Activation of Multidrug Resistance (P-Glycoprotein) mdr3/mdr1α Gene during the Development of Hepatocellular Carcinoma in Hepatitis B Virus Transgenic Mice

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Abstract

The expression of multidrug resistance (mdr) genes was investigated in the livers of transgenic mice that express the human hepatitis B virus large envelope polypeptide under the transcriptional control of a liver-specific promoter. These mice develop a storage disease due to the accumulation of a nonsecreted form of hepatitis B surface antigen in the hepatocyte. Liver cell injury is followed by a hepatocellular proliferative response, dysplasia, microscopic nodular hyperplasia, and finally hepatocellular carcinoma. The expression of mdr1, mdr2, and mdr3 genes was analyzed in livers at different stages of the disease by RNase protection assay, Western blot, and immunohistochemistry. RNase protection assay revealed that mdr3 mRNA expression was moderately increased in tissue with microscopic nodular hyperplasia and significantly overexpressed in hepatocellular carcinoma that is undetectable in earlier stages of the disease. Western blot using isoform-specific anti-mdr3 antibody demonstrated that the expression of mdr3 protein reflected the steady-state level of mdr3 mRNA. Immunohistochemical analyses using anti-mdr3 isoform-specific antibody and monoclonal antibody C219, which recognizes all the three mdr isoforms, demonstrated selective overexpression in preneoplastic foci during the stage of microscopic nodular hyperplasia as well as in neoplastic hepatocytes in hepatocellular carcinoma. No consistent activation of mdr1 and mdr2 (or occasional coactivation with mdr1) genes during hepatocarcinogenesis was observed. Our results suggest that the hepatocellular mdr3-specific activation mechanism is associated with the late events of hepatocarcinogenesis in this model. The predictable kinetics of mdr gene expression in this transgenic tumor model suggest that it is suitable for future studies of the mechanism of mdr gene activation and the possible pharmacological consequences for mdr3 gene expression of hepatocellular carcinoma.

Introduction

HCC is the most frequent malignant tumor of the liver (1). Despite the use of many therapeutic modalities, HCC remains a disease with a dismal prognosis. The median survival time is approximately 16 weeks (2). Hepatic resection, liver transplantation, and systemic chemotherapy are the major treatment modalities of liver cancers. However, one major drawback in liver cancer chemotherapy is the intrinsic drug resistance.

Many animal HCC models have been established in the literature (for reviews, see Refs. 3-5). It has been reported that the levels of mRNA encoded by the mdr gene, also known as the p-gp gene, are elevated in chemically induced liver tumors in rodents (6-8). Using an RNase protein assay, we observed that one member of the mdr gene family, mdr3 [also called mdr1a (9)], is overexpressed in tumors derived from five different HCC models, including chemical induction by diethylaminoethyl and dimethylhydrazine in C57BL/6N mice, spontaneous development in C3H/HeN mice, and transgenic mice carrying the human HBV envelope protein gene (8). Burt et al. (10) reported that transformation of rat liver epithelial cells with v-H-ras causes activation of the mdr1 gene.

The mdr family gene encodes a group of 175-190-kDa P-glycoproteins or multidrug transporters which are believed to serve as efflux pumps in expelling a number of structurally and functionally unrelated drugs from the cell (for reviews, see Refs. 11-14). Three mdr genes in rodents and two in humans have been identified by molecular cloning. Functional analyses by transfection using recombinant cDNA inserted into expression vectors demonstrated that only mdr1 and mdr3 genes, but not mdr2, can confer multidrug-resistant phenotype (MDR) in otherwise drug-sensitive cells. Expression of different MDR products in these transfected results in distinct but overlapping drug resistance profiles (15).

The observation that the mdr3 gene is activated in HCC of various origins strongly suggests that the activation mechanism is associated with a general pathway of hepatic tumor development. The stage at which the mdr

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1 The abbreviations used are: HCC, hepatocellular carcinoma; HBV, human hepatitis B virus; MDR, multidrug resistance; P-gp, P-glycoprotein; MNN, microscopic nodular hyperplasia; GC/In, ground glass/injury hepatocytes; PBS, phosphate-buffered saline; kDa, kilodalton(s); cDNA, complementary DNA; nt, nucleotide(s); FITC, fluorescein isothiocyanate; SDS, sodium dodecyl sulfate.

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gene is activated in this yet-to-be-defined pathway is unknown. This investigation was aimed at determination of mdr gene activation during hepatocarcinogenesis using a pathogenetically well-characterized tumor model. Using RNase protection and immunohistochemical analyses, we demonstrated that the mdr gene is activated at a late stage of tumor development.

Results

MDR Gene Expression in HBV Mice Determined by RNase Protection Assay. The natural history of hepatocarcinogenesis in HBV mice (lineage 50-4) follows a predictable pathogenetic program (16, 17). Expression of the envelope protein gene begins at birth. Liver injury with the appearance of ground glass hepatocytes begins at approximately 2–4 months of age. By 6 months of age, MNH foci resembling regenerative nodules are first noticeable. Most of these foci are composed of histologically benign hepatocytes, but occasionally more aggressive lesions resembling microcarcinoma are present. By 12–15 months of age, tumors with histological characteristics of hepatocellular adenomas and/or carcinomas are first detectable. By 21 months of age, liver tumors are present in virtually all mice of this lineage (16, 17).

Total RNA was extracted from liver tissue at various stages of this disease, and the RNase protection assay was used to determine the relative steady-state content of mdr transcripts using levels of albumin mRNA as reference. The probe chosen for detection of mdr1 and mdr3 mRNA was a 211-nt Pvull-KpnI (nucleotides 2561–2772 from the translation start site) fragment of mdr1 cDNA (18). Because a 56-nt sequence within the 211-nt mdr1 fragment is identical to that in the mdr3 cDNA (9, 15, 18), this probe can simultaneously detect mdr1 and mdr3 transcripts, yielding protected fragments of 211 nt (mdr1) and 56 nt (mdr3), respectively.

The steady-state content of mdr mRNA in 18 liver samples derived from different stages of carcinogenesis is shown in Fig. 1. Consistent with our previous report (8), the levels of mdr1 and mdr3 mRNA were relatively high in kidney and intestine, respectively, but both were low in normal livers (Fig. 1, Lanes 1–3, upper panel). Levels of both transcripts remained low in livers derived from animals in the 2-month age group (Fig. 1, Lanes 4–8). Histopathological evaluations of this group of animals revealed mainly GG/Hjn hepatocytes. Weak signals for mdr3 transcripts were visible in the livers of animals 5–15 months of age, in which MNH is evident (Fig. 1, Lanes 9–13). As expected, the steady-state levels of mdr3 transcripts were high in all of the HCC samples produced in HBV mice at 18–20 months of age (Fig. 1, Lanes 14–18), as well as in benign adenoma (data not shown). The levels of mdr3 mRNA were estimated to be about 15-fold elevated in HCC by a densitometric analysis of autoradiographs, using levels of albumin mRNA (Fig. 1, lower panel) as reference. The levels of albumin mRNA were not significantly different between tumor and normal liver tissues, using 28S and 18S ribosomal RNA as reference (8). Elevated levels of mdr1 transcript were occasionally found in some HBV mice at late stages of the disease (e.g., Fig. 1, Lanes 13 and 18). These results indicate that activation of the mdr3 gene is intimately linked to the development of hyperplastic nodules but is greatly enhanced in overt liver tumors.

For detection of mdr2 transcript, we used an mdr2 cDNA probe specifying the 293-nt Hincll/Nael fragment (nucleotide coordination 1875–2168) in the RNA protection assay. This probe does not cross-hybridize with mdr1 and mdr3 transcripts (19). The levels of expression of the mdr2 gene were variable in all liver samples (Fig. 1, middle panel) irrespective of the stages of disease. Individual variations of mdr2 expression in HCC were also observed previously (8). The levels of mdr2 mRNA in the livers bearing MNH and HCC are not significantly increased.

Analyses by Western Blot of the mdr3 Protein during Hepatocarcinogenesis. Anti-mdr3 antipeptide antibody was prepared from immunized rabbits. To demonstrate that this antibody recognizes mdr3 protein specifically, we first used Western blot to the membrane proteins isolated from three different tissues, i.e., kidney, liver, and intestine. Previous studies have demonstrated that the mdr1, mdr2, and mdr3 genes are preferentially expressed in these tissues, respectively (8, 20, 21). As shown in Fig. 2a, this antibody detected an overproduced membrane protein (P-gp) in intestine with a molecular weight of approximately 180,000 (see Fig. 4 for molecular weight markers). No signal was detected when preimmune serum was used (Fig. 2b). The signals on the Western blots could be blocked when the antibody was pretreated with the peptide antigen, but not with the synthetic peptides from the corresponding regions in the mdr1 and mdr2 proteins (data not shown). Similar results were obtained using a panel of murine MDR cells expressing different mdr isoforms. Immunohistochemical analysis using this antipeptide antibody demonstrated that the epithelial cells of the small intestinal villi were preferentially stained (Fig. 3a), whereas kidney (Fig. 3b) and liver (data not shown, but see Fig. 5a) specimens were only weakly stained and displayed a faint, diffuse cytoplasmic pattern. These results, taken together, suggest that this antipeptide antibody preferentially interacts with mdr3 protein. This antibody was then used to determine the expression of mdr3 protein in liver tissue from the HBV mice. As shown in Fig. 4a, the levels of mdr3 protein in normal and GC/Hjn hepatocytes were very low, but they were moderately increased at the stage of MNH and overexpressed in HCC. As a control, the preimmune serum showed no signal on the Western blot (Fig. 4b). These results demonstrate that the expression of mdr3 protein corresponds to the steady-state mdr3 mRNA during the development of HCC in HBV mice.

Analyses of mdr Gene Expression by Immunohistochemistry. As hepatocarcinogenesis arises in a progressive and perhaps polyclonal fashion, the Northern and Western blot analyses described above represent an average of heterogeneous populations of cell types in different stages of oncogenesis. To determine the expression of mdr3 gene at the cellular level, we carried out immunohistochemical staining on the liver sections at each stage of disease. Cryostat sections were incubated with anti-mdr3 antibody, followed by FITC-conjugated secondary antibody. As shown in Fig. 5, normal (a) and

* N. Savaraj, J. Y. Zhao, T. J. Lampidis, C. J. Wu, L. D. Tetter, and M. T. Kuo. Multidrug resistant cells selected with different drugs are associated with overproduction of different P-glycoproteins, submitted for publication.
GG/Inj (b) hepatocytes exhibited background fluorescence, whereas microscopic nodules (c and d) showed weak cytoplasmic staining, and HCC (d) showed strong staining (e and f). Furthermore, mdr3 protein appeared to be localized to the cytoplasm and cell membrane. Cytoplasmic localization of mdr protein in tumor cells was also reported previously (22). The staining was blocked when the antibody was pretreated with peptide antigen (Fig. 6b). Control experiments using preimmune antibody failed to stain HCC (Fig. 6c). These results suggest that the antibody was faithfully detecting the expression of mdr3 protein. Attempts to stain mdr3 protein on paraffin-embedded tissue sections were unsuccessful, perhaps owing to alterations of epitope configuration during slide preparation.

To substantiate the results presented in Fig. 5, we carried out immunohistochemical experiments using a monoclonal antibody, C219, which recognizes all three mdr isoforms. Although we expected that livers from all stages of malignancy would stain positively with this antibody due to the expression of mdr2 protein, we reasoned that if MNH exhibits elevated expression of mdr3 protein, the overall staining intensity in this lesion should be greater than in the neighboring areas in the same tissue. Likewise, livers with HCC should show stronger staining signal than those with much earlier stages of the disease.

Paraffin-embedded sections of livers from various stages of malignancy were stained with C219. Each section was flanked by a kidney section on the same slide. The kidney sections served as both positive (proximal tubules) and negative (medulla) controls (Fig. 7A). Fig. 7, B and C, shows the background levels of mdr protein in the livers of HBV mice with early stages of disease. High magnification of the field revealed that the immunoreactive mdr protein is located predominantly on the can-
Fig. 2. Western blot detection of mdr3 protein in mouse liver, kidney, and intestine tissues. Fifty µg of membrane protein prepared from different tissues were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and probed with anti-mdr3 antiserum (a) or preimmune serum (b). P-gp, P-glycoprotein.

Fig. 3. Immunohistochemical localization of mdr3 protein in tissue sections from normal mouse intestine (a) and kidney (b). Cryostat sections of fresh-frozen tissues were probed with anti-mdr3 antibody and FITC-conjugated goat anti-rabbit secondary antibody. Note that the epithelial lining of vili in the intestine is stained intensely, whereas there is only background staining in the kidney section. Kidney expresses mdr1 protein which can be detected by monoclonal antibody C219 (see Fig. 7A).

Discussions

Transgenic Liver Tumor Models and mdr Gene Expression. Liver tumors in rodents can be induced by the use of various carcinogens, toxins, dietary intervention, and drug-metabolizing enzymes, and certain strains of mice characteristically develop liver tumors spontaneously (4, 5). The lack of synchrony and predictability of hepatocellular transformation in these models makes molecular analyses of step-by-step events in hepatocarcinogenesis difficult. The development of transgenic mouse models of HCC overcomes many of these difficulties. These animals carry inherited hepatocarcinogenic determinants in a homogeneous genetic background and generate a predictable sequence of pathogenetic events leading to HCC in all mice.

The present study took advantage of a well-defined model of HCC in HBV transgenic mice which allowed us to biochemically dissect the time course of mdr gene expression at various stages of disease (Fig. 8). The immunohistochemical data basically confirm the biochemical data. In addition, we have recently analyzed the expression of the mdr gene in another transgenic mouse HCC model in which SV40 large T antigen linked to human α1-antitrypsin promoter was incorporated. The pathogenetic manifestations of HCC in this model also follow predictable kinetics (24). We found that mdr1, in addition to mdr3, was also activated during the development of HCC in this model (data not shown). The coinduction of the mdr1 gene in this tumor model may be due to the property of SV40 large T antigen as a multifunctional protein (Ref. 25 and references therein). Together, these results strongly suggest that transgenic models of HCC represent a valuable system for evaluating the molecular pathogenesis of HCC and the evolution of drug resistance in this disease.

Activation of mdr Gene during Hepatocarcinogenesis. The mechanisms underlying hepatocellular carcinogen-
Carinogenesis is complex. It is known that different carcinogens or carcinogenic regimens produce markedly different initial events of hepatocarcinogenesis. The activation of mdr3 gene in many different mouse HCC models, irrespective of carcinogenic programs, would suggest that these presumptive diverse pathways converge at a certain point of time, most likely at the late stage of tumor development. The present results, showing that the mdr3 expression is first detectable with the development of MNH but not earlier, support this idea. In this regard, the activation of the mdr3 gene may be associated with tumor promotion or progression, rather than tumor initiation.

Although hepatocellular proliferation is a characteristic of HCC, we do not believe that liver regeneration alone can explain the mdr activation in HCC, because both mdr2 and mdr3 are coordinately activated during liver regeneration, whereas only mdr3 is activated in HCC. Preliminary results show that induction of acute phase response in cultured hepatoma cell lines (Hepalclc and Hep3B) by the cytokine interleukin 6 fails to activate mdr3 expression, despite induction of a battery of acute phase genes in this system, suggesting that the induction of mdr3 expression in HCC is not merely secondary to an inflammatory response in the tumors.

Although significant progress has been made in understanding liver-specific gene expression in recent years (for review, see Ref. 29), the molecular basis of altered gene expression specifically associated with hepatocarcinogenesis remains elusive. The consistent activation of the mdr3 gene in various HCC models suggests that this gene may represent an attractive system for the investigation of gene activation following hepatocarcinogenesis. We believe that the activation mechanism is a cascade of molecular events intrinsic to the process of hepatocarcinogenesis itself. A number of independent cell lines were established from HCC originating in HBV mice. The expression of the mdr3 gene in these cell lines in culture was extinguished. Tumor developed in mice by intrasplenic injection of these cells (30) failed to reactivate the expression of the mdr3 gene. In another study, we found that the expression of MDR1 (homologue of the mouse mdr3) in human liver colon and colonic cancer is high; however, human liver metastases of colorectal tumors exhibited diminished levels of MDR1 expression. The inability of reactivation/maintenance of a high level of mdr3/MDR1 expression in these liver tumors suggests an intimate link between mdr3 activation and hepatocarcinogenesis. Furthermore, these observations suggest that future studies on the molecular mechanism of mdr gene activation would be facilitated by the use of animal models of HCC per se.

**MDR Gene Activation and Intrinsic Drug Resistance of Liver Cancers.** The results described here and in previous report (8) may have significant clinical implications, because the mouse mdr3 gene is the homologue of human MDR1, the only human MDR-related gene. High levels of MDR transcript were detected in a number of HCCs (31). In a comparative analysis of samples of HCC and surrounding nontumorous liver from a group of 21 patients, we found that 42% of patients showed overexpression of MDR1 mRNA in their tumors. Given the implication that the MDR protein in liver may function as antitumor drug transporter (32, 33), the high levels of MDR1 expression in human liver tumors are likely to be responsible for the intrinsic drug resistance of HCC; however, this remains to be critically evaluated.

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5 M. T. Kuo, unpublished results.

6 F. V. Chisari, unpublished results.

7 M. T. Kuo, D. Fan, and I. J. Fielder, unpublished observations.

Finally, the transgenic models described here may be valuable tools for preclinical studies on the role of mdr gene expression and the chemotherapeutic response of HCC to antitumor drugs. In recent years, a great deal has been learned about the pharmacological reversal of multidrug resistance in cultured cell studies (for reviews, see Refs. 34 and 35). These transgenic models of HCC may provide an in vivo system for the testing/screening of these compounds and may eventually improve the chemotherapy of human HCC.

Materials and Methods

Transgenic Animals, Tissue Collection, and Histopathological Diagnoses. Production of transgenic mice carrying the HBV envelope region (BglII-A fragment, ayw subtype)
first generation hybrids of the inbred mouse strains C57BL/6 and SJL-J. This lineage was expanded by back-crossing against the C57BL/6 parental strain.

Liver samples were harvested from mice at various ages. Multiple samples from different regions in the liver were taken, and several mice from the same stages of disease were used. Transgene-positive mice were identified by expression analysis for serum hepatitis B surface antigen as previously described (17). Animals were sacrificed by cervical dislocation. Liver tissues were excised, rinsed with buffered physiological saline, immediately frozen in liquid nitrogen, and stored at −70°C until use.

Portions of liver tissues were fixed in 10% neutral buffered Zn-formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, orcein, and Victoria blue according to standard procedure. Histopathological diagnosis was performed as described previously (17).

**Extraction of RNA and Analysis of mdr mRNA Levels.**

Liver tissues were pulverized in a porcelain mortar and pestle in the presence of liquid nitrogen and were homogenized in a guanidinium isothiocyanate solution with a loosely fitted Dounce homogenizer. RNA was isolated by the method described by Chirgwin et al. (36). The integrity of RNA was monitored by agarose gel electrophoresis of glyoxal-denatured samples followed by ethidium bromide staining and UV transillumination.

Molecular cloning of mouse mdr gene-specific cDNA, construction of templates, preparation of antisense RNA probes, and RNase protection assay of mdr mRNA levels using albumin mRNA as reference have been described previously (8).

**Preparation of Antipeptide Antibodies, SDS-Gel Electrophoresis and Western Blot.** A peptide corresponding to residues 670–686 of mdr3 protein with single letter amino acid sequence ICGPHQDRKTCSTKEAL was synthesized on a Vega Coupler 250 C synthesizer using tert-butoxycarbonyl chemistry. Synthesized peptides were deprotected and cleaved from the supporting resin with hydrogen fluoride in the presence of 5% anisole, solubilized in 26% (v/v) glacial acetic acid, and lyophilized. Peptides were analyzed by amino acid analysis and high performance liquid chromatography analyzer. Peptides with amino acid residues in the corresponding regions of mdr2 and mdr3 proteins were similarly synthesized. Peptides were reconstituted in PBS at 1 mg/ml and mixed with an equal volume of keyhole limpet hemocyanin (1 mg/ml in PBS) and 0.016 volume of 2.5% glutaraldehyde. The mixture was dried under vacuum in a Speedvec concentrator (Savant) and resuspended in PBS at 1 mg of peptide/ml.

Antibodies were produced by injecting the coupled peptides emulsified with an equal volume of incomplete Freund’s adjuvant (Sigma, St. Louis, MO) s.c. into the hind thighs (four sites) of two rabbits. Each rabbit received 1 mg of peptide/ml/injection every other week. After 4 months, rabbits were bled, and the titers were determined by enzyme-linked immunosorbent assay and/or Western blot using membrane proteins prepared from murine MDR cell lines.

Antibodies were purified by antigen immobilization on a membrane filter. Briefly, membrane vesicles (see below) prepared from mdr3-overproducing MDR cells, ARN15 line, were lysed in Laemml sample buffer, and the protein was separated by SDS-polyacrylamide gel linked to a heterologous promoter from mouse albumin gene has been described previously (17). The lineage used for this study [lineage 50-4, official designation Tg(alb-I HBV) bri 44] will be hereafter referred to as HBV mice. The founder was derived from parents that were

**Fig. 6.** Immunohistochemical staining of HCC by anti-mdr3 antibody can be blocked by peptide antigen. Anti-mdr3 antibody either pretreated for 30 min with peptide antigen (b) or without pretreatment (a) was used in immunohistochemical staining by the same procedure as described in legend to Fig. 5. Control experiment was performed as in a except using preimmune serum (c). 400x.
The proteins were electrotransferred onto a nitrocellular membrane. A portion of the membrane was cut out and Western blotted using anti-mdr3 antibody to localize the mdr3 protein on the membrane. The portion containing mdr3 protein on the remaining membrane (approximately 16 µg of protein/160 mm²) was cut out. This membrane was treated with 5 ml of PBS containing 1% of bovine serum albumin and 0.02% NaN₃ for 1 h at 22°C. One ml of serum from the immunized rabbits was added, and incubation was continued for an additional 3 h. The membrane was washed with 5 ml of PBS. Affinity-purified antibody was eluted accord-
Fig. 8. Time course of mdr gene expression in the pathogenetic program of HBV transgenic mice. Horizontal arrows, the elements involved in the natural history of the disease. Vertical arrows, the expression of given mdr genes. Thickness of arrows is meant to reflect the relative levels of expression.

References


