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Cytochrome P450 reductase, antioxidant enzymes and cellular resistance to doxorubicin

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Doxorubicin (DOX*) is one of the most potent anthracycline antibiotics used in the treatment of various hematological and solid malignancies [1]. Unfortunately, the clinical effectiveness of DOX is often limited by dose-dependent cardiotoxicity and development of drug resistance. Energy-dependent, rapid drug efflux has been shown to be a major factor in the resistance of several *in vitro* cell lines to anthracyclines [2, 3], and efflux blockers such as phenothiazines and verapamil enhance DOX retention and cytotoxicity in resistant cells [4, 5]. Several recent studies have shown that anthracycline retention does not always correlate with cytotoxicity [6–8]. Previous studies from our laboratory on DOX-resistant (P388/R-84) mouse leukemic cells have demonstrated that efflux blockers do not produce complete sensitization to DOX [5], suggesting that cellular resistance of these cells to DOX may be multifactorial.

Glutathione (GSH) has been shown to protect tumor cells from the toxic effects of several drugs including DOX [9–11]. Elevated GSH levels have been documented in several drug-resistant tumor cells [9, 10, 12]. In addition, depletion of cellular GSH has been shown to enhance chemosensitivity of resistant tumor cells against several structurally unrelated drugs [9–14]. Our recent studies indicate that despite the similar GSH content of P388 and P388/R-84 cells, DOX cytotoxicity is increased markedly in resistant cells following cellular depletion of this thiol [15]. Efflux blocking by trifluoperazine in P388/R-84 cells depleted of their GSH by incubation with D,L-buthionine-*S,R*-sulfoximine (BSO) does reduce resistance to about 92% of control [15]. These data provide further evidence that factors other than drug efflux may contribute to cellular DOX resistance of P388/R-84 cells. A complete understanding of the biochemical mechanism(s) of resistance to DOX is needed, therefore, to increase therapeutic potential of this antibiotic in resistant cells.

Anthracyclines are postulated to cause cellular damage by generation of highly reactive oxyradicals [16, 17], DNA intercalation [18] and through mediation of topoisomerase II [19]. DOX is bio-activated by one electron reduction [20] through mediation of flavin reductase(s) such as NADPH–cytochrome P450 reductase to semiquinone radical, which in the presence of oxygen can produce oxyradicals such as superoxide radical, hydrogen peroxide, and hydroxyl radical [16, 17, 20]. Thus, it is reasonable to speculate that reduced formation of free radicals in resistant cells may also contribute to DOX resistance. This can occur either by reduced activation of DOX (due to down-regulation of flavin reductases) or through enhanced scavenging of oxyradicals (due to overexpression of antioxidant enzymes) in resistant cells. To test these possibilities, we have compared the activities of NADPH–cytochrome P450 reductase and antioxidant enzymes in P388 and P388/R-84 cells.

Materials and Methods

Log-phase suspension cultures of P388 and P388/R-84 (with more than 100-fold resistance to DOX in soft agar colony-forming assay [5]) cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 I.U./mL penicillin, 100 μg/mL streptomycin, and 10 μm 2-mercaptoethanol. We have described previously other resistance characteristics of the P388 and P388/R-84 cells [5, 15].

Cell pellets, after centrifugation at 1000 rpm for 10 min, were washed twice with 10 mM potassium phosphate buffer (pH 7.0) containing 150 mM sodium chloride (PBS). Washed cells were suspended in 10 mM potassium phosphate (pH 7.0) containing 1.4 mM 2-mercaptoethanol for NADPH–cytochrome reductase, catalase and GSH peroxidase assays. For the superoxide dismutase (SOD) assay, cells were suspended in 5 mM potassium phosphate buffer, pH 7.8. Cell sonicates (30 sec × 2) were centrifuged at 14,000 *g* for 45 min to obtain the supernatant fraction.

NADPH–cytochrome P450 reductase activity was determined according to the method of Hrycay *et al.* [21] by monitoring reduction of cytochrome *c*. Catalase and GSH peroxidase activities were measured by the methods of Beutler [22] and Awasthi *et al.* [23] respectively. SOD activity was determined by the xanthine oxidase–ferricytochrome *c* assay [24], which at pH 7.8 distinguishes Cu^{2+}, Zn^{2+}- and Mn^{2+}-SODs by differential sensitivity to 1.5 mM sodium cyanide. Protein content was measured by the method of Bradford [25].

Results and Discussion

DOX is bio-activated by flavin reductases to a corresponding semiquinone radical which, in turn, produces oxyradicals in the presence of oxygen [16, 17, 20]. We have determined NADPH–cytochrome reductase activity in 14,000 *g* supernatant fractions of P388 and P388/R-84 cells, and the results are summarized in Table 1. This activity in P388/R-84 cells was 68% of that in the P388 cells. These data indicate down-regulation of flavin reductase(s) in P388/R-84 cells and suggest that bio-activation of DOX and subsequent free radical formation may be lower in these cells as compared to the P388 cells. However, further studies such as comparative estimation of DOX-stimulated oxyradicals in these cells are needed to prove this contention. In a different study, NADPH–cytochrome reductase activity in DOX-resistant MCF-7 human breast tumor cells was found to be about 85% of that in the sensitive cells [26].

Data on the activities of antioxidant enzymes in P388 and P388/R-84 cells are also presented in Table 1. GSH peroxidase activity with *t*-butyl hydroperoxide as substrate was 1.3-fold higher in P388/R-84 cells when compared to that of P388 cells. These results confirmed our earlier studies where we observed a 1.36-fold increase in this activity in P388/R-84 cells towards cumene hydroperoxide as substrate [15]. Catalase, an enzyme known to decompose hydrogen peroxide, was elevated by 2.45-fold in P388/R-84 cells as compared to that of P388 cells. These data suggest that P388/R-84 cells are better equipped to detoxify hydroxyl radical-induced hydroperoxides and hydrogen peroxide through elevated GSH peroxidase and catalase activities respectively. On the other hand, both Cu^{2+}, Zn^{2+}- and Mn^{2+}-SOD activities were comparable in P388 and P388/R-84 cells, suggesting that these cells are equally capable of scavenging superoxide radicals.

* Abbreviations: DOX, doxorubicin; GSH, glutathione; SOD, superoxide dismutase; BSO, D,L-buthionine-*S,R*-sulfoximine; P388, DOX-sensitive cells; and P388/R-84, more than 100-fold DOX-resistant cells.

Table 1. Activities of NADPH–cytochrome reductase and antioxidant enzymes in P388 and P388/R-84 cells

Enzyme activity (units/mg protein)	Cell line	
	P388	P388/R-84
NADPH–cytochrome reductase*	4.4 ± 0.05†	3.0 ± 0.15
GSH peroxidase‡	91.6 ± 6.5	119.6 ± 10.9
Catalase§	10.6 ± 0.6	26.0 ± 2.3
Cu^{2+}, Zn^{2+}-Superoxide dismutase‖	5.0 ± 0.7	5.3 ± 1.0
Mn^{2+}-Superoxide dismutase‖	1.0 ± 0.1	1.1 ± 0.2

* One unit of enzyme catalyzed the reduction of 1 nmol cytochrome *c*/min at 37°.

† Values are means ± SD of three determinations.

‡ GSH peroxidase was assayed using *t*-butyl hydroperoxide as substrate. One unit of enzyme converted 1 nmol GSH to oxidized glutathione/min at 37°.

§ One unit of enzyme utilized 1 μmol hydrogen peroxide/min at 37°.

‖ One unit of enzyme activity is defined as the amount of SOD needed to inhibit the rate of reduction of cytochrome *c* by 50%.

Differential expression of antioxidant enzymes in DOX-sensitive and -resistant tumor cells have also been reported by other investigators [26–29]. Whereas GSH peroxidase activity toward cumene hydroperoxide was reported to be elevated 12.8-fold in DOX-resistant MCF-7 human breast tumor cells, catalase activity was found to be lower in these cells [26]. Similar to the results of the present study, SOD activity was comparable in DOX-sensitive and -resistant MCF-7 cells [26]. In DOX-resistant human small cell lung cancer cell lines, catalase activity has been found to be elevated markedly [27] when compared to that in the sensitive subline. On the other hand, GSH peroxidase activity was comparable in anthracycline-sensitive and -resistant human leukemic HL60 [28] and myeloma 8226 [29] cells.

In summary, our data suggest that DOX resistance in P388/R-84 cells may result, at least in part, from reduced free radical formation by both suppression of flavin reductase(s) and overexpression of certain antioxidant enzymes such as GSH peroxidase and catalase. In addition, our results, in conjunction with other studies [26–29], indicate that flavin reductase(s) and antioxidant enzymes are differentially altered in cancer cells with acquired or *de novo* resistance to DOX. Further studies are needed, however, to elucidate the mechanism(s) by which the gene expression of these enzymes is regulated in drug-sensitive and -resistant cells.

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Multiple effects of 3,4,5,3′,4′,5′-hexachlorobiphenyl administration on hepatic cytochrome P450 isozymes and associated mixed-function oxidase activities in rainbow trout

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Polychlorinated biphenyls (PCBs) are major environmental pollutants that are potent inducers of hepatic and extrahepatic drug-metabolizing enzymes in mammals [1]. The commercial PCB mixture, Aroclor 1254, simultaneously induces the 3-methylcholanthrene (3-MC)-inducible cytochromes P450IA1 and P450IA2, and the phenobarbital (PB)-inducible cyrochromes P450IIB1 and P450IIB2 [1]. The PCB isomer, 3,4,5,3′,4′,5′-hexachlorobiphenyl (HCB), also induces both cytochromes P450IA1 and P450IA2 [2], but represses rat cytochrome P4502c (UT-A) [3].

In rainbow trout, Aroclor 1254 induces cytochrome P450 LM4b but reduces the level of the constitutive P450 isozyme, LM2 [4]. However, little is known about the effects of individual isomers of PCB on trout P450s. In this report, we examine the effects of HCB administration on the levels and catalytic activities of three previously identified constitutive P450 isozymes from rainbow trout [5], namely LMC1, LMC2 and LMC5, and on mixed-function oxidase (MFO) activities associated with these isozymes. The letters LMC stand for "liver microsomal constitutive" and the numbers 1 to 5 indicate the P450 isozymes in the order of their increasing molecular weight on sodium dodecyl sulfate–polyacrylamide gel electrophoresis [5]. LMC2 is believed to be identical to LM2 previously isolated from β-naphthoflavone (BNF)-treated trout [4, 5].

Methods

Male and female rainbow trout (*Oncorhynchus mykiss*) of the Mt. Shasta strain (14 months old) were injected i.p. with HCB in corn oil, at doses of 1 or 20 mg/kg. A control group for each sex was injected i.p. with corn oil. The fish were killed 5 days after HCB treatment, and livers were removed for tissue fractionation. Liver microsomes were prepared as previously described [5].

Hepatic microsomal protein [6], total cytochrome P450 content [7], and benzo[*a*]pyrene (BaP) hydroxylase activity [8] were measured as described. The NADPH-dependent covalent binding of aflatoxin B_1 (AFB_1) to DNA and progesterone 6β-hydroxylase activity were determined by the method of Yoshizawa *et al.* [9] and Backes *et al.* [10] respectively. All enzyme assays were done at 30° using a 30-min incubation period.

Western blot analysis of cytochrome P450 isozymes in trout liver microsomes was performed according to the method of Miranda *et al.* [11]. The data obtained in this study were analysed by Student's *t*-test.

Results and Discussion

The results of the Western blot analysis of trout liver microsomes demonstrated that HCB produces a selective repression and induction of hepatic P450 isozymes in trout (Table 1). At 20 mg/kg HCB, LMC2 levels were reduced