BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **245,** 85–89 (1998)
ARTICLE NO. RC988388

Induction of Multidrug Resistance Gene Expression in Rat Liver Cells in Response to Acute Treatment by the DNA-Damaging Agent Methyl Methanesulfonate

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Received February 25, 1998

Expression of multidrug resistance (mdr) genes encoding the P-glycoprotein (P-gp) drug efflux pump was analysed in cultured rat liver epithelial cells acutely treated by the DNA-damaging agent methyl methanesulfonate (MMS). Exposure to this alkylating agent used at 30 μg/ml for 12 or 24 h was shown to enhance mdr mRNA levels in rat liver cells without alteration of cell viability. Induction of mdr transcripts occurred through increased expression of the mdr1b gene as indicated by reverse transcriptase-polymerase chain reaction analysis using rat mdr gene-specific primers and was not associated with up-regulation of cytochrome P-450 1A1, thereby suggesting that this detoxifying enzyme and P-gp were not coordinately regulated by MMS. In addition, the DNA-damaging agent was found to enhance in a dose-dependent manner cellular efflux of the P-gp substrate rhodamine 123, which was inhibited by the P-gp inhibitor verapamil, thus providing evidence that exposure to MMS led to increased P-gp-related drug transport in rat liver cells. The up-regulation of functional P-gp expression occurring in MMS-treated liver cells may be interpreted as a part of the cellular response to DNA damage.

P-glycoprotein (P-gp) is a plasma membrane phosphoglycoprotein thought to act as an energy-dependent drug efflux pump (1). Its overexpression in tumoral cells lowers intracellular accumulation of various structurally and functionally unrelated antineoplasic agents such as anthracyclines, Vinca alkaloids, epipodophyllotoxins and taxol and, thereby conferring multidrug resistance (mdr) (2). P-gp is encoded by mdr genes, which constitute a small gene family comprizing two members in humans (MDR1 and MDR2) and three members in rodents (mdr1a, mdr1b and mdr2). Only MDR1 in humans and mdr1a and mdr1b (also known as mdr3 and mdr1 respectively) in rodents are involved in drug resistance whereas P-gp encoded by MDR2/mdr2 gene is responsible for hepatic phospholipid transport (3, 4).

P-gp overexpression has been reported to occur in various human cancers and has been correlated in some cases with failure of chemotherapeutic treatment (5, 6). In addition, P-gp is present in several normal tissues including the liver where it is thought to participate to biliary secretion of xenobiotics (7). As for various hepatic detoxication systems, P-gp expression in the liver has been shown to be induced in response to acute exposure to some xenobiotics (8). Indeed, treatments by potent chemical carcinogens such as 2-acetylaminofluorene (9) and polycyclic aromatic hydrocarbons (10) as well as by anticancer drugs such as doxorubicin (11) and mitoxantrone (12) have been demonstrated to result in P-gp overexpression in rat liver cells. The mechanisms involved in up-regulation of hepatic P-gp expression by various drugs remain largely to be determined. A role for the aromatic hydrocarbon (Ah) receptor that mediates chemical induction of drug metabolizing enzymes such as cytochrome P-450 (CYP) 1A1 (13) has been proposed since P-gp and CYP 1A1 have been shown to be co-regulated in response to some xenobiotics (8). It is however noteworthy that a feature shared by most, if not all, P-gp-inducing drugs is to lead to DNA damage. Indeed, exposure to either chemical carcinogens such as 2-acetylaminofluorene or anticancer drugs such as doxorubicin and mitoxantrone has been demonstrated to result in DNA lesions (14, 15, 16). It can therefore be hypothetized that xenobiotic-mediated up-regulation of P-gp in liver cells may be included in the cellular events triggered by chemical DNA-damaging agents. In order to

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0006-291X/98 $25.00

contribute to this hypothesis, we have analyzed in the present study mdr gene expression in cultured rat liver cells in response to acute treatment by the well-known DNA-damaging agent methyl methanesulfonate (MMS).

MATERIALS AND METHODS

Cell culture. Rat liver epithelial cells, established from normal liver of 10-day-old Sprague-Dawley rat according to the procedure of Williams et al. (17) and previously recognized as a suitable model for analyzing hepatic regulatory pathways of P-gp (10, 11), were maintained in monolayer culture in Williams E medium supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. These cells, that are not tumorigenic when injected into syngenic newborn rats (18), were passaged every week with 0.1% trypsin solution and were used for MMS treatment between passages 10 and 30.

Isolation of RNA and northern blot analysis. Total RNAs were extracted from cultured cells by the guanidinium thiocyanate/cesium chloride method of Chirgwin et al. (19). For northen blot analysis, 10 μg total RNAs were subjected to electrophoresis in a denaturing formaldehyde/agarose gel and transferred onto Hybond-N^+ sheets (Amersham, Bucks, United Kingdom). The sheets were pre-hybridized and then hybridized with ^{32}P-labeled probes. Mdr mRNAs were detected with a hamster pCHP1 probe (20) obtained from the American Type Culture Collection (Rockville, MD, USA) whereas CYP 1A1 mRNAs were analyzed with a rat CYP 1A probe (21). After hybridization, sheets were washed, dried and autoradiographed at −80°C. Equal gel loading and efficiency of transfer were checked up by staining 28S rRNAs onto the sheets using methylene blue solution (22).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Expression of mdr1a and mdr1b mRNAs was detected by the RT-PCR methods using rat mdr gene-specific primers as previously described (10). Briefly, total RNA (0.5 μg) was reverse transcribed using 200 units of Moloney murine leukaemia virus reverse transcriptase (Clontech, Palo Alto, CA, USA) and 20 pM of random hexanucleotide primers. An amount of cDNA representing 25 ng of RNA was then subjected to PCR for 25 to 30 cycles in a final volume of 50 μl using 2.5 units of Taq polymerase (Eurobio, Les Ulis, France) and mdr1a, mdr1b or β-actin gene-specific primers (10, 23). Following an initial denaturation of 2 min at 94°C, each cycle consisted of 30 sec at 94°C, 30 sec at 55°C and 60 sec at 72°C. The mdr primers used are located within highly divergent nucleotide sequences of the rat mdr genes and have been demonstrated to discriminate between the different mdr cDNAs (10). Aliquots (5 μl) of RT-PCR products were then subjected to electrophoresis in 2% agarose gel and visualised by staining the gel with ethidium bromide. Several negative control reactions were included in each experiment (e.g., PCRs carried out using water or RNA instead of cDNA). Positive controls for mdr1a and mdr1b RT-PCRs were performed using cDNAs prepared from rat hepatoma RHC1 cells known to constitutively display substantial expression of mdr1a and mdr1b genes (24).

Evaluation of MMS-induced cytotoxicity. MMS effect on cell viability was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (25). Confluent rat liver epithelial cells were incubated with various doses of MMS for 24 h and then with 0.5 mg/ml MTT for 2 h. The blue formazan product formed was dissolved in dimethyl sulfoxide and quantified by its absorbance at 540 nm using a Titertek Multiskan MCC/340 (Flow Laboratories, Puteaux, France).

Evaluation of P-gp activity. P-gp activity was assessed by analysis of cellular rhodamine 123 efflux. Indeed, the fluorescent dye rhodamine 123 is a substrate for P-gp and its transport out of the cell has been demonstrated to reflect P-gp function (26). Cells were incubated with rhodamine 123 (2 μg/ml) for 30 min, washed three times with ice-cold phosphate-buffered saline and then incubated in rhodamine 123-free medium for 1 h, in the absence or presence of verapamil (25 μM), a known inhibitor of P-gp function (1, 2). Intracellular rhodamine 123 concentration was further determined by fluorimetry using a Titertek Fluoroscan spectrophotometer (Flow Laboratories); excitation and emission wavelengths were 485 and 538 nm, respectively. Intracellular rhodamine 123 retention values were expressed as percentages of initial rhodamine 123 accumulation.

Statistical analysis. The data of MTT assays and rhodamine 123 efflux studies were analyzed by the Student's *t*-test. The criterion of significance between the means (± standard deviation) was $P<0.05$.

RESULTS

First for the determination of the cytotoxicity of MMS towards the cultured rat liver cells used, various concentrations of MMS (from 10 to 80 μg/ml) were incubated with the cells for 24 h and cell viability was assessed by the MTT assay (Fig. 1). Results indicated that MMS concentrations up to 40 μg/ml did not alter viability of the liver cells whereas higher MMS doses, i.e. 60 and 80 μg/ml, were moderately and strongly cytotoxic, respectively (Fig. 1). The 30 μg/ml non-toxic concentration was therefore retained for most of the further studies and was thus used to study the effect of several MMS-treatment times on mdr gene expression by northern blotting (Fig. 2). Hybridization with pCHP1 probe demonstrated elevated levels of 4.5 kb mdr mRNA levels in confluent liver cells exposed to MMS for 24 h whereas no mdr transcripts were clearly detected in their untreated counterparts; this effect of MMS on mdr transcripts began after a 12-h treatment and shorter exposure such as a 6-h treatment failed to obviously increase mdr mRNA levels (Fig. 2). Since the mdr pCHP1 probe used in the hybridization experiments did not discriminate between expression of the

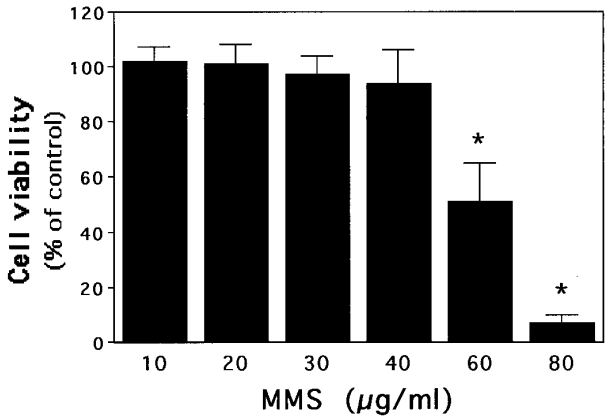

FIG. 1. Effect of MMS on cell viability. Rat liver epithelial cells were exposed to various concentrations of MMS for 24 h. Cell viability was then determined using the MTT assay as indicated in Materials and Methods. The values are expressed as percentages of cell viability found in untreated control cells and are the mean ± SD of three independent experiments in triplicate. *, $P<0.05$ compared with untreated cells.

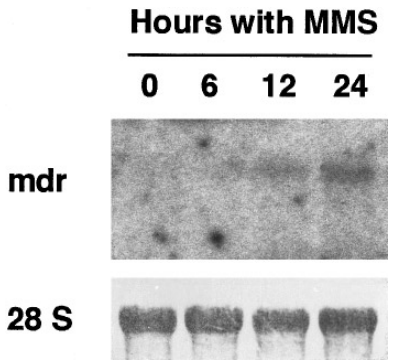

FIG. 2. Induction of mdr mRNA levels in rat liver cells by MMS treatment. Each well contained 10 μg total RNAs isolated from rat liver epithelial cells exposed to 30 μg/ml MMS for various lengths of times (0-24 h). RNAs were then transferred to Hybond N^+ sheets and hybridized with a mdr cDNA probe whereas 28S RNAs were stained using methylene blue solution.

rodent mdr1a and mdr1b genes involved in multidrug resistance (10), RT-PCR assays using rat mdr gene-specific primers were then carried out (Fig. 3). Results indicated that liver cells exposed to MMS showed a marked induction of mdr1b mRNA levels whereas similar amounts of β-actin mRNAs were found in both untreated or treated cells. By contrast, mdr1a transcripts were not detected in either untreated or MMS-treated rat liver cells whereas they were clearly evidenced in drug-resistant RHC1 cells used as positive controls (24) (Fig. 3).

The effect of MMS treatment on P-gp activity in rat liver cells was evaluated using analysis of cellular rhodamine 123 efflux (Fig. 4). Liver cells exposed to 30 μg/ml MMS for 24 h displayed lower rhodamine 123 retention levels than those found in their untreated counterparts after postincubation in rhodamine 123-free medium. Moreover, addition of verapamil strongly increased fluorescent dye retention in MMS-treated cells while it had no major effect in untreated cells (Fig. 4). The effects of various doses of MMS on cellular rhodamine 123 retention were furthermore analysed. As shown in Fig. 5, similar low rhodamine 123 retention values were observed in liver cells exposed to 30 μg/ml or 40 μg/ml MMS when compared to those found in their untreated counterparts. Treatment by 20 μg/ml MMS also resulted in decreased rhodamine 123 retention, although to a less extent whereas the use of 10 μg/ml MMS failed to alter cellular levels of the fluorescent dye (Fig. 5).

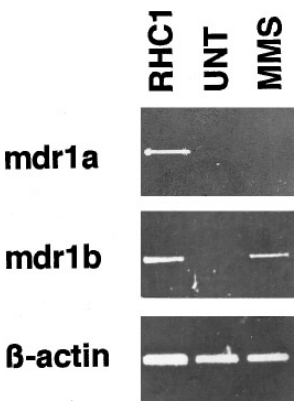

FIG. 3. Effect of MMS treatment on mdr1a and mdr1b mRNA levels in rat liver cells. Expression of mdr1a and mdr1b genes in rat liver cells either untreated (UNT) or exposed to 30 μg/ml MMS for 24 h was analysed by RT-PCR as described in Materials and Methods. Drug-resistant RHC1 cells were used as positive controls for mdr1a and mdr1b gene expression.

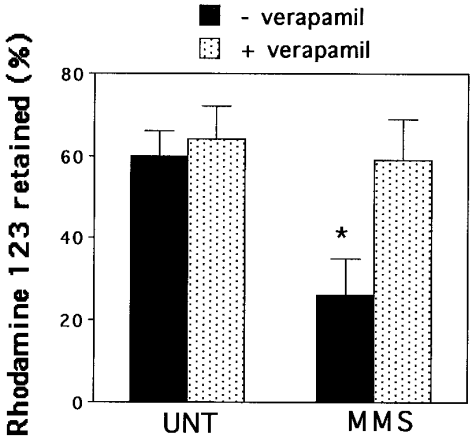

FIG. 4. Verapamil-inhibitable P-gp activity in MMS-treated rat liver cells. Rat liver cells either untreated (UNT) or exposed to 30 μg/ml MMS for 24 h were incubated with rhodamine 123 (2 μg/ml) for 30 min, washed and then incubated in rhodamine 123-free medium for 1 h, in the absence or presence of verapamil (25 μM). Intracellular rhodamine 123 concentrations were further determined by fluorimetry and were expressed as percentages of initial rhodamine 123 accumulation. The values are the mean $\pm$ SD of three independant experiments in triplicate; *, $P<0.05$ compared with untreated cells.

Finally, we examined the effect of 30 μg/ml MMS treatment on expression of CYP 1A1, a detoxifying enzyme that has been postulated to be coordinately with P-gp in the liver in response to xenobiotics (8). Exposure to MMS did not result in CYP 1A1 induction in rat liver epithelial cells while, by contrast, high levels of 2.9 kb CYP 1A1 transcripts were detected in hepatocytes treated by 3-methylcholanthrene and used here as positive controls (Fig. 6).

DISCUSSION

The results reported in the present study indicate for the first time to our knowledge that acute treatment by the DNA-damaging agent MMS lead to induction of mdr gene expression in rat liver cells. Indeed, mdr mRNA levels have been demonstrated to be up-regulated in liver cells in response to short MMS treatments

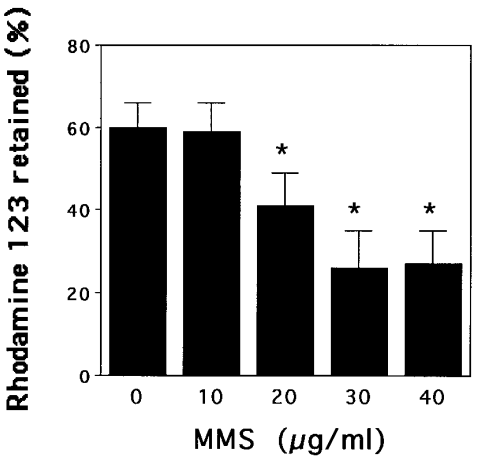

FIG. 5. Dose-dependence of P-gp activity induction in reponse to MMS treatment in rat liver cells. Rat liver cells exposed to various concentrations of MMS (0-40 μg/ml) for 24 h were incubated with rhodamine 123 (2 μg/ml) for 30 min, washed and then incubated in rhodamine 123-free medium for 1 h. Intracellular rhodamine 123 retentions were further determined by fluorimetry and were expressed as percentages of initial rhodamine 123 accumulation. The values are the mean ± SD of three independent experiments in triplicate; *, $P<0.05$ compared with untreated cells.

such as a 12 h- or a 24 h-exposure. Moreover, increased levels of mdr mRNAs in MMS-treated cells were found to be associated with enhanced efflux of rhodamine 123, that was strongly inhibited by verapamil, thus indicating it was related to increased P-gp activity. In addition, this elevated P-gp-mediated rhodamine 123 efflux was demonstrated to be linked to the MMS concentration used for treatment, thus suggesting a dose-dependent effect of this compound with respect to functional P-gp expression.

The up-regulation of mdr gene expression in MMS-treated liver cells occurred through increased expression of the mdr1b gene as evidenced by RT-PCR experiments using rat mdr gene-specific primers. Overexpression of the mdr1b gene has also been reported in rat liver cells acutely exposed to various xenobiotics such as 2-acetylaminofluorene, 3-methylcholanthrene or doxorucin (9, 10, 11). Levels of mdr1b mRNAs are also regulated in hepatocytes in various physio-pathological situations such as regeneration after partial hepatectomy, cholestasis and treatment by the steroid hormone dexamethasone (7, 27). Taken together, these data suggest that the mdr1b gene plays a pivotal role in hepatic regulatory pathways of P-gp, including P-gp induction by xenobiotics.

Induction of mdr1b gene expression in rat liver cells exposed to MMS occurred without concomitant apparent cellular toxicity. Indeed, MMS concentrations (30 or 40 μg/ml) that strongly increased P-gp activity failed to alter cellular viability as demonstrated by MTT assays. Increased functional P-gp expression occurring in response to MMS can not therefore be considered as a consequence of a major cytotoxic effect of the DNA-damaging agent. Up-regulation of mdr1b gene expression by MMS was also not associated with overexpression of CYP 1A1, therefore suggesting that this drug-metabolizing enzyme is not co-regulated with P-gp in liver epithelial cells exposed to MMS. Similarly, the anticancer drug doxorubicin that strongly increases P-gp expression in rat hepatocytes, does not alter CYP 1A1 mRNA levels (11). Taken together, these data favor the idea that some chemical inducers of P-gp expression do not interact with the Ah receptor involved in CYP 1A1 induction and, therefore, the effect of these agents on hepatic P-gp levels is probably not mediated by the Ah receptor.

The precise mechanism involved in mdr1b gene overexpression in response to MMS remains to be determined. It is however noteworthy that MMS is a well-known alkylating agent commonly used as a prototype of DNA-damaging agent (28) and therefore its effect on mdr gene expression in liver cells may be interpreted as a part of the cellular response to DNA damage. In the same way, hepatic P-gp up-regulation by chemical carcinogens or anticancer drugs may be linked to the DNA lesions that they usually induced (14, 16). The fact that P-gp overexpression in response to 2-acetylaminofluorene has recently been demonstrated to require the formation of DNA-binding metabolites (29) also fully supports this hypothesis. In addition, P-gp expression or MDR1 promoter activity have also been shown to be induced in other DNA-damaging situations such as X-ray irradiation and exposure to UV light (30, 31). P-gp expression is also increased in response to environnemental stress including heat shock and treatment by heavy metals (32). Taken together, these data indicate that P-gp up-regulation may be a part of the general response to cellular stress triggered by various

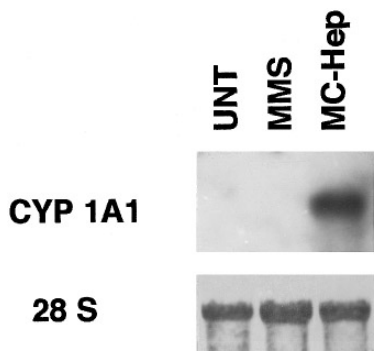

FIG. 6. Effect of MMS treatment on CYP 1A1 expression. Each well contained 10 μg total RNAs isolated from rat liver epithelial cells either untreated (UNT) or exposed to 30 μg/ml MMS for 24 h and from 3-methylcholanthrene-treated rat hepatocytes (MC-hep) used here as positive controls for CYP 1A1 expression. The RNAs were transferred to Hybond N^+ sheets and then hybridized with a rat CYP 1A probe whereas 28S RNAs were stained using methylene blue solution.

circumstances, including xenobiotic-mediated damage to DNA.

The fact that alkylating agents such as MMS can lead to increased functional P-gp expression may have clinical implications. Indeed, treatment by some of these compounds such as nitrosoureas or cyclophosphamide used as anticancer drugs that are usually not thought to be transported by P-gp (1, 2), may contribute to the development of acquired multidrug resistance in tumoral cells through up-regulation of mdr gene expression. The fact that the alkylating drug cisplatin has been recently demonstrated to enhance P-gp levels in a subpopulation of K562 human leukemia cells (33) is consistent with this hypothesis. It is also noteworthy that P-gp in the liver has been postulated to participate to biliary excretion of various xenobiotics, including anticancer drugs (7); its up-regulation following exposure to DNA-damaging agents may therefore result in enhanced biliary elimination of antitumor agents transported by P-gp and thus in decreased activity of these compounds through alteration of their pharmacokinetic.

In summary, the results reported here demonstrated that cultured rat liver epithelial cells acutely exposed to the DNA-alkylating agent MMS displayed increased functional P-gp expression through up-regulation of the mdr1b gene, therefore suggesting that xenobiotic-mediated induction of P-gp expression in the liver may be a part of the cellular response triggered by DNA damage.

ACKNOWLEDGMENTS

This work was supported by the Ligue Nationale contre le Cancer (Comité d'Ille et Vilaine). Arnaud Courtois is a recipient of a fellowship from the Association pour la Recherche sur le Cancer.

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