An anti-preS2 antibody protects human hepatocytes from hepatitis B virus infection

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Abstract

Background : **Hepatitis B virus infection is a major problem in liver transplantation. In this study, we examined the potential efficay of a recombinant adenovirus expressing an antibody against the HBV preS2 antigen (Ab-H-HBV-S2) in preventing HBV recurrent infection after liver transplantation.**

Methods : **A gene for humanized antibody against the HBV preS2 antigen was cloned into pDC315, a type 5 adenoviral shuttle plasmid. Recombinant virus was obtained by homologous recombination in the 293 packaging cells. The virus containing the Ab-H-HBV-S2 gene was transduced into the rat liver graft during cold preservation. The recombinant virus produced antibody and showed protective effects on human hepatocytes from hepatitis B virus infection in vitro.**

Results : **The recombinant virus titer determined by TCID50 analysis was 5.1×10^{10} PFU/mL. The concentration of preS2 antibody in BALB/C nude mice was 16.7 ± 10.5 µg/mL on day 3, 30.9 ± 13.6 µg/mL on day 7, and lasted for 5 weeks after the injection. At a concentration of 0.5 µg/mL or above, the preS2 antibody protected cultured human hepatocytes from hepatitis B virus.**

Conclusions : **Adenovirus-mediated gene transduction of anti-preS2 antibody in the transplanted liver may be a useful approach to prevent hepatitis B infection after liver transplantation.** (Acta gastroenterol. belg., **2009**, 72, **306-311**).

Key words : liver transplantation, hepatitis B, antibodies, genes.

In the past 4 decades, liver transplantation has witnessed major technical breakthroughs and has become an important treatment option for advanced liver diseases. With the advent of various innovative immunosuppressive agents, immunological problems that once impeded allogenic liver transplantation have now largely been resolved. In contrast, hepatitis B infection has become a major issue and a significant barrier to successful liver transplantation (1,2). Many patients with advanced liver diseases are infected with hepatitis B virus, especially in Asia. Without antiviral treatment, the rate of HBV recurrent infection in these patients could be as high as 80-100% after transplantation (3-5). About 10-30% of these patients gradually progress to acute or chronic hepatitis, cirrhosis and eventually hepatic failure.

The outer membrane of the hepatitis B virus (HBV) particle contains the HBV preS2 protein that binds to polyalbumin. The preS2 helps HBV to gain entry into liver cells via albumin receptors. In theory, blocking preS2 may prevent or minimize HBV infection (6). In this study, we tested this hypothesis by introducing an antibody against the preS2 in transplanted liver in rats. Briefly, a gene for the humanized antibody against the HBV preS2 was cloned into adenovirus. The recombinant virus successfully produced antibody against the HBV preS2 upon introduction into nude mice. We then transfected the liver graft in a model of rat liver transplantation, and confirmed the expression of anti-HBV preS2 antibody *in vivo*. Protective effects of this antibody were examined in primary human hepatocyte culture.

Materials and methods

1. *Animals*

Male Wistar rats (n = 60, 6-8 weeks, 180-220 g) and male Balb/c nude mice (n = 30, 4-6 weeks, 22-27 g) were obtained from the Experimental Animal Center of the Chinese Academy of Sciences in Shanghai. All experimental procedures were conducted in accordance with the rules that govern animal research of the Second Military Medical University.

2. *Plasmids and reagents*

Ad5 shuttle plasmid vector pDC315, frame plasmid pBHGloxΔE1-3Cre and the 293 cell line were purchased from Microbix Biosystems (Toronto, Ontario). DNA sequences encoding for variable regions of both light and heavy chains of human anti-HBV preS2 (Ad-H-HBV-S2) were synthesized by Shengneng Bocai (Shanghai, China) (7). Genes for human IgG3 light and heavy chain constant region were gifts from Dr. Wu (Shanghai Biochemistry Institute, Chinese Academy of Sciences). Purified recombinant HBV preS2 protein was kindly provided by Dr. Deng Qiang (Shanghai Biochemistry Institute, Chinese Academy of Sciences). Recombinant adenovirus containing a galactosidase (Ad-LacZ) gene was prepared in this laboratory. Goat anti-human IgG and horse radish peroxidase conjugated rabbit anti-goat antibodies were purchased from Dingguo Biotechnique Development Center (Beijing, China). Horseradish peroxidase conjugated mouse anti-human IgG was from

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Becton Dickinson (Franklin Lakes, NJ). HBsAg radioimmunoassay detection kits were from Shanghai Bluegene Biotech (Shanghai, China). Dulbcco's Modified Eagle Medium and bovine calf serum were from Gibco (New York, NY). Protein G was purchased Amersham (Piscataway, NJ).

3. *Generation of recombinant adenovirus*

Ab-H-HBV-S2 gene was cloned into pDC315. The resulting plasmid and Ad5 scaffold plasmid pBHGloxΔE1-3Cre were co-transfected into 293 cells by liposome-mediated transfection. Recombinant adenovirus was generated via homologous recombination in 293 cells. The identity of recombinant virus was confirmed by PCR and named Ad-Ab hereafter in this article. 293 cells were cultured in Dulbcco's Modified Eagle Medium containing 2% FBS. TCID50 was determined using a cytopathic effect (CPE) assay (8), at 10 days after Ad-Ab infection. Experiments were repeated twice.

4. *Expression of recombinant antibody in BALB/C nude mice*

30 nude mice were randomly divided into 3 groups : group 1 : Ad-Ab (2×10^9 pfu/mouse) ; group 2 : Ad-LacZ (2×10^9 pfu/mouse) ; and group 3 : normal saline (100 μL/mouse). Ad-Ab, Ad-LacZ and normal saline were injected through the tail vein. Serum samples of each mouse were collected at 0, 1, 2, 3, 7, 10, 14, 21, 28, 35, and 42 days after the injection. Expression level of Ab-H-HBV-S2 in the serum was determined by ELISA. Microplates were coated with HBV preS2 antigen (1 μg/well) at 4°C overnight. After washing with PBST (NaCl 8.0 g/L, KH_2PO_4 0.2 g/L, $Na_2HPO_4.12H_2O$ 2.9 g/L, KCl 0.2 g/L, Tween-20 0.5 mL/L, $C_9H_9O_2HgNaS$ 0.1 g/L ; pH 7.4), microplates were treated with 5% calf serum in PBS and washed with PBST. Ab-H-HBV-S2 and adequately diluted mouse serum were added to antigen-coated microplates. The microplates were washed after 40-min incubation at 37°C. HRP conjugated mouse anti-human IgG was added into each well and incubated for 60 minutes. HRP substrate was added and the reaction terminated after 60 min. Antibody concentration was determined with an ELISA microplate reader at 450 nm. The concentration of antibody was expressed as mean ± standard deviation (SD). The expression of human antibody in mouse serum was confirmed by Western blot with goat anti-human IgG as the primary antibody.

5. *Expression of recombinant antibody in rat transplanted liver*

Sixty rats were randomly assigned into 3 groups. Donor rats were anesthetized by diethyl ether and the liver was isolated as described by Kamada and Calne (9). After systemic administration of 100 IU of heparin, the liver was flushed with a low-pressure portal vein injection of 20 mL of 4°C Ringer's solution. Meanwhile, the suprahepatic and infrahepatic vena cava was transected to allow adequate drainage. The portal vein was then cut, and the graft was stored in Ringer's solution at 4°C. Recombinant virus was diluted to 5 ml by cold Ringer's solution, infused into the portal vein, and kept in place for 60 min before transplantation. Donor livers in the experimental group were infected with Ad-Ab virus particles (1×10^{10} pfu/liver). The livers for the other two groups were infected with Ad-LacZ (1×10^{10} pfu/liver) or treated with normal saline as controls. Reconstruction of the blood vessels and bile duct was performed with a modified Kamada cuff technique.

Rat serum was collected at 12 hours, 1, 2, 3 and 7 days after transplantation and stored at -80°C. The transplanted liver was excised 7 days after the surgery and stored in formalin (10%) for immunohistochemistry analysis.

The expression of Ab-H-HBV-S2 in the serum of recipient rats was determined with quantitative ELISA. Paraffin sections of the liver tissues were immunochemically stained for Ab-H-HBV-S2. After de-paraffinization, the sections were quenched with peroxidase, blocked with 1% BSA , and incubated with goat anti-human IgG overnight at 4°C. After washing, a horseradish peroxidase-conjugated secondary antibody (rabbit anti-goat) was applied. The signals were developed with the DAB substrate kit, and counterstained with hematoxylin.

6. *Ab-H-HBV-S2 binding to HBV*

10 μL serum from rats receiving or the recombinant virus or controls were diluted to 50 ul with 10 mM PBS (NaCl 8.7 g/L, KH_2PO_4 0.3 g/L, $Na_2HPO_4.12H_2O$ 2.9 g/L, pH 7.0). 10 ug protein G beads were added to diluted serum and 0.2 ug PreS2 was added to one Ad-Ab serum. Samples were vacillated at 4°C for 1 hour. Serum of patients containing 2×10^7 HBV particles were added. After 10 mM PBS washing and centrifugations at $200 \times g$ for 5 times, HBV DNA was extracted and thirty cycles of PCR were carried out (at 94°C for 60 s ; 55°C, 60 s ; 72°C, 180 s) with Taq. Sequence of the upstream primer was gtggtattgtgaggattcttgtc. The downstream primer was ctactgttcaagcctccaagc. HBV DNA was used as a positive control.

7. *Culture of human primary hepatocytes*

Sections of liver tissue were placed in balanced buffer (containing sodium lactate 3.10 g/L, NaCl 6.00 g/L, KCl 0.30 g/L and $CaCl_2 \cdot 2H_2O$ 0.2 g/L ; pH 7.2) in 4°C. Blood residues were cleared off with repeated vascular perfusion. Tissue was cut into roughly 1 mm^3 pieces, and incubated in 10% DMEM with 0.05% collagenase in 37°C for 30 min. The resulting cell suspension was filtered through a 200 μm porous membrane, centrifuged at $50 \times g$ at 4°C. The process was repeated 3 times. Trypan blue staining (10 mg/mL) was used to determine the survival rate of hepatocytes. Cells were cultured according to a method described by LeCluyse *et al.*[10] 2×10^6/well

hepatocytes were added into 6-well plates pre-coated with collagen IV. After cell attachment, culture dishes were gently shaken. The medium containing unattached cells was aspirated and replaced with modified Chee's medium (MCM) containing 0.1 μM dexamethasone, 6.25 μg/mL insulin, 6.25 μg/mL transferrin (10,11). Hepatocytes formed monolayers of granular epithelial cells. Occasional division was observed in hepatocyte colonies between days 5 and 10 under a light microscope. Hepatocytes displayed normal morphology and appeared undifferentiated.

8. *Effects of Ad-Ab in primary culture*

2×10^7 HBV particles (from the serum of HBV patients) were incubated for 1 hour at room temperature with human immune globulin, human HbsAb, serum from Ad-LacZ control rats, serum from Ad-Ab infected rats, or serum from Ad-Ab infected rats pre-incubated with PreS2. Mixtures were added into primary hepatocyte culture. Another 2×10^7 HBV particles were added into primary hepatocyte culture as negative control. After 16 h incubation, cells were washed with normal saline to remove excess HBV particles. The culture medium was changed daily and tested for the presence of HBsAg and HBeAg using commercially available radioimmunoassay kits. Cells were cultured for additional 10 days.

Results

1. *Generation of recombinant adenovirus*

Adenovirus shuttle plasmid pDC315 encoding for Ab-H-HBV-S2 and framework plasmid pBHGloxdeltaE1-3Cre were co-transfected into 293 cells. Recombinant adenovirus was generated by homologous recombination (Fig. 1). CMV promotor was used in the recombinant vector to control the expression. A polio virus IRES was placed between the light and heavy chains of the recombinant antibody gene. The leader signal peptide sequence was followed by the VH and VL gene segments. $TCID_{50}$ test showed that the concentration of recombinant virus was 5.1×10^{10} pfu/mL.

2. *Recombinant virus expressed in BALB/C nude mice*

Anti-pre-S2 antibody was detected by ELISA from day 2 to day 35 in the serum of the mice receiving Ad-Ab (Fig. 2). Immunoblotting displayed two protein bands, at 25 and 58 kD, respectively (Fig. 3). No expression was observed in the two control groups.

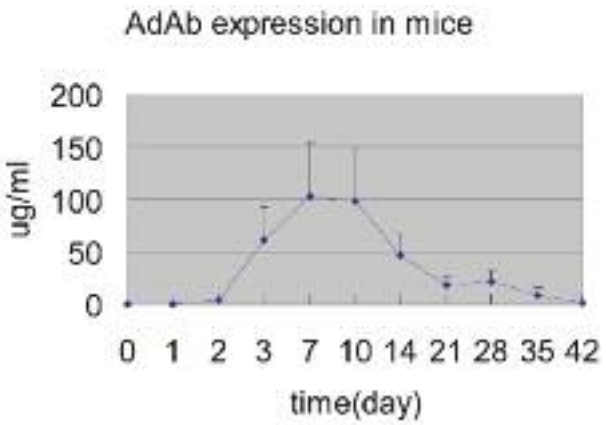

Fig. 2. — Antibody expression was detected 3 days after injection with Ad-Ab recombinant virus in an ELISA. Antibody was detectable for up to 6 weeks after the injection. No antibody was observed in the control groups.

3. *Expression of recombinant antibody in rat serum and transplanted liver*

The recombinant antibody was under detection limit at 12 hours and 1 day. The antibody was 1.7 ± 1.3 μg/mL at 2 days. Concentration of the recombinant antibody in the serum was 16.7 ± 10.5 μg/mL at 3 days after the rats received donor liver infected with Ad-Ab recombinant virus. The concentration increased to 30.9 ± 13.6 μg/mL at 7 days after the transplantation. Immunohistochemistry analysis confirmed the presence of recombinant antibody in transplanted liver (Fig. 4). No antibody was expressed in the two control groups.

4. *Ab-H-HBV-S2 binding to HBV*

Immunoprecipitation and PCR experiments showed that Ab-H-HBV-S2 could bind to HBV. Pretreatment with PreS2 prevented the binding (Fig. 5).

5. *Effects in the primary culture*

Hepatocytes formed monolayers of granular epithelial cells. A few cell divisions occurred in hepatocyte

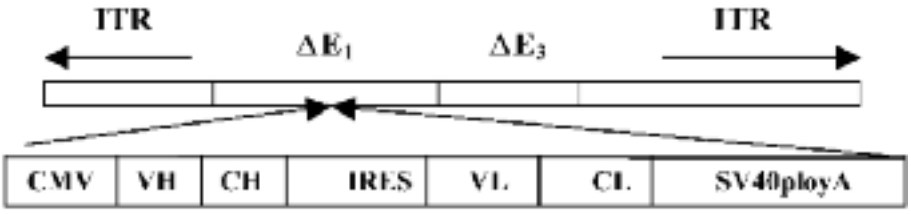

Fig. 1. — Schematic diagram of recombinant adenovirus

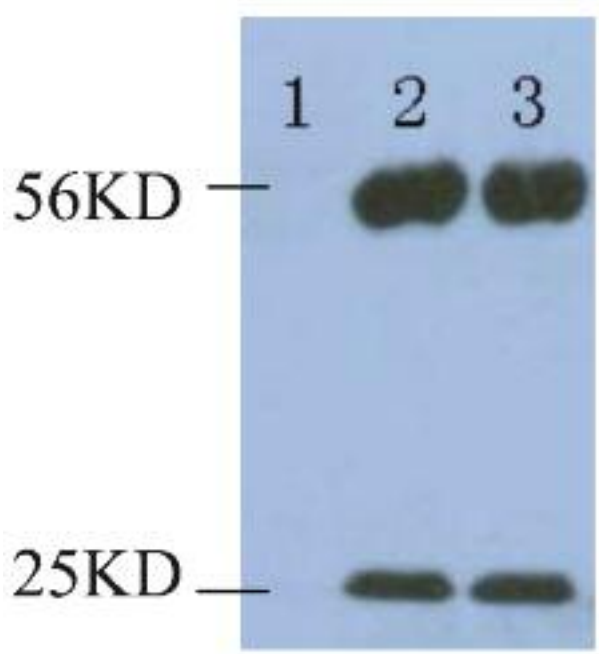

Fig. 3. — Antibody expression by immunoblotting with anti-human IgG3. Lane 1 : serum from Ad-LacZ injected mice ; lane 2 : serum from Ad-Ab injected mice ; lane 3 : human IgG3 antibody positive control.

colonies between days 5 and 10. Hepatocyte morphology remained normal and undifferentiated.

HBV infection and subsequent replication in the human hepatocytes were confirmed by measuring the kinetics of negative control HBsAg secretin. As shown in Fig. 6, the secretion rate of the HBsAg by the infected cells increased over time and reached the highest P/N ratio on day 10, indicating that HBV indeed infected the cultured human hepatocytes and replicated in the cells.

To examine whether the Ab-H-HBV-S2 neutralizes the HBV infection, the virus particles were preincubated with different concentrations of Ab-H-HBV-S2 and the mixture was added into the culture medium of primary

Fig. 5. — Ab-H-HBV-S2 binding to HBV by PCR. Lane 1 : HBV DNA positive control ; lane 2 : serum from normal rats plus HBV particles ; lane 3 : serum from Ad-LacZ infected rats plus HBV particles ; lane 4 : PreS2 /Ad-Ab plus HBV particles ; lane 5 : serum from Ad-Ab infected rat plus HBV particles ; lane 6 : molecular weight marker.

hepatocytes. Secreted HBsAg was assayed after 10 days. Human IgG and Ad-Lac infected serum were used as negative controls. HBsAb were used as positive controls. As shown in Table 1, HBsAg was not detected from the cells infected with mixture of the HBV and Ab-H-HBV-S2 (10, 1, or 0.5 μg/mL), human HBsAb (10 or 1 μg/mL). HBsAg was detected from the cells infected with the HBV and 0.01 μg/mL of Ab-H-HBV-S2, 0.1 or 0.01μg/mL of HBsAb. When the Ab-H-HBV-S2 completely neutralized by Pres2, HBsAg was detected (Fig. 6).

The in vitro neutralization assays showed that the serum containing the Ab-H-HBV-S2 protected primary hepatocyte culture from HBV infection. The minimum concentration required was 0.5 μg/mL.

Discussion

Currently available treatments for recurrent HBV infection in patients receiving liver transplant include immunization and antiviral therapies (12). Protective effects of passive immunization with the anti-HBV immunoglobulin (HBIG) are mediated by direct binding to HBV, binding to the HBV surface receptor in

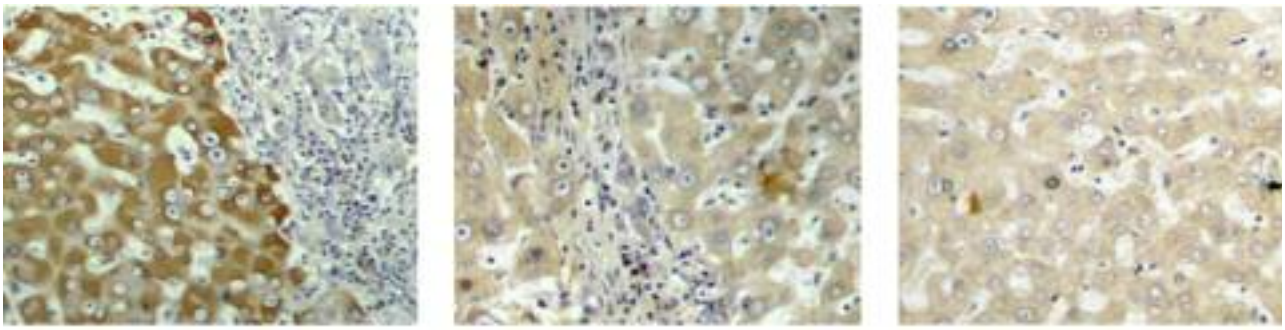

Fig. 4. — Expression of recombinant antibody in the liver. Hematoxylin staining, × 100 magnification, a) Ad-Ab recombinant adenovirus ; b) Ad-LacZ adenovirus ; c) saline control.

Table 1. — **Radioimmunoassay for HBsAg secretion by the human hepatocytes infected with antibody-treated HBV on day 10. Control P/N ratio without adding antibody was 21.4. P/N ratio of > 2.1 was considered positive**

Concentration (μg/mL)	Ab-H-HBV-S2	Ab-H-HBV-S2 + PreS2	Ad-Lac infected serum	Human IgG	Human HBsAb
10.0	1.2	22.4	24.1	22.6	0.7
1.0	1.4	17.9	19.2	11.2	0.8
0.5	0.9	14.3	15.3	29.9	9.5
0.1	10.7	11.7	16.2	24.5	10.4

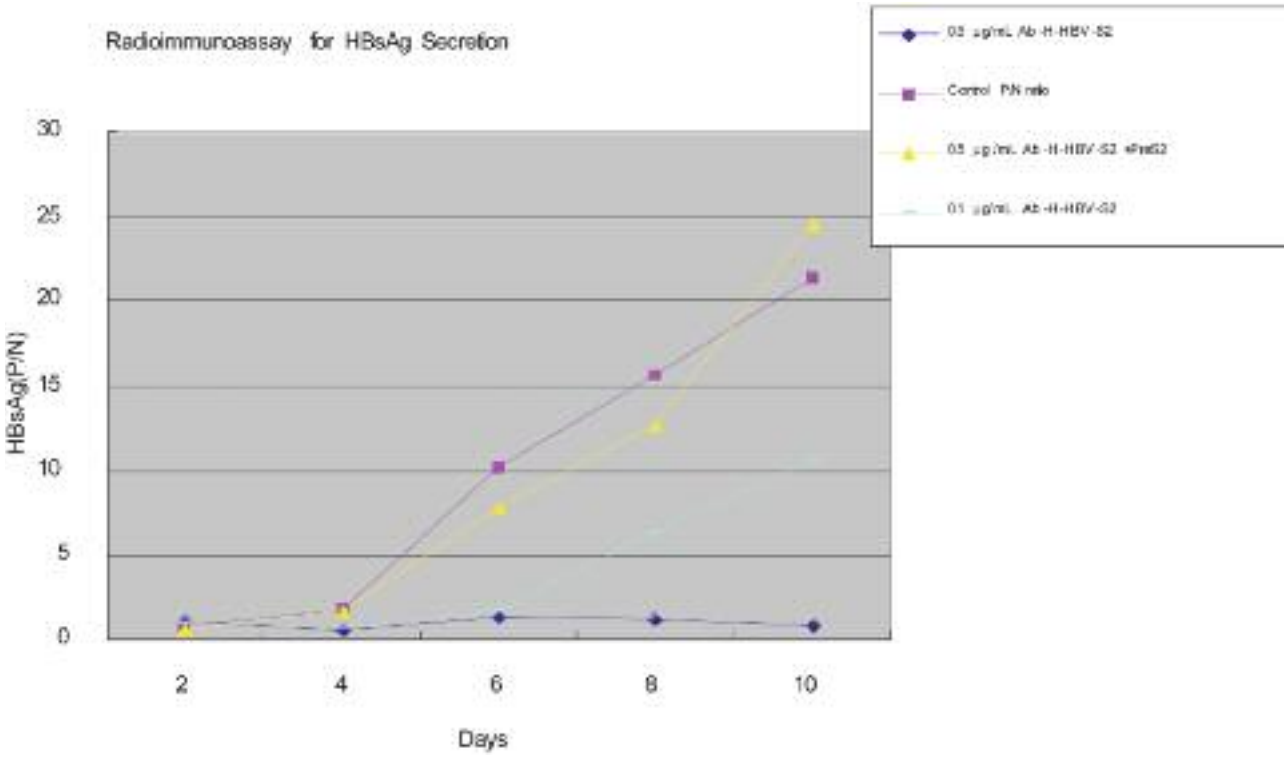

Fig. 6. — Kinetics of HBsAg secretion by HBV-infected human hepatocytes

hepatocytes, and antibody directed cell-cytotoxicity (ADCC) (13,14). Unfortunately, this treatment is rather complicated and costly. It also carries a risk of contamination with HCV, HIV and other pathogenic agents. Active immunization requires low initial viral load prior to the transplantation and the concurrent use of exogenous HBIG. In addition, the patient must not be in a severe immunosuppressive state and the efficacy of immunization is correlated with the concentration of HBIG produced (15). A major problem with many antiviral drugs is the emergence of drug-resistant HBV mutants (16,17). A combination of antiviral drugs with HBIG is a promising approach, but data regarding the long-term effects are still lacking.

The genetically engineered monoclonal antibodies specific to the surface antigens of HBV is a promising approach for the immunoprophylaxis of HBV infection (18) and may have the potential to resolve these problems. Pan *et al.* cloned the gene of human Fab against HBsAg (HB) into recombinant adeno-associated virus (AAV). They evaluated the activity of antibody HB and concluded that rAAV-HB might be a suitable method for the immunoprophylaxis of HBV infection (19). But small molecules have relatively low affinity and specificity for antigens. The main drawback of small molecule antibodies, including Fab, scFv, dsFv and mono-region antibody, is their inability, or low efficacy, in inducing ADCC due to the absence of the Fc segment (20).

Adenovirus can replicate to relatively high viral titers and can be easily purified. However, the adenovirus vector can be cleared off by specific antibodies *in vivo*. To overcome this problem, we adopted a unique approach : donor liver was infected with recombinant viruses expressing the preS2 antibody at the time of cold preservation. This procedure ensures infection of the donor liver tissue before virus vectors were introduced into the blood stream of the recipients. Expression of the HBV antibody expressed lasted for more than 1 month in nude

mice in this study as well as in our previous study (21). To further prolong the expression of the antibody, we are currently conducting a study using several neotype adenoviruses since neotype recombinant adenoviruses encoding exogenous genes have been reported to sustain gene expression for up to 1.5 years (22).

Results from the current study confirmed that the protective effects of the preS2 antibody are concentration-dependent, with a minimum of 0.5 µg/mL. This concentration is much lower than the *in vivo* recombinant anti-HBV antibody produced in rats receiving the recombinant virus. Immunohistochemistry demonstrated that antibody is expressed in liver tissue and the concentration in the liver is much higher than in other areas.

In summary, we have demonstrated that a recombinant adenovirus encoding the full-length human anti-HBV pre-S2 antibody in a transplanted liver can be used to achieve a high concentration of neutralizing antibody. This antibody protected primary human hepatocyte culture from HBV virus infection. These results suggest that recombinant antibody gene therapy through adenovirus vector may be a novel method for prevention and treatment of HBV infection recurrence after liver transplantation.

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