Cellular and Molecular Changes in the Early Stages of Chemical Hepatocarcinogenesis in the Rat

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ABSTRACT

The early cellular and molecular changes in the Solt-Farber model of hepatocarcinogenesis with and without initiation was studied by using histochemical, immunohistochemical, and in situ hybridization techniques. Increased cellularity was observed in the periductal space in both models 32 to 56 h after partial hepatectomy. These periductal cells and Ito cells were the only cells that became labeled with tritiated thymidine in the uninitiated liver model. Forty-five to 60% of the labeled periductal cells were positive for γ-glutamyltranspeptidase. From the periductal area the cells that were positive for antibody raised against oval cells (OV-6) infiltrated into liver parenchyma and were followed by desmin-positive Ito cells. The number of Ito cells in the uninitiated model 6 days after partial hepatectomy was 3.5 times higher in the area occupied by oval cells than elsewhere in the liver. The first α-fetoprotein (AFP)-positive cells appeared either as individual cells or as pseudoductal formations 32 or 56 h after partial hepatectomy at the periphery of the periductal space in both initiated and uninitiated animals. A combination of in situ and immunohistochemistry revealed that the OV-6-positive cells were AFP positive, whereas desmin-positive cells were AFP negative. Glutathione S-transferase P (GST-P) transcripts could be found mainly in OV-6-positive oval cells. Bile duct cells were positive for GST-P and negative for transforming growth factor β, whereas cells in the periductal space were positive for both of these transcripts. The GST-P-positive early preneoplastic lesions showed a similar distribution pattern as that of oval cells; the preexisting hepatocytes became trapped between small basophilic hepatocytes that showed either irregular or pseudovascular arrangement. This raises the question as to whether cells which are stem cell-like are among the target cells in the Solt-Farber model of hepatocarcinogenesis. Proliferation of transforming growth factor β,-producing, desmin-positive cells (Ito cells) and multipotent oval cells in a close proximity to each other indicates an intricate relationship between Ito cells and oval cells in liver that warrants further investigation.

INTRODUCTION

A heterogeneous population of small cells with oval or irregular nuclei is frequently induced in rat liver in the early stages of chemical hepatocarcinogenesis. This population is composed of epithelial cells with a different developmental potential ("oval cells") and other cell types of unknown phenotypic traits (1–5). The first proliferative event after certain carcinogenic regimens is observed in nondescript periductal cells and bile duct cells (4). Characterization of the periductal cells and the expanding oval cells is of considerable importance regarding the specific role and function of these cells in the early stages of hepatocarcinogenesis since it has been demonstrated that oval cells can differentiate into hepatocytes in vivo (6). One approach toward the study of this question is to compare the gene expression in both the proliferating periductal and the differentiating oval cell populations, as well as in early preneoplastic lesions. The placental isoenzyme of GST-P is one of the earliest markers of initiated hepatocytes (7–10). The appearance of cells or small groups of cells positive for GST-P are seen soon after the administration of certain hepatic carcinogens (7, 10). No information on the expression of this gene in periductal cells is presently available. However, immunohistochemical studies have revealed that GST-P is present in oval cells (11). TGF-β₁ is thought to play an important role in growth and differentiation of a variety of cell types both in vivo and in vitro (12). In the normal liver the transcripts of TGF-β₁ are present in the hepatic capsule, around the ductular structures in the portal tracts, around the central veins, and in sinusoidal lining cells (13). Furthermore TGF-β induces the differentiation of rat liver epithelial cells in vitro along the hepatocytic pathway (14) and may therefore play an important role in the differentiation of the putative hepatic stem cells in vivo. In situ hybridization technique provides the most accurate method for identification of RNA transcripts at the single cell level. When this technique is combined with immunohistochemical localization of antigens within individual cells, significant information about the spatial and temporal gene expression can be obtained. In the present study we have combined these powerful techniques in order to study both cellular and temporal expression of AFP, GST-P, and TGF-β₁ during early stages of hepatocarcinogenesis.

MATERIALS AND METHODS

Treatment of Animals. Fischer male rats (150 to 160 g) were used throughout the experiment. Diethylnitrosamine (200 mg/kg) was used for initiation as described by Solt and Farber (15) and AAF was administered by gavage (1 mg/day) for five times before and four times after partial hepatectomy. Diethylnitrosamine was omitted in the uninitiated protocol. Animals were sacrificed at the time of partial hepatectomy and 32 and 56 h and 3, 4, and 6 days after partial hepatectomy.

Histological Methods. Frozen serial sections of caudate lobe were used for staining with hematoxylin and eosin and for histochemical demonstration of GGT. In addition, tissue samples fixed in Bouin's fixative were used for hematoxylin and eosin and for histochemical demonstration of GGT. In the present study we have combined these powerful techniques in order to study both cellular and temporal expression of AFP, GST-P, and TGF-β₁ during early stages of hepatocarcinogenesis.

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GENE EXPRESSION, EARLY PRENEOPLASTIC LESIONS

Thymidine Administration. [methyl-3H]Thymidine (70 to 85 Ci/mmol; Amersham, Arlington Heights, IL), 1 μCi/g body weight, was administered i.p. to two uninitiated animals at 2 p.m., 8 p.m., 2 a.m., and 8 a.m. on the day of partial hepatectomy. These animals were sacrificed 32 h after partial hepatectomy. A similar dose of [methyl-3H] thymidine was administered to two uninitiated animals starting 32 h after partial hepatectomy. These animals were sacrificed 56 h after partial hepatectomy. Frozen sections were fixed for 20 min in paraformaldehyde and dipped in Kodak NTB-2 emulsion for autoradiography.

RESULTS

Thymidine Labeling. Thymidine labeling of the livers from the uninitiated animals sacrificed 32 h after partial hepatectomy revealed a prominent labeling of cells in the periductal space, 45% of which were GGT positive, whereas the hepatocytes remained unlabeled. By 56 h a greater number of periductal cells and bile duct cells were positive for tritiated thymidine and 60% of the labeled cells were GGT positive (Fig. 1A). Another cell population that became labeled at this time was desmin-positive Ito cells (Fig. 1B).

Automated Image Analysis. Ito cells became easily analyzable by the immunohistochemical method after the administration of AAF. Administration of corn oil alone did not have a similar effect on the morphology of the desmin-positive Ito cells. The number of Ito cells in the area occupied by oval cells by day 6 was 52 ± 3 (mean ± SD)/0.04 mm², and on the area outside the oval cells the corresponding number was 15 ± 1 (n = 5).

Histology, Histochemistry, and Immunohistochemistry. Tissue samples taken from both initiated and uninitiated animals showed an increased cellularity around the periductal space at the time of partial hepatectomy. Small cells surrounded the bile ducts in both groups 32 and 56 h after partial hepatectomy. These cells were negative for GGT, whereas the newly formed small ductal formations were positive. Crowding of the OV-6-positive bile duct cells was characteristic at this time. The "invasive" behavior of the small cells from the periductal area was evident by 56 h after the operation. A further increase of periductal cellularity and crowding of bile duct cells became even more striking by day 3 after the partial hepatectomy. The small cells infiltrated between hepatocytes either as individual cells or as groups of cells. OV-6-negative and desmin-positive small cells trailed the OV-6-positive cells deep into the liver.
acini (Fig. 2). After day 3 the space occupied by the small oval
type cells was further expanded. GGT-positive preneoplastic
foci with rounded or oval nuclei were present at the periphery
of the oval cell compartment in the initiated livers 56 h after
partial hepatectomy, but by days 3 and 4 their number increased
rapidly. GGT-positive foci composed of two to three normalized
hepatocytes were not observed. The earliest foci were
either irregular in shape or showed a pseudoalveolar arrange-
ment of small cells.

Expression of Glutathione-S-Transferase P. GST-P tran-
scripts were present in the bile duct cells of both models at the
time of partial hepatectomy. As soon as the increase in the
number of cells in the periductal space was evident, these cells
and the bile duct cells showed significant transcripts for GST-
P. Ductal formations at the perportal area and the infiltrating
mixed cell population were also positive for GST-P transcripts
(Fig. 3). In the initiated model the irregular shape of the early
preneoplastic lesions with increased transcripts became ex-
tremely conspicuous by 56 h to 3 d after partial hepatectomy
(Fig. 4A). These foci showed a similar branched distribution as
did the proliferating oval cells (Fig. 4B).

Expression of α-Fetoprotein. No AFP-positive cells were ob-
served in either model at the time of partial hepatectomy and
only a few positive cells or small ductal formations were ob-
served 32 h after the operation. They were mainly located at
the periphery of the periductal space (Fig. 5A). The thick layer
of small cells around the bile ducts as well as the bile duct cells
themselves were negative for AFP. From the periductal areas
AFP-positive cells infiltrated between hepatocytes as individual
cells or groups of cells or as pseudoductal formations. The
transcripts of AFP were concentrated on the OV-6-positive
cells, as shown in Fig. 5B. AFP- and OV-6-positive cells were
trailing by desmin-positive Ito cells which were negative for
GGT and OV-6 and did not show AFP transcripts.

Expression of TGF-β. TGF-β transcripts were absent in bile
ducts but were present in the cells located in the collagen-rich
matrix surrounding the bile ducts and the vascular formations
in the periductal space in both initiated and uninitiated models
(Fig. 6). The number of silver grains in the small OV-6-positive
ductal formations at the periphery of the periductal space was
only slightly above the background level. Both immunohisto-
chemistry with desmin antibody and in situ hybridization using
TGF-β1 probe revealed a similar linear pattern of positivity
(Fig. 7) around the oval cells. Desmin-positive cells were also
found among the collagen-producing cells in the periductal
space. Desmin-positive Ito cells formed a meshwork of cells
around the oval cells and hepatocytes (Fig. 2B). Combination
of immunohistochemistry with desmin antibody and in situ
GENE EXPRESSION, EARLY PRENEOPLASTIC LESIONS

Fig. 5. A, expression of AFP in uninitiated animal 32 h after partial hepatectomy. The first AFP-positive cells observed after operation are located mostly at the periphery of the periductal space. From this location they infiltrate between hepatocytes. Bar, 30 μm; x 300. In B, combination of in situ hybridization and immunohistochemistry 6 days after partial hepatectomy of uninitiated liver revealed that the transcripts of AFP are mainly concentrated on the OV-6-positive cells. Bar, 30 μm; x 300.

hybridization with AFP revealed a lack of AFP transcripts in desmin-positive cells (Fig. 8A). In contrast, the combination with (Fig. 8A) OV-6 antibody and TGF-β probe revealed that most of the OV-6-positive cells were negative for TGF-β transcripts (Fig. 8B).

DISCUSSION

The two major new cell populations that emerge after the partial hepatectomy in the Solt-Farber model are the oval cells and the Ito cells. Oval cells are epithelial cells with different developmental potential, among which is the capacity to differentiate into hepatocytes in vivo (1–6, 21–23), whereas Ito cells are of mesenchymal origin. Ito cells can be identified by their positive reaction to an antibody raised against the intermediary filament desmin. Similar to Ito cells, myofibroblasts are also positive for desmin, and these cells may therefore be closely related to each other (24).

The first cellular event after partial hepatectomy in the Solt-Farber model is the proliferation of both GGT-positive and -negative cells in the periductal space as well as Ito cells (Fig. 1). Some of the GGT-negative cells might represent desmin-positive cells (preliminary data), but further characterization is needed. This raises the question as to whether desmin-positive Ito cells, which coproliferate with oval cells, derive from a primitive cell population found in the periportal space. Sell and Salman (4) demonstrated the proliferation of a mixed cell population in the periductal space with elongated, oval, or convoluted nuclei after feeding AAF in a choline-deficient diet. In our study invading oval cells are surrounded by a meshwork of Ito cells (Figs. 2B and 7A). Ito cells are known to produce both basement membrane proteins and TGF-β in tissue culture, and the amount of the basement membrane proteins is dependent on the amount of vitamin A in the tissue culture media (25). No information is available concerning the vitamin A content of Ito cells in AAF-treated animals. However both vitamin A and TGF-β might play an important role in the differentiation of oval cells along the different hepatic pathways.

The AFP-positive oval cells were first observed 32 to 56 h after partial hepatectomy mainly at the periphery of the periductal space (Fig. 5A). This is the location where the terminal bile ductules, which collect the bile from the bile canaliculi, traverse the limiting membrane that surrounds the periportal space. Most probably the primitive terminal bile ductular cells give rise to the proliferating oval cells (26). The small ductal formations around the hepatic vein and bile duct in Fig. 6B might represent these proliferating and differentiating terminal hepatic ductules. They are positive for GGT and for OV-6 and also show transcripts for GST-P and AFP but seem to lack transcripts for TGF-β. This is in contrast to the type of ductal cells that proliferate after bile duct ligation and are negative for
AFP (27). Proliferation of bile duct cells and oval cells occurs simultaneously during AAF treatment and partial hepatectomy, but only AFP- and OV-6-positive oval cells infiltrate into the liver parenchyma.

GST-P is an early marker for the preneoplastic lesions (7–10). However the transcripts of GST-P are also present in the oval cells as shown in the present study (Fig. 3). We have used the expression of GST-P to trace the early morphological changes in hepatocarcinogenesis produced by the Solt-Farber protocol. As shown in Fig. 4, these lesions are present at the periphery of the area occupied by oval cells. Morphologically these early lesions show a similar branched distribution of GST-P-positive cells as do the oval cells (Fig. 4B). Only at the stage when the GST-P-positive small hepatocytes expand and fill the space occupied by the preexisting trapped hepatocytes do the preneoplastic liver lesions appear as rounded solid GST-P-positive hepatocyte conglomerates.

The close proximity of the TGF-β₁-expressing Ito cells and the multipotential oval cells raises an interesting question as to how the cytodifferentiation, growth control, and migration of the putative hepatic stem cells are regulated and what is the function of TGF-β₁ in these events. Our data agree with the earlier studies indicating the presence of TGF-β₁ message in nonparenchymal liver cells but not in the hepatocytes (28). It is possible that other factors such as vitamin A, together with TGF-β₁, play an important role in the direction of the cytodifferentiation of the oval cells in the liver.

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Fig. 7. Livers of rat from the Solt-Farber model 6 days after partial hepatectomy. In A, desmin-positive cells radiate from the peripoportal space with a linear pattern of positivity and surround the desmin negative oval cells. Bar, 120 μm; × 75. In B, a similar linear pattern of TGF-β₁ transcripts are present in the area of oval cell distribution. Bar, 120 μm; × 75.

Fig. 8. Combination of in situ hybridization and immunohistochemistry of uninitiated liver 6 days after partial hepatectomy. In A, desmin-positive Ito cells (arrows) are negative for AFP transcripts, whereas oval cells are positive. Bar, 17 μm; × 591. In B, OV-6-positive oval cells are mostly negative for TGF-β₁ transcripts, whereas the trailing cells are positive for TGF-β₁. Bar, 17 μm; × 591.
GENE EXPRESSION, EARLY PRENEOPLASTIC LESIONS


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