P-Glycoprotein Expression during Tumor Progression in the Rat Liver

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ABSTRACT

P-Glycoprotein (Pgp) has been shown to mediate multidrug resistance in tumor cell lines. Overexpression of Pgp has been detected in clinical cancer samples of many histological types. The basis and biological significance of such increases in Pgp expression are not well understood. In this study, the expression of Pgp during stepwise progression to rat liver cancer was examined to investigate the possible role of Pgp in carcinogenesis. An immunohistochemical technique was used to detect Pgp at the single-cell level, in a large number of liver nodules, hepatocellular carcinoma, and in distant metastases of the carcinomas. The results showed that distinct changes in Pgp expression occurred during stepwise liver carcinogenesis and that these changes were closely associated with the microscopic anatomy of the lesions. In contrast to γ-glutamyl transpeptidase and glutathione S-transferase-7.7, whose expression appeared to correlate with the early steps of liver carcinogenesis, Pgp expression was higher in the large hyperplastic nodules and in hepatocellular carcinomas than in the early microscopic lesions. A particularly striking finding was the consistent expression of Pgp in the lung metastases. These findings suggested that Pgp was associated with a more progressed malignant phenotype in liver carcinogenesis.

INTRODUCTION

Pgp2 is a membrane glycoprotein whose overexpression has been detected in many multidrug-resistant cell lines. Transfection studies in vitro have shown that Pgp can mediate multidrug resistance, probably through ATP-dependent drug efflux across the plasma membrane (1). Surveys of normal tissues in vivo have revealed relatively high levels of Pgp expression in diverse types of differentiated cells. These include intestinal lining epithelial cells, hepatocytes, endothelial cells, adrenal cortical cells, and striated muscle cells. The physiological function(s) of Pgp is so far unknown, although its tissue distribution is suggestive of a role in cellular transport of specific metabolites (2, 3). P-Glycoprotein has been found to be overexpressed in clinical tumor samples of a variety of histological types (4–6). In some cases, Pgp overexpression was detected upon relapse from initial response to combination chemotherapy. This may be analogous to the situation in vitro where Pgp overexpression occurred during selection for multidrug resistance (7, 8). However, in other cases, Pgp overexpression occurred without previous exposure to chemotherapy (6, 9, 10). It is possible that in this instance Pgp expression is elevated as part of the cascade of molecular events associated with tumor progression. A study of Pgp expression in an experimental tumor model in which the stepwise progression to cancer has been delineated may help to elucidate the role of Pgp in carcinogenesis.

Pgp expression has been previously studied at the mRNA level in experimental rat liver tumors produced by the Solt-Farber technique, in which carcinogenesis was initiated by an injection of diethylnitrosamine and promoted by AAFe coupled with PH (11, 12). A marked elevation of Pgp mRNA was found for both preneoplastic liver nodules and hepatocellular carcinoma, when compared with normal liver. In the present study the possible role of Pgp in carcinogenesis was further examined by using the orotic acid model of rat liver carcinogenesis. In this model, rats were initiated by PH coupled with a single injection of DMH and then promoted by continuous feeding of a 1% OA diet (13). Orotic acid is a precursor in the de novo synthesis of pyrimidine nucleotides and as such, is a cellular metabolite and not a xenobiotic. Its effect in promoting rat liver carcinogenesis is dependent on its production of a nucleotide pool imbalance (14). The orotic acid protocol resulted in sequential development of hepatic foci, hyperplastic hepatic nodules, and hepatocellular carcinoma. In previous studies using this protocol, the incidence of carcinoma after 13 months of treatment was close to 100%. Between 30 and 60% of the carcinomas metastasized to the lungs (13). Thus, this model of liver carcinogenesis was chosen to follow changes in Pgp expression at several defined steps of tumor progression. In particular, the relatively high incidence of lung metastases from the hepatocellular carcinomas facilitated the analysis of Pgp expression in metastatic lesions.

A highly specific immunohistochemical technique was used in the present study to evaluate changes in Pgp expression during rat liver carcinogenesis. Staining for Pgp was obtained by using a monoclonal antibody against Pgp (C219), in conjunction with its epitope peptide (3, 15). This allowed a detailed study of protein expression at the single-cell level, in individual preneoplastic and neoplastic lesions. In addition, the staining for Pgp was compared with that for GGT and GST-P on parallel sections. These two enzymes have been frequently found to be altered in expression during early stages of hepatic chemical carcinogenesis. In the evaluation of tissue sections, the observed changes in the expression of Pgp, GGT, and GST-P within preneoplastic and neoplastic lesions were assessed against the background of the surrounding liver which served as an internal control for “global” alterations caused by the carcinogenic treatment regimen. Our study revealed complex changes in Pgp expression during rat liver carcinogenesis which have not been demonstrated in previous studies.

MATERIALS AND METHODS

Experimental Animals. Male Fischer 344 rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 100 to 120 g, were maintained on Purina 5001 rodent laboratory chow diet and daily cycles of alternating 12-h periods of light and darkness for 1 week before the start of the experiment. Water and food were available ad libitum. The OA diet was purchased from Dhyets, Inc. (Bethlehem, PA), and was made up by incorporating 1% OA as its monosodium salt at the expense of sucrose into a semisynthetic basal diet (No. 101) (13). Both diets were color coded and were obtained as pellets. DMH was purchased from Aldrich Chemical Co. (Milwaukee, WI) and OA monosodium salt was obtained from Sigma Chemical Co. (St. Louis, MO).
Protocol for Experimental Hepatocarcinogenesis. The protocol for experimental rat liver carcinogenesis is shown in Fig. 1. Rats in the experimental group (E) were initiated by a single i.p. injection of DMH at 100 mg/kg, 18 h after a two-thirds PH. After 1 week, the rats were changed from the basal diet to one containing 1% OA and were maintained on this diet until the time of sacrifice. There were three groups of control animals. One group (C1) received an i.p. injection of 0.15 M NaCl solution after PH and was maintained on basal diet. The second group (C2) also received NaCl solution i.p. after PH but was fed the 1% OA diet. The third control group (C3) was given an injection of DMH after PH and was maintained on basal diet.

Rats were examined at intervals and sacrificed as indicated in Fig. 1. In livers without grossly visible lesions, 10 random samples were obtained for study. In livers with visible nodules, all nodules which were identified were taken for study, and in addition, random samples were taken from the remaining liver. Hepatocellular carcinomas typically occupied and distorted most of the liver. These were divided into quadrants and representative samples were taken from each quadrant.

Chemicals and Reagents. Monoclonal antibody C219 (Centocor, Malvern, PA) was used as the primary antibody for immunohistochemical detection of P-glycoprotein. C219 has been shown to bind to a linear epitope in the highly conserved COOH-terminal cytoplasmic domain of Pgp, and the amino acid sequence of the epitope has been determined through peptide mapping (15). Sequence analysis of rat genomic Pgp clones has demonstrated the presence of the C219 epitope in all three rat Pgp isoforms (16). Thus, mAb C219 should recognize all Pgp isoforms in rat tissue sections. Specificity of staining for Pgp was ensured by using the C219-epitope peptide in blocking experiments. Previous studies have shown that the presence of the epitope peptide in a large molar excess could block epitope-specific immunohistochemical staining, while nonspecific staining was not affected (3, 15). Immunohistochemical staining with mAb C219 in this study was routinely performed with a negative control in which the C219-epitope peptide was added to block epitope-specific staining.

A rabbit antiserum against GST-P from rat hyperplastic liver nodules (generously provided by Dr. Farber, Department of Pathology, University of Toronto) was used for immunohistochemical detection of GST-P. The Vector ABC reagents (Vector Laboratories, Burlingame, CA) were used in conjunction with the primary antibodies described above. Biotinylated horse anti-mouse antibody previously adsorbed with rat tissues was the second antibody for mAb C219. Biotinylated goat anti-rabbit antibody was the second antibody for the GST-P antiserum. A streptavidin-horseradish peroxidase conjugate followed by reaction with diaminobenzidine (Sigma) and hydrogen peroxide was used to localize the biotinylated label.

Histology, Histochemistry, and Immunohistochemistry. Tissue samples obtained at sacrifice were frozen in OCT (Tissuetek, Miles Inc., Elkhart, IN) by immersion in isopentane chilled with liquid nitrogen. Multiple cryostat sections (6 μm) were obtained for each tissue block so that histology, GGT histochemistry, and immunohistochemistry for Pgp and GST-P could be performed in parallel. A small number of tissue samples were not snap frozen but were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned for additional histological studies.

For histological examination, cryostat sections were briefly fixed in 10% phosphate-buffered formalin and stained with hematoxylin and eosin. For histochemical localization of GGT activity, the method of Rutenberg et al. was used (17). Cryostat sections were incubated for 45 min in a freshly prepared solution containing γ-glutamyl-4-methoxy-2-naphthylamide, Fast Blue BB salt, and glycerylglucine. After rinsing with saline, the sections were dipped in 0.1 M cupric sulfate, rinsed again with saline, washed in distilled water, and counterstained with hematoxylin. The GGT-stained sections were stored at 4°C and examined within 2 days. Bile ductules are normally positive in a histochemical reaction for GGT and were used as internal positive controls for GGT histochemistry of liver sections.

Immunohistochemical staining for Pgp and GST-P was performed on frozen sections fixed for 10 min in cold acetone. After rinsing with phosphate-buffered saline, endogenous peroxidase activity and endogenous biotin-like activity were blocked by appropriate pretreatments and immunohistochemical staining was carried out as described previously (3). Antibody C219 was used at 1.5 μg/ml in 1% bovine serum albumin in an overnight incubation (16 h) at 4°C. The polyclonal antiserum against GST-P was used at 1:150 dilution in 1% bovine serum albumin/phosphate-buffered saline, for 16 h at 4°C.

Semiquantitative Assessment of Pgp Expression by Immunohistochemistry. Previous studies performed with mAb C219 have shown a correlation between the intensity of immunohistochemical staining for Pgp in the plasma membrane and the amount of Pgp per unit membrane protein as measured by Western blot. Furthermore, in cell lines selected for progressively higher levels of multidrug resistance, the intensity of immunohistochemical staining correlated with the level of multidrug resistance, over a range of approximately 2000-fold difference in resistance (3, 18). Thus, the intensity of specific immunohistochemical staining with mAb C219 can be used for a semiquantitative assessment of the amount of Pgp per unit membrane which should in turn reflect Pgp activity. In the present study, staining of normal liver with mAb C219 revealed distinct, polarized staining of the bile canalicular surface of hepatocytes, as reported in previous studies. The intensity of Pgp staining in cells of hepatic nodules and carcinomas was found to vary from undetectable to strong staining exceeding that of normal hepatocytes. Thus, the Pgp staining of the altered hepatocytes during carcinogenesis was assessed as undetectable, weak (less intense staining than normal hepatocytes), moderate (equivalent to staining of normal hepatocytes), and strong (more intense than normal hepatocytes).

RESULTS

Progressive Development of Hepatic Lesions in Experimental Rats. Rats treated with DMH coupled with two-thirds hepatotomy and fed a diet containing 1% OA showed progressive development of preneoplastic and neoplastic liver lesions. Twenty-five experimental rats were examined at various time points during tumor development. Of the seven rats which were sacrificed after 56 or more weeks of promotion, all showed development of hepatocellular carcinoma. Three of these seven rats had metastases to the lungs. Immunohistochemical staining of a large number of hepatic foci, nodules, and primary and metastatic hepatocellular carcinoma revealed distinct changes in Pgp expression during hepatic carcinogenesis. In addition, the expression of GGT and GST-P in these lesions were studied and compared with that of Pgp. GGT and GST-P have been shown to be overexpressed by hepatocytes of hepatic foci and nodules during chemical hepatocarcinogenesis. Both enzymes are thought to be involved in hepatic detoxification pathways and contribute to the “resistant hepatocyte” phenotype which

Fig. 1. Protocol for rat liver carcinogenesis. A, treatment given to experimental and control rats. B, the eight time points at which rats were taken for examination. The experimental animals showed sequential development of preneoplastic lesions and malignant liver tumors with metastases to the lungs.
P-glycoprotein expression during liver carcinogenesis

Characterizes hepatic foci and nodules (19). Thus, a comparison of Pgp against GGT and GST-P was made to elucidate the relationship between Pgp and the resistant hepatocyte phenotype during hepatocarcinogenesis.

**Hepatic Foci.** Rats sacrificed after 5 and 10 weeks of promotion showed multiple microscopic foci of altered hepatocytes in the liver. These were readily demonstrated by GGT histochemistry as small groups of hepatocytes which stained strongly on the plasma membrane for GGT activity while the surrounding liver did not show detectable GGT activity (Fig. 2a). The number of hepatic foci per unit area of liver sections was generally comparable to that determined in previous characterization of the orotic acid model of rat hepatocarcinogenesis (20). There was no detectable change in Pgp expression in these early hepatic foci. The hepatocytes of the foci exhibited polarized staining for Pgp on the bile canalicular face of the

![GGT](image1)
![GST-P](image2)
![Pgp](image3)

**Fig. 2.** Comparison of Pgp expression with that of GGT and GST-P in liver lesions at different stages of liver carcinogenesis. **Column 1,** GGT histochemistry; enzyme activity was shown as a red reaction product. **Column 2,** immunohistochemical staining with rabbit anti-GST-P serum. **Column 3,** immunohistochemical staining for Pgp using mAb C219. For both GST-P and Pgp staining, the antigen was visualized as a brown reaction product and nuclei were counterstained with hematoxylin. Details of staining procedures were described under “Material and Methods.” Panels a,b,c: hepatic focus seen after 10 weeks of promotion, showing strong GGT activity in the plasma membrane, and strong GST-P staining in the cytoplasm, in contrast to the surrounding liver. However, the focus was not distinguished from the surrounding liver by Pgp staining. Panels d,e,f: large hepatic focus seen after 15 weeks of promotion, showing partial loss of membrane localization of GGT activity. The hepatocytes of the large focus appeared heterogeneous in Pgp expression. Panels g,h,i: 1-mm-diameter hyperplastic nodule seen after 15 weeks of promotion. There was virtually complete loss of membrane localization of GGT activity. GST-P staining was clearly reduced in the nodule, compared to the focus. Pgp expression was markedly heterogeneous within the nodule, with only a fraction of the nodule cells showing overexpression relative to the adjacent liver. Panels k,m,n: 20-mm-diameter hyperplastic nodule of the trabecular-sinusoidal type seen after 34 weeks of promotion. GGT activity was patchy in distribution through the nodule with many areas devoid of reaction product. GST-P staining was more uniform but weak. In contrast, Pgp was overexpressed by the majority of nodule hepatocytes relative to surrounding liver. **Bar,** 125 μm.
Table 1 Occurrence of hyperplastic liver nodules in rats initiated with DMH and exposed to OA for increasing number of weeks

Each line shows the number of hyperplastic nodules found by gross examination of the liver of one rat, and the size of the largest nodule found in that liver. Rats which have undergone a longer period of promotion had more nodules in their liver and/or larger nodules.

<table>
<thead>
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<th>No. of weeks of promotion</th>
<th>No. of nodules</th>
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<tr>
<td>34</td>
<td>5</td>
<td>3</td>
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</table>

* Trabecular-sinusoidal nodule, the other nodules being of the non trabecular type.

Hyperplastic Hepatic Nodules. Hyperplastic hepatic nodules were discrete, rounded masses of altered hepatocytes which measured at least 1 to 2 mm in diameter and were thus visible on gross examination. In contrast, hepatic foci were microscopic, not demarcated from the surrounding liver, and typically did not exceed 1 mm in diameter. Nodules were first observed in the liver of rats after 15 weeks of OA promotion. Between 15 and 34 weeks of promotion, there was a progressive increase in the total number of nodules observed in each liver and also an increase in their maximum size (Table 1). A total of 62 hyperplastic nodules were examined from rats sacrificed in this period.

P-Glycoprotein Expression of Hyperplastic Nodules. Two types of hyperplastic nodules were observed. They differed in gross appearance, microscopic architecture, and Pgp expression, but could not be distinguished by GGT staining. The more common type of nodule was whitish gray on gross examination. Microscopically, it consisted of closely packed, enlarged hepatocytes with inconspicuous vascular sinusoids (Fig. 3c). Pgp expression was characteristically heterogeneous, with only scattered cells within the nodules exhibiting strong staining, while most cells showed weak or undetectable staining (Fig. 2j and Fig. 3, a and b). Staining for Pgp was always membrane associated and polarized to one end of the nodular hepatocyte.

The second type of nodule was rarely found, as there were only three examples within a total of 62 hyperplastic nodules. These nodules were reddish brown on gross examination. Microscopically, the node consisted of trabeculae of altered hepatocytes separated by widened vascular sinusoids which were lined by prominent endothelial cells. There was a superficial resemblance to the normal liver, but the trabeculae of the hyperplastic nodule were haphazard in arrangement and not organized around portal triads and central veins (Fig. 3f). In contrast to the more common type of nodule described above, the majority of cells in the reddish-brown nodules stained strongly for Pgp (Fig. 3, d and e). The pattern of Pgp staining within the trabeculae recapitulated that of the normal liver.

Rats sacrificed after 15 or more weeks of promotion also had similar foci of altered hepatocytes, in addition to hyperplastic hepatic nodules which began to appear at 15 weeks (see below, “Hyperplastic Hepatic Nodules”). Compared to the foci seen at 5 and 10 weeks, those seen after 15 or more weeks of promotion had a greater average size. They were still readily demonstrated by GGT histochemistry, but the larger foci showed partial loss of membrane localization of GGT activity (Fig. 2d). The hepatocytes within these larger foci appeared heterogeneous in Pgp expression when compared against the surrounding liver, varying from no detectable Pgp to increased intensity of Pgp staining (Fig. 2f).

Fig. 3. Light microscopic appearance and Pgp staining of the two types of hyperplastic nodules in cryostat sections. Panels a,b: non trabecular nodule stained for Pgp. Many of the nodular hepatocytes had weak or undetectable staining while a minority of the nodular hepatocytes overexpressed Pgp relative to the surrounding liver. Panel c: non trabecular nodule, stained with H & E. The hepatocytes were closely-packed, with inconspicuous sinusoids. Panels d,e: trabecular-sinusoidal nodule, stained for Pgp. Most of the hepatocytes stained strongly for Pgp relative to the surrounding liver. Panel f: trabecular-sinusoidal nodule stained with H & E. The hepatocytes were organized into trabeculae which were separated by widened sinusoids with prominent endothelial cells. Bar, 125 μm.
being localized to the bile canalicular surface of the hepatocytes.

In accordance with the characteristic microscopic architecture, the two types of hyperplastic hepatic nodules that were observed were referred to as nontrabecular and trabecular-sinusoidal types.

**Hyperplastic Hepatic Nodules Obtained without Orotic Acid Promotion.** In rats treated by two-thirds partial hepatectomy coupled with an injection of DMH, dietary OA (at 1%) acted as a promoter for the development of hyperplastic hepatic nodules and hepatocellular carcinoma. Without OA promotion, initiated rats developed nodules and carcinoma with a lower incidence (13). In the present study, two rats which were initiated with partial hepatectomy and DMH and were maintained on basal diet were sacrificed and examined at 54 weeks (Table 2). One rat (no. 26) had a large, whitish nodule which measured 30 mm in greatest diameter. Microscopically, this nodule had a nontrabecular architecture and was heterogeneous in Pgp expression, with only small scattered groups of nodular hepatocytes showing strong Pgp staining. The other rat of this group (rat 25) had two large, adjacent reddish-brown nodules measuring 20 and 30 mm in greatest diameter, respectively. Microscopically, both nodules were of the trabecular-sinusoidal type, in which the majority of the nodule cells exhibited strong staining for Pgp along the bile canalicular surface. The findings in these two rats indicated that the occurrence of two types of hyperplastic nodules which were strikingly different in Pgp expression was not dependent on OA administration.

**Hepatocellular Carcinoma.** The seven rats in the experimental group which were sacrificed after 56 or 59 weeks of promotion all showed hepatocellular carcinoma (Table 2). On gross examination, carcinoma appeared as an ill-defined mass of variable color and consistency which typically occupied the entire right-anterior lobe. In some cases there was severe distortion of the liver lobes and fusion of adjacent lobes. Microscopically, hepatocellular carcinoma was distinguished from hyperplastic nodules by the increase in cellularity, cellular and nuclear pleomorphism, and increase in mitotic figures (Fig. 4a). Necrotic areas were found in some cases. The observation of hyperplastic nodular liver interposed between hepatocellular carcinoma and residual liver was consistent with hypothesis that the carcinoma has arisen within a hyperplastic nodule.

In contrast to the hyperplastic nodules, carcinomas could not be separated into two types, nontrabecular and trabecular-sinusoidal, based on microscopic anatomy. Multiple samples taken from each carcinoma showed considerable regional variations in architecture. Areas which had features of hyperplastic nodule were found contiguous to areas of carcinoma. A striking finding was the increased appearance of the trabecular-sinusoidal architecture in the carcinomas, compared with the nodules. This type of architecture was represented in every carcinoma and was often predominant over the nontrabecular architecture. The trabeculae in the carcinomas were more irregular in shape and thickness than those in the trabecular-sinusoidal nodule, varying from one-cell thickness to multiple-cell thickness. However, the trabeculae were always clearly demarcated by intervening sinusoids lined by endothelial cells (Fig. 4, a, b, and c). The level of Pgp expression also showed considerable regional variations within each carcinoma, and there was a correlation between Pgp expression and microscopic structure. Areas with features of nontrabecular hyperplastic nodule had generally weak Pgp staining while areas of carcinoma were more strongly stained. Within areas of carcinoma, the strongest expression of Pgp was found in association with a trabecular-sinusoidal architecture (Fig. 4, b and c). In some cases, a discrete area of trabecular carcinoma was seen within an area of nontrabecular carcinoma (Fig. 4d), suggesting that a trabecular organization might arise from malignant progression of a nontrabecular carcinoma. Such a trabecular zone expressed elevated levels of Pgp in comparison with the surrounding nontrabecular carcinoma, and this observation further substantiated the correlation between Pgp overexpression and a trabecular-sinusoidal architecture.

**Lung Metastases.** Three of the seven rats with hepatocellular carcinoma had metastatic lesions in the lungs (Table 2). The number of lung metastases per rat varied from 57 to 229. They varied in size from microscopic foci of a few cells to grossly visible lesions measuring 1 to 2 mm in diameter (Fig. 4e). The small metastases appeared as nests of large, atypical epithelial cells which were usually easily distinguished from the lung tissue. They were often seen to be within small blood vessels or to be extending from the vessel lumen, through the vessel wall, into adjacent lung tissue (Fig. 4f). The larger metastases typically showed a trabecular architecture and were commonly found adjacent to small blood vessels (Fig. 4g).

A total of 460 metastases were examined and every metastasis contained cells that were positive for Pgp by immunohistochemical staining, regardless of the size of the metastasis or its location with respect to the blood vessel wall. In contrast, the surrounding lung tissue did not stain for Pgp (Fig. 4, e, f, g, and h). Thus, readily detectable Pgp staining appears to be a consistent feature of the lung metastases.

There was partial loss of membrane localization of Pgp staining in some areas of the primary liver carcinomas and in some of the lung metastases (Fig. 4, c and f). This was in contrast to Pgp staining in the hyperplastic nodules which was always clearly membrane associated and polarized to one cell surface. The subcellular location of the cytoplasmic Pgp staining seen in some of the cancer cells was not clear from the light microscopic studies.

**Comparison of P-Glycoprotein Expression with GGT and GST-P Staining.** Elevation of GGT activity could be demonstrated histochemically in foci of hepatocytes within a few weeks of initiation (see above, "Hepatic Foci," and Fig. 2a). In

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**Table 2: Occurrence of hyperplastic nodules and liver carcinoma in experimental control rats after 54 or 59 weeks of promotion**

<table>
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<tr>
<th>Rat no.</th>
<th>Initiation</th>
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agreement with previous studies, the GGT-positive foci also had elevated levels of GST-P expression (19) (Fig. 2b). The "enzyme-altered" foci are thought to represent a very early stage of chemical hepatocarcinogenesis (21). These early enzyme changes were not accompanied by any detectable overexpression of Pgp (Fig. 2c).

Hyperplastic hepatic nodules showed elevated GGT activity and GST-P staining compared to the surrounding liver. It was interesting to note that, in contrast to hepatic foci, the GGT activity in nodules was almost entirely diffuse over the cytoplasm, rather than being associated with the plasma membrane (Fig. 2g). As described above, the hyperplastic nodules demonstrated two patterns of alteration in Pgp staining (Fig. 3). No corresponding difference in the pattern of GGT reaction or of GST-P staining was evident between the two types of hyperplastic nodules that were recognizable on the basis of Pgp expression and microscopic anatomy.

In hepatic nodules which exceeded about 15 mm in diameter, the histochemical staining for GGT became variable and many areas within a nodule became GGT negative (Fig. 2k). Cytoplasmic staining for GST-P was uniform but weak within the large nodules (Fig. 2m). Similarly, GGT activity and GST-P staining of the hepatocellular carcinomas were either weak or undetectable. This reduction in GGT and GST-P expression was not paralleled by a reduction in Pgp expression. Instead, there was an increase in predominance of the trabecular-sinusoidal architecture with relatively strong Pgp staining in the large hyperplastic nodules and the carcinomas (see above, "Hepatocellular Carcinoma").

Metastases of hepatocellular carcinoma to the lungs varied in their GGT reaction from a lack of staining to diffuse positive staining (Fig. 4j). Staining for GST-P was generally weak, and some of the metastases did not show detectable staining (data not shown). In contrast, every lung metastasis contained cells which stained strongly for Pgp, and metastases with no demonstrable GGT and GST-P staining were shown on parallel sections to stain clearly for Pgp (Fig. 4, h and j).

**DISCUSSION**

The mechanism of Pgp overexpression in patients' tumors is not fully understood, and may vary among different types of tumors. In solid tumors such as carcinoma of the colon, liver, or kidney, high levels of Pgp have been consistently observed in a fraction of the cases examined (4, 10). In many instances, these carcinomas have not been treated with chemotherapy, so that
the overexpression of Pgp could not be related to selection and enrichment of multidrug-resistant malignant cells. Overexpression of Pgp has also been demonstrated in different experimental models of hepatocarcinoma (11, 12, 22). These earlier studies suggest that overexpression of Pgp may be inherent to carcinogenesis under certain conditions.

The orotic acid model of rat liver carcinogenesis was used in this study to further investigate the possible role of Pgp in carcinogenesis. The features of this tumor model facilitated the study of Pgp expression at several stages of tumor progression, including metastasis to distant sites. Furthermore, OA is not a liver xenobiotic and chronic feeding of 1% OA did not appear to alter Pgp expression in the liver (data not shown). Thus the changes in Pgp expression that were observed could be reasonably attributed to the carcinogenic process itself. The use of immunohistochemical techniques allowed the examination of many preneoplastic lesions in situ, and at the single-cell level. It obviated the need for isolation of these small lesions from the surrounding liver and pooling of multiple lesions for routine RNA or protein extraction procedures. Staining for Pgp at the single-cell level revealed a correlation between cellular expression of Pgp and microscopic anatomy which would not be readily identified in biochemical studies. In the cases of hepatocellular carcinoma, assessment of Pgp expression in situ proved to be highly informative because of the marked regional variations in both microscopic anatomy and Pgp content within each carcinoma.

Our first significant finding was that the small hepatic focus, which was the earliest demonstrable lesion produced during experimental rat liver carcinogenesis, showed no evidence of change in Pgp expression (Fig. 2c). The hepatocytes of the small hyperplastic nodule which followed in appearance were characteristically heterogeneous in Pgp content, with many cells expressing undetectable or low amounts of Pgp (Fig. 2f). These findings differed from those previously obtained for preneoplastic liver nodules in the Solt-Farber model of liver carcinogenesis. Those preneoplastic nodules, obtained at 8 to 10 weeks after initiation, had markedly elevated mRNA levels for Pgp (11, 12). Several possible explanations of the disparity could be considered. Protein content was assessed at the single-cell level in the present study while mRNA levels were measured by blot hybridization previously. There is evidence to suggest that under certain circumstances protein and mRNA levels of Pgp may be separately regulated (23, 24). The liver lesions of the present study and those of the previous studies were obtained by two different carcinogenic protocols (see "Introduction"). In the Solt-Farber model used previously, PH coupled with AAF feeding was used as a brief but intense promotion for hepatocarcinogenesis. This method resulted in the rapid and synchronous appearance of a large number of liver nodules, the majority of which remodeled by differentiation into normal-appearing liver. A minority of the early nodules persisted and are thought to give rise to hepatocellular carcinoma. In the orotic acid model used in the present study, promotion was achieved by chronic feeding of 1% OA. In contrast to AAF, which is a xenobiotic and has been shown to cause rapid induction of Pgp expression in the liver (25), OA is a cellular metabolite involved in nucleotide biosynthesis and did not appear to alter Pgp expression in the liver. The high level of Pgp expression in the preneoplastic nodules of the Solt-Farber model may be specific to the use of AAF for promotion and therefore not observed when OA was used. Despite the difference in the method of promotion, the two models shared the characteristic of a high incidence of hepatocellular carcinoma at about 52 weeks after initiation, and Pgp was found to be overexpressed in the liver carcinomas in both models.

In previous studies, it has been hypothesized that Pgp overexpression might be part of the resistant hepatocyte phenotype of rat liver nodules and, together with phase II detoxifying enzymes such as GST-P, might contribute to the xenobiotic resistance of the nodule hepatocytes (12, 26). Our findings, however, indicated that there was no remarkable increase in Pgp expression in the early foci and nodules during hepatocarcinogenesis. In contrast, GGT and GST-P, two enzymes which have been linked to the resistant hepatocyte phenotype were consistently found to be overexpressed within the hepatic foci and the small hyperplastic nodules. P-Glycoprotein expression did not appear to be coordinated with that of GGT or GST-P in this model of hepatocarcinogenesis (Fig. 2). Instead, Pgp expression was closely related to microscopic anatomy, high levels of Pgp being associated with a trabecular-sinusoidal architecture in both hyperplastic nodules and hepatocellular carcinoma.

The occurrence of two distinct types of hyperplastic hepatic nodules merits discussion. Little information is available for the relatively uncommon trabecular-sinusoidal nodule which was characterized by widened sinusoids, prominent endothelial cells, and strong expression of Pgp in the majority of the hepatocytes. In the present study, such nodules were larger than the nontrabecular nodules found in the same liver or in other rat livers after the same duration of OA promotion. The development of these nodules did not appear to be a peculiar feature of OA promotion because they were also found in an initiated rat which was maintained on basal diet for 54 weeks. The two types of hyperplastic hepatic nodules with distinct differences in microscopic anatomy and pattern of Pgp expression might represent alternate intermediate steps in hepatocarcinogenesis. Although there may be a resemblance between the trabecular-sinusoidal nodule described here and certain trabecular carcinomas described by others (27), there is no clear-cut evidence to indicate that the trabecular-sinusoidal nodules were early carcinomas. While the microscopic architecture is clearly different between the two types of nodules, the hepatocytes of the two nodule types had similar cytological features, namely, a vesicular nucleus with prominent nucleoli and increase in cell size with abundant cytoplasm. After further promotion by OA, hepatocarcinoma was seen to develop in association with both types of nodules. The cells of the carcinomas have distinguishing cytological features such as nuclear pleomorphism, variability in cytoplasmic basophilia, and increase in mitotic figures. The three rats which were found to have trabecular-sinusoidal nodules showed no evidence of lung metastases, while three of seven rats which had liver carcinomas showed multiple lung metastases.

Every case of hepatocellular carcinoma obtained in this study exhibited a trabecular architecture, at least in part. The marked increase in predominance of the trabecular-sinusoidal architecture in the carcinomas, compared to the preceding hyperplastic nodules, was consistent with the idea that nontrabecular lesions developed into trabecular-sinusoidal lesions during tumor progression. This idea was further supported by the observation of trabecular carcinoma apparently having arisen within a nontrabecular carcinoma (Fig. 4d). The formation of extensive vascular sinusoids within a mass of proliferating hepatocytes would impart an increased blood supply. Thus, the development of a trabecular-sinusoidal architecture in a minority of hyperplastic
nodules and in most, if not all, cases of hepatocellular carcinoma might be a manifestation of angiogenesis during liver tumor progression (28). Although a trabecular-sinusoidal architecture has generally been considered a sign of differentiation in clinical and experimental hepatocellular carcinoma (13, 27), the observations reported here clearly suggested a different interpretation, that the development of a trabecular-sinusoidal architecture might represent one of the steps in malignant progression towards a hepatocellular carcinoma capable of metastasis.

In both hepatic nodules and carcinomas, higher levels of Pgp expression were associated with a trabecular-sinusoidal architecture than with a nontrabecular one. It is not clear if Pgp overexpression was involved in organization of hepatocytes into trabeculae separated by sinusoids, or if higher levels of Pgp expression occurred as a consequence of the increased vascularity. Nevertheless, our studies have demonstrated an increase in Pgp expression during sequential development of hepatic focus, hyperplastic nodule, and hepatocellular carcinoma. The consistent presence of Pgp in every metastasis of hepatocellular carcinoma to the lungs further supported the hypothesis that Pgp overexpression is more predominant in more advanced stages of malignant progression. The demonstration of Pgp in all metastatic lesions that were examined was in contrast with the more variable expression of GGT and GST-P in the metastases. High levels of Pgp expression might be required for one or more steps of the metastatic process, so that each lung metastasis originated from cells which have been selected for Pgp overexpression. It is not clear if Pgp expression in metastatic hepatocarcinoma was related to the microenvironment of certain tissue sites and not others (29), since metastatic liver carcinoma was not detected in other tissues than the lungs in the present study. Our results have provided new insight into Pgp overexpression in a model tumor in the absence of prior chemotherapy.

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