Effects of Hypoxia on Radiation-Responsive Stress-Activated Protein Kinase, p53, and Caspase 3 Signals in TK6 Human Lymphoblastoid Cells

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Abstract
Despite significant evidence of a role of hypoxia in cellular resistance to ionizing radiation–induced toxicity, the underlying molecular mechanisms remain unclear. This study focused on the influence of hypoxia on radiation-induced signals in TK6 human lymphoblastoid cells. Hypoxic (<10 ppm oxygen) and aerobic cells were exposed to equilethal doses of ionizing radiation, radiation dose ratio, 3:1 (hypoxia:aer). Hypoxia alone or radiation treatment under aerobic or hypoxic conditions led to increased levels of phospho-p44/42 mitogen-activated protein kinase. Levels of phospho-p38 mitogen-activated protein kinase did not change as a result of either hypoxia or irradiation. Hypoxia alone had no effect on expression of phospho-stress-activated protein kinase (SAPK), wild-type p53, or cleaved caspase 3. Irradiation under aerobic conditions resulted in an increase in the phospho-SAPK signal, whereas hypoxia suppressed the irradiation-induced increase in the level of phospho-SAPK. Both hypoxic and aerobic cells showed increases in p53 levels in response to radiation. Hypoxia blocked radiation-induced cleavage of caspase 3 and poly-ADP-ribose polymerase. Irradiation of aerobic and hypoxic TK6 cells using 6 and 18 Gy, respectively, resulted in a similar and significant increase in fraction of apoptotic cells within 24 hours postirradiation. In contrast, basal levels of apoptosis were observed at 24 hours postirradiation in aerobic and hypoxic NIH32 cells, a p53 null derivative of TK6 cells. These results suggest that radiation-induced apoptosis under hypoxia occurs independent of phospho-SAPK and caspase 3, and the p53 response is an obligatory apoptotic signal in TK6 cells. (Cancer Res 2005; 65(2): 579-86)

Introduction
Human tumors are known to differ considerably in radiosensitivity, and the etiology of such varied radiation sensitivity is a matter of intense interest. Intrinsic radiosensitivity of tumor cells has been shown to correlate with a poorer prognosis after radiotherapy (1–4). The sensitivity of tumor cells to ionizing radiation is influenced by not only their inherent radiosensitivity but also by various factors associated with the microenvironment in which they grow. A large body of published evidence points to tumor hypoxia as a major obstacle to effective treatment of tumors using ionizing radiation (5, 6) because cells exposed to radiation under hypoxic conditions are approximately thrice more resistant than when treated under aerobic conditions (7). It is estimated that approximately one half of solid human tumors have median oxygen levels <10 mm Hg prior to therapy (5, 8). Likewise, because of abnormal vasculature and compromised blood flow, which are characteristic within tumors and form the basis for tumor hypoxia, resistance to a variety of chemotherapy drugs has been reported for hypoxic cells (9).

Other signaling pathways linked to the activation of cell receptors followed by transcriptional events leading to cell proliferation, differentiation, or apoptosis are also important in the cellular response to radiation treatment. For example, members of the mitogen-activated protein kinase (MAPK) signal transduction pathways are activated in response to a variety of cellular stresses, including radiation and hypoxia (10–13). The Raf-1 and Ras proteins are among the predominant components of mitogenic signal transduction pathways. Both have been shown to be activated by radiation treatment (12, 13). In addition, radiation stimulates the activities of MAPK/extracellular signal-regulated kinase (ERK) and p44/42 MAP kinase (known also as ERK1/2), which function downstream of Raf-1 in the signaling pathway (12, 13). Depending on the cell type studied, hypoxia has been shown to stimulate components of several MAPK pathways, including p42/44 MAPK (ERK1/2), p38 MAPK, and stress-activated protein kinase (SAPK; refs. 14–16). Lastly, activation of p53 tumor suppressor protein, considered as "gatekeeper," which can lead to either cell cycle arrest and repair or apoptosis (17–20) may subsequently cause changes in the death/caspase signaling pathways that are thought to be important in the radiation response.

The influence of hypoxia on radiation-induced signal transduction pathways remains unclear. In the present study, we have compared and contrasted the expression of various signaling molecules in cells exposed to radiation under aerobic and hypoxic conditions. Cells within tumors can experience a large gradient of oxygen levels and hence the term "hypoxia" must be defined. We have used a level of hypoxia that exhibits the maximum resistance to ionizing radiation (<10 ppm oxygen). We used human lymphoblastoid TK6 cells that have wild-type p53 and have been previously shown to apoptose as a result of various cellular stresses, including radiation (21–27). Evaluation of radiation-induced effects leading to the choice between cell survival and programmed cell death or apoptosis was accomplished by following signature events of the apoptotic pathways: wild-type p53 protein, cleaved caspase 3, and cleaved poly-ADP-ribose polymerase (PARP).

The results of the present study show that hypoxia alone is a potent inducer of phospho-p42/44 MAPK, and radiation fails to
further change the level of phospho-MAPK in hypoxic cells. Hypoxia prevents radiation-induced increases in expression of phospho-SAPK, cleaved caspase 3, and cleaved PARP. Hypoxia alone has no effect on the expression of p53 protein in TK6 cells, and radiation-induced expression of p53 protein is maintained under hypoxia. Our data point to an obligatory role of wild-type p53 and suggest a caspase 3–independent mechanism of radiation-induced apoptosis under hypoxic conditions in TK6 cells.

Materials and Methods

Materials. RPMI and horse serum were purchased from HyClone (Logan, UT); penicillin-streptomycin was purchased from Life Technologies, Inc. (Grand Island, NY); Tris-HCl and NaCl were purchased from Quality Biological Inc. (Gaithersburg, MD); NP40 was purchased from Pierce (Rockford, IL); phenylmethylsulfonylfluoride was purchased from Sigma-Aldrich (St. Louis, MO); protease inhibitors mixture was purchased from Calbiochem (La Jolla, CA); sodium orthovanadate, propidium iodide, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO); Z-Val-Ala-Asp(O-methyl)-fluoromethylketone (ZVAD-fmk) was purchased from MP Biomedicals Inc. (Aurora, OH). Stock solution of ZVAD-fmk (20 mmol/L) was prepared in DMSO.

Antibodies. The following antibodies were purchased from New England Biolabs (Beverly, MA): anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-phospho-p38 MAPK (Thr180/Tyr182), anti-caspase-3, anti-cleaved caspase-3, anti-cleaved caspase-9, anti-cleaved caspase-7, anti-PARP, anti-cleaved PARP, anti-SAPK-c-jun-NH2-terminal kinase (JNK), anti-phospho SAPK/JNK (Thr185/Tyr185), and anti-rabbit IgG (Rockford, IL); phenylmethanesulfonylfluoride was purchased from Biolabs (Beverly, MA); Anti-phospho-p44/42 MAPK (Thr 202/Tyr 204), anti-phospho-p38 MAPK (Thr 180/Tyr 182), anti-caspase 3, anti-cleaved caspase 3, anti-cleaved caspase 9, anti-cleaved caspase 7, anti-PARP, anti-cleaved PARP, anti-SAPK-c-jun-NH2-terminal kinase (JNK), anti-phospho SAPK/JNK (Thr 185/Tyr 185), and anti-rabbit IgG conjugated to horseradish peroxidase. Anti-p53 (Ab-1) was purchased from Oncogene Research Products (La Jolla, CA) and goat anti-mouse IgG conjugated to horseradish peroxidase was purchased from Pierce.

Cell Culture, Hypoxia, and Radiation Treatments. Human lymphoblastoid cell lines TK6 and NH32 were used in the study. The TK6 cell line expresses wild-type p53, and is thus p53 proficient (28). The NH32 cell line is genetically homologous to TK6 cell line, with the exception that its p53 gene has been genetically inactivated by a homozygous knockout (21, 28, 29). The TK6 and NH32 cell lines were grown at 37°C as suspension cultures in glass tissue culture flasks in RPMI containing 10% horse serum and antibiotics (penicillin G potassium and streptomycin sulfate, 0.14 and 0.2 g/L, respectively) in a humidified atmosphere of 5% CO2/95% air at 37°C. The TK6 and NH32 cell lines were grown overnight in 2% serum-containing medium. Cells were made hypoxic by growing them in an Eldorado 8 60Co teletherapy unit (MDS Nordion, Ottawa, Ontario, Canada, formerly Atomic Energy of Canada, Ltd.) at dose rates between 200 and 250 cGy/min. Decay corrections were done monthly, and full electron equilibrium was ensured for all irradiations. Cells were kept on ice for 10 minutes before allowing reoxygenation. Once the cells were exposed to oxygen they were maintained on ice until lytic.

Clonogenic Survival Assay. Cell survival and viability were assessed by colony formation assay (limiting dilution; ref. 28). Immediately after irradiation, TK6 cells were seeded at 1 to 10 cells/well in 96-well plates (round-bottomed). Plates were incubated in a humidified atmosphere of 5% CO2/95% air at 37°C for 2 weeks. Colony formation was assessed 2 weeks later.

Immunoblotting. Cells were washed thrice with ice-cold PBS and lysed in lysis buffer [50 mmol/L Tris-HCl buffer (pH 8), 1.5 mmol/L NaCl, 1% NP40, PMSF 100 μg/mL, 10 μl/mL protease inhibitors mixture, and 1 mmol/L Na3VO4] to isolate whole cell extract. Proteins from whole-cell lysates were resolved by 4% to 20% gradient SDS-PAGE (Invitrogen, Carlsbad, CA) and transferred to a Protran 0.2-μm nitrocellulose membrane (Schleicher & Schuell, Keene, NH). After the transfer, the membrane was washed 3 × 5-minute washes with TBS containing 0.1% Tween (TBS-T). The membrane was blocked overnight with 5% nonfat dry milk (Safeway brand) in TBS-T and immunoblotted with the desired primary antibody at 1:1000 dilution (for all New England Biolabs antibodies) and 1:30 dilution [for p53 (Ab-1) antibody] at room temperature for 90 minutes. The membrane was washed at ambient temperature with TBS-T 4 × 10-minute washes followed by immunoblotting at a 1:12,000 dilution (for all New England Biolabs primary antibodies), and 1:10,000 dilution (for p53 primary antibody) of an appropriate horseradish peroxidase–coupled secondary antibody. The membrane was washed 4 × 10-minute washes with TBS-T, and the immunoreactive protein bands were revealed by LumiGLO chemiluminescent detection according to the manufacturer’s protocol (New England Biolabs). The protein level quantification and data analysis were done on a Macintosh G3 computer using the public domain NIH Image program (developed at the U.S. NIH and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Flow Cytometric Analysis. TK6 and NH32 cells were grown in 2% serum for 24 hours at 37°C. Because hypoxic cells are ∼3× more resistant to radiation, hypoxic and aerobic cells were irradiated with a 3:1 dose ratio, respectively (186 Gy). Apoptotic cells at different time points were determined by DNA labeling with propidium iodide hypotonic solution and flow cytometric analysis (31). Aliquots containing 3 × 105 cells/mL of radiation-treated cells were centrifuged (1000 rpm, 5 minutes, at ambient temperature) and washed twice with 5 mL PBS. The centrifuged cell pellet was gently resuspended in 1.5 mL hypotonic fluorochrome solution (propidium iodide 50 μg/mL in 0.1% sodium citrate plus 0.1% Triton X-100). The samples were placed at 4°C in the dark overnight before the flow cytometric analysis. The propidium iodide fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA).

Results

Effect of Hypoxia or Aerobic Radiation on Phosphorylation of p42/44 MAPK. To evaluate the effects of hypoxia on radiation-induced modifications of various anti- and proapoptotic signals, it was important to first determine the doses of radiation that result in a comparable cell survival under hypoxia and air. The radiation dose response experiments indicated that hypoxic and aerobic TK6 cells receiving a radiation dose ratio of 3:1 show similar clonogenic cell survival response (Fig. 1). Phosphorylation and activation of p42/44

Figure 1. Radiation dose response of TK cells under hypoxia or in air. Cells were grown overnight in 2% serum-containing medium. Cells were made hypoxic by gassing nitrogen for 90 minutes. Hypoxic and aerobic cells were irradiated using indicated radiation doses and incubated for 24 hours at 37°C. Clonogenic survival of hypoxic TK6 cells was determined by DNA labeling with propidium iodide hypotonic solution and flow cytometric analysis (31). Aliquots containing 3 × 105 cells/mL of radiation-treated cells were centrifuged (1000 rpm, 5 minutes, at ambient temperature) and washed twice with 5 mL PBS. The centrifuged cell pellet was gently resuspended in 1.5 mL hypotonic fluorochrome solution (propidium iodide 50 μg/mL in 0.1% sodium citrate plus 0.1% Triton X-100). The samples were placed at 4°C in the dark overnight before the flow cytometric analysis. The propidium iodide fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA).
MAPK ERK have been associated with enhanced cell proliferation and cell survival. Hypoxia (<10 ppm oxygen) or aerobic irradiation (6 Gy) treatment led to an increase in phospho-ERK level within 30 minutes after treatment (Fig. 2A). Whereas aerobic radiation treatment induced a ~2 fold increase in ERK phosphorylation 2 hours after treatment, hypoxia alone caused almost 3-fold increase in level of phospho-ERK after 2 hours. No further change in the level of phospho-ERK was observed following continued exposure to hypoxia alone (for at least additional 90 minutes; Fig. 2B). The total levels of ERK were unchanged. Phospho-ERK levels were unchanged in the hypoxic cells exposed to radiation (18 Gy) when compared with the change observed in cells treated with hypoxia alone (Fig. 2C).

Effects of Hypoxia or Radiation on Cleavage of Caspase 3 and PARP. Caspase 3 is a terminal caspase associated with apoptosis. The cleavage of caspase 3 results in its catalytic activation followed by cleavage and inactivation of several cell survival and DNA-repair proteins including PARP. Similar to the effects on phospho-SAPK (Fig. 3), hypoxia alone caused no changes in the levels of cleaved caspase 3 and cleaved PARP (Fig. 5A). Irradiation of aerobic cells resulted in cleavages of caspase 3 and PARP at 4 hours postirradiation (Fig. 5B, Air). Phospho-SAPK in TK6 cells (Fig. 3A). Aerobic irradiation (6 Gy) led to an increase in level of phospho-SAPK (~3.5-fold; hypoxic cells) (Fig. 3B). In contrast, hypoxic cells revealed minimal to no effect on the level of phospho-SAPK at various times postirradiation (Fig. 3B; quantification and assessment of significance of changes was done using the NIH Image program as described in Materials and Methods). The total levels of SAPK in aerobic and hypoxic cells remained unchanged. No changes in the levels of p38 MAPK (phosphorylated and nonphosphorylated) were seen as a result of aerobic irradiation, hypoxia treatment, or irradiation of hypoxic cells (data not shown).

Effects of Radiation on p53 Protein Expression under Aerobic and Hypoxic Conditions. The p53 tumor suppressor protein, the "gatekeeper" protein, plays important roles in signaling pathways leading to either cell cycle arrest and repair or apoptosis. Here we asked whether p53 protein levels are regulated in TK6 cells under hypoxia or postirradiation in aerobic and hypoxic cells. Hypoxia alone had no effect on the level of wild-type p53 in TK6 cells (Fig. 4A), whereas equilethral doses of radiation in aerobic (6 Gy) and hypoxic (18 Gy) cells caused similar increases in the levels of wild-type p53 at 90 to 120 minutes postirradiation (120 minutes; aerobic cells ~3.5-fold; hypoxic cells ~3.2-fold; Fig. 4B). To verify if p53 induction is a radiation-specific cytotoxic signal in TK6 cells, we compared expression of p53 in aerobic and hypoxic cells exposed to 3 Gy of radiation, a dose resulting in a significantly higher cell death in air as compared with hypoxia (Fig. 1). Radiation treatment of aerobic but not hypoxic cells caused an increase in level of p53 at 90 to 120 minutes postirradiation (3 Gy: ~3.2-fold versus minus IR; Fig. 4C). No further increase in p53 was observed using a higher dose of radiation (6 Gy), most likely due to high toxicity under aerobic conditions (greater than 98%). These results show a link between radiation-induced p53 expression and cell death in TK6 cells.

Effects of Hypoxia or Radiation on Cleavage of Caspase 3 and PARP. Caspase 3 is a terminal caspase associated with apoptosis. The cleavage of caspase 3 results in its catalytic activation followed by cleavage and inactivation of several cell survival and DNA-repair proteins including PARP. Similar to the effects on phospho-SAPK (Fig. 3), hypoxia alone caused no changes in the levels of cleaved caspase 3 and cleaved PARP (Fig. 5A). Irradiation of aerobic cells resulted in cleavages of caspase 3 and PARP at 4 hours postirradiation (Fig. 5B, Air); quantification and assessment of significance of changes was done using the NIH Image program as described in Materials and Methods. The total levels of phospho-MAPK in hypoxic TK6 cells. A. Cells were grown overnight in 2% serum-containing medium and treated with either hypoxia (top) or radiation (6 Gy; bottom) and lysed at indicated time points. Normalized protein contents were quantified with anti-MAPK (p22/44) and anti-phospho MAPK (p42/44) antibodies. B. The immunoreactive phospho-MAPK bands shown in A were quantified. Columns, mean from three different experiments; bars, SD. C. Cells were grown overnight in 2% serum-containing medium and made hypoxic by gassing with nitrogen. The hypoxic cells were irradiated (18 Gy) and lysed at the desired time points. Normalized protein contents were quantified with anti-CEIN MAPK antibody. The immunoreactive phospho-MAPK bands were quantified. Columns, mean from three different experiments; bars, SD. Radiation had no effect on the level of phospho-MAPK (p42/44) in hypoxic TK6 cells.

Figure 2. Hypoxia or aerobic irradiation enhances phosphorylation of MAPK (p42/44) in TK6 cells. A. Cells were grown overnight in 2% serum-containing medium and treated with either hypoxia (top) or radiation (6 Gy; bottom) and lysed at indicated time points. Normalized protein contents were quantified with anti-MAPK (p22/44) and anti-phospho MAPK (p42/44) antibodies. B, The immunoreactive phospho-MAPK bands shown in A were quantified. Columns, mean from three different experiments; bars, SD. C, Cells were grown overnight in 2% serum-containing medium and made hypoxic by gassing with nitrogen. The hypoxic cells were irradiated (18 Gy) and lysed at the desired time points. Normalized protein contents were quantified with anti-phospho MAPK antibody. The immunoreactive phospho-MAPK bands were quantified. Columns, mean from three different experiments; bars, SD. Radiation had no effect on the level of phospho-MAPK (p42/44) in hypoxic TK6 cells.
described in Materials and Methods). Pretreatment of aerobic cells with a general caspase inhibitor ZVAD-fmk (40 mol/L, 30 min) showed elimination of basal level of cleaved PARP in nonirradiated cells, but had no effect on radiation-induced PARP cleavage (Fig. 5C).

Under hypoxia, radiation effects on caspase 3 and PARP were markedly suppressed (Fig. 5B, Hypoxia), implying that hypoxia causes a potent blockade of radiation-induced caspase activation.

Changes in caspase 7 cleavage, another downstream effector, were similar to those of caspase 3 (data not shown).

Flow Cytometric Analysis of Radiation-Induced Apoptosis in Aerobic and Hypoxic TK6 and NH32 Cells. To ascertain that radiation treatments of aerobic and hypoxic TK6 cells (hypoxic: aerobic radiation dose ratio, 3:1) caused similar levels of apoptosis, hypoxic and aerobic cells were treated with 18 and 6 Gy of ionizing radiation, respectively. The fractions of sub-G1 phase cells indicative of apoptotic cells were determined at 4, 24, and 48 hours after radiation treatment. Unirradiated aerobic and hypoxic TK6 cells showed no change in the number of apoptotic cells at various time points, whereas irradiation under air and hypoxia resulted in similar and significant increases in apoptotic cell populations at 24 and 48 hours postirradiation (Fig. 6A). Next, we measured radiation-induced apoptosis in NH32 cells, a p53 null derivative of TK6 cells. In contrast to TK6 cells, both aerobic and hypoxic NH32 cells showed basal levels of apoptosis at 24 hours postirradiation, and only a slight increase in apoptosis was observed at 48 hours postirradiation (Fig. 6B). These data provide evidence of a direct link between p53 expression and radiation-induced apoptosis in aerobic and hypoxic TK6 cells.

Discussion

Earlier studies have shown that exposure of mammalian cells to ionizing radiation activates intracellular signaling pathways leading to apoptotic or other forms of cell death, depending on

Figure 3. Hypoxia prevents radiation-induced phosphorylation of SAPK in TK6 cells. Cells were grown overnight in 2% serum-containing medium and made hypoxic by gassing with nitrogen. The aerobic cells and hypoxic cells were irradiated and lysed at the desired time points. Normalized protein contents were immunoblotted with anti-phospho SAPK and anti-SAPK antibodies. A, expression of phospho-SAPK in cells under hypoxia for indicated time points. B, expression of phospho-SAPK at indicated times postirradiation of aerobic cells (Air, 6 Gy) and hypoxic cells (Hypoxia, 18 Gy). Representative of at least three independent experiments in each panel.

Figure 4. Radiation induces wild-type p53 protein expression in both aerobic and hypoxic TK6 cells. Cells were grown overnight in 2% serum-containing medium and made hypoxic by gassing with nitrogen. The aerobic and hypoxic cells were irradiated and lysed at the desired time points. Normalized protein contents were immunoblotted with anti-p53 antibody. A, expression of p53 protein in cells under hypoxia for indicated time points. B, expression of p53 protein postirradiation in aerobic cells (Air, 6 Gy) and hypoxic cells (Hypoxia, 18 Gy). C, comparison of p53 protein expression in aerobic and hypoxic cells postirradiation using equal doses of radiation. Representative of at least three independent experiments in each panel.
the cell type studied (32–35). The exposure of cells to hypoxia induces cell signaling response as well, mainly promoting tumor growth but also leading to apoptosis (36–38). Some studies claim that hypoxia alone is not a stimulus for apoptosis (39, 40). Other studies, on the other hand, report the induction of apoptosis by hypoxia via two independent pathways (41, 42). Previous studies on TK6 cells have shown that hypoxia decreased the number of radiation-induced DNA single-strand breaks (43) but had no effect on DNA lesions leading to point mutations (44). The present study focused on the effects of hypoxia (<10 ppm oxygen) on the radiation-induced changes in the expression of known anti- and proapoptotic signals in TK6 cells exposed to equilethal radiation doses in air and hypoxia.

Our observations in TK6 cells showing similar cell survival after radiation treatment with 3:1 dose ratio under hypoxic and aerobic conditions (Fig. 1) correlate with an oxygen enhancement ratio of ~3. Phospho-p42/44 MAPK/ERK and phospho-SAPK/JNK are believed to have opposing effects on cell death, with the phospho-SAPK promoting cell death (33, 45). We show that hypoxia treatment per se is sufficient to cause a significant and sustained stimulation of phospho-ERK in TK6 cells, resulting in the maximum attainable level within ~2 hours (Fig. 2B and C). Exposure of the hypoxic cells to a highly cytotoxic dose of radiation (Note: 9 Gy under hypoxia causes approximate 97% decline in the clonogenic cell survival, Fig. 1) causes no further change in the level of phospho-ERK (Fig. 2C). It is possible that activation of pMAPK pathway under hypoxia may have implications for hypoxic radiation resistance. Activation of pMAPK under hypoxia may in some way alter radiation-induced activation of phospho-SAPK and thus contribute to the radiation resistance caused by hypoxia. We are currently exploring this possibility.

We show that hypoxia does not affect phospho-SAPK, but it clearly suppresses radiation-induced phosphorylation of SAPK in TK6 cells (Fig. 3). It seems that a cross-talk between hypoxia- and radiation-signaling pathways may negatively impact the radiation effect on phospho-SAPK. Because the SAPK pathway is both inflammatory and apoptotic, it is also likely that the phospho-SAPK expression may not be a pertinent radiation-responsive cell death signal in hypoxic TK6 cells as mentioned above.

Earlier studies have reported the influence of p53 tumor suppressor protein status on cellular radiosensitivity, with some studies reporting increased radiosensitivity in mutant p53-expressing cell lines (46, 47), whereas others concluding wild-type p53-containing cells are significantly more radiosensitive than mutant p53 cell lines (48, 49). The role of p53 in affecting cellular radiosensitivity is attributed to its function in the cellular response to DNA damage (17–20). p53 is activated in response to various DNA-damaging processes and agents (17, 50–52), and p53 activity regulates multiple cellular processes including cell cycle checkpoints and programmed cell death. A checkpoint arrest provides time for cellular repair to proceed and as such can enhance cell survival and limit mutagenic events following DNA damage (53). It has been previously
suggested that radiation-induced tumorigenesis is enhanced in the absence or reduction of p53 as a result of increased DNA instability at various loci, such as those for tumor suppressor genes (52). This hypothesis is further supported by the fact that mutations in the p53 tumor suppressor gene are found in more than 50% of human tumors (47). Levels of p53 tumor suppressor protein have been previously reported to be induced by radiation treatment (52, 53) as well as by hypoxia (54–57). Here we examined the changes in wild-type p53 protein levels in aerobic and hypoxic TK6 cells. Our results show that hypoxia alone did not induce a change in p53 level, whereas p53 protein levels were similarly increased in irradiated aerobic and hypoxic TK6 cells. It is evident from Fig. 1 that equal doses of radiation produce higher survival rates in hypoxic versus aerobic cells. These results correlate to the changes seen in levels of p53 protein in aerobic and hypoxic cells following equal doses of radiation (Fig. 4C) in which p53 levels were significantly induced in aerobic cells but not in hypoxic cells. These results suggest that induction of the wild-type p53 protein is a radiation-specific stimulus in aerobic and hypoxic TK6 cells. Is this a biologically relevant response in TK6 cells? In NH32 cells, a p53 null derivative of TK6 cells, we observed a significantly delayed and only mild induction of apoptosis under aerobic and hypoxic conditions (Fig. 6B). Recent studies have shown that TK6 cells but not WT-K-1 human lymphoblastoid cells (with mutant p53) exposed to nitric oxide undergo significant apoptosis involving p53-mediated elevation of apoptosis protease-activating factor-1 and inhibition of X-chromosome–linked inhibitor of apoptosis (58). Whether radiation induces a similar response in aerobic and hypoxic TK6 cells remains to be determined.

Because p53 is known to have a major role in the induction of apoptosis as a result of radiation or hypoxia treatment (25–27,59), we examined downstream effectors of the death/caspase pathway in TK6 cells irradiated under aerobic and hypoxic conditions. In an earlier study, Inanami et al. (35) have shown hypoxia to have different effects on radiation-induced changes in different cell lines. They reported that hypoxia significantly attenuated the X-ray–induced activation of caspase 3 in HL60 cells but not in MOLT-4 cells. Hypoxia similarly affected the time-dependent increase of apoptotic cells in HL60 cells correlating with caspase activation, but not in MOLT-4 cells (35). In the present work, the effect of hypoxia on the radiation-induced changes in the caspase/death pathways correlated with its effect on the SAPK/JNK pathway. Hypoxia inhibited the radiation-induced increase in cleavage of caspase 3, caspase 7, and PARP in TK6 cells (Fig. 5). These data suggest that similar to phospho-SAPK, caspase 3 is differentially regulated in irradiated aerobic and hypoxic cells, and cleavage of caspase 3 is not a necessary signal for radiation-induced death in hypoxic cells.

These findings led us to our next step of the study to determine whether radiation-induced changes in signaling molecules can be correlated with apoptotic cell death. Hypoxia treatment alone caused no change in population of apoptotic TK6 cells compared with control aerobic cells. Consistent with the radiation-specific p53 response, both aerobic and hypoxic

![Graphs showing apoptosis induction](image-url)
cells irradiated with 6 and 18 Gy, respectively, showed a similar time-dependent increase in the fraction of apoptotic cells (Fig. 6d). Aoki et al. (34) reported similar results for L5178Y cells irradiated with 5 Gy under aerobic conditions and 15 Gy under hypoxia. Unlike the results reported by Inanami et al. (35), radiation-induced cleavage of caspases 3 and 7 and of PARP in aerobic but not hypoxic TK6 cells does not correlate with cell survival results, which are similar in both aerobic and hypoxic cells. It also does not correlate with the flow cytometry apoptotic assay because both aerobic and hypoxic cells irradiated with 6 and 18 Gy, respectively, showed a similar time-dependent increase in the fraction of apoptotic cells. Hypoxia itself did not induce or inhibit apoptosis in TK6 cells. It can be concluded that radiation-induced apoptosis under hypoxic conditions in TK6 cells is independent of caspase 3 and PARP cleavage.

The results of the present study comparing TK6 cells exposed to radiation in air and under hypoxia are summarized in Fig. 7. We conclude that (a) hypoxia causes significant and sustained expression of phospho-p42/44 MAPK, and radiation also induces expression of phospho-p42/44 MAPK in aerobic cells but it is ineffective in hypoxic cells; (b) radiation-induced apoptosis in hypoxic cells is independent of phospho-SAPK and activated caspase 3; and (c) wild-type p53 is not a target of hypoxia but it plays an important role in radiation-induced apoptosis in both aerobic and hypoxic TK6 cells.

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References
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