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Carcinogen-induced *mdr* overexpression is associated with xenobiotic resistance in rat preneoplastic liver nodules and hepatocellular carcinomas

(multidrug resistance/xenobiotic resistance/P glycoprotein)

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ABSTRACT **We have previously reported the isolation of a human breast cancer cell line resistant to doxorubicin (adriamycin; Adr^R MCF-7 cells) that has also developed the phenotype of multidrug resistance (MDR). MDR in this cell line is associated with increased expression of *mdr* (P glycoprotein) gene sequences. The development of MDR in Adr^R MCF-7 cells is also associated with changes in the expression of several phase I and phase II drug-detoxifying enzymes. These changes are remarkably similar to those associated with development of xenobiotic resistance in rat hyperplastic liver nodules, a well-studied model system of chemical carcinogenesis. Using an *mdr*-encoded cDNA sequence isolated from Adr^R MCF-7 cells, we have examined the expression of *mdr* sequences in rat livers under a variety of experimental conditions. The expression of *mdr* increased 3-fold in regenerating liver. It was also elevated (3- to 12-fold) in several different samples of rat hyperplastic nodules and in four of five hepatomas that developed in this system. This suggests that overexpression of *mdr*, a gene previously associated with resistance to antineoplastic agents, may also be involved in the development of resistance to xenobiotics in rat hyperplastic nodules. In addition, although the acute administration of 2-acetylaminofluorene induced an 8-fold increase in hepatic *mdr*-encoded RNA, performance of a partial hepatectomy either before or after administration of 2-acetylaminofluorene resulted in a >80-fold increase in *mdr* gene expression over that in normal untreated livers. This represents an important *in vivo* model system in which to study the acute regulation of this drug resistance gene.**

Multidrug resistance (MDR) occurs in tumor cell lines that develop resistance to a single agent but often develop cross-resistance to a wide range of structurally dissimilar agents whose presumed mechanisms of action differ markedly (1–5). Studies in MDR cell lines have shown that resistance is frequently associated with defects in drug accumulation (6, 7) as well as overexpression of high molecular mass (130–170 kDa) membrane glycoprotein(s) (4, 6, 8, 9). This membrane protein is thought to be involved in the active efflux of drug from MDR cells. Several laboratories, including our own, have isolated DNA sequences that apparently encode this membrane protein (designated P glycoprotein or *mdr*) and have demonstrated their amplification and overexpression in MDR animal and human cell lines (10–15). The role of the *mdr*-encoded gene product as a putative drug efflux pump is supported by studies demonstrating the ability of this protein to bind to antineoplastic drugs (16–18). Moreover, DNA sequence analysis has demonstrated homologies between portions of *mdr*-encoded cDNAs and several bacterial membrane transport proteins (19–21).

We have previously reported the isolation of a human MCF-7 breast cancer cell line that was selected for resistance to doxorubicin [adriamycin-resistant (Adr^R) MCF-7] and that exhibits the phenotype of MDR (22, 23). MDR in these Adr^R MCF-7 cells is associated with a defect in drug accumulation as well as amplification and overexpression of *mdr* gene sequences (15, 22, 23). Resistance in these cells is also associated with changes in the expression of several phase I and phase II drug-detoxifying enzyme activities, which are remarkably similar to those associated with the development of xenobiotic-resistance in a Solt–Farber model of carcinogenesis (24). In this model system, exposure of rats to carcinogens induces macroscopic liver foci referred to as hyperplastic liver nodules (HNs). HNs are preneoplastic lesions, most of which regress over several weeks to months into normal-appearing hepatic parenchyma. A few lesions, however, eventually progress to malignant hepatomas (25, 26).

One of the interesting features of rat HNs has been the finding that these foci are resistant to the cytotoxic effects of many xenobiotics (27) and that the development of resistance is associated with a number of biochemical changes in rat HNs (1, 28). In a previous report, we compared biochemical changes that developed in Adr^R MCF-7 cells with those that develop in rat HNs (23). In both systems, exposure to a single agent results in the selection of cells that are cross-resistant to a wide variety of structurally different toxins (27–30). Moreover, resistance in both systems is associated with decreased toxin accumulation (6, 7, 31, 32), decreased expression of the phase I metabolizing enzyme aryl hydrocarbon hydroxylase (33–35), and increased expression of several phase II drug-conjugating enzymes, including an anionic glutathione *S*-transferase (GST-P or π; EC 2.5.1.18) (27, 36, 37) and UDP glucuronyl transferase I (38).

Since decreased toxin accumulation is a property of both model systems of resistance, we examined *mdr* gene expression in rat HNs using a human cDNA probe obtained from Adr^R MCF-7 cells. The studies presented in this report demonstrate that xenobiotic resistance induced by carcinogens in rat HNs is associated with increased *mdr* gene expression, a gene that is associated with resistance to antineoplastic agents.

Abbreviations: WT, wild-type parental MCF-7 cells; Adr^R, doxorubicin (adriamycin)-resistant MCF-7 cells; MDR, multidrug resistance; *mdr*, gene encoding P glycoprotein; 2-AAF, 2-acetylaminofluorene; HNs, hyperplastic liver nodules; DEN, diethylnitrosamine; GST-P, anionic isozyme of glutathione *S*-transferase.

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MATERIALS AND METHODS

Materials. 2-Acetylaminofluorene (2-AAF) was obtained from Aldrich. Deoxycytidine 5'-[α-^{32}P]triphosphate (400 Ci/mmol; 1 Ci = 37 GBq), deoxyguanosine 5'-[α-^{32}P]triphosphate (400 Ci/mmol), and uridine 5'-[α-^{32}P]triphosphate (800 Ci/mmol) were obtained from Amersham. The pGEM3 vector and Riboprobe Gemini System II were obtained from Promega Biotec (Madison, WI) and reagents for nick-translation were from Bethesda Research Laboratories. Both systems were used according to instructions provided by the manufacturer.

Cell Culture. Wild type (WT) and Adr^R MCF-7 human breast cancer cell lines were maintained in improved minimum essential medium containing 5% fetal bovine serum as described (22).

Animals and Treatment. Male Fischer 344 rats (Charles River Breeding Laboratory) weighing 150–175 g were used in these experiments. HNs were generated essentially as reported by Solt and Farber (24). Briefly, diethylnitrosamine (DEN) was injected once intraperitoneally (200 mg/kg). Beginning 2.5 weeks later, 2-AAF was given intragastrically once daily (20 mg/kg) on 3 consecutive days; this was followed by partial hepatectomy on the next day. Using this protocol, large HNs form within 6–8 weeks after the initial treatment. In many animals, frank hepatocellular carcinomas eventually develop. These cancers were isolated 6–14 months after 2-AAF treatment. The nodules or cancers were dissected away from the surrounding tissue, frozen immediately on dry ice, and stored at −80°C. Samples of regenerating liver were obtained from animals receiving a partial hepatectomy only, without prior carcinogen treatment. To study the acute effect of 2-AAF on normal liver, animals were treated with 2-AAF intragastrically once daily (20 mg/kg) for 2 or 3 consecutive days. Animals were sacrificed at various times following the initiation of the treatment protocol, and the livers were removed and stored as described above. To determine the effect of liver cell proliferation and carcinogen treatment on gene expression, some rats received a partial hepatectomy either after or before treatment with 2-AAF without prior initiation with DEN. Under these treatment conditions liver cancer is not induced unless animals are also initiated with DEN or another carcinogen (39).

Nucleic Acid Analysis. Frozen liver samples were first ground into a fine powder with mortar and pestle while on dry ice. RNA was isolated from MCF-7 cells and liver samples using guanidinium isothiocyanate followed by cesium chloride centrifugation as described (40, 41). Poly(A)$^+$ RNA was obtained using oligo(dT)-cellulose chromatography. Slot blot analysis was performed by applying total cellular RNA directly to nitrocellulose filters as described by the slot blot manufacturer (Schleicher & Schuell). RNA transfer blot analysis was performed following electrophoresis of RNA on a 1% formaldehyde/agarose gel, staining with ethidium bromide to check for equality of RNA loading, and then transferring the size-fractionated RNA to nitrocellulose filters (42). RNA filters were prehybridized, hybridized, and washed as described (42).

The probes used in these studies include a human *mdr* cDNA probe, pADR-1, which we isolated from a cDNA library made from RNA from Adr^R MCF-7 cells using a hamster probe homologous to P glycoprotein sequences (12, 15). pADR-1 was subcloned into pGEM3 and used to make a radiolabeled RNA probe using T7 RNA polymerase. A probe coding for the rat anionic GST-P, pGP5, present in rat HNs was kindly provided by M. Muramatsu (37).

RESULTS

Increased *mdr* Transcripts in Regenerating Liver, HNs, and Hepatocellular Carcinomas. A map of the human *mdr* cDNA sequence isolated from a cDNA library constructed from Adr^R MCF-7 cells is shown in Fig. 1*A* (15). This clone hybridized to a 4.8-kilobase (kb) mRNA that was overexpressed in Adr^R MCF-7 cells compared to WT MCF-7 cells (Fig. 1*B*). This sequence also detected a 4.8-kb mRNA species in normal rat liver. When RNA was obtained from rat livers 18 hr after partial hepatectomy and probed with pADR-1, there was a slight increase in the concentration of *mdr*-encoded RNA (Fig. 1*B*). Thus, stimulation of hepatic cell growth by partial hepatectomy resulted in increased basal *mdr* gene expression.

RNA was also obtained from pooled rat HNs taken from a single animal and examined for the expression of *mdr* RNA. As shown in the RNA transfer blot analysis in Fig. 1*B*, the level of the 4.8-kb *mdr* RNA was increased in rat HNs relative to the expression in normal liver. The expression of *mdr* appeared to be increased relative to the level in regenerating liver. Thus, the induction of these preneoplastic lesions several weeks after carcinogen treatment of animals resulted in a marked increase of *mdr* RNA above the basal level. The expression of this gene was also examined in a single hepatocellular carcinoma that subsequently formed in one of the animals. The level of *mdr* RNA in this cancer, examined 14 months after the initial induction regimen, was also increased relative to the level in normal liver (Fig. 1*B*). Thus, the carcinogen-induced increase in *mdr* gene expression was stable over time and apparently remained elevated during the subsequent transformation of the preneoplastic nodule to the malignant cancer.

In a similar manner, we also examined the expression of an anionic GST-P in these samples using a rat cDNA probe (37). As shown in Fig. 1*C*, there was a marked increase in the expression of anionic GST-P in the preneoplastic nodules and in the cancer relative to the expression in normal rat liver. This finding is consistent with previous observations in which GST-P protein (36) and mRNA (37) were found to be

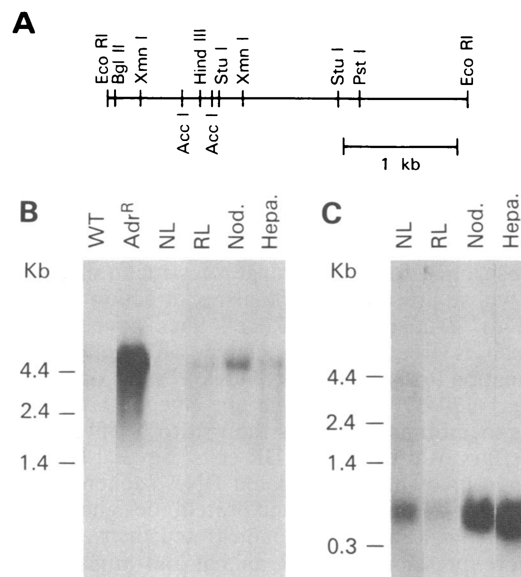

FIG. 1. Expression of *mdr* (*B*) or GST-P (*C*) mRNA in normal, preneoplastic, and neoplastic liver tissue. (*A*) Map of the human *mdr* cDNA sequence pADR-1. In *B*, ^{32}P-labeled pADR-1 was hybridized to the filter. In *C*, a ^{32}P-labeled cDNA probe, pGP5, was used to determine GST-P expression. Each lane in the RNA transfer blot analysis contains 10 μg of poly(A)$^+$ RNA. Lane labels are normal rat liver (NL), 18-hr regenerating liver (RL), nodules (Nod.), and hepatocellular carcinoma (hepa.). Molecular mass markers from an RNA ladder (Bethesda Research Laboratories) are indicated.

increased in rat HNs and hepatomas. Thus, *mdr* and GST-P gene expression were increased in both of these states. In contrast to the increase in *mdr* RNA following partial hepatectomy noted above, we found no increase in GST-P RNA in regenerating liver at any time (12–72 hr) following partial hepatectomy alone in the absence of carcinogen treatment.

Quantitation of *mdr* Transcripts in HNs and Hepatocellular Carcinomas. To obtain a more accurate indication of the level of *mdr* mRNA in rat HNs and carcinomas, slot blot hybridization was performed using total cellular RNA as described in *Materials and Methods*. As shown in Fig. 2, there was a 3-fold increase in *mdr* mRNA concentration in regenerating liver 18 hr after partial hepatectomy relative to that present in normal hepatic parenchyma. Thus, as noted in the previous section, increased hepatic cell proliferation alone resulted in increased *mdr* gene expression. In other experiments we have found that the level of *mdr* RNA increased in a time-dependent manner following partial hepatectomy. The maximum increase (3-fold) occurred 18–24 hr after surgery (data not shown).

The expression of this gene was also examined in pooled samples of HNs obtained from three additional animals 6–8 weeks following treatment with 2-AAF and partial hepatectomy. In the HNs from one of these animals *mdr* gene expression was increased 10-fold relative to its expression in normal liver, whereas the expression of this gene was increased 4- and 5-fold in the HNs from the other two animals. In addition, hepatomas were isolated from five individual animals. In one of these tumors, there was no difference in the level of *mdr* RNA relative to the level in normal liver. However, in each of the remaining four samples there was an increase in *mdr* gene expression (3- to 12-fold) relative to that in normal hepatic parenchyma. For comparison, we have also shown the hybridization results using the same amount of RNA from WT and Adr^R MCF-7. In the Adr^R MCF-7 cells, which are 200-fold more resistant to doxorubicin than the WT MCF-7 cells, there was >120-fold more *mdr* mRNA relative to that present in the drug-sensitive WT MCF-7 cell line. Although the increase in *mdr* expression in rat HNs and hepatomas was less than that in the highly resistant Adr^R cell line, the range of the increase (3- to 12-fold) was as great or greater than the 2-fold increase in *mdr* RNA that we have previously noted in a drug-resistant MCF-7 subline that was 6-fold resistant to doxorubicin (15).

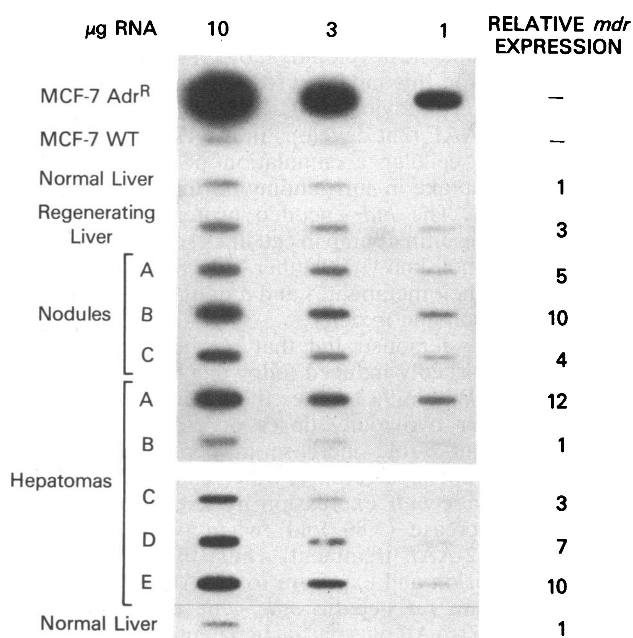

FIG. 2. Quantitation of *mdr* transcripts in nodules and hepatocellular carcinomas. Slot blots were made containing 10, 3, or 1 μg of total cellular RNA from HNs (6–8 weeks after partial hepatectomy) and hepatocellular carcinomas (>6 months after partial hepatectomy). The blot was hybridized to the cDNA, pADR-1, which was radiolabeled by nick-translation. The far right column indicates the relative increase in *mdr* expression in each sample relative to normal liver.

Effects of Acute Carcinogen Exposure on Hepatic *mdr* Expression. Since the relative expression of *mdr* was elevated in HNs and hepatic cancers when sampled weeks to months after the initiation of therapy, we next looked at the acute effects of 2-AAF administration on the regulation of *mdr* gene expression. In this experiment, an animal was treated with three daily doses of 2-AAF and then sacrificed on the fifth day. As shown in the RNA transfer blot in Fig. 3*A*, there was a moderate increase in the level of *mdr* mRNA in rat liver when assayed on the fifth day after the first dose of 2-AAF compared to that present in normal rat liver. Thus, acute exposure to 2-AAF induced an increase in *mdr* gene expression in rat livers.

We next examined the time course of the effects of 2-AAF on *mdr* mRNA. These experiments consisted of four separate treatment groups that are schematically depicted in Fig. 4. Animals in group A were treated with 2-AAF for 3 consecutive days. This is the same dose and schedule of 2-AAF that was used in the induction of rat HNs studied previously. However, in this experiment the animals were not treated with DEN, to initiate carcinogenesis, nor did they receive a partial hepatectomy. Treatment group B examined the effect of two daily doses of 2-AAF on *mdr* gene expression. The other two treatment groups examined the effects of concomitant partial hepatectomy when performed either after (group C) or before (group D) treatment with 2-AAF. In each of these experiments, animals were sacrificed at various times after the first dose of 2-AAF and the levels of hepatic *mdr* and GST-P mRNA in each sample were analyzed by slot blot hybridization (Fig. 3*B*).

In group A, following three daily doses of 2-AAF, there was no detectable increase in *mdr* mRNA on day 3 (12 hr after the last dose) or on day 4 (24 hr after the last dose) of the treatment schedule. However, on day 5 (48 hr after the last dose of 2-AAF) there was an 8-fold increase in the level of hepatic *mdr* RNA. Thus, acute administration of 2-AAF resulted in an increase in hepatic *mdr* gene expression. Similar results were obtained in treatment group B, in which only two daily doses of 2-AAF were given to animals. Though there was no increase in *mdr* RNA in this treatment group on day 4 (48 hr after the last dose), there was a similar 8-fold increase in the expression of this gene on day 5. Thus, two doses of 2-AAF are sufficient to induce hepatic *mdr* expression. In both experiments, the induction of *mdr* gene expression was somewhat delayed and occurred on the fifth day following the first dose of 2-AAF.

The combination of 2-AAF and partial hepatectomy produces a more marked enhancement of *mdr* gene expression. Whereas partial hepatectomy alone induced a 3-fold increase in *mdr* RNA (Figs. 2 and 3*B*) and 2-AAF alone produced an 8-fold increase (Fig. 3*B*, group A, day 5), the two treatments combined produced a >80-fold increase in *mdr* gene expression when assayed on day 5. The level of *mdr* RNA in group C animals approached the level present in Adr^R MCF-7 cells, which are 200-fold resistant to doxorubicin (Fig. 3*B*) (15). The marked enhancement of *mdr* gene expression also occurred in animals treated with a partial hepatectomy prior to receiving 2-AAF (group D). Thus, 2-AAF treatment and partial hepatectomy apparently act synergistically to enhance hepatic *mdr* gene expression.

The same RNA samples obtained from the acute induction experiments outlined in Fig. 4 were probed with the rat

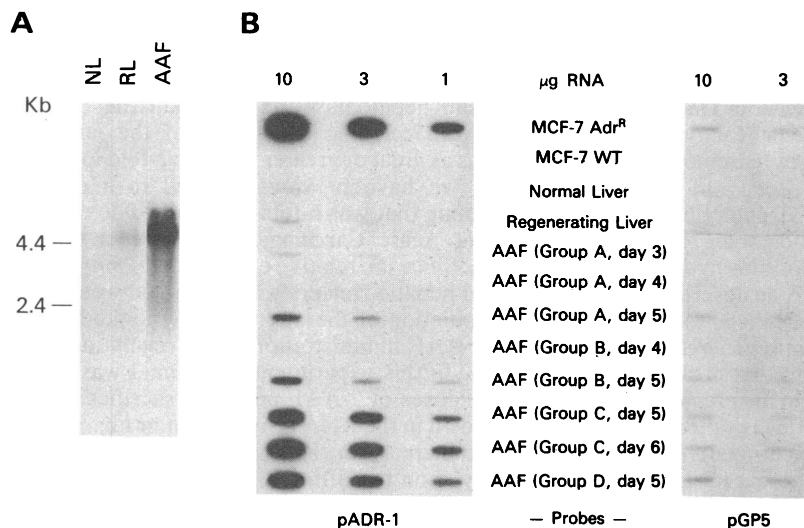

FIG. 3. Effect of acute 2-AAF treatment on *mdr* (*A* and *B*) and GST-P (*B*) mRNA expression in rat liver. For RNA transfer blot analysis each lane contained 10 μg of poly(A)$^+$ RNA isolated from normal liver (NL), 18-hr regenerating liver (RL), or rats treated with 2-AAF for 3 consecutive days (liver sample removed on day 5). For quantitative slot blot analysis total cellular RNA was isolated from Adr^R and WT MCF-7 cells, normal and regenerating liver, and 2-AAF-treated rat livers and analyzed. The protocol for 2-AAF treatment is shown in Fig. 4. The probes used were pADR-1 (*mdr*) and pGP5 (GST-P).

anionic GST-P cDNA. As shown in Fig. 3*B*, in contrast to the results with the *mdr* gene, treatment with 2-AAF alone or in combination with partial hepatectomy had essentially no effect on GST-P expression in rat livers by day 5 or 6. Thus, these two genes are regulated quite differently in rat hepatocytes.

DISCUSSION

We have previously reported that MCF-7 breast cancer cells selected for doxorubicin resistance possess many features that are similar to those induced by carcinogens in rat HNs (23). In both model systems, selection by exposure to a single toxin produces cellular changes that result in the development of resistance to the selecting agent as well as cross-resistance to a wide range of structurally dissimilar toxins (2–5, 27, 43). Resistance in both systems is associated with decreased intracellular accumulation of toxin (6, 7, 31, 32) as well as decreased activity of the phase I activating enzyme aryl hydrocarbon hydroxylase and increased expression of phase II drug-conjugating isozymes GST-P and UDP glucuronyl transferase I (22, 23, 27–37). Thus, several important features associated with the development of resistance to carcinogens and other hepatotoxins in rat HNs are also

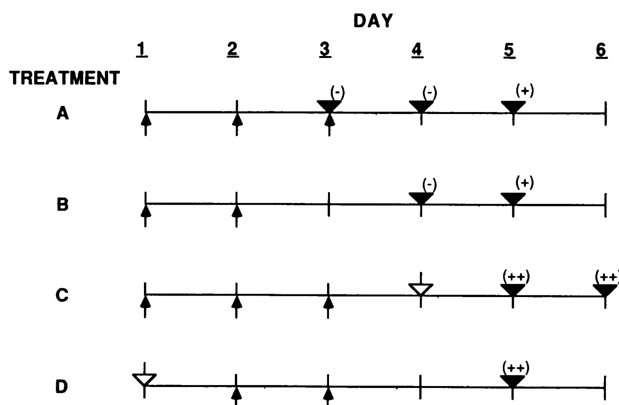

FIG. 4. Outline of the protocol for acute 2-AAF treatment and summary of results. There were four separate treatment groups (A–D). Small arrows (↑) indicate a single intragastric dose of 2-AAF (20 mg/kg). Open arrows (⇩) indicate that a partial hepatectomy was performed. Large solid arrowheads (▼) indicate the day on which the liver sample was taken. The (+) or (++) indicates the degree to which *mdr* expression is increased, whereas (−) indicates no change in *mdr* expression.

associated with the development of antineoplastic drug resistance in MCF-7 cells. The parallel biochemical changes noted in these two models are further strengthened by the finding that the MCF-7 cells that were selected for resistance to doxorubicin are also cross-resistant to the carcinogen benzo[*a*]pyrene.

The studies presented in this report demonstrate that *mdr* gene sequences are expressed in normal rat liver and that the expression of this gene is increased (3-fold) in hepatocytes of regenerating liver. Moreover, the expression of this gene is also elevated (3- to 12-fold) in rat HNs as well as in four of the five malignant hepatomas that we studied relative to the level of expression in normal liver. The increase in both states is equal to or greater than that observed in regenerating liver. Thus, the increase in *mdr* expression in rat HNs and in hepatomas is not simply a reflection of enhanced hepatocyte growth.

The relationship between the increase in *mdr* gene expression in rat HNs and the development of xenobiotic resistance that is observed in this model system is as yet unclear. As mentioned earlier, previous studies have shown that the resistance to 2-AAF that develops in the HNs is associated with decreased cellular accumulation of this carcinogen relative to the uptake in surrounding normal hepatic parenchyma (31, 32). The *mdr*-encoded protein is thought to function as a drug efflux pump in cell lines selected for MDR; however, it is not known whether this protein can bind carcinogens or their metabolites and modulate their intracellular accumulation and toxicity.

We have also demonstrated that rat hepatic *mdr* gene expression is markedly induced following acute administration of 2-AAF. Within 96 hr after the administration of the first of three (or two) daily doses of 2-AAF, there was approximately an 8-fold increase in hepatic *mdr* RNA. Moreover, a partial hepatectomy, which causes only a 3-fold increase in hepatic *mdr* expression by itself, resulted in a much larger increase (>80 fold) when performed either before or after 2-AAF treatment. Thus, the stimulation of hepatic cell division and exposure to 2-AAF act synergistically to modulate rat hepatic *mdr* gene expression. The combined use of 2-AAF plus partial hepatectomy under these conditions leads to severe inhibition of liver cell proliferation (24) with arrest and accumulation of the hepatocytes late in the G_1 phase of the cell cycle (44). Thus, this study demonstrates that *mdr* gene expression can be acutely regulated in cells. Whether the increase in *mdr* RNA is due to transcriptional or posttranscriptional regulation is unknown. Since the level of expression of this gene is a critical determinant of antineoplastic drug resistance, understanding the cellular

factors involved in its regulation represents an important research priority. The large increase in *mdr* RNA in the liver of rats treated with 2-AAF plus partial hepatectomy offers a readily available *in vivo* model system in which to study the control of *mdr* gene expression.

Enhanced *mdr* gene expression can now be added to the list of biochemical changes associated with the development of xenobiotic resistance in rat HNs as well as with MDR in Adr^R MCF-7 breast cancer cells. These findings may have important clinical implications and suggest a possible explanation for the *de novo* resistance to chemotherapy that is a common problem in tumors associated with increased carcinogen exposure, such as colon cancer. Indeed, elevated levels of the anionic GST-P protein (45) and GST-P RNA (J. Moscow and K.H.C., unpublished data) and high levels of *mdr* RNA (46) have been found in colon cancer samples. Perhaps the *de novo* resistance to chemotherapy in colon cancer is due, in part, to carcinogen-induced changes similar to those found in rat HNs and Adr^R MCF-7 cells, including changes in expression of phase I and phase II drug detoxification genes and enhanced *mdr* gene expression.

Note. Since submission of this manuscript, another study has appeared that also demonstrates an increase in *mdr* RNA in rat HNs and hepatomas (47).

C.R.F. is a Pharmacology Research Associate of the National Institute of General Medical Sciences.

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