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Changes in Expression of Genes Encoding Antioxidant Enzymes, Heme Oxygenase-1, Bcl-2, and Bcl-xl and in Level of Reactive Oxygen Species in Tumor Cells Resistant to Doxorubicin

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Abstract—The relationship between expression of genes encoding key antioxidant enzymes, heme oxygenase-1, Bcl-2, and Bcl-xl and change in production of reactive oxygen species (ROS) resulting from development of resistance of cancer cells K562, MCF-7, and SKOV-3 to the prooxidant chemotherapeutic agent doxorubicin (DOX) has been studied. Significant increase in mRNA level and activity of Mn-superoxide dismutase (Mn-SOD), catalase, and selenium-dependent glutathione peroxidase-1 (GPx-1) and reduced ROS level was found in resistant K562/DOX and SKVLB cells. In contrast, no change in ROS level was observed in MCF-7/DOX cells in parallel with decrease in Mn-SOD and catalase mRNAs and corresponding activities concurrently with high increase in GPx-1 mRNA and activity. As a result of the development of resistance, a similarity was found between the change in ROS level and the change in *ho-1* and *bcl-2* gene expression, whereas elevation of *bcl-xl* gene expression was observed in all three types of resistant cells. Particular features of development of adaptive antioxidant response as well as redox-dependent change in *bcl-2* gene expression under formation of DOX resistance of cancer cells of different genesis are discussed.

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Key words: drug resistance of cancer cells, doxorubicin, reactive oxygen species, antioxidant enzymes, heme oxygenase-1, Bcl-2, Bcl-xl

Multidrug resistance (MDR) of tumor cells, which significantly decreases the efficiency of chemotherapy, is of multifactorial nature. Several different mechanisms of MDR are known such as enhanced expression of genes encoding some transmembrane transporters (P-gp, MRP, LRP, and BCRP), elevated level of the detoxification system significantly associated with increased level of expression of glutathione S-transferase genes, decreased expression of the gene encoding topoisomerase II with decrease in activity of the enzyme, and changes in expression of genes controlling apoptosis. These factors block genetically programmed cell death [1].

However, the role of free-radical processes in the development of MDR is poorly known [2, 3], despite it being well known that the effect of prooxidant chemotherapeutics on tumor cells is usually associated with development of drug resistance [4]. Reactive oxygen species (ROS) can activate expression of redox-sensitive

Abbreviations: bp) base pairs; DOX) doxorubicin; HO-1) heme oxygenase-1; MDR) multidrug resistance; ROS) reactive oxygen species; RT-PCR) reverse transcriptase-coupled polymerase chain reaction; SOD) superoxide dismutase.

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genes, such as the genes encoding key antioxidant enzymes: catalase (*cat*), selenium-dependent glutathione peroxidase-1 (*gpx-1*), and Mn-superoxide dismutase (*sod 2*) [5], thus regulating the cellular antioxidant defense. Nonetheless, the character of expression of these genes in development of drug resistance remains poorly known. Elevated expression of the heme oxygenase gene (*ho-1*) is regarded as an adaptive and protective response to oxidative stress [6]. The protein HO-1 is induced by several factors including H_2O_2, NO, and UV radiation [7, 8], and potentiates antioxidant status of the cell [9]. In this connection, it is important to study changes in expression of the quick response gene *ho-1* in development of drug resistance of tumor cells.

It is worth noting that ROS influence the transcription level of the Bcl-2 protein family controlling development of programmed cell death [10, 11]. The expression status of this family of proteins in many ways determines sensitivity of cells to apoptosis-inducing factors. High correlation between elevated expression of the genes encoding the antiapoptotic proteins Bcl-2 and/or Bcl-xl on one hand, and increased malignization degree, tumor growth progression, and development of resistance to chemotherapy on the other hand was found in many tumor types [11]. It has been demonstrated that Bcl-2 is distinctly associated with cellular antioxidant status. In particular, mice knocked-out in gene *bcl-2* demonstrated increased sensitivity to prooxidants [12]. However, the mechanism by which the proteins Bcl-2 and Bcl-xl contribute to drug resistance in cancer cells remains poorly understood.

In the present work the character of adaptive antioxidant response is studied in tumor cells of various geneses (K562, MCF-7, SKOV-3) resistant to chemotherapeutic drug with prooxidant effect, doxorubicin (DOX), via evaluation of expression of genes encoding the key antioxidant enzymes and HO-1 and simultaneous evaluation of the link with *bcl-2* and *bcl-xl* gene expression.

MATERIALS AND METHODS

Cell culture conditions. The following cell lines were used in the study: human erythroleukemia K652 (kindly provided by T. M. Grinchuk, Institute of Cytology, Russian Academy of Sciences, St. Petersburg); human breast carcinoma MCF-7 and human ovarian carcinoma SKOV-3 (Institute of Carcinogenesis, Cancer Research Center, Russian Academy of Medical Sciences); DOX-sensitive cells (K562/S, MCF-7/S, SKOV-3 with IC_{50} = 0.005, 0.008, and 0.2 μg/ml, respectively); DOX-resistant cells (K562/DOX, MCF-7/DOX, SKVLB with IC_{50} = 4.0, 4.1, and 4.5 μg/ml, respectively). The K562 cells were grown in suspension culture in RPMI 1640 medium (Sigma, USA), whereas MCF-7 and SKOV-3 cells were grown as monolayers in DMEM medium (Sigma). All cells were grown in cellular medium supplied with 10% heat-inactivated fetal calf serum (Gibco BRL, UK), 2 mM of L-glutamine, 100 U/ml of penicillin, and 50 μg/ml of streptomycin at 37°C and in a humid atmosphere containing 5% CO_2. Cell resistance to DOX was achieved by stepwise elevation of the cytostatic concentration in the culture medium.

Evaluation of ROS formation. Cells were grown up to $5 \cdot 10^5$/ml, and then 5 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA) (Aldrich Chem. Co, USA) was added followed by incubation for 1 h at 37°C with subsequent cell disintegration by sonication and centrifugation at 28,000*g*. The level of ROS (H_2O_2) in cell supernatants was evaluated on a Hitachi MPF-4 spectrofluorimeter (Hitachi, Japan) from fluorescence of 2′,7′-dichlorofluorescein (DCF) (λ_{ex} = 488 nm and λ_{em} = 530 nm) produced from DCF-DA [13]. H_2O_2 at concentration 25 μM was taken as the control.

RT-PCR analysis. RNA was isolated using an RNAwiz kit (Ambion, Canada) according to the manufacturer's protocols. About 5 μg of total RNA of each specimen was used as a template for synthesis of the first cDNA chain by the reverse transcriptase Superscript II (Invitrogen, USA). PCR conditions: 94°C for 3 min; 30-40 cycles: 94°C for 30 sec, 55-64°C for 20 sec, 72°C for 30 sec; 72°C for 5 min. Electrophoresis of PCR products was conducted in 1.5-2.0% agarose gel followed by densitometry. Gel analysis was performed using the BioCaptMW software (Vilber Lourmat).

The following primers were used: for Mn-SOD – 5′-AGCTATTTGGAATGTAATCAACTGG-3′ (forward) and 5′-TAAGCAACATCAAGAAATGCTACA-3′ (reverse); for Cu,Zn-SOD – 5′-TGGGCAAAGGTGGAAATGAA-3′ (forward) and 5′-GCGATCCCAATTACACCACAA-3′ (reverse); for catalase – 5′-GCAGATACCTGTGAACTGTC-3′ (forward) and 5′-GTAGAATGTCCGCACCTGAG-3′ (reverse); for GPx-1 – 5′-GCCTGGGCTCCCTGCGGGGCAAGGT-3′ (forward) and 5′-TACGAAAGCGGCGGCTGTACCTGCG-3′ (reverse); for Bcl-2 – 5′-AGATGTCCAGCCAGCTGCACC-3′ (forward) and 5′-GGCATGTTGACTTCACTTGTG-3′ (reverse); for Bcl-xl – 5′-AGCAACCGGGAGCTGGTGGTCGAC-3′ (forward) and 5′-GACTGAAGAGTGAGCCCAGCAGA-3′ (reverse); for HO-1 – 5′-ATGGCCTCCCTGTACCACATC-3′ (forward) and 5′-GCGAAGACTGGGCTCTCCT-3′ (reverse); for β-actin – 5′-CCACGAAACTACCTTCAACTCC-3′ (forward) and 5′-TCGTCATACTCCTGCTTGCTGATCC-3′ (reverse).

Determination of activity of antioxidant enzymes. This activity was determined using a Hitachi-220A spectrophotometer (Hitachi) in supernatants of cell lysates prepared by ultrasonication followed by centrifugation at 28,000*g* [14].

Activities of Cu,Zn-SOD and Mn-SOD were determined from inhibition of cytochrome *c* reduction in the

xanthine–xanthine oxidase system assayed at 550 nm [15, 16], where an amount of the enzyme necessary for 50% decrease in reduction of cytochrome *c* was taken for the activity unit of Cu,Zn-SOD and Mn-SOD. Sodium cyanide (1 mM; Sigma) was used as an inhibitor of Cu,Zn-SOD when measuring Mn-SOD activity. Catalase activity was determined by the reduction of H_2O_2 (Aldrich) at 240 nm [17]. Glutathione peroxidase activity (GPx) was evaluated in a system coupled with glutathione reductase from the rate of NADPH oxidation (Sigma) at 340 nm [18], using H_2O_2, cumene hydroperoxide, or tert-butyl hydroperoxide (Sigma) as substrates [19]. The amount of enzyme necessary for the reduction of 1 μmol H_2O_2 or oxidation of 1 μmol NADPH per min was taken as one activity unit of catalase or GPx, respectively. Protein was determined by the method of Lowry [20].

Chemicals from Sigma, Aldrich, Invitrogen, and Ambion were used in the study.

Statistical data processing was carried out by Student's *t*-test. The data are presented as mean ± standard deviation.

RESULTS

Evaluation of intracellular ROS level. Intracellular ROS level in sensitive and resistant cells was evaluated by fluorescence of DCF formed from DCF-DA as a result of the action of cellular acetoesterases and oxidation of endogenous H_2O_2, enabling wide use of this method for detection of intracellular H_2O_2 level [13].

A decrease in H_2O_2 level was found to occur during the development of resistance to DOX in resistant K562/DOX cells (1.8-fold) and SKVLB (1.5-fold) in comparison with sensitive cells, whereas virtually no change in H_2O_2 level was detected in MCF-7/DOX cells (Fig. 1). No significant difference was found between sensitive and resistant cells in all three lines when H_2O_2 was used at the concentration of 25 μM as a control of maximum DCF response.

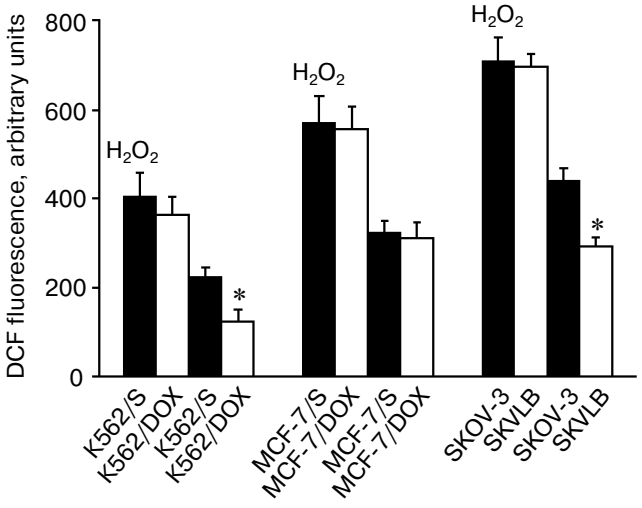

Fig. 1. ROS level in DOX-sensitive (K562/S, MCF-7/S, and SKOV-3) and resistant (K562/DOX, MCF-7/DOX, and SKVLB) cells ($n = 7$). 25-μM H_2O_2 was used as a control. * $p < 0.01$ (statistically significant changes in relation to sensitive cells).

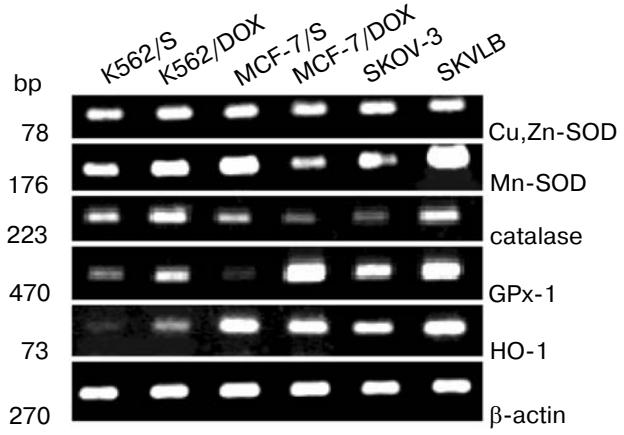

Fig. 2. Electrophoregram of RT-PCR products from DOX-sensitive (K562/S, MCF-7/S, and SKOV-3) and resistant (K562/DOX, MCF-7/DOX, and SKVLB) cells. The level of β-actin mRNA was used as a positive control.

Evaluation of mRNA level and activity of antioxidant enzymes. We found that the cells K562, MCF-7, and SKOV-3 undergoing development of DOX resistance demonstrate oppositely directed changes in the levels of mRNA of key antioxidant enzymes (Fig. 2): Mn-dependent isoform of superoxide dismutase (Mn-SOD) catalyzing dismutation of $O_2^{\cdot-}$ into less reactive H_2O_2 in mitochondria, as well as catalase and selenium-dependent GPx-1 catalyzing decomposition of produced H_2O_2 and, in the case of GPx-1, detoxification of organic hydroperoxides [21].

Significant changes were found in expression level of the *sod 2* gene (Figs. 2 and 3a). Significant increase in the level of mitochondrial Mn-SOD mRNA was found in resistant K562/DOX cells (twofold) and SKVLB cells (threefold) compared with sensitive cells (Fig. 3a). However, a profound (fourfold) decrease in the level of Mn-SOD mRNA was observed in the MCF-7/DOX cells. The same changes were found in activity of Mn-SOD in resistant cells of each line compared with the sensitive cells (table). Maximum gain in Mn-SOD activity was registered in SKVLB cells (3.5-fold) and to a lesser extent (2.5-fold) in K562/DOX cells. In contrast, the Mn-SOD activity was significantly (fivefold) decreased in MCF-7/DOX cells.

However, we did not find significant difference in Cu,Zn-SOD mRNA levels between resistant and sensitive cells of all three lines (Fig. 2). However, change in activi-

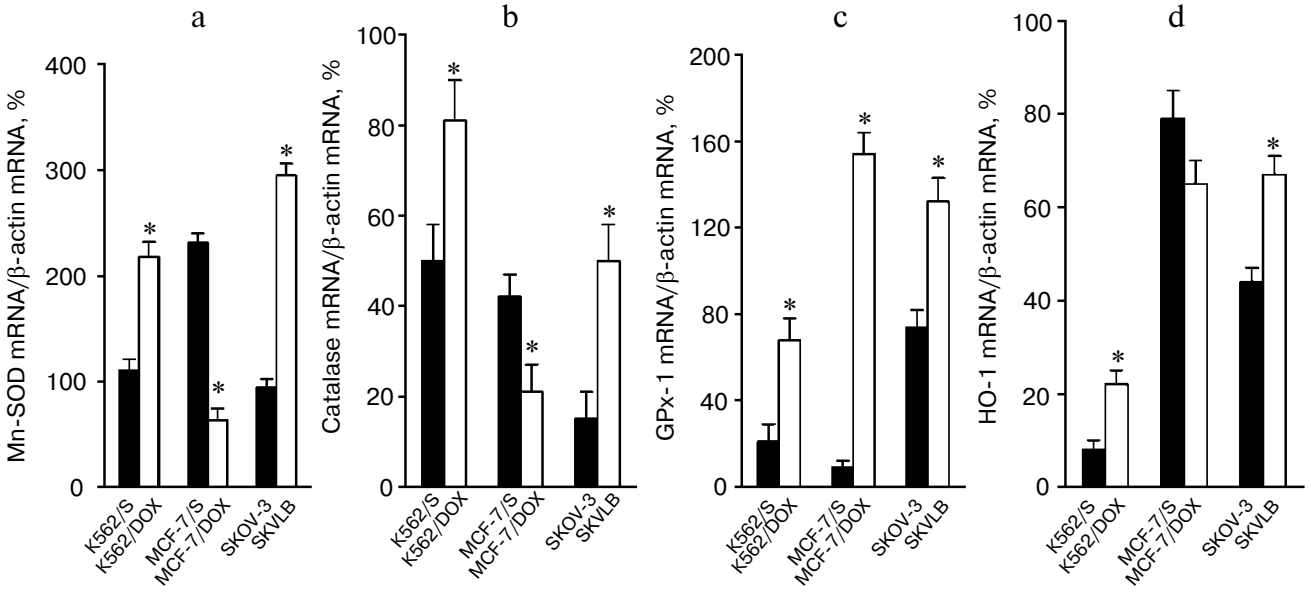

Fig. 3. Levels of Mn-SOD (a), catalase (b), GPx-1 (c), and HO-1 (d) mRNAs in comparison to the level of β-actin mRNA from the data of densitometry ($n = 3$, * $p < 0.05$).

ty of Cu,Zn-SOD was statistically significant in resistant K562/DOX cells compared to the sensitive ones (table).

The level of catalase mRNA was significantly (3.3-fold) higher in resistant SKVLB cells and to lesser degree (1.5-fold) in K562/DOX cells compared to the corresponding sensitive cells (Figs. 2 and 3b). In contrast, the catalase mRNA level was twofold decreased in MCF-7/DOX. The same changes were also observed in catalase activity (table). In particular, expressed elevation (twofold) in this activity was found in SKVLB cells, whereas in K562/DOX cells it was only 1.3-fold. In contrast, the catalase activity was decreased (2.7-fold) in MCF-7/DOX cells.

Significantly enhanced expression of the *gpx-1* gene was observed in resistant cells of all three lines (Figs. 2 and 3c). The maximum growth (17-fold) of the GPx-1 mRNA level was found in MCF-7/DOX cells, to lesser degree in K562/DOX cells (3-fold) and SKVLB cells (1.8-fold). Similarly, MCF-7/DOX cells demonstrate the greatest gain in activity of GPx to H_2O_2, as well as to cumene hydroperoxide and tert-butyl hydroperoxide (3.5-, 6-, and 16-fold, respectively). The same gain was 1.7-, 2.8-, and 2.7-fold in K562/DOX cells and 1.6-, 1.9-, and 2.3-fold in SKVLB cells compared to the sensitive cells (table). It is worth noting that tert-butyl hydroperoxide is a specific substrate for GPx-1, and cumene hydroperoxide for Se-independent GPx (glutathione transferase) [21].

Evaluation of HO-1 mRNA level. The established significant increase in expression of the *ho-1* gene (Figs. 2 and 3d), which is a marker for adaptation to oxidative stress [6], might serve as confirmation to significant

Activities of antioxidant enzymes in sensitive and doxorubicin-resistant cells K562, MCF-7, and SKOV-3

Cell line	Cu,Zn-SOD, U/mg protein	Mn-SOD, U/mg protein	Catalase, μmol/min per mg protein	Glutathione peroxidase, nmol/min per mg protein		
				H_2O_2	cumene hydroperoxide	tert-butyl hydroperoxide
K562/S	10.1 ± 0.6	4.11 ± 0.91	12.9 ± 1.1	5.35 ± 0.68	3.13 ± 0.48	4.11 ± 0.71
K562/DOX	13.1 ± 1.1*	10.3 ± 1.3**	16.8 ± 1.2*	9.11 ± 0.48**	8.74 ± 0.58***	11.3 ± 1.1***
MCF-7/S	7.53 ± 0.86	6.02 ± 1.2	9.72 ± 1.02	4.05 ± 0.32	1.92 ± 0.48	1.12 ± 0.25
MCF-7/DOX	8.74 ± 1.10	1.22 ± 0.48**	3.61 ± 0.88**	14.2 ± 0.8***	11.4 ± 0.6***	18.2 ± 1.2***
SKOV-3	13.3 ± 1.1	4.61 ± 0.74	6.43 ± 1.12	13.2 ± 1.2	4.51 ± 0.95	3.22 ± 0.58
SKVLB	12.7 ± 1.2	16.4 ± 1.3***	13.2 ± 1.2**	21.3 ± 1.1**	8.53 ± 1.38*	7.33 ± 1.21**

Note: Differences between resistant and sensitive cells are statistically significant: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The data are given as mean ± standard deviation ($n = 5$-7).

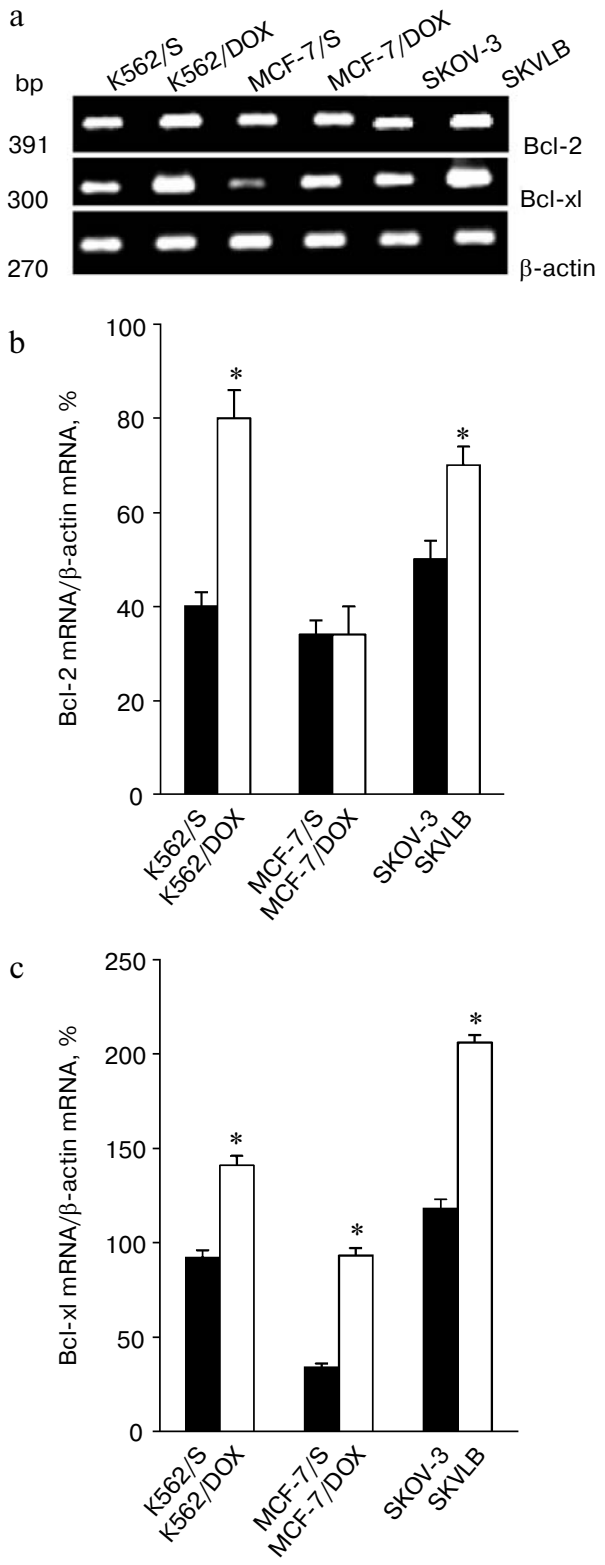

Fig. 4. RT-PCR of Bcl-2 and Bcl-xl in DOX-sensitive (K562/S, MCF-7/S, and SKOV-3) and resistant (K562/DOX, MCF-7/DOX, and SKVLB) cells. a) Electrophoregram of RT-PCR products. The level of β-actin mRNA was used as positive control. b, c) Evaluation of Bcl-2 and Bcl-xl mRNA levels, respectively, in relation to the level of β-actin mRNA using the data of densitometry (n = 3-4). * $p < 0.05$.

change in antioxidant status of K562/DOX cells. The level of HO-1 mRNA in K562/DOX cells about three times exceeded that for sensitive K562/S cells (Fig. 3d). At the same time, lesser increase (1.5-fold) in HO-1 mRNA level was observed in resistant SKVLB cells, and virtually no effect was observed in MCF-7/DOX cells.

Evaluation of Bcl-2 and Bcl-xl mRNA levels. A decrease in the intracellular ROS level was accompanied by noticeable change in expression of the *bcl-2* gene during development of resistance of K562 and SKOV-3 cells. It was found that the Bcl-2 mRNA level was elevated to a greater extent in resistant K562/DOX cells (2-fold) compared to SKVLB cells (1.4-fold) (Figs. 4a and 4b). At the same time, no change in the level of Bcl-2 mRNA was found in the MCF-7/DOX cells. In contrast, change in expression of the *bcl-xl* gene in resistant cells was opposite (Figs. 4a and 4c). Elevation in the Bcl-xl mRNA was observed in all three lines of resistant cells compared to the sensitive ones. Maximum increase in Bcl-xl mRNA was in MCF-7/DOX cells (2.5-fold) and to lesser extent in SKVLB cells (1.7-fold) and K562/DOX cells (1.5-fold).

DISCUSSION

The cytotoxic effect of DOX is largely associated with prooxidant activity caused by the presence of quinone in its structure. Under the effect of some flavoproteins, such as NADPH:cytochrome P-450 reductase, NADH-dehydrogenase, and xanthine oxidase, catalyzing one-electron reduction, a free-radical semiquinone form of doxorubicin is produced, which can be recycled in the presence of molecular oxygen to form superoxide anion with subsequent generation of a ROS cascade including H_2O_2 and highly reactive $^{\bullet}OH$ radicals, whose production is significantly accelerated in presence of Fe^{2+} [22].

Oxidative stress caused by DOX leads to activation of lipid and protein peroxidation and DNA degradation [23]. At the same time, DOX can cause a significant decrease in activities of antioxidant enzymes, such as catalase, SOD, and GPx [24], resulting from oxidative modification of their amino acid residues, as well as aggregation and fragmentation of protein molecules due to the oxidative stress [25].

The development of drug resistance of tumor cells, which is a multifactorial process, might involve free-radical processes leading to development of adaptive antioxidant response [14, 26].

We have demonstrated that significant changes in expression of genes encoding key antioxidant enzymes take place during the development of DOX resistance in three cell lines of different genesis: human erythroleukemia K562, human ovarian adenocarcinoma SKOV-3, and human breast adenocarcinoma MCF-7; these changes might cause changes in intracellular ROS level.

The lines studied possess different initial sensitivity to cytotoxic effect of DOX (IC_{50} = 0.005, 0.008, and 0.2 μg/ml for K562/S, MCF-7/S, and SKOV-3 cells, respectively) and virtually the same level of formed resistance (IC_{50} = 4.0, 4.1, and 4.5 μg/ml for K562/DOX, MCF-7/DOX, and SKVLB cells, respectively), but demonstrate diverse types of adaptive antioxidant response.

Significant growth of Mn-SOD, catalase, and GPx-1 expression was found in resistant K562/DOX and SKVLB cells, which might contribute to an observed decrease in H_2O_2 level in these cells, because concerted growth of Mn-SOD, catalase, and GPx-1 expression is an effective mechanism blocking the growth of ROS level [27]. Significant elevation of both the mRNA level and activity of mitochondrial Mn-SOD rather than the cytosolic Cu,Zn-SOD in these cells is probably due to the fact that, unlike expression of *sod 1* gene, which is commonly constitutive, expression of the *sod 2* gene depends on the redox status of the cell and can be induced by various factors [28, 29].

However, unlike resistant SKVLB cells, in which a preferable growth of catalase mRNA level and activity was found, the K562/DOX cells are characterized by higher mRNA level and activity of GPx-1, an enzyme which is more specific to H_2O_2 than catalase [30]. Moreover, unlike catalase preferably localized in peroxisomes, GPx-1 is found both in cytosol and particularly in mitochondrial matrix [31]. This makes it highly functional in catabolism of H_2O_2. GPx-1 also possesses activity against organic peroxides [21], which heightens its role in antioxidant defense under the influence of oxidative stress.

In turn, development of resistance of MCF-7 cells to DOX leads to quite another balance between changes in expression of genes encoding key antioxidant enzymes: a significant growth of GPx-1 mRNA level and significant growth of GPx-1 activity against both H_2O_2 and organic hydroperoxides (cumene hydroperoxide and tert-butyl hydroperoxide) is observed with decrease in both Mn-SOD and catalase mRNA and activity. These data are confirmed by data from the literature [14, 32]. Others have reported on elevation of Mn-SOD activity in MCF-7/DOX cells [33]. In connection with this, one can accept as correct the supposition of [14] (which assumes different stages of development of adaptive antioxidant response due to peculiarities in development of MCF-7 cell resistance to DOX). High level of GPx-1 mRNA may be considered as an effect of specific activation of *gpx-1* gene expression in MCF-7/DOX cells. Clear proof for this supposition is the virtual absence of changes in expression of quick response gene *ho-1* in MCF-7/DOX cells (elevated expression of this gene is interpreted as adaptive and protective response to oxidative stress) [6]. In contrast, significant growth of the HO-1 mRNA level in resistant K562/DOX cells and, to lesser degree, in SKVLB cells corresponds to the ROS level decrease in these cells and seems to reflect the level of adaptive antioxidant response.

It is worth noting that HO-1 or Hsp32 (being heat shock protein) is induced by a wide range of factors including H_2O_2 [7] and, providing formation of bilirubin and inducing expression of ferritin, possesses antioxidant effects [9, 34]. Bilirubin may serve as a trap for peroxyl radicals [35], whereas induction of ferritin as the intracellular depot of iron decreases the risk of the development of oxidative stress [36]. At the same time, HO-1 can probably characterize development of drug resistance. In particular, overexpression of the *ho-1* gene in neurons is a factor associated with resistance to oxidative stress caused by glutamate [37].

Note that changes in both the antioxidant system and intracellular ROS level in resistant cells are accompanied by conspicuous changes in expression of antiapoptotic protein genes belonging to the Bcl-2 family: Bcl-2 and Bcl-xl. We have observed a coincidence between the growth in Bcl-2 mRNA level and change in the intracellular ROS level. The growth in Bcl-xl mRNA level was detected in all three types of resistant cells.

The data on significant decrease in cell resistance to DOX, when antisense oligonucleotides to Bcl-2 mRNA are used [38], is evidence for a significant role of Bcl-2 in development of tumor cell resistance to DOX. However, it was shown in some studies that protective effect of Bcl-2 is associated with reduced production of ROS [39] and inhibition of lipid peroxidation [40]. Along with an inhibitory effect of Bcl-2 on cytochrome *c* release from mitochondria [41], this blocks development of apoptosis. It was also found that the use of antisense oligonucleotides to Mn-SOD mRNA might lead to elevated ROS production [42] and decreased expression of *bcl-2* and *bcl-xl* genes [43], thus indicating an association between their expression and both ROS level and antioxidant status of the cell.

The data appear to indicate tighter association between expression of the *bcl-2* gene and the level of antioxidant response in development of resistance of K562, MCF-7, and SKOV-3 cells to the prooxidant action of DOX compared to that of the *bcl-xl* gene. This may be a result of difference in redox-dependent regulation of cell response.

Thus, formation of resistance of K562, MCF-7, and SKOV-3 cells to DOX demonstrates a specificity of development of adaptive antioxidant response and different degree of redox-dependent regulation of expression of the genes *bcl-2* and *bcl-xl* depending on the cell type.

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