# 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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Received October 3, 2006; Accepted November 6, 2006

**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-resictance 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

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*Key words:* hepatocarcinogenesis, DRH rats, liver cell transplantation, preneoplastic hepatocytes, GST-P

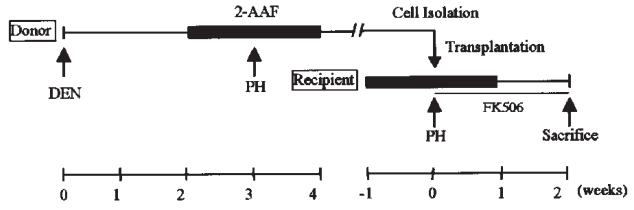


Figure 1. Experimental design for carcinogen treatment and cell transplantation. Donor rats were treated with DEN at a dose of 100 $\mu$g/g body weight, a 14 day 2-AAF diet 2 weeks later and then underwent PH at the 3rd week. Hepatocytes, including normal and preneoplastic ones, were isolated from the donor livers between the 6th and 8th week. Recipient rats were also treated with a 2-AAF diet and PH, and the hepatocytes were infused into the portal vein immediately after PH. All recipient rats were given FK506 after transplantation, and sacrificed 2 weeks later.

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 $\mu$g/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 mesenteric vein of the recipient animals using a 27-gauge stainless syringe needle immediately after PH. In the first experiment, 6-8-week-old, male Donryu and DRH rats were used as the recipients, and were treated with 2-AAF (in the case of recipients, 0.02% both for Donryu and DRH) and PH, followed by a normal diet for one week (Fig. 1). The rats were divided into 6 groups (Table I): Group A, Donryu rats without cell transplantation; Group B, Donryu with transplantation of Donryu hepatocytes; Group C, Donryu with transplantation of DRH hepatocytes; Group D, DRH rats without cell transplantation; Group E, DRH with transplantation of Donryu hepatocytes; Group F, DRH with transplantation of DRH hepatocytes. In the second experiment, 6-8-week-old, female Donryu rats treated with 2-AAF/PH received transplantation of male DRH hepatocytes ($10^6$ hepatocytes per animal). All recipients received FK506 (Fujisawa, Osaka, Japan) intramuscularly at a dose of 0.2 $\mu$g/g body weight per day after cell transplantation and were sacrificed 2 weeks later.

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

| | | | | | | | Oval cell | | | GST-P (+) lesions | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Exp | Group | Recipient | Sex | Donor | Sex | n | I | II | III | No/cm$^2$ | Diameter (mm) |
| Exp. 1 | A | Donryu | M | - | - | 5 | 0 | 0 | 5 | 0 | 0 |
| | B | Donryu | M | Donryu | M | 8 | 0 | 1 | 7 | 30.7±12.7 | 1.17±0.93 |
| | C | Donryu | M | DRH | M | 7 | 0 | 0 | 7 | 23.6± 6.8 | 1.06±0.83 |
| | D | DRH | M | - | - | 5 | 5 | 0 | 0 | 0 | 0 |
| | E | DRH | M | Donryu | M | 7 | 6 | 1 | 0 | 31.7±12.1 | 0.62±0.60[a] |
| | F | DRH | M | DRH | M | 8 | 7 | 1 | 0 | 29.9±12.1 | 0.45±0.54[a] |
| Exp. 2 | | Donryu | F | DRH | M | 3 | 0 | 1 | 2 | 45.4±16.4 | 1.03±1.10 |

[a]Comparison against Group B or C. $^*p<0.05$.

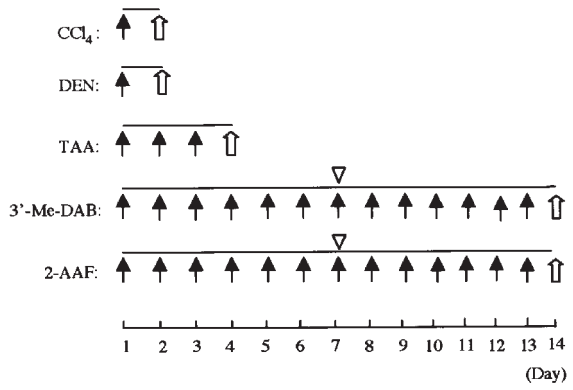


Figure 2. Treatment with hepatotoxic substances (arrows). The animals exposed to 3'-Me-DAB or 2-AAF were subjected to PH on day 7 (open arrowheads). Rats were sacrificed (open arrows) for examination of hepatic histology and serum AST/ALT.

*Effect of 2-AAF on liver regeneration*. Donryu and DRH rats were given the diet containing various concentration of 2-AAF for 7 days, performed PH on day 7 and sacrificed on various days after PH. Bromodeoxyuridine (BrdU) was given at a dose of 50 $\mu$g/g body weight 1 h before sacrifice.

*Treatment with hepatotoxins or hepatocarcinogens*. DRH and Donryu rats were treated with either a single dose of $CCl_4$ (dissolved in corn oil, 5 ml/kg body weight via a gastric tube), a single dose of DEN (dissolved in 0.9% NaCl, 250 mg/kg body weight by intraperitoneal injection), 3 daily doses of thioacetamide (TAA) (400 mg/kg body weight by intraperitoneal injection), 13 daily doses of 3'-Me-DAB (dissolved in corn oil, 50 mg/kg body weight via a gastric tube) or 13 daily doses of 2-AAF (dissolved in corn oil, 25 mg/kg body weight via a gastric tube) (Fig. 2). The rats that received either 2-AAF or 3'-Me-DAB were subjected to PH on day 7. The animals were sacrificed one day after treatment with $CC1_4$ or DEN, 3 days after the start of treatment with TAA and 2 weeks after the start of treatment with 3'-Me-DAB or 2-AAF. All livers were examined histologically and blood samples were also taken at the time of sacrifice to determine serum aspartate aminotransferase (AST) and alanine aminotrasferase (ALT) levels using standard laboratory techniques.

*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-$\mu$m 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% $H_2O_2$ 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), and the sections were counter-stained with hematoxylin. The numbers and average size of GST-P positive preneoplastic hepatocytic lesions were then analyzed under a microscope. For the BrdU stained sections, the frequency of the BrdU labeled hepatocytes were microscopically counted, and the BrdU labeling index (LI) was determined for each animal.

*Microdissection and PCR*. For detection of male cells transplanted into the female livers, the *sry2* gene, a marker for the Y chromosome (14), was amplified from DNA isolated from the dissected tissue sections. Using the GST-P immunostained sections, GST-P(+) colonies were isolated by the laser capture microdissection system (Olympus, Tokyo, Japan) and incubated in a proteinase K solution (0.5 mg/ml) for 2 h at 37˚C, followed by further incubation for 30 min at 95˚C. DNA was isolated, and PCR was then performed to amplify *sry2* using the primers, 5'-GGAGAGAGGCACAAGTTGGC-3' (forward) and 5'-CTTCAGTCTCTGCGCCTCT-3' (reverse). The PCR products were electrophoresed on agarose gels, followed by staining with ethidium bromide.

*Statistical analysis*. A statistical analysis was performed using the Student's t-test, and p-values of $<0.05$ were considered to be statistically significant.

## Results

*GST-P (+) lesions after transplantion with preneoplastic hepatocytes*. The health of most Donryu rats (Groups A, B and C) that survived 2 weeks after PH deteriorated, while most of the DRH rats (Groups D, E and F) that survived remained healthy. The livers of the Donryu rats treated with 2-AAF plus PH without transplantation (Group A) contained no GST-P (+) colonies, although a very few single GST-P(+) hepatocytes were seen (data not shown). The number of GST-P(+) lesions/cm$^2$ in each section did not differ much among the groups that had received the transplantation (Table I). On the other hand, the livers of the Donryu that had received transplantation with either Donryu (Group B) or DRH cells (Group C) showed large GST-P(+) colonies (Table I and Fig. 3A). In contrast, GST-P(+) lesions were smaller in the DRH livers, regardless of the origin of the donor cells (Fig. 3A). Most Donryu livers in Groups A, B and C showed strong oval cell proliferation, a marker of severe hepatic damage (15-17), whereas it was much less prominent in the DRH livers (Groups D, E and F) (Fig. 3B). When oval cell proliferation was categorized into 3 grades (grade I, no or very weak involvement in zone 1 of the hepatic lobule (18); grade II, extensive involvement in zone 1; grade III, involvement in zones 1 and 2), most Donryu livers were grade III, while most DRH livers were grade I (Table I).

*Donor origin of GST-P(+) lesions after transplantation with preneoplastic hepatocytes*. When PCR using the primers for the *sry2* gene was performed on DNA isolated from livers of male DRH and female Donryu rats, the specific 135-base pair band was detected for the male DNA but not for the female DNA (Fig. 4). PCR analysis using DNA isolated from all 7 GST-P(+) foci in the female Donryu livers that received transplantation of the male DRH cells detected the specific band, indicating that GST-P(+) lesions in the recipient livers were derived from the donor cells.

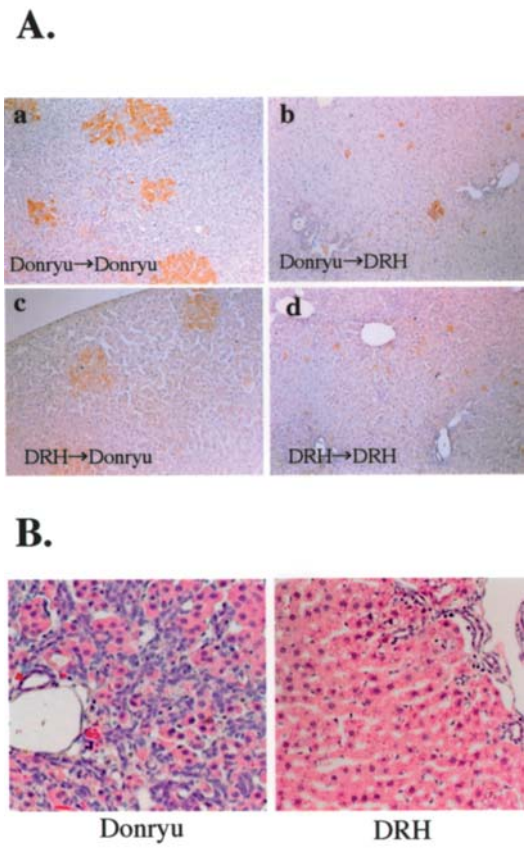


Figure 3. GST-P immunostaining of recipient livers (A). In the case of recipient Donryu, large GST-P(+) foci can be seen after transplantation of either Donryu (a) or DRH (c) hepatocytes, while recipient DRH livers show small GST-P(+) foci or single cells after transplantation of either Donryu (b) or 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).

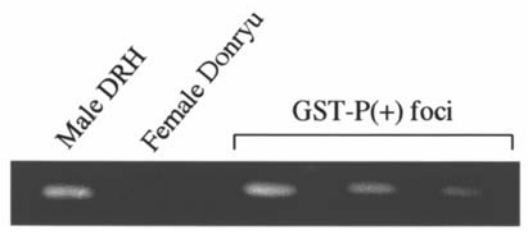


Figure 4. PCR using DNA isolated from untreated livers of male DRH and female Donryu rats, and from GST-P(+) foci in female Donryu recipient livers transplanted with male DRH hepatocytes.

*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

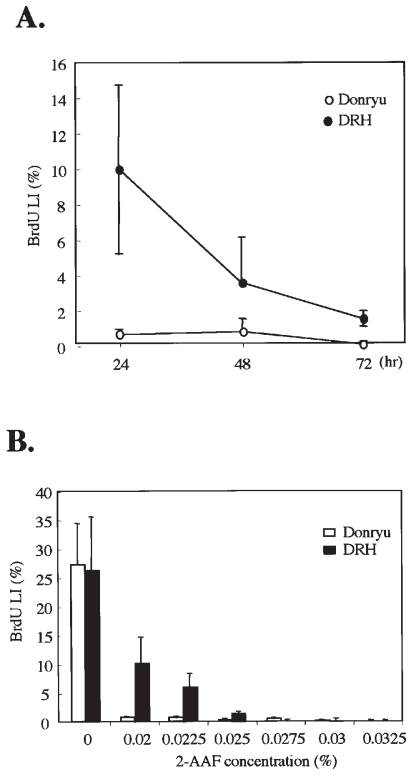


Figure 5. Effect of 2-AAF feeding on BrdU LI of hepatocytes after PH. BrdU LI after PH in Donryu (open circles) and DRH (closed circles) that were fed with the 0.02% 2-AAF diet for 1 week (A). N=3 at each time-point. BrdU LI 24 h after PH in the Donryu (open columns) and DRH (closed columns) given the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

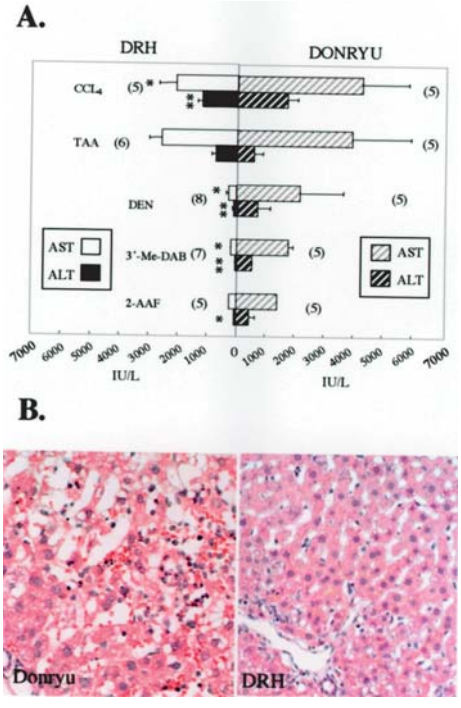


Figure 6. ALT (open columns) and AST (closed columns) levels after exposure to hepatotoxic substances (A). Note that the values are generally high in the Donryu and low in the DRH. Vertical bars represent standard deviation. The difference between the Donryu and DRH values is significant ($^{*}p<0.05$, $^{**}p<0.01$). 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 $CC1_4$, 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 hepatocytes themselves. In this transplantation experiment, the colonies formed within the recipient livers appear to be of donor origin, because the *sry2* gene, a Y chromosome marker, could be detected by PCR in the dissected GST-P(+) colonies of the female recipients that received transplantation of male cells (Fig. 4).

The BrdU LI of hepatocytes after PH was lowered by treatment with 2-AAF. When compared to Donryu and DRH rats, such growth-inhibitory effect of 2-AAF on hepatocytes was much weaker in DRH rats (Fig. 5). Furthermore, the degree of oval cell proliferation was much less prominent in the DRH than the Donryu livers after 2-AAF feeding and PH (Fig. 3B and Table I). Oval cells, originating from cells present in the canal of Herring or from blast like cells located next to bile ducts, proliferate under pathological conditions in which hepatocyte proliferation is inhibited due to severe injury (15-17). It is then probable that the DRH livers might be damaged to a lesser degree after 2-AAF/PH treatment compared to the Donryu livers. In the Solt-Farber model, preneoplastic hepatocytes are much more resistant to 2-AAF cytotoxicity than normal hepatocytes and can thereby proliferate by responding to PH growth stimulation, while normal hepatocytes cannot proliferate due to 2-AAF toxicity (3). It is then conceivable that in the DRH livers, the normal hepatocytes may be resistant to the 2-AAF toxicity and can therefore proliferate by responding to the growth stimulus. Under such condition, hepatic mass may be restored by the division of normal hepatocytes, which in turn attenuates the growth stimulus, and thus prevents neoplastic hepatocytes from efficiently forming colonies.

In the present study, DRH hepatocytes were demonstrated to be not only resistant to 2-AAF cytotoxicity, but also other chemicals, suggesting that DRH hepatocytes may possess a mechanism resistant to a wide variety of hepatotoxins. The pathways leading to hepatocyte death by toxic substances are complex, diverse processes, which may vary depending on chemicals. In most cases, the reactive metabolites generated in hepatocytes may destroy cellular structures and functions either by direct covalent binding to nucleic acids, proteins and lipids or the formation of reactive free radicals. Recent evidence further suggests that the death may be triggered by the activation of intrinsic apoptotic pathways which may be controlled by the balance between proapoptotic factors such as TNFα, TGFß and oxygen radical and prosurvival factors such as IL-6 and TGFα (19). Most of these factors are generated by non-parenchymal cells such as Kupffer cells and stellate cells. Although the mechanism of resistance to toxic substances in DRH hepatocytes has remained obscure so far, the observation that resistance is particularly evident for 2-AAF, 3'-Me-DAB and DEN, but modest for $CC1_4$ 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

This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and a Grant-In-Aid for Cancer Research from the Japanese Ministry of Health, Welfare and Labour. We are grateful to Mr. Satake for his technical assistance and the Fujisawa Therapeutical Corporation for providing FK506.

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