

# The effect of mycophenolate acid on hepatitis B virus replication in vitro

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**OBJECTIVE:** To use 2.2.15 cell line to determine the effects of mycophenolate acid (MPA) on hepatitis B virus (HBV) replication and viral protein synthesis in vitro.

**METHODS:** The 2.2.15 cells were treated with different concentration of MPA (1–50 µg/ml) for 12 days. HBsAg and HBeAg were detected in the supernatant fluid by ELISA and intracellular HBV DNA was analyzed quantitatively by slot blot hybridization.

**RESULTS:** MPA could suppress the expression of HBsAg and HBeAg, and the higher concentration of MPA induced lower expression of HBsAg and HBeAg. The suppression rates of MPA for HBsAg and HBeAg at a concentration of 50 µg/ml were 34.2% and 24.1% respectively. The expression of HBV DNA was only 49% as compared with controls when treated with MPA at a concentration of 50 µg/ml.

**CONCLUSIONS:** Mycophenolate acid can suppress the expression of HBsAg and HBeAg as well as the replication of HBV DNA in the 2.2.15 cell. The suppressive degree is dose-dependent.

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**Key words:** mycophenolate acid; hepatitis B virus; cell line

## Introduction

Hepatitis B virus (HBV) reinfection after liver transplantation is still a major cause of graft failure within the first year after transplantation.<sup>[1]</sup> The factors that led to severe posttransplantation recurrence of hepatitis B are not well understood. It is generally accepted that administration of immunosuppressive agents, graft rejection and operation stress are related to HBV reinfection.<sup>[2–4]</sup> In this study, the 2.2.15 cell line was used to examine the effects of mycophenolate acid (MPA) on HBV replication and viral protein synthesis in vitro. Based on the findings in this study, we can

choose suitable protocols of immunosuppression for HBV-related liver graft recipients.

## Methods

### Drugs

Three-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), with the store concentration of 5 mg/ml was solved by PBS solution. Mycophenolate acid (MPA, Roche Pharmaceuticals Ltd., USA) at the store concentration of 5 mg/ml was solved by culture medium.<sup>[5]</sup>

### Cell culture

The 2.2.15 cell line was kindly presented by the Institute of Infectious Diseases, Zhejiang University Medical School, Hangzhou, China. It was cultured in a DMEM medium, which was supplemented with 10% fetal bovine serum, 0.03% glutamine, and 200 µg/ml G418. The pH value of the medium was adjusted to 7.0 by solid NaHCO<sub>3</sub> and it was filtered by negative pressure. The 2.2.15 cells were applied to further experiment when they

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stably produced HBsAg and HBeAg in the supernatant fluid and the P/N value was more than 5.

### MTT assay

The 2.2.15 cells were trypsinized to single cell suspending solution and were diluted to  $5 \times 10^4$ /ml (about 20 ml) after cell count using a hemocytometer. The cells were seeded into 96-well tissue culture dishes,  $200 \mu\text{l}$  ( $10^4$  cells) in each well.

After 24 hours, the cells were treated at different concentration of MPA for 12 days. The concentration of MPA was divided into 12 gradients from 5 to 5000  $\mu\text{g}/\text{ml}$ , each being added into 3 parallel wells. During the period of incubation, the culture medium was removed every 4 days and replaced with a fresh medium containing MPA. On the 12th day MTT agent ( $20 \mu\text{l}$  5 mg/ml) was added to each well at 4 hours before ending of the culture. After culture for 4 hours at  $37^\circ\text{C}$ , DMSO was added to each well to dissolve MTT crystal in live cell.<sup>[6]</sup> The OD value of each well was detected at 570 nm in a Microplate Reader. The cell survival rate was calculated with the following formula: cell survival rate = OD value of drug well/OD value of control well  $\times 100\%$ .

### MPA intervened experiment

The 2.2.15 cells were diluted to  $2.5 \times 10^4$ /ml (about 100 ml) and seeded into 24-well tissue culture dishes,  $5 \times 10^4$  cells (2 ml) in each well. After 24 hours, the cells were treated at different concentration of MPA for 12 days. The concentration of MPA was divided into 6 gradients (1  $\mu\text{g}/\text{ml}$ , 2  $\mu\text{g}/\text{ml}$ , 5  $\mu\text{g}/\text{ml}$ , 10  $\mu\text{g}/\text{ml}$ , 20  $\mu\text{g}/\text{ml}$ , 50  $\mu\text{g}/\text{ml}$ , depending on the concentration for clinical application), and each concentration was added into 3 parallel wells. Three wells with untreated cells were used as controls. During the incubation period, the culture medium was removed every 4 days and replaced with a fresh medium containing MPA. The removed supernatant fluid was stored at  $-20^\circ\text{C}$  for subsequent analysis of HBsAg and HBeAg. At the end of incubation, the cells were collected and stored at  $-80^\circ\text{C}$  for analysis of intracellular HBV DNA.

### Detection of HBsAg and HBeAg by ELISA

HBsAg and HBeAg were detected by ELISA (Xinchuang Technology Limited Cooperation, Xiamen, China), and the OD value was detected at 450 nm/630 nm in a Microplate Reader. Then suppression rate of HBsAg and HBeAg was calculated: Suppression rate (%) = (OD value of drug well/OD value of negative control well - OD value of control well/OD value of negative control well) / (OD value of control well/OD value of negative control well - 2.1).

### HBV DNA analysis

HBV DNA analysis was adopted by slot blot hybridization. The DNA extracted from cells was equally divided into 2 parts, one supplied for HBV DNA hybridization, and the other for  $\beta$ -actin hybridization. HBV DNA or  $\beta$ -actin probe with DIG-label was prepared by random primer labeling. The template of HBV DNA or  $\beta$ -actin probe was synthesized by PCR. The relative value of HBV DNA was determined by densitometry after X-ray film exposure. Value R stands for the ratio of HBV DNA treated with drug to HBV DNA in control well. Value R = (density of HBV DNA in drug well/density of  $\beta$ -actin in drug well) / (density of HBV DNA in control well/density of  $\beta$ -actin in control well).

## Results

### MTT

The 2.2.15 cell survival rates after 12 days incubation at different concentration of MPA is shown in Fig. 1. The concentration of MPA in which the

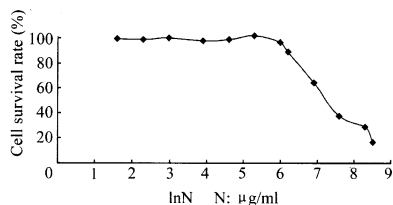
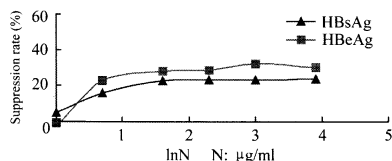
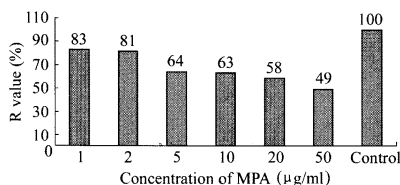


Fig. 1. Toxicity of concentration of MPA to the 2.2.15 cell (N: concentration of MPA).

**Table.** The suppression rates of HBsAg and HBeAg treated with MPA (%)

	MPA concentration ( $\mu\text{g/ml}$ )					
	1	2	5	10	20	50
Suppression rates of HBsAg	5.00	15.93	22.57	23.56	23.23	24.05
Suppression rates of HBeAg	-2.64	22.57	27.78	28.77	32.11	30.20

**Fig. 2.** Suppression rates of HBsAg and HBeAg treated at different concentration of MPA (N: concentration of MPA).**Fig. 3.** Expression of HBV DNA treated at different concentration of MPA.

cell survival rate is above 95% was 400  $\mu\text{g/ml}$ .

### Effect of MPA on HBsAg and HBeAg synthesis in the 2.2.15 cells

The suppression rates of HBsAg and HBeAg after 12 days incubation at different concentration of MPA are shown in Table and Fig. 2.

### Effect of MPA on HBV DNA in the 2.2.15 cells

Mycophenolate acid can suppressed the replication of HBV DNA. The expression of HBV DNA was only 49% compared with controls at a concentration of 50  $\mu\text{g/ml}$  (Fig. 3).

## Discussion

The HBV-transfected cell line 2.2.15 provides an effective model for study of gene expression and modulation of HBV DNA and the effect of antiviral drugs on HBV replication and mechanism of drugs. The 2.2.15 cells can express all the viral markers of HBV, including HBV DNA, HBsAg, HBeAg, HBcAg, HBV DNA polymerase. Moreover, Dane's particles can be detected in supernatant fluid, which is similar to the replication, transcription and expression of HBV in vivo. HBsAg and HBeAg are dissolvable proteins presenting in supernatant fluid and can be detected easily, economically and quickly. Hence they serve as indicators for screening antiviral drugs. But HBV DNA is the direct and sensitive indicator for viral replication.

The concentration of MPA in this experiment depended upon the usual concentration in clinical application of MMF. According to the results of MTT, MPA under the experimental concentration has no toxicity to the 2.2.15 cell. Because MMF exerts its immunosuppressive effect after it converts to MPA in the intestinal tract or serum. MPA was adopted in our experiment. The results show that mycophenolate acid can suppress expression of HBsAg as well as replication of HBV DNA in the 2.2.15 cell. The suppressive degree is dose-dependent.

Immunosuppressive agents, such as steroid, can not only promote replication of HBV DNA by suppressing the immune function of host, but also directly stimulate replication of HBV DNA by connecting to steroid-responsive element in HBV genome. The effects of immunosuppressive agents on HBV replication in vivo firstly depends on their effects in vitro. Gong's research showed that MPA can suppress replication of HBV DNA in hepatocyte culture, but this phenomenon was not proved in the 2.2.15 cell.<sup>[7]</sup> It is known that MPA has wide antiviral effects on both DNA virus (cytomegalovirus, herpes simplex virus type 1) and RNA virus (yellow fever virus, coxsackie B3 virus, and influenza A virus).<sup>[48]</sup> In addition, MPA has anti-HIV effect,<sup>[9]</sup> the mechanism of which is to block the synthesis of GPT and to suppress the function of retro-transcriptase. Retro-transcriptase is essential for HIV to synthesize provirus. HBV is similar

to HIV in the process of replication, but whether the mechanism of MPA suppressing HBV is the same as HIV remains to be further studied. Mycophenolate mofetil also may show synergistic anti-hepatitis C virus properties when used with interferon<sup>[10]</sup> or ribavirin.<sup>[11]</sup>

Based on the results that MPA can suppress replication of HBV DNA *in vitro*, we consider whether it is practicable to substitute MMF with azathiopurine to reduce or delay HBV reinfection after transplantation. It is reported that immune rejection does not increase when MMF takes the place of azathiopurine if recipients are tolerable, as confirmed in our clinical practice.<sup>[12]</sup> However, the clinical effect of substituting MMF with azathiopurine on HBV reinfection needs further study.

### Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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