Low selection of preneoplastic hepatocytes after treatment with the 2-acetylaminofluorene diet-partial hepatectomy regimen in the liver of hepatocarcinogenesis-resistant DRH strain rats

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Abstract. In hepatocarcinogenesis-resistant DRH rats, preneoplastic hepatocytic lesions are smaller than those of usual rats during carcinogenesis. When preneoplastic hepatocytes from DRH and Donryu (original strain of DRH) were reciprocally transplanted into the livers of DRH and Donryu treated with 2-acetylaminofluorene (2-AAF) diet/two-thirds hepatectomy (PH), the Donryu cells formed small colonies within the DRH liver, whereas the DRH cells formed large colonies within the Donryu liver. The DRH liver showed less degree of oval cell proliferation after treatment with 2-AAF and PH, and DRH hepatocytes were more resistant to the growth-inhibitory effect of 2-AAF after PH. Furthermore, DRH hepatocytes were generally resistant to cytotoxicity of hepatotoxins. The tissue environment of the DRH liver, therefore, is less effective for selective growth of preneoplastic hepatocytes during the carcinogen treatment, which is probably a major cause of the hepatocarcinogenesis-resistance in DRH rats.

Introduction

Hepatocarcinogenesis can be separated into three distinct stages: the initiation stage, where rare initiated hepatocytes capable of progressing towards hepatocellular carcinomas (HCC) are generated; the promotion stage, where the initiated cells clonally expand their populations to form preneoplastic lesions; and the progression stage, where rare cells within the initiated cell populations may progress further to malignancy (1,2). Preneoplastic hepatocytes usually show gene expression patterns distinct from those of normal hepatocytes, which may favor their selection for outgrowth. During expansion of preneoplastic hepatocyte populations, a number of genetic/epigenetic changes in oncogenes and tumor suppressor genes may occur, resulting in progression to HCC.

Chemical hepatocarcinogens may be both genotoxic and cytotoxic against hepatocytes. Therefore, exposure to them may not only induce genetic changes causative for the neoplastic transformation of hepatocytes, but may also induce hepatic tissue damage and generate the growth stimuli associated with tissue repair, thereby promoting proliferation of preneoplastic hepatocytes (1). The hepatocarcinogenesis model developed by Solt and Farber (3) consists of a single dose of diethylnitrosamine (DEN) and subsequent dietary treatment with 2-acethylaminofluorene (2-AAF), during which a two-thirds partial hepatectomy (PH) is performed. In this model, DEN-induced initiated preneoplastic hepatocytes are resistant to 2-AAF hepatotoxicity and can selectively proliferate after PH to form colonies, whereas normal hepatocytes cannot, because their growth is suppressed under the influence of 2-AAF. Thus, the growth stimulus causes the rapid expansion of preneoplastic hepatocyte populations, which eventually progress to HCC (4,5).

DRH rats, a strain established by the inbreeding of Donryu rats that showed resistance to hepatocarcinogenesis on a diet containing 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) for more than 20 generations, are known to be highly-resistant to hepatocarcinogenesis (6-9). Such resistance was reported to be related to two highly significant clusters of quantitative trait loci on rat chromosomes 1q and 4q which determine the number, size and areas of glutathione-S-transferase (GST-P, a marker for preneoplastic hepatocytes) positive foci as well as GST-P mRNA levels (10,11). On the other hand, essentially similar levels of DNA adducts of 3'-Me-DAB metabolites are generated in livers of DRH and Donryu after being fed the 3'-Me-DAB diet (7), suggesting that the DRH livers may have the capacity to convert procarcinogens to active metabolites. It has also been shown that, when treated with the DEN/3'-Me-DAB diet/PH regimen, the size of preneoplastic hepatocyte colonies is significantly smaller in DRH than Donryu rats, although the number of the lesions
does not differ so much, suggesting that the clonal expansion of the initiated cells may not be as efficient in the case of DRH (9). However, whether such growth suppression is due to intrinsic properties of the preneoplastic hepatocytes or dependent on the hepatic tissue environment has not been clarified so far.

To address the resistance mechanism to hepatocarcinogenesis in DRH rats, we transplanted preneoplastic hepatocytes from Donryu or DRH rats into the livers of the DRH and Donryu strains, respectively, applied the Solt-Farber regimen and then compared the growth of the transplanted cells. In this model, the hepatic tissue environment of the two strains can be compared in terms of the growth of preneoplastic hepatocytes (12,13). We found that reduction of growth capacity of hepatocytes and histological damage were less severe in the DRH than the Donryu livers after treatment with 2-AAF and PH, suggesting that DRH hepatocytes may be resistant to the toxicity of the carcinogen, and thereby the tissue environment in the DRH liver may be less effective for selection of preneoplastic hepatocyte populations. We further investigated whether DRH hepatocytes were also resistant to hepatotoxic substances other than 2-AAF.

Materials and methods

Carcinogen treatment and cell transplantation. Male Donryu and DRH rats were purchased from Charles River (Yokohama, Japan) and Seac Yoshitomi (Fukuoka, Japan), respectively. All procedures performed on the animals were approved by the Asahikawa Medical College Animal Experiment Committee. In the donor rats for hepatocyte transplantation, DEN was administered intraperitoneally at a dose of 100 μg/g body weight at the age of 5 weeks, two weeks later, a diet containing 2-AAF was given for a period of up to 14 days, and PH was performed 1 week after starting the 2-AAF treatment (Fig. 1). Although the 2-AAF concentration in the diet was 0.02% for Donryu rats, it was 0.03% for the DRH rats, because only small preneoplastic lesions were generated by the 0.02% 2-AAF diet in the case of DRH. Six to eight weeks later, hepatocytes including both normal and preneoplastic cells were isolated from the livers by collagenase perfusion, purified by Percoll centrifugation and suspended in phosphate-buffered saline (PBS). Aliquots of these cells (10^6 cells per animal) were then infused into the portal vein immediately after PH. All recipient rats were given FK506 after transplantation, and sacrificed 2 weeks later.

Table I. Degree of oval cell proliferation and number and size of GST-P (+) foci.

<table>
<thead>
<tr>
<th>Exp</th>
<th>Group</th>
<th>Recipient</th>
<th>Sex</th>
<th>Donor</th>
<th>Sex</th>
<th>n</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>No/cm²</th>
<th>Diameter (mm)</th>
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</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td>A</td>
<td>Donryu</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Donryu</td>
<td>M</td>
<td>Donryu</td>
<td>M</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>30.7±12.7</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Donryu</td>
<td>M</td>
<td>DRH</td>
<td>M</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>23.6±6.8</td>
<td>1.06±0.83</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>DRH</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>DRH</td>
<td>M</td>
<td>Donryu</td>
<td>M</td>
<td>7</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>31.7±12.1</td>
<td>0.62±0.60*</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>DRH</td>
<td>M</td>
<td>DRH</td>
<td>M</td>
<td>8</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>29.9±12.1</td>
<td>0.45±0.54*</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>Donryu</td>
<td>M</td>
<td>M</td>
<td>Donryu</td>
<td>M</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>45.4±16.4</td>
<td>1.03±1.10</td>
</tr>
</tbody>
</table>

*Comparison against Group B or C. **p<0.05.
Histology and immunohistochemistry. Hepatic tissue samples were perfusion-fixed with phosphate-buffered 4% formaldehyde solution, and tissue samples from all hepatic lobes were embedded in paraffin, cut into 4-μm thick sections and stained with hematoxylin and eosin. For immunohistochemistry of GST-P, deparaffinized tissue sections were microwaved in 0.01 M citrate buffer (pH 6.0) for 10 min in an electric oven, treated with 3% H2O2 dissolved in ethanol for 60 min and then incubated with the 1/6,000 diluted GST-P antibody (MBL, Nagoya, Japan) overnight at 4°C. For BrdU immunohistochemistry, the sections were treated with 0.2 N NaOH before application of the anti-BrdU monoclonal antibody (1/50 dilution, Becton-Dickinson). Antibody binding was visualized by the avidin-biotin method using a Histofine kit (Nichirei, Tokyo, Japan) overnight at 4˚C. For BrdU immunohistochemistry, the sections were treated with 0.2 N NaOH before application of the anti-BrdU monoclonal antibody (1/50 dilution, Becton-Dickinson). Antibody binding was visualized by the avidin-biotin method using a Histofine kit (Nichirei, Tokyo, Japan) overnight at 4˚C. For BrdU immunohistochemistry, the sections were treated with 0.2 N NaOH before application of the anti-BrdU monoclonal antibody (1/50 dilution, Becton-Dickinson). Antibody binding was visualized by the avidin-biotin method using a Histofine kit (Nichirei, Tokyo, Japan) overnight at 4˚C.
Effect of 2-AAF on proliferating capacity of hepatocytes. After PH the BrdU LI started to increase from 18 h, reached the peak at 24 h and declined thereafter both in untreated Donryu and DRH rats as previously described (9) (data not shown). When Donryu and DRH rats were given 0.02% 2-AAF diet for 7 days and then subjected to PH, the BrdU LI peaked at 24 h after PH in DRH rats, while there was almost no increase at 24, 48 and 72 h in Donryu rats (Fig. 5A). The BrdU LI was then compared between Donryu and DRH rats

Figure 3. GST-P immunostaining of recipient livers (A). In the case of recipient Donryu, large GST-P(+) foci can be seen after transplantation of either Donryu (a) or DRH (c) hepatocytes, while recipient DRH livers show small GST-P(+) foci or single cells after transplantation of either Donryu (b) or DRH (d) cells (x40). Severe oval cell proliferation in a recipient Donryu liver (left), and mild changes in a recipient DRH liver (right) (B) (x100).

Figure 4. PCR using DNA isolated from untreated livers of male DRH and female Donryu rats, and from GST-P(+): foci in female Donryu recipient livers transplanted with male DRH hepatocytes.

Figure 5. Effect of 2-AAF feeding on BrdU LI of hepatocytes after PH. BrdU LI after PH in Donryu (open circles) and DRH (closed circles) that were fed with the 0.02% 2-AAF diet for 1 week (A). N=3 at each time-point. BrdU LI 24 h after PH in the Donryu (open columns) and DRH (closed columns) groups given the diet containing various concentrations of 2-AAF (B). N=5 for each bar.

Figure 6. ALT (open columns) and AST (closed columns) levels after exposure to hepatotoxic substances (A). Note that the values are generally high in the Donryu and low in the DRH. Vertical bars represent standard deviation. The difference between the Donryu and DRH values is significant (*p<0.05, **p<0.01). Numbers in parenthesis represent the numbers of rats used. Hepatic necrosis induced by DEN in Donryu and DRH livers (B). Note that with DEN the degree of necrosis is much less severe in the DRH than in the Donryu rats (x100).
at 24 h after PH, when they were given the diet containing various concentration of 2-AAF (Fig. 5B). The BrdU LI of hepatocytes was almost completely inhibited by the diet containing more than 0.02% 2-AAF in Donryu rats at 24 h after PH, while it was increased even at 0.025% 2-AAF in DRH rats.

Acute hepatic injury. DRH hepatocytes were more resistant than their Donryu counterparts to all 5 hepatotoxic substances tested. In the case of CC14, serum AST and ALT levels in the DRH rats were about 50 and 70%, respectively, of those in the Donryu rats, and in the case of TAA, the AST levels in the DRH rats were about 65% of those of the Donryu rats (Fig. 6A). On the other hand, with DEN, 2-AAF and 3'-Me-DAB, the respective values were only around 10%, generally paralleling the degree of hepatic necrosis (Fig. 6B).

Discussion

DRH livers have been reported to show smaller sized preneoplastic lesions than those in normal rat livers during treatment with hepatic carcinogens (9-11). In the present study, transplanted DRH preneoplastic hepatocytes were found to form large colonies like their Donryu counterparts within the Donryu livers, while transplanted Donryu preneoplastic hepatocytes formed small colonies like their DRH counterparts within the DRH livers (Fig. 3A and Table I), clearly demonstrating that the low expansion of preneoplastic hepatocyte colonies in the DRH livers is dependent on the hepatic environment rather than a property of the preneoplastic hepatocyte colonies in the DRH livers has remained obscure so far, the observation that resistance is particularly evident for 2-AAF, 3'-Me-DAB and DEN, but modest for CC14 and TAA, suggests that DRH hepatocytes may be resilient to some specific death pathway(s).

In conclusion, DRH resistance to hepatocarcinogenesis seems to be mainly based on their resistance to the cytotoxicity of chemical carcinogens, which may result in low selection for the growth of preneoplastic hepatocyte populations.

Acknowledgements

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References


