Effect of Hypoxia on the Doxorubicin Sensitivity of Human MCF-7 Breast Cancer Cells

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ABSTRACT – Intrinsic or acquired resistance to chemotherapeutic drugs is one of the major obstacles to effective cancer treatment. Hypoxia is widespread in solid tumors as a consequence of decreased blood flow in the tumor-derived neovasculature. The recent finding of a link between hypoxia and chemoresistance prompted us to investigate whether hypoxia induces doxorubicin resistance in human MCF-7 breast cancer cells. Low oxygen concentration decreased the doxorubicin sensitivity in MCF-7 cells. The expression of p-glycoprotein, a major MDR-related transporter, and those of apoptosis-related proteins (anti-apoptotic Bcl-2, Bcl-XL and pro-apoptotic Bax) were not altered by hypoxia in MCF-7 cells. Intracellular uptake of doxorubicin was significantly decreased under hypoxic conditions. Decreased cellular uptake of doxorubicin under hypoxia may contribute to causing doxorubicin resistance in these cells. The use of agents that can modulate the doxorubicin uptake for adjuvant therapy may contribute to improving the therapeutic efficacy of doxorubicin in breast cancer patients.

Key words – Hypoxia, Doxorubicin, Chemotherapy, Multidrug resistance

Intrinsic or acquired resistance to chemotherapeutic drugs is one of the major obstacles to effective cancer treatment. The most frequent form of resistance observed in cancer patients is multidrug resistance (MDR), which is characterized by cross-resistance to a wide variety of structurally unrelated drugs, including the anthracyclines, some vinca alkaloids, and the epipodophyllotoxins.[1] Several mechanisms of MDR have been identified, including the overexpression of the ATP-binding cassette (ABC) superfamily of transporters, which function as pumps to extrude anticancer drugs from cancer cells. Among the ABC transporters frequently overexpressed in drug-resistant cancer cells are multidrug resistance protein (MRP) and P-glycoprotein.[2]

Hypoxia is widespread in solid tumors as a consequence of decreased blood flow in the tumor-derived neovasculature. Low oxygen levels in a range of solid tumor types has correlated tumor hypoxia with advanced stage, poor response to chemotherapy and poor prognosis. *In vitro* cell culture studies have also shown that hypoxia induce resistance to a wide range of chemotherapeutic drugs including etoposide, vincristine and doxorubicin in human neuroblastoma, melanoma and lung adenocarcinoma cells.[3-5]

At present, it remains unclear how low oxygen levels surrounding tumors confer drug resistance. After prolonged exposure to hypoxia, increased expression of p-glycoprotein or MRP was observed in certain types of cancer cells, including prostate[6] and vascular endothelial cells,[7] suggesting the putative role of drug efflux pumps. However, the expression of MDR-related proteins was not changed in glioma,[8] neuroblastoma[9] and colon cancer cells[10] cultured under hypoxic conditions. In breast cancer cells, hypoxia induced resistance in MCF-7 cells to mitoxantrone without changing the protein levels of these drug transporters.[11] These differing results may be due to the difference among tissue origins, cell types or culture conditions.

Doxorubicin is a weak base chemotherapeutic agent that is commonly used in combination chemotherapy to clinically treat breast cancer patients. In the current study, we sought to examine whether hypoxia induces doxorubicin resistance in MC-7 breast cancer cells.

Materials and Methods

Materials

Doxorubicin was obtained from LKT Laboratories (Minneapolis, MN). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO). Antibodies to human p-glycoprotein were obtained from Calbiochem (La Jolla, CA). Antibodies to human Bax, Bcl-2 and Bcl-x were purchased from Cell signaling Technologies (Beverly, MA), Santa Cruz Biotechnology (Santa Cruz, CA) and BD Pharmingen (San Diego, CA). All other chemicals

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were of reagent grade and used without further purification.

Cell lines and Cultures

The human MCF-7 breast cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in 1:1 mixture of DMEM/F12 medium, supplemented with 10% heat-inactivated fetal calf serum (FBS) (Hyclone, Logan, UT) and 100 units/ml each of penicillin and streptomycin, in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. When hypoxic experiments were performed, hypoxia was stimulated in a chamber with a gas mixture of 0.1% O_2, 5% CO_2 and 94.9% N_2 by placing the cells in the hypoxia chamber (Forma scientific).

Immunoblotting

Treated cells were scraped from the culture, washed twice with PBS and incubated for 15-30 min on ice in lysis buffer containing 150 mM NaCl, 10 mM Tris, 0,2%Triton X-100, 0.3% NP-40, 0.2 mM Na_3VO_4 and protease inhibitors (pH 7.4) (Roche). After centrifugation at 16,000 g for 15 min at 4°C, supernatants were collected and the protein concentration in each was measured by the Bradford method. Aliquots of supernatants containing equal amounts of protein were boiled in SDS-reducing buffer for 5 min, electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk and probed with specific primary antibodies, followed by incubation with appropriate peroxidase-conjugated secondary antibodies. Blots were developed with ECL Plus reagent (Amercham, Arlington Heights, IL) according to the manufacturer's protocol.

Cellular uptake of doxorubicin

Cells seeded on 24-well plates were treated with increasing doses of doxorubicin. At 4–h postincubation, cells were harvested, washed three times with ice-cold PBS to remove extracellular doxorubixcin, and then lyzed with 1% Triton-X in PBS. Aliquots were taken from cell lysates and subjected to spectrofluorometric analysis for determining doxorubicin concentration at Ex 488 nm/Em 570 nm in a fluorescence microplate reader (Molecular Devices, Gemini EM). Extracts from untreated cells were used as blank. Doxorubicin concentration was expressed as ng doxorubicin equivalents per 100 μg of protein.

Cytotoxicity assay

Cells were seeded into 96-well plates. The next day varying doses of doxorubicin were added, and the cells were incubated

for 24 h under normoxic or hypoxic conditions, after which cell growth and viability were measured using MTT. The ability of cells to form formazan crystals by active mitochondrial respiration was determined using a microplate spectrophotometer (Molecular Devices) after dissolving the crystals in DMSO.

Statistical Analysis

Statistically significant differences between values obtained under different experimental conditions were determined using two-tailed unpaired Student's t-tests.

Results and Discussion

To examine the effect of hypoxic culture condition on the doxorubicin sensitivity of MCF-7 cells, we treated MCF-7 cells with increasing doses of doxorubicin under normal or low oxygen concentrations. We found that incubation of MCF-7 cells with $10 \sim 20000$ nM doxorubicin under normal oxygen concentration inhibited cell growth in a dose-dependent manner (Fig. 1A): 1000 and 20,000 nM doxorubicin inhibited MCF-7 cell growth 39.4% and 48.1% after 24-h incubation. In contrast, the treatment of same doses of doxorubicin inhibited MCF-7 cell growth 11.6% and 20.3% under hypoxic con-
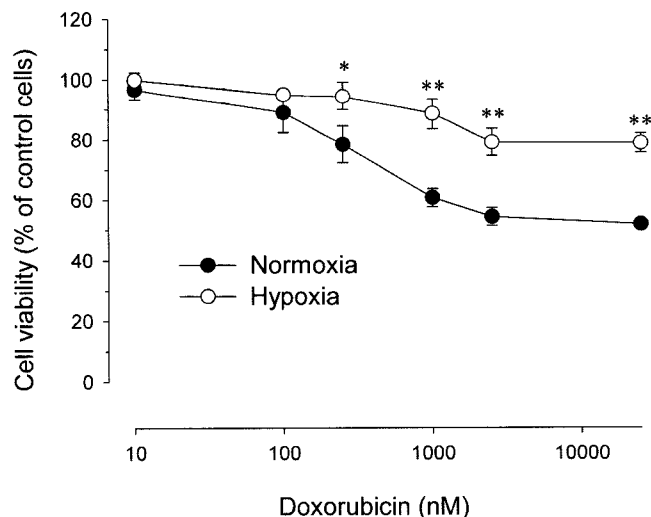

Figure 1–Comparison of the cytotoxic effects of doxorubicin in MCF-7 cells cultured under normoxic or hypoxic conditions. Cells were seeded at a density of 5×10^3 cells/well in 96-well plates and, starting 24 h later, were incubated with varying doses of doxorubicin under normoxic (5% O_2) or hypoxic (0.1% O_2) conditions. Hypoxic conditions were generated by placing the cells in the hypoxia chamber as described in the text. At 24-h postincubation with doxorubicin, their growth and viability of cells were determined by MTT assay. Results are expressed as percentage growth (mean ± S.D. of triplicate wells) relative to untreated cells (*$p < 0.05$; **, $p < 0.01$ by unpaired t-test (n = 3)).
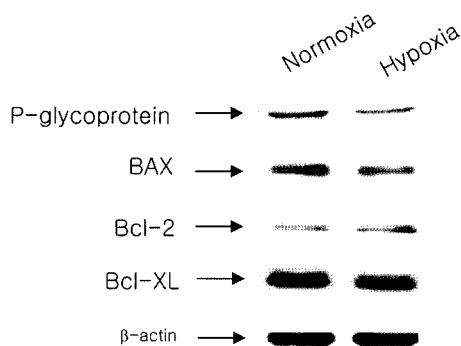
Figure 2—Immoblot analysis of p-glycoprotein, bcl-2, bax and bcl-XL proteins in MCF-7 cells cultured under normoxic or hypoxic conditions. Cell lysates were obtained from exponentially growing cells and subjected to immunoblotting with appropriate antibodies.

Figure 3—Intracellular accumulation of doxorubicin in MCF-7 cells cultured under normoxic or hypoxic conditions. Cells plated at 24-well dishes were incubated with indicated doses of doxorubicin for 4 h. Cells were harvested, washed three times with PBS, lyzed with 1% Triton X-100, and processed for quantitative determination of intracellular concentration of doxorubicin as described in the text (**$p < 0.01$, normoxic vs. hypoxic conditions by unpaired t-test ($n = 3$)).

ditions (Fig. 1). These data indicate that hypoxia induces doxorubicin resistance in MCF-7 breast cancer cells.

To investigate whether the induced doxorubicin resistance was associated with increased expression of major MDR transporters, we performed immunoblot analysis with p-glycoprotein antibodies. Expression of p-glycoprotein was not altered by hypoxia in MCF-7 cells, suggesting that a change in P-glycoprotein expression is unlikely to contribute to the resistance caused by hypoxia (Fig. 2).

Cytotoxicity of chemotherapeutic drugs such as doxorubicin is mediated primarily by induction of apoptosis in cancer cells.[12] Susceptibility of cells to initiate apoptotic cell death is affected by changes in the expression of apoptosis-related proteins including Bcl-2, Bax and Bcl-xL.[13] It prompted us to check a possibility that the increased doxorubicin resistance in MCF-7 cells exposed to hypoxia was caused by the changes in the expression of anti-apoptotic Bcl-2, Bcl-XL and pro-apoptotic Bax proteins. As shown in Fig. 2, hypoxic culture condition did not cause any significant changes in the expression of these proteins.

Earlier study demonstrated that hypoxia decreased the cellular uptake of mitoxantrone due to the acidification of media.[11] The uptake of weakly basic anticancer drugs can be decreased by acidification of the solid tumor milieu, leading to chemoresistance.[14] When we compared the intracellular doxorubicin concentration after 4-h incubation by measuring the fluorescence intensity caused by intracellular doxorubicin, we found that it was 32% decreased by hypoxia in MCF-7 cells (Fig. 3). These data suggest that decreased cellular uptake may be one factor responsible for doxorubicin resistance in hypoxia-induced MCF-7 cells.

Our data in the present study indicate that the susceptibility to doxorubicin of MCF-7 cells was greatly influenced by hypoxia. It seems unlikely that the changes in the expression of
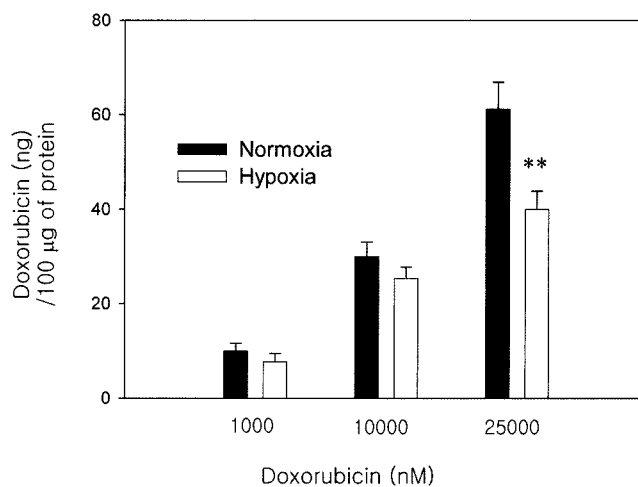
MDR- or apoptosis-related proteins caused doxorubicin resistance in hypoxia-induced MCF-7 cells. Decreased cellular uptake of doxorubicin appears to be one of factors causing doxorubicin resistance in these cells. While not studied here, however, we cannot exclude the possibility that other factors are also involved in mediating doxorubicin resistance. For example, the expression of other MDR-related proteins such as BCRP (Breast cancer resistance protein) might be increased in cells exposed to hypoxia.

Tumor hypoxia usually occurs at a distance of 100-200 μm from blood vessels and seems to be strongly associated with tumor progression and resistance to chemo- and radiotherapy.[15] Therefore, searching for reagents that can increase the doxorubicin sensitivity of cells under hypoxia may provide one effective means to improve the therapeutic response in patients with solid tumor.

Acknowledgements

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