

Synergistic Effect of Hepatitis B Virus and Aflatoxin B₁ in Hepatocarcinogenesis in Tree Shrews

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Abstract

An animal experiment with tree shrews was performed to detect the synergistic effects of hepatitis B virus (HBV) infection and dietary aflatoxin B₁ (AFB₁) in hepatocarcinogenesis. Adult healthy tree shrews (*Tupaia belangeri chinensis*) were divided into four groups: Group A (HBV+ AFB₁)—animals were infected with human HBV serum at first, then fed AFB₁ diluted with milk, 150 ug/kg.bw/day, 6 days/week for 105 weeks. Group B (HBV)—animals were infected with human HBV as Group A, but no AFB₁ treatment. Group C (AFB₁)—animals were treated with AFB₁ as Group A but no HBV infection. Group D—animals were treated neither with human HBV nor AFB₁. During the experiment, blood samples and liver biopsies were taken regularly from all animals in each group. All the animals were sacrificed on the 160th week when the experiment ended. The samples of sera and liver tissues were checked for HBV markers and histological changes. Hepatocellular carcinomas (HCCs) were found only in Group A and Group C, with incidences of 47% and 30% respectively. The average time for HCC occurrence in Group A and Group C was 120.3 ± 16.6 and 153.3 ± 5.8 weeks respectively ($P < 0.01$). Even though no HCC occurred in Group B, 1 animal which died before the end of the experiment showed two large hepatocellular nodules. These results showed that there is synergistic effect between HBV and AFB₁ in tree shrews' hepatocarcinogenesis, even though the hepatocarcinogenic effect played by HBV alone is rather weak.

Ann Acad Med Singapore 1999; 28:67-71

Key words: Animal model, Hepatocellular carcinoma

Introduction

Primary hepatocellular carcinoma (HCC) is one of the commonest cancers in some parts of the world including several areas in China. The geographically distinct distribution of HCC indicates there are some special factors which play key roles in hepatocarcinogenesis.¹ Even though evidence from epidemiology and laboratory research have shown that hepatitis B virus (HBV) and aflatoxin B₁ (AFB₁) are the two most important factors in hepatocarcinogenesis, little is known about how they interact with each other. Yan and colleagues² reported on experiments with tree shrews, a species of small primate, that there was a synergic effect between HBV and AFB₁ in hepatocarcinogenesis. The present study,

by a series of progressive observations during the experiment of tree shrews' hepatocarcinogenesis, added further information to their work.

Materials and Methods

Animals and Treatments

Adult tree shrews (*Tupaia belangeri chinensis*) of both sexes were obtained from Kunmin Medical Biology Institute, Chinese Science Academy, their body weights were 100 to 160 g each. The animals were raised in stainless steel cages individually and fed with basic diet, fruits, eggs, and given tap water. Before starting the experiment, all animals were screened by testing the sera alanine aminotransferase (ALT), hepatitis B virus

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surface antigen (HBsAg) and liver biopsy. Healthy animals were then divided into 4 groups as follows:

Group A (HBV+AFB₁): Animals were inoculated with 0.5 ml of human HBV infected sera twice, at two-days interval, intravenously and intraperitoneally respectively. Two weeks later, their sera were checked weekly for HBV infection. The HBV positive animals were randomized into Group B (see below for details), the rest were allocated to Group A and received AFB₁ 6 weeks after HBV inoculation. AFB₁ was added to milk before being fed to the animals, 150 ug/kg.bw/day, 6 days a week until 105th week of the experiment.

Group B (HBV): The sera inoculated to animals and the procedure of inoculation were similar to that of Group A. The animals in this group, which were all positive for HBV infection at the beginning, received no further treatment except their sera were tested and liver biopsy was done regularly.

Group C (AFB₁): All the animals were treated with AFB₁ alone by the same procedure as that for Group A.

Group D (negative control): Animals were raised normally, neither HBV nor AFB₁ were given.

During the experiment, samples of blood (every 2 to 4 weeks) and liver biopsy (every 15 weeks) were collected from all animals in each group regularly to test for HBV infection. The experiment ended on the 160th week and all the remaining animals were sacrificed. Liver tissues from every biopsies and autopsies before or at the end of the experiment were examined histopathologically and immunohistochemically.

Serum for Inoculation

The infected serum for inoculating animals in Group A and Group B was obtained from a patient carrying HBV. This serum was positive for HBsAg, HBeAg and HBcAb.

Reagents

AFB₁ was purchased from Sigma, USA. DMSO (for dissolving AFB₁) was from FARCO, Hongkong. The anti-HBs antibody (#ZM-0122) was made by Zymed Laboratories Inc, USA. The immunohistochemical kits (#KIT-9200) were made by Maxim Biotech Inc, USA. The procedures for testing were according to the kits' instructions.

Treatment Of Liver Tissues

A part of each liver tissue from biopsies and autopsy were frozen in liquid nitrogen and then kept in -80°C. The rests were fixed in buffered formalin solution and then embedded in paraffin for histological examination.

Statistical Analysis

Differences of HCC incidence among groups and between sexes were analysed by the Chi-square (χ^2) test. Differences of the average time for HCC appearing were

analysed by the Student's t-test.

Results

General Condition

The first case of HCC appeared on the 99th week of the experiment, at which point of time the remaining animals in each groups were counted as "effective numbers". The effective numbers of animals were 21 for Group A, 6 for Group B, 10 for Group C and 9 for Group D. The causes of death were mostly secondary to venepuncture or liver biopsy before the 99th week, HCC or generalized weakness after the 99th week.

AFB₁ Intake and HBV Infection

By feeding AFB₁ for 105 weeks, the average amount of AFB₁ for each animal in Group A and Group C was 11.6 ± 0.9 mg and 11.4 ± 0.8 mg respectively. The difference was not statistically significant by the Student's t-test ($P > 0.05$).

The markers for HBV infection in sera or in liver tissues were not constantly positive in animals from Group A and Group B, but all of them were at least two times positive during the experiment.

Incidence of HCC

HCC occurred only in animals from Group A and Group C. The HCC incidence was higher in Group A than in Group C, and the average time for HCC appearance was significantly shorter (Table I).

No HCC occurred in Group B or in Group D. However, one animal in Group B which died on the 130th week did have two large proliferating nodules with diameters of 0.3 cm and 0.5 cm respectively on the surface of the liver (see below for details).

Histopathology of HCC and Proliferation Foci/Nodules Of Liver Cells

No difference was found histopathologically on the HCCs from Group A and Group C, both by naked eyes and by microscope. The sizes of HCC were from 4 cm in diameter to as small as a grain of rice. Most of them were multicentric, poor or moderately differentiated. The histological types of the HCC were trabecular, adenomatous or mixed (Fig. 1). Proliferation foci/nodules and atypical proliferation of liver cells were often seen around HCC. There was no marked fibrosis in the livers of tree shrews from all groups and no metastasis in any case of HCC.

The proliferation foci/nodules were common in Groups A, B and C. They appeared much more frequently in Groups A and C, and much earlier in Groups A and B. They were composed by different types of cells such as clear cells (positive for PAS staining), fat cells, acidophilic or basophilic cells. Usually these foci/nodules appeared around the 105th week of the experiment.

TABLE I: HCC IN GROUPS A AND C

	Incidence of HCC	Weeks for HCC to appear ($\bar{x} \pm s$)
Group A (HBV + AFB ₁)		
Total	66.7% (14/21)	120.3 $\pm$ 16.6*
Male	50.0% (5/10)	115.0 $\pm$ 10.5
Female	81.8% (9/11)	123.2 $\pm$ 19.1**
Group C (AFB ₁)		
Total	30.0% (3/10)	153.3 $\pm$ 5.8*
Male	16.7% (1/6)	160.0 $\pm$ 1.3
Female	50.0% (2/4)	150.0 $\pm$ 0.0**

* $P < 0.01$; ** $P < 0.05$

HCC: hepatocellular carcinoma; HBV: hepatitis B virus; AFB₁: aflatoxin B₁; $\bar{x}$: sample mean; s: standard deviation



Fig. 1. Hepatocellular carcinoma from a tree shrew in Group C. H & E 10 $\times$ 10

However, in a few animals from Groups A and B the foci/nodules appeared as early as the 30th to the 50th week. Some early clear cell foci disappeared automatically later on, but some of them existed continuously and mixed with some other kinds of cells such as acidophilic or basophilic liver cells during the late stage of the experiment. Even though no HCC appeared in Group B, there were many proliferation foci/nodules in this group. Big nodules were seen in one animal which died before the end of the experiment as mentioned above. Under light microscope these nodules consisted of irregularly arranged multi-layer liver cells, mixed with a few clear cells, and some liver cells formed small adenoid structure (Fig. 2). Thin fibrous septa were seen in some cases of this group, and piecemeal necrosis were seen occasionally.

A few cases in Group D showed proliferation of liver cell cord as two cells in thickness. But neither focus/nodule nor HCC appeared in this group throughout the experiment. There was one animal which died from the follicular type of malignant lymphoma near the end of the experiment.

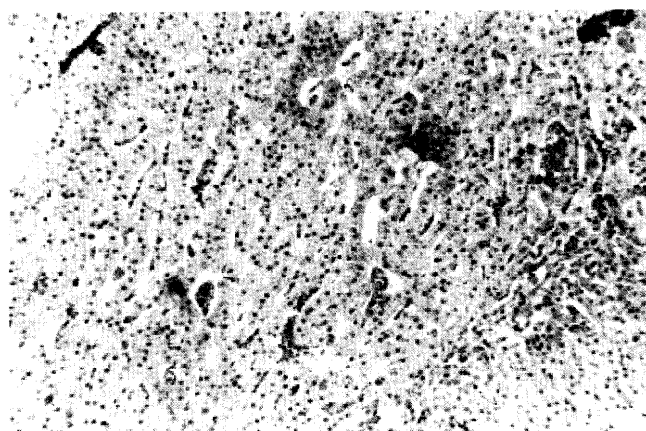


Fig. 2. Nodule from a tree shrew in Group B showing irregular arrangement of the proliferating liver cells with multi-layer and small adenoid structure H & E 10 $\times$ 10.

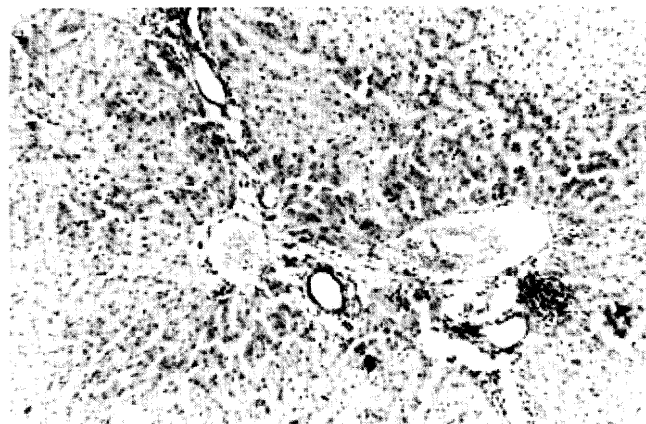


Fig. 3. Inflammatory infiltration and fibrosis in the portal area of a tree shrew in Group B. H & E 10 $\times$ 10.

Other Alterations

Alterations such as liver cell necrosis, inflammatory infiltration and fibroplasia in portal area (Fig. 3) were demonstrated in parts of animals from Groups A, B and C. The acute and chronic hepatitis were generally mild in Groups A and B, but occasionally these alterations were pronounced. In a few animals from Group A and C, liver cells showed acidophilic changes and acidophilic body. One animal in Group A which had HCC later on showed "ground glass cells" with HBsAg positive in its early biopsy.

Discussion

Our results showed that the HCC incidence was higher and the average time for HCC occurrence was shorter in Group A which was treated with both HBV and AFB₁ than in Group C which was treated with AFB₁ alone. These results provided further support for the existence of the synergistic effect between HBV and AFB₁ in tree shrew's hepatocarcinogenesis. Even though no HCC occurred in Group B which was treated with HBV alone, proliferation foci/nodules appeared quite early and frequently in this group, and unusually large nodules

were occasionally seen in this group. These findings indicate that HBV is probably capable to induce liver tumour, even though the ability is quite weak when it plays alone. Our results are consistent with that of Lunn et al³ who studied hundreds of cases of HCC in Taiwan recently by comparing the relative risk of AFB₁-DNA adducts in liver tissue and that of HBsAg in sera. Their results showed that the highest risk factor was individuals with both markers, the second risk factor was AFB₁-DNA adducts alone, and the last one was HBsAg alone. They believe therefore that there might be a viral-chemical interaction in hepatocarcinogenesis.

The clear cell foci which were quite characteristic in our observation were also seen in the research on synergistic effect between woodchuck hepatitis virus (WHV) and AFB₁ by Bannasch et al.⁴ We agree with their speculation that, since this and other kinds of precancerous foci/nodules appeared in the animals no matter what treatment (WHV and/or AFB₁) was given, these two oncogenic agents might carry a similar underlying molecular mechanism which is mainly responsible for their synergistic effect.

It is yet unclear as to why there is a male predominance in human HCC incidence. Our results showed that the incidence of HCC in female tree shrews was higher than males in both Groups A and C. This may be explained on the basis that female tree shrews are more sensitive to AFB₁ than males. Our previous study showed a similar tendency." Results from Reddy et al⁶ also support this presumption. They found that the HCC incidence in male and female tree shrews were 3/6 (50%) and 6/6 (100%) respectively after feeding them with AFB₁ for 172 weeks. The tree shrew's gender difference on HCC carcinogenesis is quite different from some other animals such as rats and its significance needs further study.

On the other hand, by using HBsAg transgenic mice to study chemical-induced hepatocarcinogenesis, Dragani et al⁷ found that the incidence of liver proliferation nodules and tumours in male HBV (+) mice was higher than that of male HBV (-) mice after treatment with 4-dimethylaminoazobenzene (DAB) or diethylnitrosamine (DEN) but there was no difference between HBV (+) females and HBV (-) ones. However, there was no such clear tendency between male and female tree shrews in our study. The difference between our results and that of Dragani et al might be due to the different species of animals and experimental methods.

During this experiment we observed several kinds of liver cell alterations such as inflammation, degeneration, and proliferation in Groups A, B and C. Even HBsAg positive "ground glass" liver cells in a few animals from Group A were observed. All these alterations are similar to the alterations often seen in human liver diseases. This indicates that the tree shrew model is

quite suitable in studying hepatocarcinogenesis in human beings.

It is still poorly understood as to how HBV and AFB₁ interact in hepatocarcinogenesis. The traditional view is that the effect of HBV in inducing liver cancer is related to liver cell damage, necrosis and subsequent regeneration after its infection. From serial liver biopsies, our observations are consistent with this hypothesis. We found the inflammatory cell infiltration, liver cell necrosis and regeneration were more active in the animals which developed HCC later on. Recently, by using mice transgenic with both TGF- α and HBsAg, Jakubezak et al⁸ found that the rate of liver cell proliferation, HCC incidence and average time for HCC formation were raised. These results might also suggest that the effect of HBV in inducing HCC is related to its ability of inducing liver cell proliferation. However, results from Petersen et al⁹ were different. By transplanting woodchuck hepatocytes into the livers of a kind of mice that lacked T and B lymphocytes and then infecting the mice with woodchuck hepatitis virus (WHV), they found that WHV is not only capable of establishing persistent noncytotoxic infection but also could cause more preneoplastic lesions. These results might challenge the traditional concept of "HBV infection $\rightarrow$ immune reaction of host liver cells $\rightarrow$ hepatitis $\rightarrow$ HCC". Besides altering the expression of carcinogen-metabolizing enzymes such as cytP450s, the other possible mechanism could involve the interaction of HBV with AFB₁.¹⁰ Furthermore, the effect of suppressive gene p53 might also be important. By using male transgenic mice, Ghebranious et al¹¹ found that the HCC incidence was 100% (7/7) in mice treated with all the three factors of p53+/-, HBV+ and AFB₁+. If any one of the factors was absent, the incidence dropped.

This paper reports only a part of the results from this study. The results on testing oncogenes, suppressive genes and growth factors will be reported in subsequent publications.

Acknowledgements

We would like to thank Dr D Luo and Dr XL Luo for their help with the experiment, and Ms G H Huang and Mr C Ou for their histological technical assistance. We also wish to thank Ms XL Deng and Mr HP Huang for maintaining the animals.

This project was supported by Chinese National Research Grant 39260033 and Guangxi (China) Science Research Grant 96-32006.

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