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Acidity is one of the main mechanism for hypoxia triggering chemoresistance to mitoxanthrone (MX) in the human breast cancer MCF-7 cell line

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Summary

Background: The development of multidrug resistance (MDR) and subsequent relapse while receiving therapy is one of the major obstacles to effective cancer treatment. Hypoxia is associated with the development of resistance to chemotherapeutic agents in tumors. The aim of this study is to investigate the effects and mechanisms of hypoxia on the chemoresistance in breast cancer cells. *Materials and Methods:* Cell viability assay, flow cytometry assay, reverse transcription-PCR, and Western blotting for the kinetic changes of mRNA and protein of hypoxia-inducible factors-1α (HIF-1α) and ATP-binding cassette sub-family G member 2 (ABCG2) were used to study the effects of hypoxia on the chemosensitivity of mitoxanthrone (MX) of MCF-7 and MCF-7/MX breast cancer cell lines subjected to hypoxia. *Results:* Hypoxia (1% O_2) 18 hours decreased the MX sensitivity of MCF-7 cells. Although the levels of mRNA and protein of HIF-1α and ABCG2 in MCF-7 and MCF-7/MX cells were affected under 1% oxygen tension, however, a specific inhibitor of ABCG2, Ko143 did not affect the MX sensitivity of MCF-7 cells subjected to hypoxia for 18 hours. The acidified media (pH 6.5) by hypoxia significantly decreased the retention of MX in MCF-7 cells. *Conclusion:* Acidity resulting from the hypoxia may cause chemoresistance to MX through a decrease of intracellular MX. As such, targeting the highly acidic microenvironments of tumors may hold therapeutic promise as a means of overcoming the chemoresistance of cancer.

Key words: Hypoxia; Hypoxia-inducible factors-1α (HIF-1α); Chemoresistance; ATP-binding cassette sub-family G member 2 (ABCG2); Acidity.

Introduction

Breast cancer, the most frequently diagnosed cancer and the leading cause of cancer-related deaths in women worldwide, accounts for 23% of the total cancer cases and 14% of cancer deaths [1]. According to a cancer registration report from the Taiwan Ministry of Health and Welfare (MOHW), the incidence rate for breast cancer was the highest for any cancer, with 33 women being diagnosed with the disease and 5.67 women dying from it on a daily basis [2]. Although breast cancer is responsive to a wide array of single and combination chemotherapy regimens, some initially responsive breast cancer patients may relapse and develop resistance to multiple anticancer agents with different structures and mechanisms of action, leading to metastases and eventual death from the disease [3]. As such, insights at the cellular and molecular levels may broaden our understanding of the development of chemoresistance and hold therapeutic promise against the cancer.

The development of multidrug resistance (MDR) and subsequent relapse when receiving therapy is a widespread problem in patients with breast cancer [4]. MDR is caused by multidrug transporters (MDTs), which can recognize a wide variety of chemical substrates and pump them out of cells to reduce intracellular levels of the substrates, thereby causing treatment failure [5]. MDTs, which are also called ATP-binding cassette transporters (ABC-transporters), utilize the energy of adenosine triphosphate (ATP) hydrolysis to conduct biological reactions [6]. The P glycoprotein (Pgp) and the multidrug resistance-related protein (MRP) are two typical ABC transporters and have been found to be overexpressed in breast cancer cells [7, 8]. Meanwhile, the discovery of ATP-binding cassette sub-family G member 2 (ABCG2) has explained the puzzle of non-Pgp and non-MRP-mediated mechanisms seen in some tumors [9]. ABCG2 is expressed in several kinds of tumors, including breast cancer, gastric carcinoma, hepatocellular carcinoma, endometrial carcinoma, colon cancer, small cell lung cancer, and melanoma [10], and is associated with resistance due to its active extrusion of diverse therapeutic com-

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pounds, e.g., mitoxantrone, methotrexate, topotecan, camptothecins, flavopiridol, antifolates, and SN38 [11]. The use of chemotherapy-based anthracyclines, including doxorubicin, epirubicin, etc., is a standard approach for the adjuvant treatment of breast cancer [12]. Importantly, these compounds are substrates for ABCG2. Although the actions of ABCG2 are similar to those of Pgp and MRP in that they involve a cycle of substrate transport and ATP hydrolysis, it is unknown how such transport develops in breast cancer.

Hypoxia is one of the main features of solid tumors [13, 14]. About 25%–40% of invasive breast cancers exhibit hypoxic regions [15]. Low oxygen stress in a growing tumor may induce numerous hypoxia-responsive genes to cause cellular changes leading to a more aggressive phenotype and resistance to chemotherapy and radiotherapy, resulting in a poor prognosis [13-16]. Hypoxia inducible factor-1 (HIF-1), a heterodimeric transcription factor, is composed of an inducible α subunit and a constitutively expressed β subunit [17]. Under normoxia, HIF-1α is degraded by proteosomes, but hypoxia prevents this degradation, leading to increased intracellular HIF-1α protein levels, the formation of HIF-1, and the activation of HIF-1 target genes important for cell survival, metabolic adaptation, and angiogenesis. Therefore, HIF-1α represents the main mediator of the hypoxic response [18]. The overexpression of HIF-1α is detected in many solid tumors, including breast cancer [18, 19]. If hypoxia is the main factor in making the cancer more difficult to treat and is associated with chemotherapy failure, additional hypoxia should up-regulate HIF-1α, which would consequently drive target genes, including MDR-related genes, to produce chemoresistance. The MDR1 gene which encodes Pgp contains a hypoxia response element (HRE) for HIF-1 binding, and the increasing expression of Pgp in hypoxia confers resistance to doxorubicin [20]. There is also a HRE in the promoter of ABCG2, and hypoxia promotes the expression of ABCG2 in several cell lines, including progenitor cells [21]. The present authors are interested in knowing whether the hypoxia of breast cancer cells confers resistance by up-regulating ABCG2 to pump out many chemotherapeutics, thus resulting in the chemoresistance. On the other hand, if hypoxia induced ABCG2 is not the cause of the chemoresistance, are there any other mechanisms that are responsible for it?

In a previous study [22], the authors demonstrated that MCF-7/MX cells showed lower retention of MX and higher survival rates when exposed to MX in comparison with MCF-7 cells because the MCF-7/MX cells overexpressed ABCG2, which pumped out the MX, supporting the conclusion that ABCG2 represents a mechanism of drug resistance in cancer cells. Several MX-induced genes such as ATP synthase, cytochrome c oxidase subunit VIc (COX6C), HIF-1α, and ABCG2 were identified by the use of cDNA microarray screening. Since hypoxia is related to chemoresistance, the aims of this study were, firstly, to investigate the effects of hypoxia on the chemoresistance of MCF-7 and MCF-7/MX cells to MX. Secondly, the authors also sought to explore the underlying mechanisms of hypoxia-related chemoresistance. The present results suggested that the responses of these two cell lines subjected to hypoxia were very different and that the effect of hypoxia leading to the chemoresistance was not mediated by the inducible ABCG2 but by the acidification, which decreased intracellular concentrations of MX to increase the survival of MCF-7 cells under hypoxia. Targeting the highly acidic microenvironments of tumors could thus hold therapeutic promise as a means of overcoming clinical drug resistance.

Materials and Methods

The ABCG2-overexpressing breast cancer cell line MCF7/MX and its parental line MCF7 were kindly provided by Dr. M. Barrand (University of Cambridge, UK). These cells were cultured as mono-layers in complete medium, including RPMI 1640 medium, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Furthermore, 80 nM MX was included in the complete medium to maintain the chemoresistance of the MCF-7/MX cells. The MCF-7/MX cells were cultured in drug-free medium for at least three days before the experiments.

To investigate the effects of hypoxia, the authors chose 1% as the oxygen setting for the hypoxia experiments [23, 24]. MCF-7 and MCF-7/MX cells were incubated in a 5% CO_2/1% O_2 balanced with N2 atmosphere in a CO_2–O_2 hypoxia incubator for the hypoxic condition. An oxygen meter was used to calibrate and confirm the hypoxia treatment.

Exponentially growing MCF-7 and MCF-7/MX cells (10^6) were incubated for 30 minutes with 10μM MX in complete medium and then washed with ice cold PBS to remove the MX-containing medium. The cells were then re-suspended in drug-free fresh medium and incubated for 90 minutes. After then being washed three times more with ice-cold PBS, the cells were kept on ice before analysis on a flow cytometer equipped with a standard argon laser at 488 nm excitation and with a 530 nm band pass (FL1).Data were analyzed with Cell Quest software.

Total RNA was extracted from MCF-7 and MCF-7/MX cells by using TRIzol reagent and then treated with DNase according to the manufacturer's instructions. RNA was quantified and quality assessed using a NanoDrop ND-1000 and a 2100 bioanalyzer, respectively. cDNAs were synthesized by using a SuperScript II reverse transcriptase kit based on the manufacturer's protocol and were used to analyze the expression levels of genes after PCR amplification. All PCR products were normalized to β-actin. Primers were designed by using the online "Primer 3" software package (http://www-genome.wi.mit.edu/ cgi-bin/primer/primer3_www.cgi) and synthesized by MWG Biotech. 5 μg of total RNA was used for generating single-stranded cDNA. The cDNA was then used for PCR amplification. The primer sets for PCR amplification were as follows: ABCG2 (product size 429 bps) forward 5′-TTATCCGTGGTGTGTCTGGA-3′, reverse 5′-CCTGCTTGGAAGGCTCTATG-3′; HIF-1 α (product size 542 bps) forward 5′- TGCTCATCAGTTGCCACTTC -3′, reverse 5′-ACCACCTATGACCTGCTTGG -3′; β-actin (product size 644 bps) forward 5′-50-ACGTTATGGATGATGATATCG-3′, reverse 5′-CTTAATGTCACGCACGATTTC-3′. PCR was carried out with 1 μL of cDNA as follows: hot start: 1 cycle, initial denatura-

tion at 94 °C for 5 minutes followed by 25–30 amplifying cycles of 94 °C for 30 seconds; annealing temperature 55–59°C for 30 seconds, 72°C for 1 minute followed by a final elongation at 72°C for 7 minutes. After amplification, 18 μL of each RT-PCR product was mixed with 3 μL 6x DNA loading dye and run in a 2% agarose gel immersed in 1x TAE buffer solution plus ethidium bromide (0.5mg/L) using 70 mV voltage. Gels were then placed in a UV cabinet, and the specific bands were visualized with ethidium bromide (EB). Gels were then placed in a UV cabinet, and the specific bands were visualized with EB and photographed under UV light. Reverse transcription-polymerase chain reaction (RT-PCR) reactions without reverse transcriptase yielded no specific bands. The fluorescence of bands of HIF-1α, BCRP, and other molecules were quantified with the aid of NIH Image J 1.63 (a free download from http://rsb.info.nih.gov/ij/ download/).

Cells were lysed in whole lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.5–8.0, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 100 μg/mL PMSF, 1 μg/mL aprotinin), and protein concentrations were determined using the Bio-Rad protein assay. Equal amounts of cell lysates from MCF-7 and MCF-7/MX cells were subjected to SDS-poly-acrylamide gel electrophoresis, and then transferred to 0.45 μm nitrocellulose membranes. Membranes were blocked with 5% skimmed milk-PBS/0.1% Tween 20 for an hour prior to an overnight incubation at 4°C with primary antibodies (anti-HIF-1α antibody, 1:1000; anti-ABCG2, 1:500; and β-tubulin, 1:200, all diluted in 5% skimmed milk in PBS/0.1% Tween 20). The membrane was then incubated with HRP-conjugated secondary antibody. Following successive washes, membranes were developed using an enhanced ECL detection system. β-tubulin was used as an internal control.

Equal amounts of MCF-7 and MCF-7/MX cells were seeded into each well of 96-well plates in triplicate in RPMI 1640 supplemented with 10% fetal bovine serum and incubated in a humidified atmosphere at 37 °C. After the cells attached to the wells (36 hours), the cells were stimulated with 100 μL of serially diluted MX to achieve final concentrations of 0–1000 μM for an additional four hours. These cells were then incubated under hypoxia conditions for a further 12 or 18 hours before being challenged with 100 μL of serially diluted MX to achieve final concentrations of 0 to 1000 μM. The drug-containing medium was aspirated, followed by the addition of 200 μl of MTT (3-(4,5-dimethylthiazol-2-il)-2,5-diphenyl tetrazolium bromide) [27] into the medium (final concentration of 0.5 mg/ml). The plate was incubated for 3 hours at 37°C, the MTT solution carefully aspirated from each well, and the formazan crystals were dissolved in 150 μl dimethyl sulfoxide (DMSO) per well. The plates were then read immediately at 540 nm on a scanning multi-well spectrophotometer (ELISA reader), and from these reading the fraction of surviving cells in each well compared to control (untreated cells) could then be calculated. All the data points represent the mean value of a minimum of six wells. At least four such experiments were performed for each cell type.

Results

MCF-7/MX cells are known to show chemoresistance to MX [25]. To examine if hypoxia could influence the chemosensitivity of the tumor cells to MX, cells were incubated either in normoxia or hypoxia for 12 or 18 hours. Figure 1A clearly shows that the survival rate of MCF-7 cells treated with 500 μM of MX ranged from 42.1 ± 3.2 % under normoxia to 59.4 ± 3.4% under hypoxia for 12 hours, and to 82.3 ± 5.5% under hypoxia for 18 hours, suggesting that the cancer cells developed chemoresistance to the cytotoxic effects of MX when they were subjected to 18 hours of hypoxia. In contrast, hypoxia did not affect the chemosensitivity of MCF-7/MX cells to MX.

To investigate whether the induced chemoresistance to MX of MCF-7 cells was associated with increased expression of HIF-1α and major MDR transporters in hypoxia, the authors performed kinetic RT-PCR and immuno-blot analysis with HIF-1α and ABCG2 mRNAs and antibodies. Figure 2 demonstrates that the expression levels of the mRNA and protein of ABCG2 in the MCF-7/MX cells remained relatively constant during the entire 18 hours period of hypoxia. However, the mRNA expression of ABCG2 in the MCF-7 cells was low at time zero and steadily increased. There was no detectable ABCG2 protein in response to hypoxia until 18 hours when a clear band of 70-kDa appeared. Expression levels of basal levels of HIF-1α mRNA and protein were higher in the MCF-7 cells than they were in the MCF-7/MX cells. However, the changes in the expression levels of the mRNA and protein of ABCG2 were not in accordance with those of HIF-1α.

As MX has a fluorescent property and is one of the ABCG2 substrates, MCF-7 cells showed higher retention of MX (Figure 3, 1st bar) when exposed to MX in comparison with MCF-7/MX cells (Figure 3, 2nd bar) as determined by flow cytometry under normoxia. When the ABCG2 specific inhibitor Ko143 was added, the sensitivity to MX was restored in the MCF-7/MX cells (Figure 3, 3nd bar). To investigate the functions of hypoxia-induced ABCG2, MCF-7 cells were subjected to hypoxia for 18 hours either with (Figure 3, 5nd bar) or without Ko143 (Figure 3, 4nd bar);the cells in both treatment conditions showed reduced retention of MX, suggesting that hypoxia-induced chemoresistance to MX is irrelevant to the induced ABCG2 in MCF-7 cells. It is well-known that extracellular pH in human tumors, including breast cancers, has been found, through measurement with microelectrodes, to be significantly acidic, in the range from 6.2 to 7.0 [26]. Therefore, if the hypoxia-induced low retention of MX in MCF-7 cells was not caused by the over-expressed ABCG2, could the hypoxia-induced acidification of the medium have been the main cause for the low retention and chemoresistance observed in this study? At first, the pH of the complete media under normoxia (20% O_2) and hypoxia (1% O_2) for 18 hours was measured, and the values were found to be 7.2 and 6.5, respectively. After being exposed to the complete media with pH values of 7.2 and 6.5 for 18 hours, the MX retention rates in the MCF-7 cells were 51.6 ± 3.1% (pH 7.2) and 23.1 ± 3.8 (pH 6.5).

Discussion

In the present experiments, subjecting cancer cells to 1% oxygen for 18 hours rendered MCF-7 cells more resistant

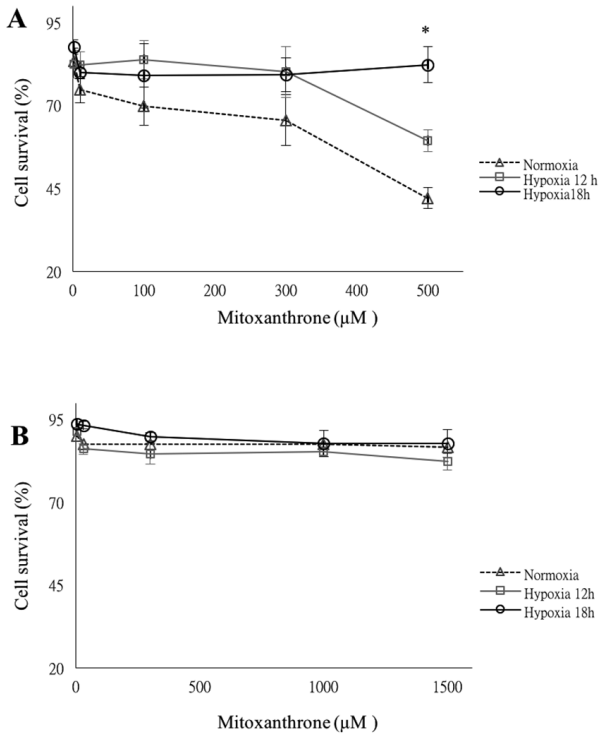

Figure 1. — Effect of hypoxia on the chemosensitivity of MCF-7 and MCF-7/MX cells to MX.MCF-7 cells and MCF-7/MX cells were seeded into 96-well plates and kept in an incubator at 37°C in 5%CO2 and 95% air for 48 hours. MCF-7 and MCF-7/MX cells were treated in triplicate with serially diluted MX at 1, 10, 100, 100, and 1000 μM. For hypoxia experiments, the cells were kept in an incubator at 37°C in 5%CO2 and 95% air for 36 hours. Before commencing each experiment, the medium was replaced by a fresh medium previously maintained under 1% oxygen tension in a hypoxia incubator for 1 hour. These cells were then incubated under hypoxia for a further 12 or 18 hours before being challenged with 100 μL of serially diluted MX to achieve final concentrations of 0 to 1000 μM for 4 hours, and the cell viability was then detected by the MTT assay. *$p < 0.05$ (n =6).

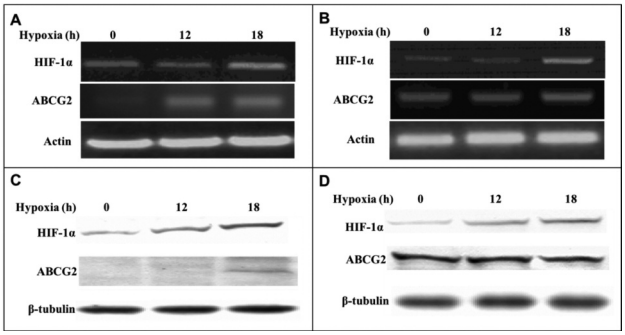

Figure 2. — Hypoxia effects on the levels of mRNA and protein in MCF-7 and MCF-7/MX cells. MCF-7 cells and MCF-7/MX cells were incubated in a hypoxia incubator for periods of 0, 12, and 18 hours. Before commencing each experiment, the medium was replaced with a fresh medium previously maintained under 1% oxygen tension in a hypoxia incubator for 1 hour. Levels of the mRNA of HIF-1α and ABCG2 in MCF-7 cells (A) and MCF-7/MX cells (B) subjected to normoxia and hypoxia for 12 and 18 hours. As resolved by SDS-PAGE, HIF-1α and ABCG2 migrated as 92-kD and 70-kD bands, respectively, in the MCF-7 and MCF-7/MX cells. Figures 4 C and D show that in response to hypoxia, the level of HIF-1α protein in the MCF-7 cells and MCF-7/MX cells increased steadily for 18 hours. Although the initial protein level of ABCG2 was low and there was no obvious change until 18 hours when a band with 70-kDa could be seen clearly, the level of ABCG2 was relatively stable in response to hypoxia. The levels of HIF-1α and ABCG2 protein were detected using an anti-human HIF-1α antibody (HIF-1α; 1:1000) and anti-human ABCG2 antibody (1:1000), respectively. β-tubulin (β-tubulin; 1:200) was used as an internal control (n = 3).

to MX-induced cell death, whereas hypoxia did not significantly affect the changes in chemosensitivity of MCF-7/MX cells to MX. These findings support the conclusion that tumor hypoxia is associated with resistance to chemotherapeutic agents [13]. The present data also indicated that MCF-7 cells and MCF-7/MX cells possess different mechanisms against MX under hypoxia. Next, if hypoxia activates numerous transcriptional pathways relevant to resistance to chemotherapeutics, the authors asked whether HIF-1α, a central mediator in hypoxia-associated tumor development and therapeutic resistance [27], was the key to triggering the chemoresistance because assessments of tumor oxygenation and HIF-1α expression patterns could be used to determine tumor chemosensitivity [28]. There were three important findings in this study. First, the expression levels of HIF-1α mRNA and protein in both types of cells were up-regulated in response to hypoxia. Second, the baseline levels of HIF-1α mRNA and protein were higher in the MCF-7 cells than in the MCF-7/MX cells, and these differences may be due to the chronic treatment with MX affecting HIF-1α expression [27]. Third, the elevation of HIF-1α expression preceded that of ABCG2 in response to hypoxia, suggesting that HIF-1α protein may enhance the transcription rate of the ABCG2 by binding to HRE, which is harbored in the promoter region of the ABCG2 gene[21].

If hypoxia drove the sensitive cancer cells to develop the resistance to MX through the activation of HIF-1α to up-regulate the expression of ABCG2, the inconsistent changes in the expression of the mRNA and protein of ABCG2 in accordance with those of HIF-1α, and the lower expression of ABCG2 in MCF-7/hypoxic cells in this study, seemingly could not completely explain the developing chemoresistance of MCF-7 cells to MX under hypoxia. Therefore, a functional study by flow cytometry was conducted and showed that MCF-7 cells retained much more MX when exposed to MX in comparison with MCF-7/MX cells under normoxia (Figure 3), indicating that ABCG2 actively ex-

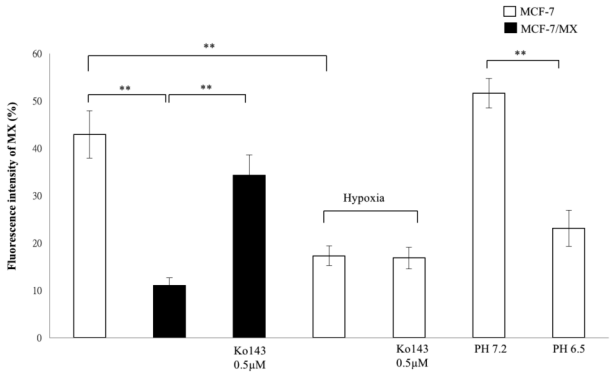

Figure 3. — Effects of pH on breast cancer cells during exposure to MX as determined by flow cytometry under normoxia and hypoxia. Exponentially growing MCF-7 cells and MCF-7/MX cells were collected and transferred to FACS tubes. The cells were incubated with 10 μM MX in serum-free medium for 30 minutes, centrifuged at 1,000 rpm for 5 minutes at 4°C, and washed with ice-cold PBS to remove the MX-containing medium. After discarding the supernatants, the cells were kept on ice, and the pellets were resuspended in 0.5 ml PBS immediately before analysis on a FACScan flow cytometer with a standard argon laser for 488 nm excitation and with 530 nm band pass (FL1). Hypoxia: cells were exposed to 1% O2 for 18 hours. Ko143: a specific ABCG2 inhibitor, cotreated with MX. pH 7.2 and 6.5: The pH of a complete medium was titrated to pH 7.2 and 6.5. All flow cytometric data were analyzed with the Summit Offline software program (n = 4). (*$p < 0.0005$)

truded the drug [9]. A specific and novel ABCG2 inhibitor, Ko143 [29], significantly increased the MX retention of MCF-7/MX cells. Surprisingly, however, treatment with Ko143 had a negligible influence on the retention of MX in MCF-7/hypoxic cells. Therefore, these results and others [26, 29, 30] support the notion that hypoxia can cause lower intracellular MX concentration without inducing the expression of ABCG2, suggesting that other mechanisms may be responsible.

The tumor microenvironment is hypoxic and has high acidity [31]. It has been demonstrated that extracellular pH plays an important role in drug resistance and malignant progression [26, 30-33]. If hypoxia is the main cause of the more acidic environment, it is reasonable to ask whether pH may play a crucial role in affecting the transport of chemotherapeutic drugs through the cellular membrane, thus leading to chemoresistance? To analyze the influence of hypoxia on the pH values of the cells and medium, MCF-7 cells and complete medium were incubated under hypoxia for 18 hours. The media of hypoxic samples had a pH of 6.5 compared to the 7.2 pH of the media under normoxia. A retention assay showed that the 51.57±3.1 % intracellular concentration of MX at pH 7.4 was decreased to 23.0 ± 3.8% at pH 6.8 in MCF-7 cells, indicating that the lower intracellular MX concentration may lead to decreased cytotoxicity under hypoxia through the changes of acidity instead of the hypoxia-induced ABCG2. There are at least three points to support the present findings. Firstly, the transport of MX through the cellular membrane was significantly influenced by the degree of acidity[30, 33, 34]. Secondly, MX is a weakly basic drug that may become concentrated in more acidic compartments, thus hindering it from reaching its intracellular target. Since hypoxia induces a more acidic environment, this may hamper the passive influx of MX into cells and cause a decrease in cytotoxicity [35]. Thirdly, the cytotoxicity to MX is increased at higher pH levels [36]. These findings further demonstrate that the effect of hypoxia leading to the chemoresistance was not mediated by the inducible ABCG2 but by the acidification, which decreased intracellular concentrations of MX to increase the survival of MCF-7 cells under hypoxia.

Conclusion

Hypoxia decreased the MX sensitivity of MCF-7 cells and showed no effects on that of MCF-7/MX cells. Hypoxia enhanced the mRNA and protein expression of HIF-1α, which inconsistently up-regulated the expression of ABCG2 in both MCF-7 and MCF-7/MX cells. Ko143, a specific inhibitor of ABCG2, did not increase the sensitivity of MCF-7 cells subjected to hypoxia for 18 hours while they were also exposed to MX. Rather, it was the acidity resulting from the hypoxia that triggered the chemoresistance to MX through a decrease of intracellular MX. Targeting the acidic microenvironments of tumors may hold therapeutic promise as a means of overcoming the chemoresistance of cancer.

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