0 Gy.

Phosphorylation of MEK1/2 and Elk-1. Because both 2 cGy and 6 Gy stimulated ERK1/2 phosphorylation, we determined if the upstream MEK1/2 and the downstream effector, Elk-1, were activated. Using antibody against phosphorylated MEK1/2 protein, we found that both 2 cGy and 6 Gy of X-rays enhanced phosphorylation of MEK1/2, although phosphorylation was more significant in cells irradiated with 6 Gy (Fig. 5B). IP-kinase assay revealed that the level of Elk-1 protein was significantly increased by 2.5-fold and 4-fold by 2 cGy and 6 Gy irradiation, respectively (Fig. 5, C and D).

Effects of Inhibitors on ERK1/2 Phosphorylation and Enhanced Cell Proliferation. The role of MEK1/2 in ERK1/2 activation was examined using a specific inhibitor for MEK, PD98059 (Fig. 6). We found that the administration of 10 μM of PD98059 inhibited the phosphorylation of ERK1/2 in cells irradiated with 6 Gy of X-rays, but its suppressive effect was less effective in cells exposed to 2 cGy of X-rays. The effects of the inhibitor for EGFR tyrosine kinase, AG1478, on ERK1/2 phosphorylation were also examined. Treatment of X-rays. The effects of the inhibitor for EGFR tyrosine kinase, AG1478, on ERK1/2 phosphorylation were also examined. Treatment of X-rays.

**Fig. 4. Dose-dependent phosphorylation and activation of ERK1/2 and p53.** A. exponentially growing cells were irradiated with various doses of X-rays as indicated. To compare the maximum level of each protein, they were incubated for 2 h (for p53 and phosphorylated p53), 4 h (for p21WAF1/CIP1), or 5 h (for ERK1/2 and phosphorylated ERK1/2) before extraction of the proteins, respectively. B, band intensity of the blots was measured densitometrically, and relative amount of each protein was calculated. arabic numbers, phosphorylated ERK1/2; ◊, p53; ©, p21WAF1/CIP1; arabic numbers, phosphorylated ERK1/2.

**Fig. 3. Activation of ERK1/2, JNK1/2, and p38 by X-irradiation and UV in normal human diploid cells.** A. Western blot analysis of ERK1 and ERK2. Blots were probed with anti-ERK1/2 antibody (top panel) or with antibody-recognizing phosphorylated forms of ERK1/2 (bottom panel). B. Western blot analysis of JNK1 and JNK2. Blots were probed with anti-JNK1/2 antibody (top panel), with antibody-recognizing phosphorylated forms of JNK1/2 (middle panel), or with antiphosphorylated c-Jun (bottom panel), C. Western blot analysis of p38. Blots were probed with anti-p38 antibody (top panel), with antibody-recognizing phosphorylated forms of p38 (middle panel), or with antiphosphorylated ATF-2 (bottom panel). C, control; X, 5 h after 4 Gy X-rays; U, 1 h after 10 J/m² of UV.

**Fig. 5. Phosphorylation of MEK1/2 and Elk-1 after X-irradiation with 2 cGy and 6 Gy.** A. phosphorylation of ERK1/2 was determined 5 h after X-irradiation at 2 cGy or 6 Gy. B, phosphorylation of MEK1/2 was determined 5 h after X-irradiation at 2 cGy or 6 Gy. Blot was probed with anti-MEK1/2 antibody (top panel) or with anti-phosphorylated MEK1/2 (bottom panel). C, phosphorylation of Elk-1 was determined 5 h after X-irradiation at 2 cGy or 6 Gy. Phosphorylated ERK1/2 was immunoprecipitated from the cell lysate, and Elk-1 phosphorylation was assayed in vitro using Elk-1-GST fusion protein as described in “Materials and Methods.” Phosphorylation of Elk-1 was determined by Western blot analysis. Blot was probed with anti-Elk-1 antibody (top panel) or with antiphosphorylated Elk-1 (bottom panel). Elk-1, purified Elk-1-GST fusion protein. D, blot indicated in Fig. 9C was scanned densitometrically, and relative amount of phosphorylated Elk-1 was calculated.

**DISCUSSION**

In the present study, we demonstrate that low-dose ionizing radiation stimulates proliferation of normal human diploid cells. Because the effect was observed in cells irradiated with very low doses of X-rays, a limited range of low doses was suggested to activate certain signal transduction pathways as described in the rodent cells (52). We used antibodies specifically recognizing the activated form of MAPKs, in which specific threonine and tyrosine are doubly phosphorylated. Although detectable levels of all three of the MAPKs were observed in normal human diploid cells, X-ray irradiation stimulated phosphorylation of ERK1/2, but it did not induce phosphorylation of JNK1/2 and p38. In contrast, when cells were exposed to 10 J/m² of UV light, all of these MAPKs were activated and downstream effec-
tors c-Jun and ATF-2 proteins were phosphorylated, indicating that the three pathways are intact in these cells. The results contrast previous studies showing that ionizing radiation causes activation of not only ERK1/2 but also JNK1/2 and p38 (43, 46, 47). However, these studies used tumor-derived cells, and therefore, our study emphasizes that the type of MAPKs activated by ionizing radiation differs depending on the cell type and that ionizing radiation is a strong stimulator for ERK1/2 in normal human diploid cells.

We found that X-ray irradiation at low doses, between 2 cGy and 5 cGy, activated ERK1/2 as efficiently as doses of ≥4 Gy (Fig. 4). The time course experiments showed that phosphorylation of ERK1/2 continued up to 10 h after irradiation (data not shown). Although previous reports have proven that high-dose radiation activates EGFR followed by the stimulation of Ras, Raf, and MEK1/2 (42–46), the present results implicated that the same mechanism may be involved in ERK1/2 activation by low-dose radiation. As shown in Fig. 6, both 10 μM of PD98059 and 0.2 and 0.4 μM of AG1478 inhibited the activation of ERK1/2 by 6 Gy of X-rays. Similar suppressive effects of PD 98059 and AG1478 were observed in cells irradiated with 2 cGy, although there was less effect on PD98059 treatment. These results indicated that both 6 Gy and 2 cGy of X-rays transduce the signal to ERK1/2 via activation of EGFR and MEK1/2.

Activated ERK1/2 phosphorylated Elk-1, which involves in the induction of the growth-related genes, suggesting that stimulative effects of low-dose irradiation were mediated by ERK1/2 activation. Involvement of ERK1/2 activation in growth stimulation by low dose was confirmed by the results presented in Figs. 7 and 8. First, two kinds of inhibitors, PD98059 and AG1478, were used, and they both suppressed phosphorylation of ERK1/2 and increased cell proliferation. Second, the induced expression of the ERK2 gene potentiated a stimulative effect, whereas the forced expression of the antisense ERK2 gene abrogated enhanced cell growth by low-dose irradiation, completely. Recent studies have reported that ERK1/2 phosphorylate not only transcription factors but also other protein kinases and upstream mediators, such as p90RSK and EGFR and SOS (53). Thus, our results indicate that activation of ERK1/2 by low-dose radiation result in increased proliferation of cells through phosphorylation and activation of such growth-related factors.

In contrast to ERK1/2, accumulation of p53 did not occur with X-ray doses <50 cGy. The evidence obtained thus far indicates that p53 accumulation is dependent on DNA strand breaks (23, 24). An X-ray dose of 10 cGy is estimated to cause approximately five initial double strand breaks per nuclei, and the initial double strand breaks are predicted to be rejoined within ~10 min of the irradiation (54). Single strand breaks are repaired much faster than double strand...
breaks (54). As shown in Fig. 1, X-rays doses <10 cGy will not kill very many cells, suggesting that most of the damage is repairable. Although it is difficult to estimate the exact number of DNA strand breaks remaining, cells irradiated with ≤10 cGy may not have unrepaired DNA breaks, which are lethal to cells and lead to the accumulation of p53 protein. As shown in Fig. 4, ERK1/2 are activated at these doses. Although previous studies have indicated that MAPKs accumulate and activate p53 protein (55), our results suggest that ERK1/2 alone are not sufficient to accumulate and activate p53 protein in normal human cells. Accumulated p53 is activated as a transcription factor and stimulates transcription of a group of genes including the p21WAF1/CIP1 gene. The present results confirmed that accumulated p53 induced p21WAF1/CIP1, but it was observed with doses >1 Gy. It can be concluded that very low doses of ionizing radiation stimulate only ERK1/2, and enhance cell proliferation, whereas higher doses activate not only ERK1/2 but also p53, which antagonizes the proliferative effect of ERK1/2 activation and results in cell cycle arrest.

Several studies have shown that ERK1/2 activation leads to an induction of the genes involved in cell proliferation, suggesting that the activation of ERK1/2 has cytoprotective effects. Our previous study found that oncogenic RAF and Ras alleviated the lethal effects of irradiation (56). A recent study also showed that inhibition of MAPK potentiated the cell killing by ionizing radiation (47). Although the mechanism of the cytoprotective effect of ERK1/2 is unknown, a recent study has shown that ERK1/2 phosphorylate Histone H3, of which phosphorylation is hypothesized to be involved in the transcriptional activation of immediate-early genes through chromatin remodeling (57–60). Therefore, it is very likely that activation of ERK1/2 by a limited range of low doses of X-rays before subsequent higher-dose irradiation may induce gene expression related to DNA damage repair or cell survival or facilitate DNA repair by remodeling the chromatin structure. Thus, the present results provide the possibility that the activation of ERK1/2 may be one mechanism of low-dose effects in normal human diploid cells.

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REFERENCES


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