Pergamon

Biochemical Pharmacology, Vol. 47, No. 2, pp. 421–424, 1994.

0006-2952/94 $6.00 + 0.00

Status of glutathione and glutathione-metabolizing enzymes in menadione-resistant human cancer cells

(*Received* 15 *July* 1993; *accepted* 17 *September* 1993)

Abstract—Cloned menadione (MD)-resistant human breast cancer cell lines have been developed and characterized with respect to glutathione (GSH) content and GSH-metabolizing enzymes. Increases in the activities of γ-glutamyltranspeptidase and glutathione-*S*-transferase were demonstrated in the absence of alterations in the GSH content of two cloned MD-resistant cell lines. The MD-resistant cells also displayed alterations in their growth kinetics, possessing longer doubling times and increased fractions in the G1/O phase of the cell cycle as compared to parental MD-sensitive cells. The possible mechanisms for the resistance to MD, including an increase in repair of MD-induced DNA damage, are discussed.

Key words: menadione, drug resistance, glutathione, γ-glutamyltranspeptidase, DNA damage, glutathione-*S*-transferase

Detoxification of xenobiotics is attributed, in part, to conjugation reactions catalyzed by enzymes belonging to the phase II drug biotransforming proteins such as glutathione-*S*-transferase (GST)* [1]. GST allows for the detoxification of chemicals via conjugate formation with the tripeptide molecule glutathione (GSH). Indeed, the alteration in the genetic expression of GST serves as an indicator of drug resistance. Several drug-resistant cell lines developed *in vitro* exhibit an increase in GST activity as one mechanism of resistance [2–4].

Menadione (2-methyl-1,4-napthoquinone; MD) is a simple napthoquinone molecule that has been utilized widely as a model for studies of oxidative stress. Since several clinically used antineoplastic agents contain in them the quinone moiety, MD-resistant human breast cancer cell lines were developed in order to investigate the contributory role(s) of the quinone moiety to drug resistance. Since GSH is one of the cellular targets of MD and since conjugate formation between MD and GSH has been documented previously [5], we initiated our studies by examining the status of GSH, GST and another GSH metabolizing enzyme, γ-glutamyltranspeptidase (GGT), in these MD-resistant MCF-7 cells. We report here the increase in the enzymatic activity of GST and GGT in the absence of an alteration in the cellular GSH pool in the MD-resistant sublines. Furthermore, drug uptake studies indicate that these resistant sublines take up more radiolabeled MD, with a greater proportion of the radioactive drug associated with the acid-soluble fraction. This redistribution phenomenon suggests that MD is rapidly metabolized by a yet unknown mechanism to possibly an inactive form. The changes described above constitute part of the multifactorial mechanisms of resistance of human cancer cells against the redox cycling agent, MD.

Materials and Methods

MD, glycyl-glycine, 1-chloro-2,3-dinitrobenzene (CDNB) and *p*-nitroanilide were obtained from the Sigma Chemical Co. (St. Louis, MO). The development of menadione-resistant MCF-7 cells, a human breast cancer cell line, was patterned after the pulse drug treatment described previously [6]. Briefly, log phase MCF-7 cells (in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and 100 μg/mL kanamycin) were exposed to 200 μM MD for 1 hr, after which cells were harvested and reseeded in drug-free medium at 10,000 cells/25 cm² culture flasks. After a 3- to 4-week incubation at 37°, cells that survived this dose of MD were harvested by pancreatin and expanded into a cell line; then the pulse treatment was repeated as described above. Ten pulse treatments were performed over a 12-month period of time. Subsequent pulsing did not increase the degree of resistance any further. The heterogenous MD-resistant MCF-7 cells were subsequently cloned by dilution into multiwell plates as previously described [7]. Twelve MD-resistant subclones were obtained and the level of resistance of these subclones to MD was assessed by the trypan blue dye exclusion assay [8]. Two MD-resistant sublines, named herein as BX-200 and HX-200, were chosen for further characterization.

The colony formation assay [9] was used to evaluate the IC_{50} values of MD (concentration of drug that inhibits 50% of colony formation) in MCF-7, BX-200 and HX-200 cells. MD uptake by the cell lines was assessed by exposure of cells to 630 nM [^{3}H]MD (2.4 Ci/mmol; Moravek Corp., Brea, CA) and processed as previously described [10]. Evaluation of the total cellular GSH pool was performed using the method described by Griffith [11]. The bulk GST activity was assessed as described in Ref. 12 using CDNB as the substrate, while GGT activity was measured in whole cells based on the method described by Griffith *et al.* [13]. All reagents utilized in the present study are of the highest quality available. ANOVA (Newman Keuls Multiple Comparison Test) was performed for comparison of values from more than two groups, using the Clinfo statistical program. Values were considered statistically significant if $P < 0.01$.

Results and Discussion

Menadione, a simple napthoquinone, has been widely used as a model compound to study the phenomenon of oxidative stress, resulting from the repeated reduction–oxidation (redox cycling) of MD culminating in the production of reactive oxygen species such as the hydroxyl radical [10]. Several mechanisms of cytotoxic action of MD have been proposed. These include the induction of macromolecular damage, disruption of calcium homeostasis, alteration of pyridine nucleotide pools and depletion of cellular thiol levels [9]. Most of the above reported cellular effects of MD are thought to be a consequence of the oxidative stress induced by MD. Therefore, MD also serves as an excellent model agent to evaluate the molecular

* Abbreviations: GST, glutathione-*S*-transferase; GSH, glutathione; GGT, γ-glutamyltranspeptidase; and MD, menadione.

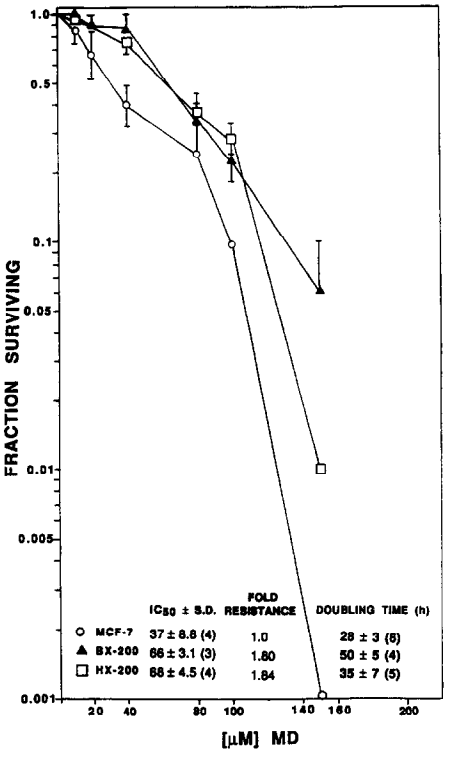

Fig. 1. Cytotoxicity profile of MD in MCF-7, BX-200 and HX-200 cells. The magnitude of MD resistance in BX-200 and HX-200 cells compared with MCF-7 cells was assessed by the colony formation assay [9]. MCF-7 cells were seeded at 1500 cells/60 mm dish, while BX-200 and HX-200 cells were seeded at 3000 cells/60 mm dish. The cell lines were exposed to the indicated MD concentrations for 1 hr, replated and incubated for 8 days in drug-free medium. The IC_{50} values were determined in triplicate, whereas the determination of the doubling times was performed in duplicate. The numbers in parentheses represent the number of times the experiments were performed.

changes in cells that have acquired resistance to oxidant-stress inducing agents. To our knowledge, this is the first reported study of human cancer cells resistant to MD. Figure 1 shows the cytotoxicity profile of MD in MCF-7, BX-200 and HX-200 cells. Both MD-resistant subclones exhibited a relatively low degree of resistance against MD (i.e. ~1.8-fold). The development of low level resistance against this agent is not surprising; MD-resistant Chinese hamsters cells developed and isolated by two independent groups of investigators [14, 15], utilizing the continuous exposure technique, also exhibit resistance against MD ranging from 1.5- to 3-fold. The pulse method of developing drug-resistant cells offers an advantage over the continuous drug exposure technique in that the former may more closely simulate the clinical situation. Therefore, biochemical changes that have occurred in these *in vitro* drug-resistant cell populations may provide more relevant information in terms of clinical drug resistance.

As shown in Fig. 1, MD-resistant subclones showed different growth kinetics as compared with the parental MCF-7 cell line. Both BX-200 and HX-200 cells exhibited longer doubling time than MCF-7 cells. Flow cytometric analysis using the propidium iodide staining technique indicated that a greater fraction of BX-200 and HX-200 cells were in the G1/O phase (58.0 ± 1.0 and 59 ± 2.6%, respectively) as compared with MCF-7 cells (49 ± 2.7%). This was accompanied by a decrease in the percentage of the resistant cell populations present in the S and G2/M phases. The collective changes in the doubling time and cell-cyle distribution in the MD-resistant MCF-7 cells would likely alter the response of these cells to other chemotherapeutic agents, in particular those agents that are cell cycle-specific.

Oxidative stress induced by exposure of cells to quinones has been suggested and demonstrated to contribute significantly to the cytotoxic actions of quinoid compounds. Therefore, modulation of the pathways that would either reduce the formation of MD-mediated free radical formation or increase the detoxifying capacity of the cells would most likely represent potential mechanisms of MD resistance. To investigate this possibility, we evaluated the status of one of the documented cellular targets of MD, glutathione (GSH), in the MD-resistant sublines. The depletion of thiol pools by MD is thought to be due to either the nucleophilic attack of GSH by MD resulting in conjugate formation or as a result of the extensive amounts of free radicals produced during the reduction–oxidation process of MD [5]. In general, it is thought that GSH constitutes one of the important cellular detoxification defenses against various xenobiotics, including the electrophilic quinone molecules. Indeed, increases in the GSH pool have been documented to be a mechanism of resistance of certain antitumor agents [16, 17]. Other investigators, on the other hand, have reported decreased GSH pools in their resistant cell lines [2]. Our MD-resistant sublines provide a system to evaluate the role of GSH in the acquisition of MD resistance. As indicated in Table 1, BX-200 and HX-200 cells did not exhibit statistically significant alterations in the GSH levels ($P > 0.01$). A possible explanation for unaltered GSH pools in the MD-resistant MCF-7 cells is discussed below.

GST, a phase II drug-metabolizing enzyme, catalyzes the conjugation of GSH with xenobiotics, thereby inactivating the xenobiotics for export out of the cell [1]. Due to this action of GST, this enzyme has been widely studied for its role in drug resistance. Several drug-resistant cell lines that were examined demonstrate an increase in GST activity. We examined the status of GST in our MD-resistant sublines. Both BX-200 and HX-200 cells exhibited higher bulk GST activity compared with MCF-7 cells (i.e. 1.8- and 1.4-fold, respectively; Table 1).

Conjugate formation between GSH and xenobiotics has been known to be a form of detoxification. However, thiodione [18], the end product of the GST-catalyzed

Table 1. Status of GSH and GSH-related enzymes in parental and MD-resistant MCF-7 cells

Cell line	Total GSH (nmol/mg)	GST (nmol/mg/min)	GGT (nmol/mg/hr)
MCF-7	55 ± 4 (21)	4.33 ± 0.23 (12)	5.93 ± 0.44 (9)
BX-200	65 ± 5 (22)	7.9 ± 0.30 (10)	10.90 ± 0.78 (10)
HX-200	74 ± 6 (21)	6.24 ± 0.43 (11)	12.47 ± 0.72 (9)

Values are means ± SD. The numbers in parentheses represent the number of times the assays were performed.

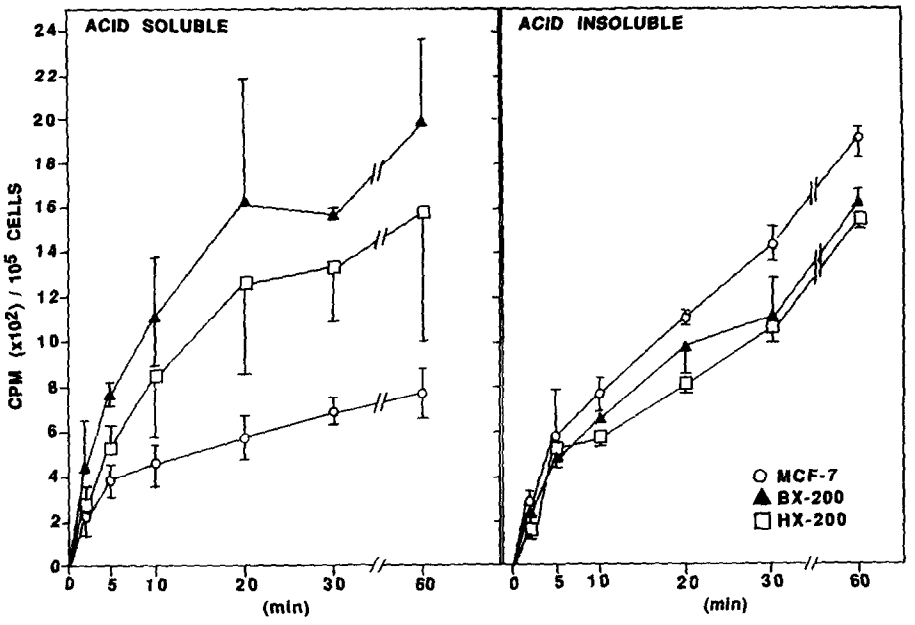

Fig. 2. Time course of [^{3}H]MD uptake in MCF-7 and MD-resistant MCF-7 cells. MCF-7, BX-200 and HX-200 cells (2.5×10^5 cells) were exposed to 630 nM [^{3}H]MD for the indicated periods of time. The radioactivity associated with the acid-soluble and acid-insoluble fractions was then evaluated, as previously described [10]. Values shown are the means ± SD calculated from two separate experiments done in duplicate.

conjugation reaction between MD and GSH, has the ability to undergo redox cycling and has been shown to possess a similar redox potential as the parental MD [5, 19, 20]. Therefore, the increase in GST activity in MD-resistant cells may not represent a detoxification mechanism, since the product of the conjugation reaction is as potent as the parental MD.

γ-Glutamyltranspeptidase (GGT) catalyzes the removal of the γ-glutamyl moiety from GSH or GSH-xenobiotic conjugates [21]. This activity of GGT, in conjunction with that of the enzyme dipeptidase, results in the provision of constituent amino acids for the synthesis of GSH [13, 22]. This function, as discussed later, may likely be one reason why the γ-glutamyltranspeptidase activity in BX-200 and HX-200 cells was increased as compared with the parental MCF-7 cells (Table 1; $P < 0.01$). Other investigators have examined GSH and GGT levels in drug-resistant cells; results from studies performed by Ahmad *et al.* [16] documented the parallel increase in GSH levels and GGT activity in L-PAM resistant cells. On the other hand, increased GST activity was coupled with lowered GGT activity in Adriamycin®-resistant MCF-7 cells, which was associated with a compromised cellular GSH pool [2]. The results presented in our study further corroborate the notion that GGT expression serves not only as a cellular differentiation protein but, more importantly, as an index of drug resistance [23].

It is interesting to note that our MD-resistant MCF-7 cells, strictly speaking, exhibit neither of the above phenomena. The unaltered GSH levels in the MD-resistant subclones are likely due to the existence of an equilibrium between supply (by GGT) and demand. It is possible that the increase in GGT activity in the MD-resistant cells could ensure sufficient levels of GSH for utilization in the repair of DNA damage engendered by MD; GSH has been implicated in the DNA repair process [24, 25]. Indeed, we have noted that the absolute level of MD-induced double-strand DNA breaks in the two cloned MD-resistant cell lines was significantly less (*i.e.* 40–50% less than the breaks produced in MCF-7 cells)* than that produced by MD in the parental MCF-7 cells. Furthermore, results from preliminary studies of the kinetics of repair in the resistant cells indicated that the rate of repair in the resistant cells was higher than that measured in parental MCF-7 cells.* Collectively, the increased GGT activity in conjunction with the decreased numbers of DNA breaks in the MD-resistant cells is consistent with a role of GSH in an enhanced repair process in the resistant cells.

MD uptake mutants were eliminated from the present study. We evaluated the ability of BX-200 and HX-200 clones to take up MD as compared with the parental MCF-7 cells by utilizing radioactively labeled MD. A time course of MD uptake (Fig. 2) suggests that, first, BX-200 and HX-200 cells take up more MD than MCF-7 cells (total cpm/10^5 cells in a 1 hr [^{3}H]MD exposure: MCF-7 = 2670; BX-200 = 3600; HX-200 = 3140); second, the [^{3}H]MD taken up by the resistant subclones seems to be redistributed in a way that a greater proportion of the radioactive drug is associated with the acid-soluble fraction in both MD-resistant MCF-7 cell lines.

Obviously, more questions arise as a result of this study. The identity of the acid-soluble associated radioactive MD awaits further investigation. Identification of this entity would enable us to elucidate the potential cellular mechanism(s) responsible for protecting cells from macromolecular damage engendered by MD. Conjugate formation, catalyzed by the phase II system, may, in some instances, result in the further bioactivation of xenobiotics. In fact, a novel ATP-dependent GSH-*S*-conjugate transmembrane pump has been demonstrated recently in various cell types [26]. This pump aids in the final export of GSH-*S*-conjugates out of the cell. As was suggested by Ishikawa [1], the role of this export pump in drug resistance deserves further examination. Because MD is known to form a conjugate with GSH, our MD-resistant MCF-7 cells provide a good model to evaluate the role of this export pump in the acquisition of chemotherapeutic drug resistance by human cells.

Acknowledgements—This work was supported by 1R29CA53618 from the National Cancer Institute, National

* Ngo EO and Nutter LM, unpublished results.

Institutes of Health; L.M.N. is the recipient of a Junior Faculty Award JFRA 423 from the American Cancer Society.

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