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Up-regulation of antioxidant enzymes and coenzyme Q_{10} in a human oral cancer cell line with acquired bleomycin resistance

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Abstract
Bleomycin (BLM) is an anti-cancer drug that can induce formation of reactive oxygen species (ROS). To investigate the association between up-regulation of antioxidant enzymes and coenzyme Q_{10} (CoQ_{10}) in acquired BLM resistance, one BLM-resistant clone, SBLM24 clone, was selected from a human oral cancer cell line, SCC61 clone. The BLM resistance of SBLM24 clone relative to a sub-clone of SCC61b cells was confirmed by analysis of clonogenic ability and cell cycle arrest. CoQ_{10} levels and levels of Mn superoxide dismutase, glutathione peroxidase 1, catalase and thioredoxin reductase 1 were augmented in SBLM24 clone although there was also a mild increase in the expression of BLM hydrolase. Suppression of CoQ_{10} levels by 4-aminobenzoate sensitized BLM-induced cytotoxicity. The results of suppression on enhanced ROS production by BLM and the cross-resistance to hydrogen peroxide in SBLM24 clone further demonstrated the development of adaptation to oxidative stress during the formation of acquired BLM resistance.

Keywords: *Bleomycin, resistance, coenzyme Q_{10}, antioxidant enzymes, reactive oxygen species, hydrogen peroxide.*

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Introduction

Many antioxidant enzymes in the aerobic organisms are essential for the protection against oxidative stress. Superoxide dismutase (SOD) catalyses the dismutation of superoxide radicals to hydrogen peroxide and oxygen. CuZnSOD (SOD1) and MnSOD (SOD2) are primarily localized in cytosol and mitochondrial matrix, respectively. Hydrogen peroxide is further detoxified by catalase, glutathione peroxidase (GPx) and peroxiredoxin. There are four major GPx isoenzymes in human cells [1]. GPx1 is a major form of GPx that is expressed in both cytosol and mitochondria [2]. Oxidation of thioredoxin during the catalytic cycle of peroxiredoxin or reduction of protein disulphide is reduced back by thioredoxin reductase (TrxR), which also exists as cyosolic form (TrxR1) or mitochondrial form (TrxR2) [3]. Expression or activities of these antioxidant enzymes could be elevated in response to various conditions of oxidative stress [1,4,5]. Increased expression of antioxidant enzymes in normal tissues of transgenic mice can be protective against acute toxicity of anti-cancer drugs that produce oxidative stress [6]. Elevation of few antioxidant enzymes, such as MnSOD and TrxR, have also been implicated in the resistance to certain anti-cancer drugs in human cancer cells [7,8].

Coenzyme Q, which is endogenously synthesized, is an essential mitochondrial electron carrier in the mitochondrial electron transport chain. It is present in the form CoQ_{10} in human cells. CoQ is widely distributed in most sub-cellular compartments, although it is enriched in the mitochondrial inner

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membrane [9]. The reduced form of CoQ, ubiquinol, is known to be important in suppressing lipid peroxidation [9], but its oxidized form, ubiquinone, can also exert antioxidative function by scavenging superoxide radicals in the mitochondria [10]. Moreover, CoQ_{10} can inhibit apoptosis independent of its antioxidant function [11]. It has been shown that acute treatment of the anti-cancer drug camptothecin could induce biosynthesis of endogenous CoQ_{10} in human cancer cells in association with concurrent increase in the production of reactive oxygen species (ROS), which might be important for survival of cancer cells in response to camptothecin-induced cell death [12]. Furthermore, our recent work demonstrated that the direct exposure of hydrogen peroxide (H_2O_2) to the human 143B cell line enhanced CoQ_{10} levels in a time- and dose-dependent manner [13].

Bleomycin (BLM) is an anti-cancer drug, an antibiotic with glycopeptides structure originally derived from *Streptomyces*, used to treat various types of human cancers, especially squamous cell carcinomas and malignant lymphomas. Because it does not cause myelosuppression, it is widely used in combination chemotherapy [14]. BLM can enhance intracellular levels of ROS [15,16] and induce cell cycle arrest at G_2/M phase [17]. BLM binds to DNA with its biothazole ring and can cause strand breaks independent of ROS [18], but ROS generated from BLM can also cause strand breaks or lead to oxidative DNA damage [19]. The target of BLM in cells is not limited to nuclear DNA, as BLM can also augment lipid peroxidation [20] and damage mitochondrial DNA [21]. The major hypothesis for BLM resistance is the up-regulation of BLM hydrolase (BLMH) that can inactivate BLM by hydrolysis of a peptide structure on BLM [14]. However, it has been indicated that the increase of BLMH was not always enhanced in all BLM-resistant clones [22] or the degree of BLM inactivation did not correlate with the high BLM resistance [23]. Moreover, that hypothesis could not explain the cross-resistance between BLM and ionizing radiation observed in previous reports [22,23]. Other mechanisms of BLM resistance have also been indicated [14,24]. Therefore, additional mechanisms or multiple changes for BLM resistance should exist.

Although it has been known that BLM can cause oxidative stress, whether up-regulation of antioxidant enzymes or molecules could occur during the development of acquired BLM resistance has never been investigated before in the literature. In the present study, we hypothesized that up-regulation of antioxidant enzymes or CoQ_{10} could be involved in the acquired resistance to BLM, leading to the gain of adaptation to oxidative stress in cancer cells. A BLM-resistant clone with high resistance to BLM was first selected from a human oral squamous cell carcinoma cell line, SCC61 cells, after long-term culture of SCC61 cells in BLM. Levels of major antioxidant enzymes and endogenous CoQ_{10} were then investigated. ROS levels in response to acute BLM treatment were also examined. The possible cross-resistance of the BLM-resistant clone to H_2O_2 was further explored to demonstrate the possible development of adaptation to oxidative stress during the acquisition of BLM resistance.

Materials and methods

Drugs and chemicals for cell treatment

The 15-mg potency of bleomycin hydrochloride was purchased from Nippon Kayaku Co. (Tokyo, Japan). H_2O_2 at the grade of Suprapur® in the form of 30% (w/w) solution was purchased from Merck (Darmstadt, Germany). 4-Aminobenzoate (4AB) in the form of sodium salt was purchased from Sigma (St. Louis, MO).

Cells and cell culture

The SCC61 squamous cell carcinoma cell line, which was obtained from Dr Ralph Weichselbaum, was derived from retromolar trigone of patients without receiving radiotherapy or chemotherapy [25]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum and 0.4 μg/ml of hydrocortisone.

Selection of BLM-resistant clones

BLM-resistant cells from SCC61 cells were initially selected by intermittent treatment of SCC61 cells with 0.2 μg/ml of BLM for 12 weeks. Single colonies were isolated by using the plastic cloning cylinders (Bel-Art Products, Pequannock, NJ). SCC61b and SBLM24 clones, from SCC61 and BLM-resistant SCC61 cells, respectively, were selected. SCC61b was therefore a sub-clone of SCC61 cells. SBLM24 cells were maintained in medium without BLM after selection.

Clonogenic assay

The clonogenic assay was performed to access the ability of single cells to propagate to colonies with at least 50 cells in each colony [26]. Cells were plated as 200–300 cells per 6-cm dish and grown overnight. Cells were treated with BLM for a certain period of time and medium without BLM was replaced after the treatment. Colonies were then stained with 2% crystal violet dissolved in 95% ethanol after another 11–14 days. The concentration of drug that could inhibit 50% of colonies survived, IC_{50}, was obtained by using the Softmax Pro software (Molecular Devices, Sunnyvale, CA) with a 4-parameter-fit curve.

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Analysis of cell cycle arrest by flow cytometry

Cells were treated with BLM for 24 h for cell cycle analysis at the dose of 10 μg/ml. Cell density was controlled so that control cells were not in confluent state when harvested to avoid higher proportions of cells in the G_0 or G_1 phase. Trypsinized cells and any floating cells were pooled together. Cell pellets from 1×10^6 cells were possessed and stained with 50 μg/ml propidium iodide, as described by Chiou et al. [27]. Different phases of cell cycle were analysed by the FACSCalibur (Becton Dickinson, San Jose, CA). The fractions of G_1, S and G_2/M phases were determined by the CellQuest software (Becton Dickinson) and ModFit LT software (Verity Software House, Topsham, ME).

Western blot analysis and antibodies

To detect protein levels of antioxidant enzymes, cells were homogenized in 50 mM potassium phosphate buffer (pH 7.4) containing 0.05% Triton X-100 and the protease inhibitors prepared from the Complete Protease Inhibitor Cocktail Tablets (Roche, Mannheim, Germany). The supernatant after 10-min $1000 \times g$ centrifugation was used for Western blot analysis. Protein concentrations were determined by the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). Procedures of Western blot analysis were modified from published methods [28]. In brief, after the separation of proteins by electrophoresis with the separation gel containing 12.5% acylamide, the proteins were transferred to nitrocellulose membranes (PerkinElmer, Branchburg, NJ). Membranes were blocked, incubated with primary antibodies at 4°C overnight and then reacted with horseradish peroxidase (HRP)-labelled secondary antibodies at room temperature for 2 h. Actin was detected as a loading control. Abundance of proteins was detected by using chemiluminescence detection kits that reacted with HRP. The SuperSignal West Femoto Maximum Sensitivity Substrate from Pierce (Rockford, IL) with higher sensitivity was used for the detection of CuZnSOD and TrxR1, whereas The Immobilon Western Chemiluminescent HRP Substrate from Millipore (Billerica, MA) was used for the detection of other proteins. The chemiluminescence of protein bands was photographed and quantified by a chemiluminescence imaging system, LAS-3000 (Fujifilm Life Science, Tokyo, Japan). The primary antibodies used were mouse monoclonal antibodies against CuZnSOD (R&D Systems, Minneapolis, MN), catalase (LabFrontier, Seoul, Korea), TrxR1 (LabFrontier) and actin (Chemicon, Temecula, CA); and rabbit polyclonal antibodies against MnSOD (Upstate, Lake Placid, NY), GPx1 (Abcam, Cambridge, UK) and TrxR2 (LabFrontier). The secondary antibodies used were HRP-labelled goat anti-mouse IgG and goat anti-rabbit IgG antibodies from Chemicon.

Enzymatic activity assays for CuZnSOD, MnSOD, catalase and GPx

Enzymatic activity assays for antioxidant enzymes were carried out as previously described [16] by using the DU-800 spectrophotometer (Beckman, Fullerton, CA). For SOD activity assay, cells were homogenized in 50 mM potassium phosphate buffer (pH 7.8) and sonicated. Whole homogenate was then used. One unit of SOD was defined as the amount of protein that could inhibit rate of nitroblue tetrazolium (NBT) reduction by superoxide to 40% of blank rate, which was about half of the maximal inhibition rate when using the homogenate of SCC61b cells, and obtained by using the Softmax Pro software (Molecular Devices, Sunnyvale, CA) with a 4-parameter-fit curve derived from results on samples with eight different concentrations. MnSOD activity was measured in the presence of sodium cyanide, whereas CuZnSOD activity was calculated as the difference between total SOD activity, which was the SOD activity in the absence of sodium cyanide, and MnSOD activity. On the other hand, supernatant from $1000 \times g$ centrifugation of sonicated cell homogenates prepared in 50 mM potassium phosphate buffer (pH 7.4) was used for the activity assays of catalase and GPx. The protein concentration was determined by the Bio-Rad Protein Assay Kit.

Detection of CoQ_{10} in cells

Endogenous levels of CoQ_{10} in cells were analyzed as previously described [13] by using a high-performance liquid chromatography (HPLC) with coulometric array detectors (CoulArray HPLC, model 5600) from ESA Inc. (Chelmsford, MA). In brief, cells in the amount of 2×10^6 were homogenized in 0.1 ml of 50 mM sodium phosphate buffer (pH 7.4) and then mixed with 0.1 ml of 2.5% sodium dodecyl sulfate [29]. The homogenate was further mixed with 0.2 ml ethanol containing 10 mg/L butylated hydroxyanisole after the addition of 10 μl retinyl acetate (10 μg/ml), which was used as an internal control. The mixture was then further extracted and analyzed for CoQ_{10} as for plasma using our previously established procedures and HPLC conditions [30].

Detection of intracellular ROS by flow cytometry

ROS were detected by using the 2-[6-(4'-hydroxy) phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (HPF) as the probe [31] coupled with flow cytometric analysis, which was modified from our previous established method [13]. HPF is a novel fluorescent probe that detects highly reactive ROS by forming fluorescent products after *O*-dearylation reaction

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upon the reaction of HPF with highly reactive ROS [31]. HPF was purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). Cells grown in 12-well culture plates were incubated with HPF (20 μM) at 37°C for 30 min in a pre-warmed incubation buffer, which was a modified Hanks' balanced salt solution that consisted of 5.37 mM KCl, 137 mM NaCl, 8.33 mM glucose, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4•7 H_2O, 1 mM $MgCl_2$•6H_2O, 1 mM $CaCl_2$•H_2O, and 10 mM HEPES adjusted to pH 7.2. Attached cells were then trypsinized, mixed with culture medium, and centrifuged. After removing culture medium, the cell pellet was resuspended in the cold incubation buffer. ROS levels were monitored by the fluorescent intensity from 10,000 cells with a flow cytometer, FACSCalibur (Becton Dickinson), using 488 nm and 515–545 nm as the excitation and emission wavelength, respectively. We first used control SCC61b cells without HPF as the negative control to calibrate the machine to make the background fluorescence below 10. The mean of fluorescence per cell for the cell population with fluorescence above 10 was obtained by the CellQuest software.

Detection of mRNA levels of BLMH by real-time polymerase chain reaction

Steady-state mRNA levels of human *BLMH* gene were evaluated by performing a real-time polymerase chain reaction (PCR) with the ProbeLibrary (UPL) platform provided by Roche (Mannheim, Germany), which has been described in details in our recent work for other genes [13]. Total RNA was isolated by the Trizol reagent (Invitrogen, Carlsbad, CA) and cDNA was produced by the Reverse-iT First Strand Synthesis Kit (ABgene, Epsom, UK). Real-time PCR was performed by using the LightCycler TaqMan Master Kit (Roche) and the LightCycler System 1.0 (Roche). *ACTB*, the gene coding for β-actin, was used as the reference gene. The probes with the structure of locked nucleic acid (LNA) and primers for each probe were selected by using the ProbeFinder software (www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). The number of probe used for *BLMH* and *ACTB* was 25 and 64, respectively. The expression of *BLMH* was normalized by that of *ACTB* in each sample and the relative amount of normalized *BLMH* mRNA to one calibrator sample, one of SCC61b sample, was obtained by the method of relative quantification in the LightCycler software 4.05 (Roche).

Statistical analysis

Data were analysed by the SPSS software (Chicago, IL). One-way analysis of variance (ANOVA) and two-tailed student *t*-test were used to determine the significance of difference among multiple groups and between two groups, respectively. Bonferroni method was used to obtain *p*-values between any two groups in multiple comparisons. Data were represented as mean ± SD. Statistical significance was considered when *p*-values were smaller than 0.05.

Results

SBLM24 clone exhibited a high degree of resistance to BLM relative to SCC61b clone

Results of the clonogenic assay showed that IC_{50} of BLM on survival of colonies from individual cells in SCC61b and SBLM24 were 0.08 and 1.19 μg/ml, respectively (Figure 1). Therefore, the degree of resistance to BLM, estimated by the extent of augmentation in IC_{50} relative to SCC61b, in SBLM24 clone has approximately increased 15-fold. Next, since cell cycle arrest at G_2/M phase was a characteristic action of BLM, the ability of SBLM24 cells to suppress bleomycin-induced G_2/M arrest was evaluated. There was a dramatic induction of G_2/M arrest in SCC61b cells after 24-h BLM treatment at the dose of 10 μg/ml, but this effect was completely abolished in SBLM24 cells (Figure 2). However, non-confluent control cells of SBLM24 cells had a greater percentage of G_1 phase and lower fractions at S and G_2/M phases compared with SCC61b cells at basal conditions, which was consistent with slower growth rate of SBLM24 clone compared with SCC61b clone (data not shown).

Protein levels and activities of multiple antioxidant enzymes were augmented in SBLM24 clone

To investigate whether protein levels of multiple antioxidant enzymes were altered in SBLM24 cells

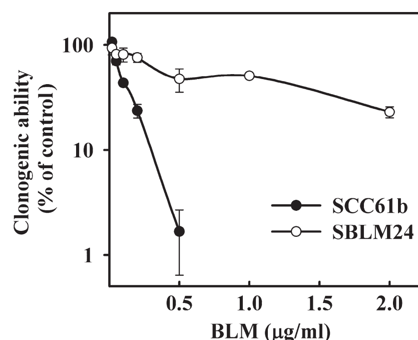

Figure 1. Evaluation of the sensitivity of SBLM24 clone to BLM in comparison with SCC61b clone by the clonogenic assay. There were three replicates for each data point. The y-axis was in log scale. Following 24-h BLM treatment at different doses, the IC_{50} of SCC61b and SBLM24 on surviving fraction was 0.08 and 1.19 μg/ml, respectively. There was no colony survived at the doses beyond 0.5 μg/ml for SCC61b clone.

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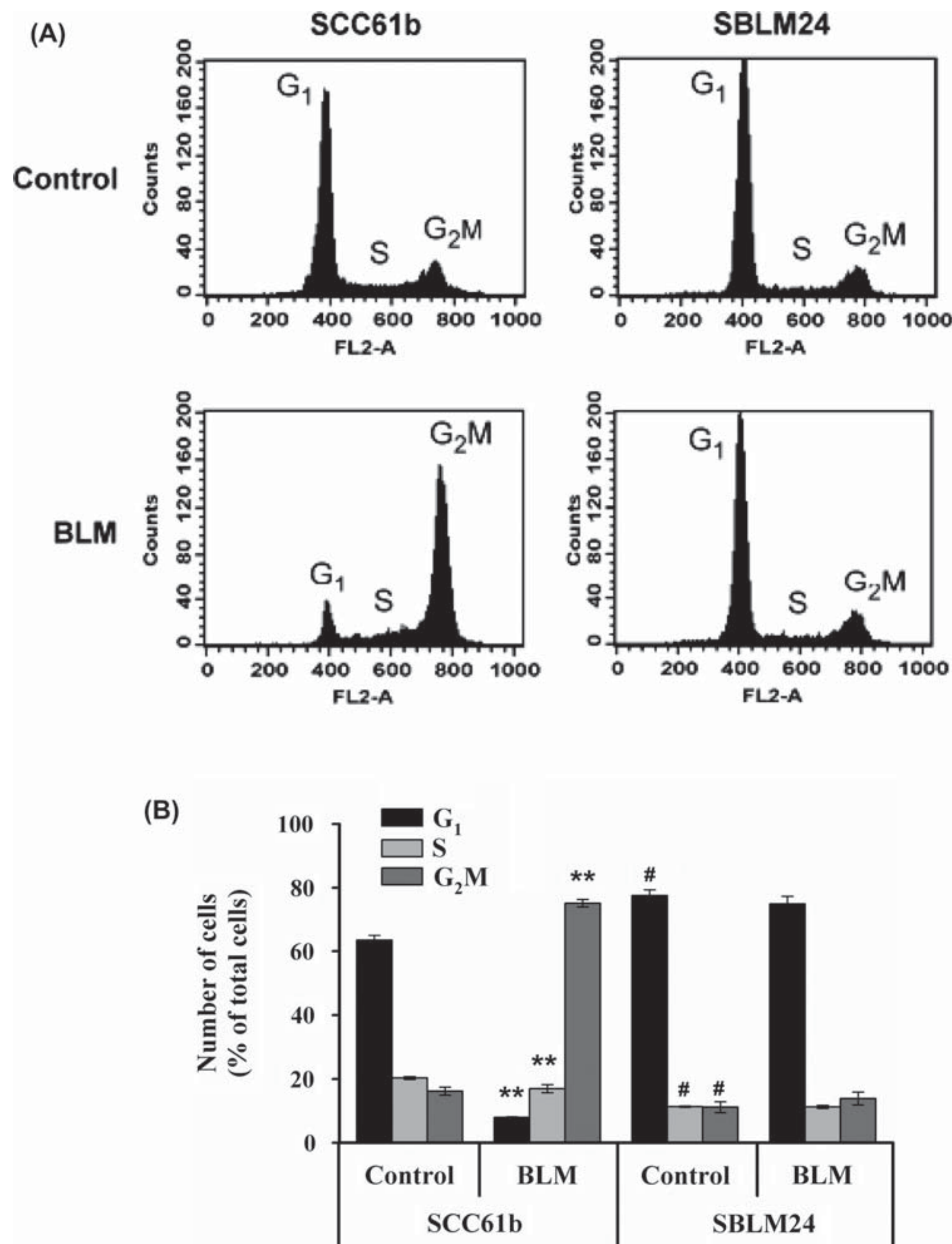

Figure 2. The resistance of SBLM24 clone to BLM-induced cell cycle arrest at G_2/M phase. (A) Representative histograms of cell cycle distribution from flow cytometric analysis in SCC61b and SBLM 24 clones before and after 24-h BLM treatment at the dose of 10 μg/ml. (B) Results of cell cycle analysis from four replicates. There was a significant G_2/M accumulation and decrease in G_1 phase in SCC61b clone, but not SBLM24 cells. ** $p < 0.001$ for the comparison between BLM-treated group and controls of SCC61b cells; $^{\#}p < 0.005$ for the comparison between controls of SCC61b and SBLM24 cells.

compared with SCC61b cells, Western blot analysis was first performed (Figure 3). There was a significant increase in protein levels of CuZnSOD, MnSOD, GPx1, catalase and TrxR1, but not TrxR2, in SBLM24 cells compared with SCC61b cells. The statistical significance for the extent of increase was less for catalase than for other proteins. Furthermore, activities assays confirmed that activities of CuZnSOD, MnSOD, total GPx and catalase were also significantly elevated in SBLM24 cells (Table I). The activities have increased ~ 7.7-, 3.2-, 5.2- and 1.5-fold for CuZnSOD, MnSOD, GPx and catalase, respectively.

Endogenous CoQ_{10} levels were elevated in SBLM24 clone

To further explore the possible involvement of CoQ_{10} in BLM resistance, we examined whether endogenous levels of CoQ_{10} in BLM-resistant clones were altered. The CoQ_{10} levels in SCC61b and SBLM24 cells were 42.8 ± 4.3 and 59.9 ± 5.4 ng/10^6 cells, respectively

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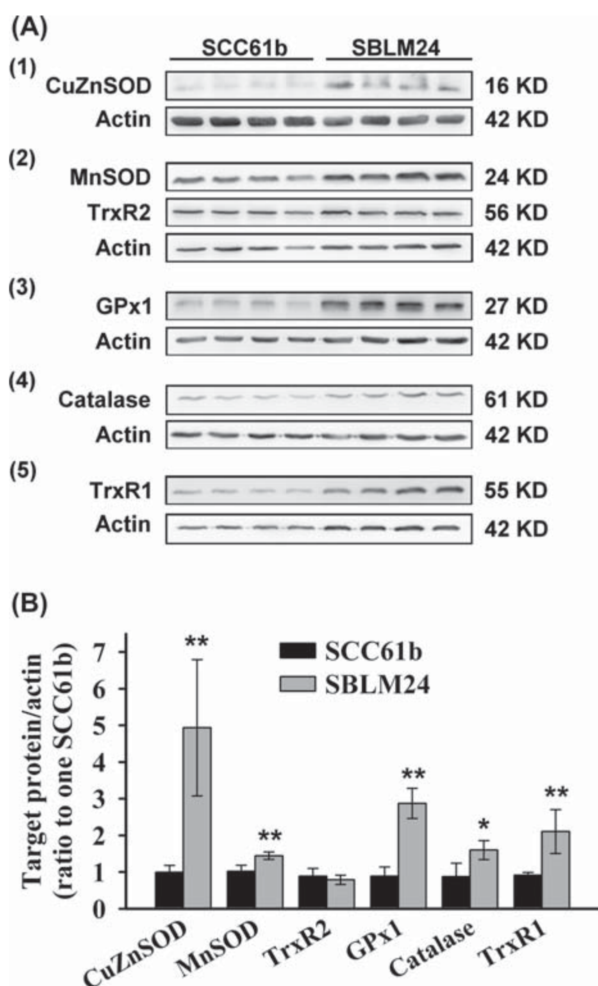

Figure 3. Protein levels of antioxidant enzymes in SCC61b and SBLM24 clones detected by Western blot analysis. (A) Five different nitrocellulose membranes, (1)–(5) blots, were used to detect protein levels of different antioxidant enzymes. There were four replicates for each clone. Actin, which was used as a normalizing control, was detected for each blot. (B) The ratio of the chemilunicescent density for each antioxidant enzyme normalized by actin in each replicate relative to one SCC61b control was obtained. Results from four replicates in SCC61b and SBLM24 clones were compared. * $0.01 \le p < 0.05$ vs SCC61b; ** $p < 0.01$ vs SCC61b.

(Table I). The results showed that endogenous levels of CoQ_{10} per cell were significantly increased in SBLM24 cells compared with SCC61b cells.

BLM-induced cytotoxicity was enhanced by 4AB

To examine the role of CoQ_{10} in BLM-induced cytotoxicity, an inhibitor of CoQ biosynthesis that can inhibit polyprenyl-4-hydroxybenozate transferase [32] and has been shown to suppress CoQ_{10} levels in human cells [12] was used. The condition that could decrease approximately half of CoQ_{10} levels in SCC61b and SBLM2 clones without affecting cell growth were found to be two consecutive 24-h treatment of 4AB at the dose of 100 μM 48 h before the confluence of cell growth. The sensitivity of SCC61 and SBLM24 clones to the acute BLM treatment for 24 h at the dose of 2 μg/ml in the absence or presence of 4AB treatment was evaluated by counting cell number and presented as percentage to one control in each clone. As shown by Figure 4, SBLM24 clone with high BLM resistance was indeed less sensitive to growth inhibitory effect of the acute BLM treatment. Moreover, 4AB treatment enhanced growth inhibition by BLM relative to control groups in both SCC61 and SBLM24 clones. However, the significance of increase in SBLM24 was greater as there was 8% and 15% more inhibition by 4AB in SCC61b and SBLM24 clones, respectively.

The enhancement of ROS production by BLM was suppressed in SBLM24 clone

Because we have previously shown that BLM could increase intracellular ROS levels in human cells [16], it would be important to examine whether BLM-induced ROS production could be attenuated in SBLM24 cells. As demonstrated by Figure 5, ROS levels were significantly augmented after 18-h BLM treatment at the dose of 10 μg/ml. This augmentation was completely attenuated in SBLM24 clone. Moreover, the basal ROS levels without the presence of BLM were also lower in SBLM24 cells compared with SCC61b cells (Figure 5). The results indicated that up-regulation of antioxidant enzymes and CoQ_{10} levels in SBLM24 cells were strongly associated with a reduction of basal ROS levels and resistance to BLM-induced oxidative stress in SBLM24 cells.

SBLM24 clone was much more resistant to H_2O_2 than SCC61b clone

To further demonstrate the adaptive changes in response to oxidative stress in SBLM24 clone, clonogenic assays were conducted after H_2O_2 treatment at different doses for 3 h. Figure 6 showed that BLM-resistant SBLM24 cells also exhibited a high degree

Table I. Activities of antioxidant enzymes and intracellular levels of CoQ_{10} in SCC61b and SBLM24 clones.

Clone	CuZnSOD (units/mg)	MnSOD (units/mg)	GPx (nmol/min/mg)	Catalase (nmol/min/mg)	CoQ_{10} (ng/10^6 cells)
SCC61b	9.6 ± 6.9 (5)	28.1 ± 7.9 (5)	13.1 ± 1.8 (4)	33.9 ± 5.8 (3)	42.8 ± 4.3 (6)
SBLM24	73.6 ± 14.6 (5)**	90.5 ± 11.8 (5)**	68.1 ± 6.3 (4)**	49.9 ± 6.2 (3)*	59.9 ± 5.4 (6)**

Data were presented as mean ± SD (number of replicates). * $0.01 \le p < 0.05$ vs SCC61b; ** $p < 0.01$ vs SCC61b.

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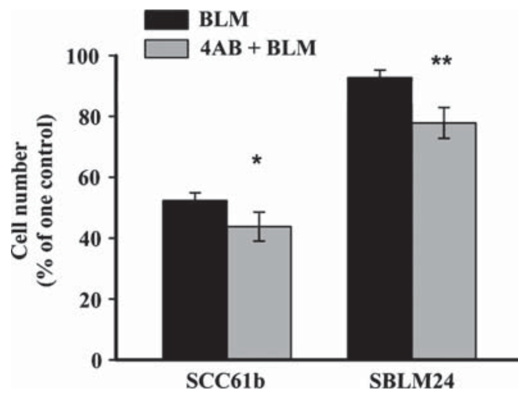

Figure 4. Effect of 4AB on the sensitivity of cells to acute BLM-induced cytotoxicity. Cells were treated with BLM at the dose of 2 μg/ml for 24 h for the "BLM" group" 24 h before cells reached confluence. For the 'BLM + 4AB' group, cells were pre-treated with 4AB at the dose of 100 μM 24 h before BLM treatment and then followed by BLM treatment in combination with second 4AB treatment. Cell number at the end of BLM treatment was counted to evaluate the cytotoxicity and data were presented as a percentage to one control sample without any treatment for each clone. There were four replicates in each group. $^{*}0.01 \leq p < 0.05$ for the comparison between BLM only group and BLM plus 4AB group in SCC61b clone; $^{**}p < 0.005$ for the comparison between BLM only group and BLM plus 4AB group in SBLM24 clone.

of cross-resistance to H_2O_2. IC_{50} of H_2O_2 on survival of colonies in SCC61b clone and SBLM24 clone were 75 and 460 μM, respectively. Therefore, there was a 6-fold increase in the resistance of SBLM24 cells to H_2O_2 relative to that of SCC61b cells.

Steady-state mRNA levels of BLMH were up-regulated in SBLM24 clone

Because up-regulation of BLMH has been a major hypothesis for BLM resistance indicated in the literature, mRNA levels of *BLMH* were checked in SCC61b and SBLM24 clones. Protein levels were not detected because the protein expression was too low to be detected by commercially available antibodies. Results

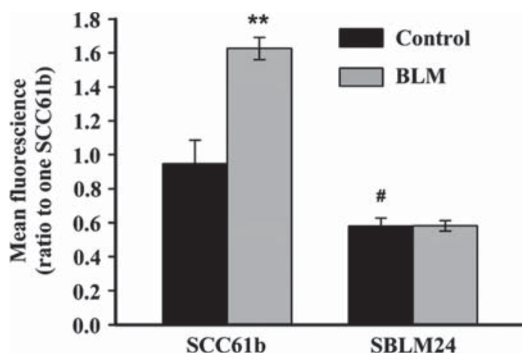

Figure 5. Intracellular ROS levels after BLM treatment. ROS levels were enhanced after 18-h BLM treatment at the dose of 10 μg/ml in SCC61b clone, but not in SBLM24 clone. There were three replicates in each group. $^{**}0.01 \leq p < 0.001$ for the comparison between BLM-treated group and controls of SCC61b cells; $^{\#}p < 0.005$ for the comparison between controls of SCC61b and SBLM24 cells.

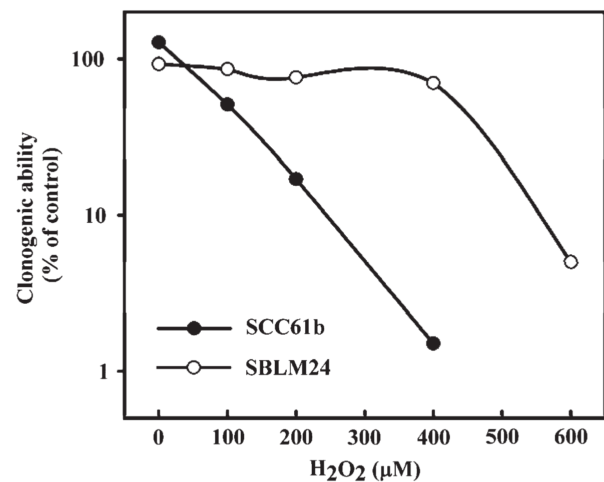

Figure 6. The cross-resistance of SBLM24 clone to H_2O_2 evaluated by clonogenic assays. Following 3-h H_2O_2 treatment, the IC_{50} of SCC61b and SBLM24 was 75 and 460 μM, respectively. There was no colony survived for SCC61b at the dose of 600 μM. Each data point was the average of two replicates.

of quantitative real-time PCR showed that there was a 1.5-fold increase in mRNA levels of *BLMH* in SBLM24 clone compared with SCC61b clone (Figure 7).

Discussion

We have selected a BLM-resistant oral cancer cell line with strong resistance to BLM after long-term culturing the cells in BLM. Our results demonstrate for the first time that up-regulation of antioxidant enzymes and CoQ_{10} levels are associated with the development of acquired BLM resistance and indicate that these alterations play an important role in the suppression of BLM-induced ROS formation and the cross-resistance to H_2O_2 in BLM-resistant cells. Although BLM has been known to cause oxidative stress for a long time, the development of adaptation to oxidative stress as an additional mechanism for BLM resistance in human cancer cells is first indicated by our current

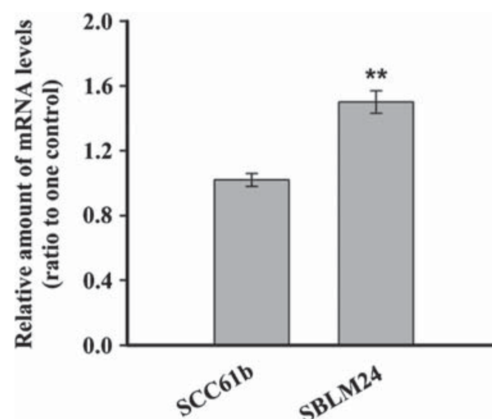

Figure 7. Results of real-time PCR analysis for mRNA levels of *BLMH*. There were five replicates in each group. $^{**}p < 0.01$ vs SCC61b.

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findings, even though up-regulation of BLMH is also involved in our model.

We have previously reported that acute BLM treatment increased expression of GPx1 and GPx activities, but not MnSOD, CuZnSOD or catalase, in both normal and transformed human lung fibroblast cells [16]. In contrast to selective enhancement of GPx activities in the acute effect of BLM, a panel of multiple antioxidant enzymes was up-regulated in SBLM24 cells during the development of strong BLM resistance in this study. It implies that the coordination of multiple antioxidant enzymes, rather than an action of a single enzyme, should be important for the development of acquired resistance of SCC61 cells to BLM-induced oxidative stress. Moreover, this kind of phenomenon has never been found in human cancer cells with acquired resistance to any kind of chemotherapeutic agents previously, although the potential roles of enhanced expression of MnSOD in native resistance of cancer cells to etposide [7] and increased TrxR activities in acquired resistance of cancer cells to doxorubicin [8] have been previously reported . It is interesting to note that Zuckerman et al. [23] actually have previously speculated alterations of free radical scavenging pathways as a possible mechanism for BLM resistance in cancer cells by discussing the findings of Matsuda et al. [33] on the gain of BLM resistance in *Escherichia coli* after induction of MnSOD by paraquat. However, this issue has never been further perused in their model or other studies until our current study.

This is also the first study to report up-regulation of endogenous CoQ_{10} levels in cancer cells developing acquired resistance to an anti-cancer drug. It has been shown that BLM treatment increased CoQ_{10} content in normal liver tissues of hamsters [34]. Brea-Calvo et al. [12] also showed that acute treatment of several anti-cancer drugs, camptothecin, doxorubicin, etoposide and methotrexate, could lead to augmentation of CoQ_{10} levels in human cancer cells. Suppression of CoQ_{10} biosynthesis by 4AB enhanced camptothecin-induced ROS formation and apoptosis. Moreover, induction of *COQ7* for biosynthesis of CoQ_{10} through activation of NF-kB, a transcription factor that can be activated by oxidative stress, was important for the survival of cancer cells in response to acute champtothecin treatment [35]. Because our recent work showed that oxidative stress induced by H_2O_2 treatment could augment CoQ_{10} levels in human cells possibly by inducing expression of several genes in the terminal biosynthetic pathway of CoQ [13], it was reasonable to expect that chronic oxidative stress during long-term BLM treatment could increase CoQ_{10} levels in SBLM24 clone. Furthermore, our current results on the sensitization of BLM-induced cytotoxicity by 4AB with greater extent in SBLM24 clone also supported the notion that CoQ_{10} should partially, although not fully, contribute to the protection against BLM-induced cytotoxicity. On the other hand, although ubiquione in mitochondria can be reduced back to ubiquinol by functional electron transport chain, several reductases, such as TrxR1, in cytosol might be important for regeneration of ubiquinol in cytosol [9]. Increased levels of TrxR1, therefore, might cooperate with antioxidant function of CoQ_{10} in SBLM24 cells. Moreover, the concurrent enhancement of MnSOD and CoQ_{10} levels suggest an important role of mitochondria in BLM resistance, such as the protection of SBLM24 cells against BLM-induced apoptosis.

The reduction of BLM-enhanced ROS production and the cross-resistance to H_2O_2 demonstrated the gain of adaptative alterations in response to oxidative stress in SBLM24 clone. Because BLMH only acts on a specific peptide structure, the acquisition of this adaptation should be unrelated to BLMH. Although our results also showed increased expression of BLMH in SBLM24 clone, the possible involvement of up-regulation of antioxidant enzymes and CoQ_{10} levels in acquired BLM resistance should not conflict with the BLMH hypothesis since both mechanisms could co-exist. The reasons are that the moderate increase in BLMH expression (1.5-fold) might not sufficiently account for the high BLM resistance (15-fold) in SBLM24 clone and that the gain of adaptation to oxidative stress might attenuate cell death or cell cycle arrest resulting from BLM-induced oxidative stress in SBLM24 clone. Moreover, the phenomenon of acquired ability to resist oxidative stress during development of BLM resistance might be related to the mechanism of the cross-resistance of BLM-resistant cell lines to ionizing radiation or etoposide [22,23] and the cross-resistance between cyanomorpholino derivative of doxorubicin and BLM, which was related to glutathione metabolism [36], reported in other studies.

Because BLM can induce DNA strand breaks [37], it can cause G_2M arrest [17] since DNA strand breaks leads to inhibition of Cdc25 through different pathways [38]. However, it is also well known that oxidative stress can result in G_2M arrest [39,40]. ROS has been indicated to regulate the cell cycle via mechanisms related to modulation of protein phosphorylation/dephosphorylation or protein ubiquitination [40]. Therefore, adaptation to oxidative stress in SBLM24 cells might partially contribute to suppression of G_2M arrest induced by BLM and, therefore, BLM resistance. The direct relationship between antioxidant enzymes and regulation of cell cycle is not well established so far, but it has been shown that over-expression of MnSOD promoted the exit of G_2/M arrest in irradiated cells and protected against ionizing radiation-induced cell death independent of p53 [41]. On the other hand, the results of cell cycle analysis indicated that SBLM24 clone exhibited slight but significant arrest at G_1 phase at basal levels. The

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mechanism is currently unknown, but it possibly resulted from lower basal ROS levels because it has been indicated that ROS is important in promoting the signals for cyclin-dependent kinases and retinoblastoma protein in G_1 phase [42].

Taken together, our current findings have provided new insights into the role of oxidative stress in BLM resistance in addition to the action of BLMH and may be implicated in the possible cross-resistance of BLM-resistant cancer cells to other chemotherapeutic agents or radiation therapy that also can cause oxidative stress during cancer therapy. Moreover, the coordinated up-regulation of different antioxidant enzymes localized either in cytosol or mitochondria, in addition to the increase of CoQ_{10} levels, indicates the involvement of both cytosolic and mitochondrial events in BLM resistance.

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Declaration of interest

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