Hypoxia Increases Resistance of Human Pancreatic Cancer Cells to Apoptosis Induced by Gemcitabine

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Abstract

Purpose: Hypoxia, frequently found in the center of solid tumor, is associated with resistance to chemotherapy by activation of signaling pathways that regulate cell proliferation, angiogenesis, and apoptosis. We determined whether hypoxia can increase the resistance of human pancreatic carcinoma cells to gemcitabine-induced apoptosis by activation of phosphatidylinositol 3′-kinase (PI3K)/Akt, MEK/mitogen-activated protein kinase (extracellular signal-regulated kinase) [MAPK(Erk)] kinase (MEK)], and nuclear factor κB (NF-κB) signaling pathways.

Experimental Design: We evaluated the phosphorylation of Akt and MAPK(Erk), DNA binding activity of NF-κB, and apoptosis induced by gemcitabine in L3.6pl human pancreatic cancer cells under normoxic and hypoxic conditions. We then examined the effects of the PI3K inhibitor LY294002, MEK inhibitor U0126, and the epidermal growth factor receptor tyrosine kinase inhibitor PKI 166 on these signaling pathways and induction of apoptosis.

Results: Hypoxic conditions increased phosphorylation of Akt and MAPK(Erk) and NF-κB DNA binding activity in L3.6pl cells. The activation of Akt and NF-κB was prevented by LY294002, whereas the activity of MAPK(Erk), but not NF-κB, was inhibited by U0126. The increased activation of Akt, NF-κB, and MAPK(Erk) was inhibited by PKI 166. Under hypoxic conditions, L3.6pl cells were resistant to apoptosis induced by gemcitabine. The addition of LY294002 or PKI 166 abrogated cell resistance to gemcitabine, whereas U0126 only partially decreased this resistance.

Conclusions: These data demonstrate that hypoxia can induce resistance of pancreatic cancer cells to gemcitabine mainly through the PI3K/Akt/NF-κB pathways and partially through the MAPK(Erk) signaling pathway. Because PKI 166 prevented the activation of PI3K/Akt/NF-κB and MAPK(Erk) pathways, the combination of this tyrosine kinase inhibitor with gemcitabine should be an effective therapy for pancreatic cancer.
oxygen tension in the microenvironment of the cells. Cells growing as monolayers in vitro are exposed to an ample supply of oxygen, whereas cells growing in vivo in a three-dimensional configuration have heterogeneous exposure to oxygen. Specifically, cells growing in the center of rapidly proliferating tumors have a lower tension of oxygen, i.e., hypoxic conditions.

Hypoxia, a common condition in zones of rapidly proliferating tumors, influences signaling pathways such as those controlling cell proliferation, angiogenesis, and apoptosis (24), and it has been shown to associate with resistance to chemotherapy and radiation therapy and hence poor prognosis (25). Because multiple signaling molecules, such as PI3K/Akt, MEK/MAPK(Erk), and IκB/NF-κB are modulated under hypoxic conditions in several type of the cells (26), we designed experiments to determine whether hypoxia can activate these signaling molecules in human pancreatic cancer cells and thus render the cells more resistant to apoptosis induced by gemcitabine.

Materials and Methods

Cell Lines and Culture Conditions. The highly metastatic L3.6pl human pancreatic cancer cell line (7, 8) was maintained in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), and a penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD; Ref. 27). Adherent monolayer cultures were maintained on plastic and incubated in an atmosphere containing 5% CO2 and 95% air at 37°C (normoxic condition). The cultures were free of Mycoplasma and the following pathogenic murine viruses: reovirus type 3; pneumonia virus; Theiler’s encephalitis virus; Sendai virus; minute virus; mouse hepatitis virus; lymphocytic choriomeningitis virus; ectromelia virus; and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD). The cultures were maintained no longer than 10 weeks after recovery from frozen stocks.

Reagents. PKI 166, a novel EGFR tyrosine kinase inhibitor of the pyrrolo-pyrimidine class that inhibits phosphorylation of the intracellular domain of the EGFR tyrosine kinase, was synthesized and produced by Novartis Pharma, AG (Basel, Switzerland; Ref. 28). PKI 166 affects the EGFR kinase by inhibiting a number of them at submicromolar concentrations, e.g., c- abl, c-src, kinase-insert domain-containing; Ref. 28).

The following antibodies and inhibitors were purchased from Cell Signaling Technology (Beverly, MA): anti-Akt and anti-phospho-Akt (serine 473); anti-p44/42 MAPK antibody and anti-phospho-p44/p42 MAPK (Thr202/Tyr204); anti-EGFR antibody, anti-phospho-EGFR (Tyr845); PI3K inhibitor LY294002; and MEK inhibitor U0126.

Induction of Hypoxia. Tumor cells were incubated in a hypoxic incubator (Precision Scientific, Winchester, VA) with 1% O2 balanced by CO2 and nitrogen. Cells were plated in culture dishes for 72 h before placement in hypoxic conditions. When the cultures reached 70% confluence, fresh medium was added, and the dishes were incubated under normoxic or hypoxic conditions for 12 h. At that time, all cells excluded trypan blue dye (>95%) and exhibited no morphological changes on light microscopy. To prevent changes in pH, the medium was buffered to pH 7.35 with 20 mM 2-(N-morpholinoo)ethanesulfonic acid and 20 mM tris(hydroxymethyl)aminomethane (29).

Electrophoretic Mobility Shift Assay (EMSA). The L3.6pl pancreatic cancer cells were incubated under normoxic or hypoxic conditions, and nuclear extracts were prepared according to the method described previously (29). Nuclear extracts (10 μg) were incubated with 1 μg of poly(deoxyinosinic-deoxycytidylic acid) (Amersham Bioscience Corp., Piscataway, NJ) in a binding buffer for 30 min at 4°C. 32P-labeled double-strand NF-κB oligonucleotides or specificity protein-1 (SP-1) oligonucleotides were used as probes, and end-labeled probe (30,000 cpm) was added to the mixture. The binding reaction was allowed to proceed for 30 min at room temperature. The competition assay was performed with 50-fold excess of unlabeled NF-κB and SP-1 oligonucleotides. The supershift experiments were performed with anti-p65, anti-p50, or control antibodies (Promega, Madison, WI). The reaction mixture was analyzed on 4% polyacrylamide gels containing 0.25% Tris-borate EDTA buffer.

Western Blotting. After the various treatments, the cells were washed with ice-cold PBS containing 1 mM sodium orthovanadate, harvested in 100 μl of a lysis buffer (20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, and 0.15 unit/ml aprotinin), and centrifuged to recover insoluble protein. Lysates were resolved on 7.5% SDS-PAGE and transferred onto 0.45-micron nitrocellulose membranes. The membranes were blocked with 3% BSA in Tween-Tris-buffered saline [0.1% Tween 20, 20 mM Tris-HCl (pH 7.5), and 150 mM NaCl] and incubated overnight at 4°C with the indicated antibodies. Immunolabeling was detected by enhanced chemiluminescence (Amersham Bioscience Corp.) according to the manufacturer’s instructions (27).

Detection of Apoptosis. Apoptosis was identified by flow cytometry analysis of the cell cycle after DNA staining with propidium iodide (Sigma Chemical Co., St. Louis, MO) as described previously (30). Cells were grown in 6-well plates to 70% confluence. The culture medium was replaced with fresh medium, and the cells were placed in normoxic or hypoxic conditions for 12 h. Floating cells were collected, and the attached cells were harvested in PBS/EDTA (5 mM EDTA in PBS). All cells were then centrifuged for 5 min at 200 × g, the supernatant was discarded, and the cell pellet was suspended in 0.5 ml of PBS, using pipetting to break clumps. The cell suspension was then transferred to 4.5 ml of 75% cold ethanol. Ethanol-fixed cells were centrifuged for 5 min at 200 × g and washed once with 5 ml of PBS. Finally, total cell pellets were suspended in 1 ml of propidium iodide staining solution (20 μg/ml propidium iodide and 0.2 mg/ml RNase in PBS) and incubated for 30 min at 37°C. Samples were analyzed by flow cytometry (FACS Calibur; Becton-Dickinson, Mountain View, CA). Apoptosis was measured as the percentage of cells with a DNA content lower than that of cells in G0-G1 in the propidium iodide intensity-area histogram plot (30).

Statistical Analysis. The significance of the results was determined by Student’s t test.
Epidermal Growth Factor (EGF) Stimulates Tyrosine Phosphorylation of EGFR and MAPK(Erk) in L3.6pl Human Pancreatic Cancer Cells under Normoxic Conditions.

In the first set of experiments, we determined whether EGFR and its major downstream protein kinase cascades, such as Akt and MAPK(Erk), are phosphorylated by EGF stimulation. L3.6pl cells were cultured to 70% confluence and then incubated under normoxic conditions for 15 min in serum-free medium containing various concentrations of EGF (0, 5, 10, and 20 ng/ml). Phosphorylation of EGFR, MAPK(Erk), and Akt was detected by Western blot analysis with specific antibodies. Whereas EGF increased the phosphorylation of EGFR and MAPK(Erk) in a dose-dependent manner, expression of total EGFR and MAPK(Erk) proteins was unchanged. On the other hand, phosphorylation of Akt and total Akt protein expression were unchanged from those of unstimulated cells (Fig. 1A).

PKI 166 Prevents EGF-Induced Phosphorylation of EGFR, MAPK(Erk), and Akt under Normoxic Conditions.

Next, we determined whether PKI 166 inhibits EGF-stimulated tyrosine phosphorylation of EGFR, MAPK(Erk), and Akt (unchanged basal level). Pretreatment of cells with different concentrations of PKI 166 (0, 0.3, 1.0, and 3.0 μM) for 2 h before a 15-min treatment with EGF (20 ng/ml) prevented induction of EGFR and MAPK(Erk) phosphorylation by EGF in a dose-dependent manner (0.3–3.0 μM/μl). Levels of Akt phosphorylation unchanged by EGF stimulation were also inhibited by PKI 166. PKI 166 had no effect on the expression of total EGFR, MAPK(Erk), or Akt protein (Fig. 1B).

Hypoxia Induces Akt and MAPK(Erk) Phosphorylation and NF-κB Activation, but not EGF Phosphorylation, and SP-1 Activation. We next studied the effect of hypoxic conditions on the phosphorylation of EGFR, MAPK(Erk), and Akt and the activation of NF-κB and SP-1. Cells were incubated under hypoxic conditions (1% O₂) for different periods of time. After each indicated incubation period, the cells were collected, and total and nuclear proteins were extracted. Phosphorylation of EGFR, Akt, and MAPK(Erk) was analyzed by Western blot analysis (Fig. 2A). Activation of NF-κB and SP-1 was assessed by EMSA (Fig. 2B).

Phosphorylation of Akt and MAPK(Erk) was increased 6 h after the initiation of hypoxic treatment and reached a maximum by 12 h, when the level of phosphorylation began to decrease. In contrast, hypoxia did not change the phosphorylation of EGFR. Total expression of Akt, MAPK(Erk), and EGFR proteins was unchanged. NF-κB DNA binding activity was increased 6 h after initiation of hypoxia and reached maximum activity by 12 h. Using unlabeled wild-type oligonucleotides in competition and specific antibodies against both P65 and P50 antibodies in supershift assay, EMSA results indicated that the DNA binding activities in the cell were specific to NF-κB sites and that the NF-κB complex contained both P65 and P50 subunits. In contrast, under hypoxic conditions, SP-1 DNA binding activity was unchanged. These data indicate that, under hypoxic conditions, Akt, MAPK(Erk), and NF-κB can be activated independently of increased EGFR phosphorylation. Conditioned medium from cells treated under hypoxic conditions failed to induce Akt, MAPK(Erk), or NF-κB activation in the L3.6pl cells grown under normoxic conditions (data not shown).

Results

Epidermal Growth Factor (EGF) Stimulates Tyrosine Phosphorylation of EGFR and MAPK(Erk) in L3.6pl Human Pancreatic Cancer Cells under Normoxic Conditions. In the first set of experiments, we determined whether EGFR and its major downstream protein kinase cascades, such as Akt and MAPK(Erk), are phosphorylated by EGF stimulation. L3.6pl cells were cultured to 70% confluence and then incubated under normoxic conditions for 15 min in serum-free medium containing various concentrations of EGF (0, 5, 10, and 20 ng/ml). Phosphorylation of EGFR, MAPK(Erk), and Akt was detected by Western blot analysis with specific antibodies. Whereas EGF increased the phosphorylation of EGFR and MAPK(Erk) in a dose-dependent manner, expression of total EGFR and MAPK(Erk) proteins was unchanged. On the other hand, phosphorylation of Akt and total Akt protein expression were unchanged from those of unstimulated cells (Fig. 1A).

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Hypoxia-Induced Akt Phosphorylation and NF-κB Activation Requires PI3K, Whereas MAPK(Erk) Phosphorylation Requires MEK. We studied the signaling mechanism by which hypoxic conditions induced Akt, MAPK(Erk), and NF-κB activation. To determine whether PI3K plays a role in these events, we added the specific PI3K inhibitor LY294002 to cells cultured under hypoxic conditions. The addition of LY294002 for 12 h prevented hypoxia-induced Akt phosphorylation (Fig. 3A)

![Fig. 2 A](image1)

Western blot analysis of phosphorylated (P-) and total epidermal growth factor receptor, Akt, and mitogen-activated protein kinase (extracellular signal-regulated kinases 1/2) proteins in L3.6pl cells under hypoxic conditions. Cells were cultured to reach 70% confluence. Thereafter, the culture medium was replaced with fresh medium and serum, and the cells were transferred to normoxic (21% O2) or hypoxic (1% O2) conditions for the indicated periods. Cells were then collected and subjected to lysis, and proteins were detected by Western blot analysis. B, nuclear factor κB (NF-κB) and SP-1 activity in cells under hypoxic conditions was determined by electrophoretic mobility shift assay. L3.6pl cells were cultured to 70% confluence. The culture medium was then replaced with fresh serum and medium, and the cells were transferred to normoxic (21% O2) or hypoxic (1% O2) conditions for the indicated periods. Nuclear proteins were then collected, and NF-κB and SP-1 activity was detected by electrophoretic mobility shift assay. Unlabeled wild-type oligonucleotides (cold probe) in competition assays were used to determine the specificity of DNA binding to the NF-κB and SP-1 sites. Supershift assays using NF-κB P65 and P50 antibodies indicated that the NF-κB complex contained both P65 and P50 subunits. The data are from one representative experiment of four.

![Fig. 3 A](image2)

The effects of the phosphatidylinositol 3'-kinase inhibitor LY294002 and the MEK inhibitor U0126 on L3.6pl cells and on nuclear factor κB activity in L3.6pl cells are shown. L3.6pl cells were cultured under hypoxic conditions for 12 h and phosphorylated (P-) and total proteins were detected by Western blot analysis. B, nuclear proteins were collected, and nuclear factor κB activity was detected by electrophoretic mobility shift assay. The data are from one representative experiment of four.
NF-κB activation (Fig. 3B) in a dose-dependent manner but did not prevent MAPK(Erk) phosphorylation. Incubating the cells with the MEK inhibitor U0126 prevented MAPK(Erk) phosphorylation but did not affect Akt and NF-κB activities. These data suggest that hypoxic conditions stimulate the PI3K/Akt/NF-κB signaling pathway and the MEK/MAPK(Erk) signaling pathway by independent mechanisms.

Induction of Akt and MAPK(Erk) Phosphorylation and NF-κB Activation by Hypoxia Can Be Prevented by PKI 166. Cultures at 70% confluence were grown under normoxic or hypoxic conditions. Fresh medium containing various concentrations of PKI 166 (0, 0.3, 1.0, and 3.0 μM) was added to the cells, which were then incubated for 12 h under either normoxic (20% O₂) or hypoxic (1% O₂) conditions. The cells were collected and subjected to lysis, and phosphorylated (P-) and total proteins were detected by Western blot analysis. A, the cells were then collected and subjected to lysis, and phosphorylated (P-) and total proteins were detected by Western blot analysis. B, 12 h later, nuclear proteins were collected, and nuclear factor κB activity was detected by electrophoretic mobility shift assay. The data are from one representative experiment of four.

Hypoxia Protects L3.6pl Cells from Apoptosis Induced by Gemcitabine by PI3K. Because PKI 166 inhibits both PI3K/Akt/NF-κB activation and MEK/MAPK(Erk) phosphorylation, we determined which signaling pathway protects cells from apoptosis induced by gemcitabine. L3.6pl cells cultured under normoxic or hypoxic conditions were incubated with gemcitabine and the PI3K inhibitor LY294002 or the MEK inhibitor U0126, and apoptosis was analyzed by flow cytometry. As shown in Fig. 6, the cell-protective effect against apoptosis induced by gemcitabine under hypoxic conditions was completely reversed by LY294002, whereas treatment with U0126 produced a partial effect. These results indicate that the protective effects of hypoxia against induction of apoptosis by gemcitabine are mediated mainly by the PI3K/Akt/NF-κB pathways and depend only partially on MEK/MAPK pathways.

Discussion

A hypoxic microenvironment is commonly found in the central region of solid tumors. Because hypoxia in tumors is...
associated with poor prognosis, resistance to chemotherapy and radiation therapy, and increased metastatic potential, targeting hypoxia response pathways is of potential therapeutic value (24). PI3K/Akt, a key downstream mediator of many receptor tyrosine kinase signaling pathways involved in cell proliferation, migration, and inhibition of apoptosis, is phosphorylated under hypoxic conditions (26, 31). Similarly, MAPK(Erk), which regulates cell proliferation in response to various growth factors, has been shown to be phosphorylated under hypoxic conditions independent of interaction with its ligands (31). In our experiments, phosphorylation of both Akt and MAPK(Erk) was increased under hypoxic conditions, but phosphorylation of EGFR was unchanged. Phosphorylation of Akt was prevented by the PI3K inhibitor LY294002, and phosphorylation of MAPK(Erk) was prevented by the MEK inhibitor U0126, whereas the EGFR tyrosine kinase inhibitor PKI 166 decreased the phosphorylation of EGFR as well as that of Akt and MAPK(Erk).

In our experiments, EMSA performed with the NF-κB probe in human L3.6pl pancreatic carcinoma cells demonstrated that the DNA binding activity of NF-κB was increased in hypoxic cells. Others have reported that NF-κB DNA binding activity is up-regulated in the majority of clinical specimens of human pancreatic adenocarcinomas and human pancreatic cancer cell lines (19). Our data demonstrate that NF-κB DNA binding activity is inhibited by the EGFR tyrosine kinase inhibitor PKI 166 and the PI3K inhibitor LY294002, but not the MEK inhibitor U0126, indicating that the activation of NF-κB is through upstream signal transduction cascades that include PI3K/Akt activated under hypoxic conditions. A previous report revealed that Akt activity is required for the activation of IκB kinase for IκB phosphorylation that results in NF-κB activation (32).

Our present data show that, under hypoxic conditions, L3.6pl cells are resistant to apoptosis mediated by gemcitabine. The resistance correlated with increased phosphorylation and activation of Akt, MAPK(Erk), and NF-κB. Under hypoxic conditions, inhibition of PI3K by PKI 166 or LY294002 prevented the activation of Akt/NF-κB signaling pathway and resistance to gemcitabine-induced apoptosis. Inhibition of MEK did not affect apoptosis as much as PI3K/Akt/NF-κB inhibition. These data suggest that activation of the PI3K/Akt/NF-κB pathway is the main mechanism responsible for antiapoptotic effects.

Fig. 5 The effects of hypoxia and PKI 166 on apoptosis induced by gemcitabine. L3.6pl cells were cultured to reach 60–70% confluence. The culture medium was then changed to medium with or without 100 nm gemcitabine in the presence of PKI 166 or DMSO (0 μM PKI 166). The cells were incubated under normoxic (N) or hypoxic (H) conditions for 12 h. A, cell cycle and DNA content of the cells were analyzed by flow cytometry. The data are the means of four independent experiments. B, apoptosis was assessed as the percentage of cells with a DNA content lower than that of cells in G1-G0. The data are the means ± SE of four independent experiments (*, P < 0.05).

Fig. 6 The effect of the phosphatidylinositol 3'-kinase inhibitor LY294002 and the MEK inhibitor U0126 on apoptosis induced by gemcitabine. L3.6pl cells were grown in vitro to reach 60–70% confluence. The culture medium was replaced with complete medium containing 100 nM gemcitabine, PKI 166, LY294002, U0126, or DMSO, and cells were incubated in normoxic (N) or hypoxic (H) conditions. Apoptosis was assessed as the percentage of cells with a DNA content lower than that of cells in G1-G0 as analyzed by flow cytometry. The data are the means ± SE of four independent experiments (*, P < 0.05).
The mechanism of the activation of PI3K and MEK in hypoxic cells is unclear. Hypoxia-induced Akt activation in PC12 cells can be prevented by treatment with actinomycin D or cycloheximide, suggesting that de novo protein synthesis is required (30). In our experiment, however, the conditioned medium from hypoxic cells failed to induce Akt, MAPK(Erk), or NF-κB activation and increase the resistance to apoptosis induced by gemcitabine in L3.6pl cells grown under normoxic conditions.

Receptor tyrosine kinase is a platform for the recognition and recruitment of a specific complement of signaling relay proteins and nonreceptor tyrosine kinases (33–36). Thus, it is possible that the hypoxic effects are mediated by the recruitment of specific signaling relay proteins and nonreceptor tyrosine kinases. For example, the adaptor protein shc, an immediate substrate of tyrosine kinases, may play an important role in linking hypoxic conditions to downstream signaling events, such as induction of hypoxic inducible factor (37). The IC50 for inhibiting EGFR phosphorylation by PKI 166 is reported to be 10 nM (28), but in our experiment, PKI 166 inhibited PI3K/Akt/NF-κB and MEK/MAPK(Erk) at a micromolar concentration. As stated previously, relatively high concentrations of PKI 166 may inhibit not only EGFR tyrosine kinase but also other tyrosine kinases (28) that phosphorylate and stimulate the PI3K/Akt/NF-κB and MEK/MAPK(Erk) pathways.

Although we could not elucidate how PKI 166 inhibited the activation of these signaling pathways, we found a novel therapeutic effect of PKI 166 on abrogating the increased resistance to apoptosis induced by gemcitabine under hypoxic condition, which might happen frequently in growing pancreatic cancer tumor in vivo.

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References

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