Lack of Albumin as Genotypic Marker of Preneoplastic Analbuminemic Rat Hepatocytes Transplanted within Albumin-positive Liver

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ABSTRACT

In analbuminemic rats, preneoplastic hepatocytes lack the capability to produce albumin. On the other hand, the hepatocytes of F1 hybrids born from parents of analbuminemic rats and normal rats retain their capability to produce albumin, since the analbuminemia is inherited as a recessive trait in rats. We isolated hyperplastic nodule cells from Nagase's analbuminemic rats and transplanted them into the livers of F1 hybrid rats by infusion into the mesenteric vein. The host rats were subjected to a short term dietary 2-acetylaminofluorene and underwent a two-thirds partial hepatectomy to promote the growth of preneoplastic hepatocytes. Within 10 to 13 days after transplantation, many albumin-negative hepatocytic nodules were formed in the albumin-positive host livers. Almost all the albumin-negative nodules expressed conventional biochemical markers for preneoplastic hepatocytes. Eight to 9 weeks after the transplantation, almost the same number of albumin-negative nodules were observed as on days 10-13. However, roughly a half of the albumin-negative nodules showed no biochemical markers. The results indicate that the majority of early preneoplastic lesions revert to become phenotypically normal after removal of the promoting stimuli.

INTRODUCTION

Focal proliferation of carcinogen-altered hepatocytes (enzyme altered, hyperplastic foci, or nodules) are almost universally seen during the preneoplastic stage of spontaneous or experimentally induced hepatic carcinogenesis (1-3). Although carcinogen-altered hepatocytes have various biochemical markers (3), such as deficiency in the activities of ATPase and glucose-6-phosphatase, and the increase in the activities of GGT3, EH, and GST-P, such enzymatic alterations seem to be epigenetic because a majority of the hyperplastic nodules lose these activities and revert to become phenotypically normal after cessation of the promoting stimuli (4-8). If the carcinogen-altered hepatocytes had a genotypic marker which was retained throughout the carcinogenesis, it would be possible to accurately investigate the fate of hyperplastic nodules and sequential events during hepatic carcinogenesis.

Laishes et al. (9, 10) demonstrated that hepatocytes isolated from the hyperplastic nodules were transplantable to the liver of syngeneic rats. They found that the cells of hyperplastic nodules infused into the portal vein form colonies within the liver of the hosts which had received dietary 2-AAF and a two-thirds partial hepatectomy. Hunt et al. (11) applied allo-specific membrane antigens as a genotypic marker in the transplantation system using Fisher 344 rats as a donor and Wistar/Furth × Fischer 344 F1 rats as a host. They demonstrated that most colonies of enzyme-altered hepatocytes formed in the host livers are of a donor origin.

In the present study, we developed a new genotypic marker system by using analbuminemic rats (NAR) as a donor and Sprague-Dawley rats × NAR F1 rats (SD × NAR F1) as a host. In the liver of NAR, hyperplastic nodules and hepatocellular carcinomas are immunochemically negative for albumin (12). On the other hand, since the analbuminemia of NAR is inherited as a recessive trait (13), virtually all hepatocytes of SD × NAR F1 rats retain the capability to produce albumin. In a preliminary study (14), we isolated the hepatocytes of NAR, including nodule cells, and then transplanted them into the liver of SD × NAR F1 which had received the 2-AAF diet plus partial hepatectomy, strong promoting stimuli for proliferation of nodule cells (15). In this system, many albumin-negative hyperplastic nodules were formed within the albumin-positive liver of the SD × NAR F1. These nodules were easily detected by immunostaining for albumin using paraffin-embedded tissue sections. In this study, we investigated the phenotypic changes of transplanted nodule cells by combination of genotypic and biochemical markers.

MATERIALS AND METHODS

Animals and Diets. SD rats were obtained from Clea Japan (Kanagawa) and NAR from Sasaki Institute (Tokyo, Japan). SD × NAR F1 rats were made by mating female SD rats and male NAR. All animals were housed in plastic cages in an air-conditioned room at 24°C with a 12-h light and 12-h dark cycle. Rats were given a chow diet (Oriental Yeast Co., Tokyo, Japan) and water ad libitum.

As NAR were found among SD of Clea Japan (13), we first tried to transplant preneoplastic hepatocytes of SD to the livers of NAR, but such experiments proved to be always unsuccessful. Then, to test the transplantability of the liver cells between NAR and SD or between NAR and SD × NAR F1, the mixed lymphocyte reaction was carried out using spleen cells isolated from each strain. Compared to the autologous reaction, a high blastogenic reaction was observed between SD (stimulator) and NAR (responder), and vice versa. On the other hand, there was no significant difference from the control values of the mixed lymphocyte reaction between NAR (stimulator) and SD × NAR F1 (responder), and vice versa. Thus, male NAR (initially weighing 147-188 g) and male SD × NAR F1 (200-294 g) were used as a donor and host for the transplantation experiment, respectively.

Chemicals and Antiseria. Diethylnitrosamine was purchased from Wako Pure Chemical Industries (Osaka, Japan); 2-AAF from Katayama Chemical Co. (Osaka, Japan); collagenase (type I) from Sigma Chemical Co. (St. Louis, MO); anti-rat albumin antibodies from Cooper Biochemical, Inc. (Malvern, PA); biotinylated goat anti-rabbit IgG antibodies and avidin-biotin-peroxidase complex from Vector Laboratories, Inc. (Burlingame, CA). Anti GST-P and GGT antibodies were gracious gifts from Dr. Sato (Department of Biochemistry, Hirosaki University School of Medicine, Japan) (16) and from Dr. Taniguchi (Department of Biochemistry, School of Medicine, University of Osaka, Japan) (17), respectively. Anti-EH antibodies were raised in rabbits using EH isolated by the method of Lu et al. (18) as the antigen.

Transplantation of Liver Cells. Hyperplastic nodules were induced in NAR (donors) by Solt and Farber's regimen (15). At the 6th week after the start of the regimen, the liver cells were isolated by collagenase perfusion (19) (Fig. 1). Viability of the isolated cells was about 85%. The percentage of nodule cells in the liver cell suspension was estimated by immunostaining for GST-P. At this time, the livers of the other NAR treated with the same regimen were histologically examined. Host rats (SD × NAR F1) were given dietary 2-AAF (0.02%) for 2 weeks
TRANSPLANTATION OF PRENEOPLASTIC HEPATOCYTES

Donor: NAR

Cell Isolation

Ph

Transplantation

Host: SD × NAR F1

Control: SD × NAR F1

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 (WEEKS)

Fig. 1. Schematic representation of experimental protocol. DEN, diethylnitrosamine; PH, partial hepatectomy.

from 1 week before the time of transplantation. They were subjected to a two-thirds partial hepatectomy, and then immediately infused with the liver cells into the mesenteric vein. As the control, some SD × NAR F1 were treated with 2-AAF plus a partial hepatectomy without any cell infusion. Host and control rats were sacrificed under light ether anesthesia at 10–13 days and 8–9 weeks after transplantation (or partial hepatectomy).

Immunohistochemistry. The livers were fixed by perfusion with periodate-lysine-paraformaldehyde fixative (20) for 5 min, sliced into 2-mm thick sections, washed in cold phosphate-buffered saline containing 7.5% sucrose for 1 h, dehydrated in graded ethanols, penetrated with benzene overnight, and embedded in paraffin. Serial thin sections were immunostained for albumin, GST-P, and EH by the ABC method (21) and for GGT by the PAP method (22), and then counterstained with 1% methyl green.

The whole area of the immunostained sections were photographed, and negatively- or positively-stained areas were marked on the prints by examining the sections under a microscope. The number of nodules per cm² was determined using an image analyzer (Kontron, West Germany).

RESULTS

The Livers of Donor (NAR). The hepatocytes of untreated NAR (6 weeks after birth) were almost all negative for albumin, however a small number of albumin-positive cells were seen by careful examination (Fig. 2) as described by Makino et al. (12). The majority of these albumin-positive hepatocytes were mostly observed as singlets or doublets, sometimes forming clusters consisting of less than 20 cells. The frequency of the albumin-positive cells was 1.2 cells/cm². Six weeks after the start of the carcinogenic regimen, the livers of NAR contained many hyperplastic nodules with a diameter of less than 3 mm. Most of these nodules showed increased staining for GST-P (Fig. 3A), EH, and GGT, while being virtually negative for albumin (Fig. 3B). The cell suspension dissociated from the donor livers contained 11.2–11.5% GST-P-positive cells. On the other hand, albumin-positive hepatocytes increased to about 400 times (454 cells/cm²) by the treatment with the carcinogenic regimen. They

Fig. 2. The liver of NAR immunostained by anti-rat albumin antibodies. Although a majority of hepatocytes are negative for albumin, an albumin-positive cell is seen. × 100.

Fig. 3. Hyperplastic nodules induced in NAR. Nodule cells are positive for GST-P (A), but are negative for albumin (B). × 100.
were seen not only in the surrounding area of hyperplastic nodules but also within the nodules.

The Livers of Host Rats (SD x NAR F1). Liver cells of untreated SD x NAR F1 were almost all for albumin whereas a small number of albumin-negative cells were observed (20.6 cells/cm²) (Fig. 4). They were usually present as singlets or doublets whereas some formed clusters consisting of less than 20 cells.

Ten to 13 days after the infusion, many hyperplastic nodules were formed with the host livers (Table 1). The nodules were sharply demarcated from the surrounding liver tissue and usually consisted of more than 100 basophilic hepatocytes (Fig. 5A). Most of the nodules (97%) were negative for albumin (Fig. 5B). The expression of albumin and biochemical markers were examined on 448 nodules seen in representative liver sections of three rats. Of these, 438 nodules were negative and 10 were positive for albumin. Of the albumin-negative nodules, 98.6% were positive for GST-P (Fig. 5C), 94.1% positive for EH (Fig. 5D), 60.3% positive for GGT, and 59.4% were positive for all three markers. Only 0.4% were negative for all three markers, indicating that virtually all albumin-negative nodules showed at least one enzyme alteration (Table 2).

Eight to 9 weeks after infusion, almost the same number of albumin-negative areas were observed per cm² of the cross-section of the livers as seen on days 10–13 (Fig. 6A, Table 1). However, the albumin-negative areas were variable for the intensity of the stains for GST-P, from strongly positive to negative (Fig. 6B). Even when positively stained, most of them showed weak or partial staining for GST-P, EH, and GGT (Fig. 7, A–D). Of the albumin-negative areas, only 45.2% were

<table>
<thead>
<tr>
<th>Periods after transplantation</th>
<th>Experimental group</th>
<th>Albumin-negative areas</th>
<th>GST-P-positive areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>10–13 Days</td>
<td>Control (4)</td>
<td>0.4 ± 0.4</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Host (8)</td>
<td>92.1 ± 40.6</td>
<td>93.1 ± 40.1</td>
</tr>
<tr>
<td>8–9 Weeks</td>
<td>Control (5)</td>
<td>4.0 ± 3.1</td>
<td>3.6 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Host (4)</td>
<td>97.7 ± 35.2</td>
<td>47.0 ± 13.6</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of rats included in the mean.
* Mean ± SD.
* Significantly different from the value for the control; P < 0.01.
* Significantly different from the value for the control; P < 0.05.

Fig. 4. The liver of SD × NAR F1. Almost all hepatocytes are positive for albumin whereas a cluster of albumin-negative hepatocytes (arrow) is seen. × 100.

Fig. 5. The liver of SD × NAR F1 13 days after the transplantation. Nodules in an H & E-stained section (A) are negative for albumin (B), and positive for GST-P (C) and EH (D). × 20.
positive for GST-P, 43.3% positive for EH, and 22.4% positive for GGT. About one-half of the nodules were negative for all three markers (Table 2). Although some of the albumin-negative areas appeared as typical hyperplastic nodules (less than 5%), a majority of those did not exhibit any constraint against their surroundings and appeared to be almost normal hepatic tissue (Fig. 7, A–D).

The Livers of Control Rats. Only a small number of hyperplastic nodules were seen in the liver of control rats, which received a regimen of 2-AAF plus a partial hepatectomy without a cell infusion, either at 10–13 days or at 8–9 weeks after a partial hepatectomy (Table 1). Although these nodules showed positive stain for GST-P, EH, and GGT to a varied degree, all of them were found to be positive for albumin. On the other hand, the number of albumin-negative hepatocytes was increased in these rats as compared to untreated SD × NAR F1. Such albumin-negative cells were usually observed as singlets or doublets but occasionally formed clusters of less than 80 cells. None of these cells showed positive staining for GST-P, EH, or GGT.

DISCUSSION

This paper describes an improved system, originally reported by Laishes and Farber (9), for the intrahepatic transplantation of presumptive preneoplastic hepatocytes. The purpose of the present experiment was to elucidate the applicability of albumin as a genotypic marker of presumptive preneoplastic hepatocytes. Use of genotypic markers is advantageous for the investigation of hepatic carcinogenesis, because the expression of the conventional biochemical markers in preneoplastic hepatocytes is heterogeneous and variable depending on different factors. For example, although elevated GGT (23) or GST-P (16) activity has been widely seen in foci or nodules induced by various chemicals, the activity is not seen in the lesions induced by the peroxisome proliferator (24). It is also described that X-ray induced foci show a deficiency in ATPase activity but rarely exhibit an elevated GGT activity (25). Furthermore, it is known that nodules strongly express the markers during exposure to carcinogens or promoting stimuli whereas they gradually lose such markers after removal of the carcinogenic or promotional treatment (4–8).

Validity of the present model is dependent on the following assumptions: (a) the preneoplastic and neoplastic hepatocytes of NAR are always albumin-negative during carcinogenesis, and (b) those of SD × NAR F1 are always albumin-positive. According to the first assumption, Makino et al. (12) reported that the nodules and hepatocellular carcinomas induced by chemicals are always negative for albumin in NAR. However, they also reported that a small number of albumin-positive hepatocytes are present within the liver of NAR, and when administered 3’-methyl-4-aminobenzene or 2-AAF, such cells significantly increased in number. We also observed albumin-positive cells not only in the surrounding of the nodules but also within the nodules. However, as the number of such cells within the nodules is very small, it must be very rare for them to form albumin-positive nodules within the host liver, if at all.

### Table 2
**Expression of albumin and biochemical markers in hepatocytic nodules in the host livers**

<table>
<thead>
<tr>
<th>Period after transplantation</th>
<th>Albumin-negative</th>
<th>GGT</th>
<th>EH</th>
<th>GST-P</th>
<th>Albumin-positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 Days (3)*</td>
<td>438</td>
<td>260</td>
<td>149</td>
<td>3</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Count %</td>
<td>(59.4)</td>
<td>(34.0)</td>
<td>(0.7)</td>
<td>(4.6)</td>
<td>(0.0)</td>
<td>(0.7)</td>
</tr>
<tr>
<td>8 Weeks (3)</td>
<td>527</td>
<td>99</td>
<td>109</td>
<td>8</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>Count %</td>
<td>(18.8)</td>
<td>(20.7)</td>
<td>(1.5)</td>
<td>(4.2)</td>
<td>(0.8)</td>
<td>(1.3)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of rats examined.
* Percentage of distribution of expression of biochemical markers in albumin negative nodules.

![Fig. 6. Eight weeks after the transplantation. Of seven albumin-negative nodules seen in (a), five are positive but two negative for GST-P (b). × 32.](image)
Various results have been reported concerning the capability of albumin production of preneoplastic or neoplastic hepatocytes in normal rats. Schwarz et al. (26) described that although large nodules induced by diethylnitrosamine and phenobarbital contained reduced amounts of albumin mRNA, small foci induced by diethylnitrosamine and those induced by 4-dimethylaminoazobenzene showed the same or higher levels of albumin production than the surrounding liver tissue. Petropoulos et al. (27) reported that albumin mRNA level increased in proneoplastic cells induced by ethionine and a choline deficient diet. On the other hand, Sell et al. (28) reported that albumin level is decreased in transplantable hepatocellular carcinomas. We observed that the foci and nodules induced by Solt and Farber's regimen (15) always stained positive for albumin to the same or increased extent as the surrounding hepatic tissue whereas hepatocellular carcinomas usually stained more weakly than the surrounding liver in SD × NAR F1 (data not shown). Thus, it seems possible to conclude that albumin is expressed throughout hepatic carcinogenesis though its level may be varied.

In the present study, we occasionally observed albumin-negative hepatocytes in the livers of the control SD × NAR F1 rats which had not received liver cell transplantation. Such albumin-negative cell populations were apparently different from foci or nodules because none of them showed the biochemical markers. Furthermore, most of them consisted of less than 20 cells in cross-sections and were usually smaller than the nodules of transplanted cells. Therefore, the presence of such albumin-negative cells in the host liver does not seem to hamper the efficiency of the present model.

The present system enabled us to detect the transplanted nodule cells by the immunohistochemical method using formalin-fixed paraffin-embedded hepatic tissues. It is also easy to investigate not only the wide areas of liver sections but also other properties of the individual nodules such as the morphology and expression of conventional biochemical markers using continuous sections.

Ten to 13 days after transplantation, virtually all albumin-negative nodules expressed at least one of the biochemical markers, indicating that, of the infused hepatocytes, only the nodule cells selectively grow and give rise to nodules within the host liver. However, 8–9 weeks after transplantation, a majority of the albumin-negative areas were weakly and partially positive, or even negative for biochemical markers. Such areas appeared to be morphologically normal. Only a small number of the areas clearly expressed the markers and showed constraint figures against the surrounding tissue in H & E-stained sections. These observations confirmed the previously published results (4–8) that, for the early nodules, two options are available, either remodeling to a normal phenotype or persisting as typical hyperplastic nodules. The results of this report indicate that a fairly large number of nodules completely lose the
biochemical abnormality. This transplantation system will be useful for the continued investigation of such carcinogen-altered populations, including the study of the sequence of changes leading to neoplasia as well as the process of reversion towards the morphologically normal state.

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