Over-expression of uPA increases risk of liver injury in pAAV-HBV transfected mice

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Abstract

AIM: To investigate the relationship between over-expression of urokinase plasminogen activator (uPA) and hepatitis B virus (HBV) related liver diseases in a transgenic mouse model.

METHODS: Albumin-tetracycline reverse transcriptional activator and tetO-uPA transgenic mice were generated respectively through pronuclear injection and crossed to produce the double transgenic in-alb-uPA mice, for which doxycycline (Dox)-inducible and liver-specific over-expression of uPA can be achieved. Hydrodynamic transfection of plasmid adeno-associated virus (AAV)-1.3HBV was performed through the tail veins of the Dox-induced in-alb-uPA mice. Expression of uPA and HBV antigens were analyzed through double-staining immunohistochemical assay. Cytokine production was detected by enzyme linked immunosorbent assay and α-fetoprotein (AFP) mRNA level was evaluated through real-time quantitative polymerase chain reaction.

RESULTS: Plasmid AAV-1.3HBV hydrodynamic transfection in Dox-induced transgenic mice not only resulted in severe liver injury with hepatocarcinoma-like histological changes and hepatic AFP production, but also showed an increased serum level of HBV antigens and cytokines like interleukin-6 and tumor necrosis factor-α, compared with the control group.

CONCLUSION: Over-expression of uPA plays a synergistic role in the development of liver injury, inflammation and regeneration during acute HBV infection.

INTRODUCTION

Hepatitis B virus (HBV) infection causes a necroinflammatory liver disease of variable duration and severity, with a high risk of developing cirrhosis and hepatocellular carcinoma. The immune response to HBV-encoded antigens is responsible both for viral clearance and for disease pathogenesis during HBV infection\[1\]. However,
the roles of urokinase plasminogen activator (uPA)/uPAs receptor (uPAR) systems as important inflammatory mediators have not yet been well investigated in acute and chronic hepatitis B, a common inflammatory disease in China[3]. Clinical studies almost focused on the correlation of uPA levels with the liver disease severity in hepatitis B patients. And the role of uPA in the HBV-induced liver injury, especially in the early stage, is less investigated.

uPA is one kind of plasminogen activator that catalyzes the conversion of plasminogen to plasmin. Together with uPAR, uPA participate in fibrinolysis, innate and adaptive immunity, and pathology[4,5]. In cancer cells, the effects of uPA and uPAR were thought to be related to cell migration[6], metastasis[7], and a more recent role of uPA in cancer growth has emerged[8]. The levels of uPA and uPAR have been found to be increased in tissues, plasma and other body fluids of cancer patients and to be markers of cancer development and metastasis, such as in patients with colon adenocarcinoma[9], lymphomas and leukemia[10].

The tetracycline (Tet)-inducible expression system is one of the most prominent and widely-used systems, which allows relatively stringent, reversible, and quantitative regulation of transgene expression in a wide range of cells in culture as well as in transgenic animals[11,12]. It consists of two parts: the ligand-dependent transactivator tetracycline reverse transcriptional activator (rtTA) as the effector and a tetO-cytomegalovirus (CMV) minimal promoter cassette regulating the expression of the transgene as the responder[13]. When doxycycline (Dox) is present, rtTA binds to the tetO-sequence and induces expression of the target gene[14,15]. Together with a tissue-specific promoter, it can result in transgene expression in a temporally and spatially defined fashion.

In this study, an effective inducible and liver specific uPA expression mouse model was constructed in which the murine uPA expression was controlled by rtTA which is regulated by murine albumin enhancer/promoter. Through administration of Dox, the inducible expression of uPA specifically in mouse liver can be achieved with lower mortality. Then hydrodynamic injection of pAAV-1.3HBV, which contained inverted terminal repeat elements of adeno-associated virus (AAV) and 1.3 copies of HBV genome/ayw subtype, was performed to mimic the acute HBV infection. The mouse liver showed specific and inducible expression of uPA. Plasmid AAV-1.3HBV transfection in Dox-induced transgenic mice resulted in sever liver injury, higher HBV antigen and cytokine expression compared to the control group. These data further indicated for the first time in mice that the overexpression of uPA may have accelerative role in the development of liver injury, inflammation and liver regeneration during acute HBV infection.

MATERIALS AND METHODS

Plasmid construction
For liver-specific expression of rtTA, the transgenic construct albumin-rtTA was generated, which has rtTA gene under the control of the liver-specific albumin promoter and was based the plasmid pTet-on (Clontech Lab, Inc). To introduce appropriate restriction sites in pTet-on, linker sequences were designed as follows, Tet-on-linker-F: 5'-CTAGGATATCAGCTAGGTGACCCGCCG- GGCCCGG-3' and Tet-on-linker-R: 5'-ATATCCGGC GCGGCGGGCGTGACACACTGATGATC-3'. The linkers were annealed at 95°C for 10 min and then were digested with EcoRI and SphI and ligated to pTet-on digested with the same restriction enzymes, and the construct was named pTet-on-link. The albumin promoter fragment and enhancer fragment (Genbank accession no. AC140220.4) were separately amplified by polymerase chain reaction (PCR) using genomic DNA extracted from C57BL/6 mouse liver as the template. The primers for albumin enhancer were Alb-En-FP: 5'-GCCG AGCTCTGCGCGGCTAGTCTTCTTACGTAG-3' and Alb-En-RP: 5'-GGGTTAAGGATCCCAAGCT GTGAG-3'. The primers for albumin promoter were Alb-Pro-FP: 5'-CGGGATCCACAGCTCCAGAT GGCAACATAC-3' and Alb-Pro-RP: 5'-TTTGGC CAGAGGTATGCGGTTGGT-3'.

The albumin enhancer PCR product was digested with BamHI I and cloned into pGEM-7ZF, then the albumin promoter sequence was inserted behind the enhancer at the site of BamHI I, and the plasmid was named p7ZF-Albumin, which was confirmed by restriction enzyme digestion analysis and DNA sequence analysis. Then p7ZF-Albumin was digested by SalI I and KpnI I, and the released 223bp fragment was ligated to pTet-on-link digested by EcoRI V and KpnI I, to yield the recombinant construct named pTet-on-Albumin.

For rtTA responsive expression of uPA, the transgenic construct pTRE2-uPA was generated which is based on the plasmid pTRE2 containing tetO. The uPA cDNA and uPA exon 11 was amplified by reverse transcription polymerase chain reaction (RT-PCR) and PCR from the total RNA and genomic DNA extracted from the kidney of C57BL/6 mouse, respectively. For uPA DNA, the primers were uPA-cDNA-F: 5'-GGGATCCATGCGATGATGGTACCGGGCCCGGC GGGCGGTG-3' and uPA-cDNA-R: 5'-GGGATCCATGCGATGAGGAG-3'. The primer sequences were designed as follows, Tet-on-linker-F: 5'-CTAGGATATCAGCTAGGTGACCCGCCGCCGGGCGTGACACACTGATGATC-3' and Tet-on-linker-R: 5'-ATATCCGGGC GCGGCGGGCGTGACACACTGATGATC-3'. The PCR product was ligated to pMD18T and the construct pMD18T-uPA cDNA was confirmed by sequence analysis. The uPA exon 11 was amplified by PCR from the genomic DNA template, the primers were uPA3'-F: 5'-CGGGATCCATGCGATGAGGAG-3' and uPA3'-R: 5'-GGGATCCATGCGATGAGGAG-3'. The PCR product was ligated to the SalI site of pMD18T-uPA cDNA to yield the construct pMD18T-uPA and sequence was confirmed. Then the uPA (cDNA + exon 11) fragment was subcloned into the Pvu II and Xho I sites of pTRE2 to yield pTRE2-uPA.

Generation and PCR analysis of the albumin-rtTA and tetO-uPA transgenic mice
The albumin-rtTA and tetO-uPA transgenic mice were generated in C57BL/6 × CBA F1 zygotes using standard
pronuclear injection, which was performed by Shanghai Research Center for Biomodel Organisms. For microinjection, the 6034 bp fragment of transgene albumin-rtTA and the 5739 bp fragment of transgene tetO-uPA were excised from the vector backbone of pTet-on-albumin by XhoI digestion and pTRE3-uPA by PvuI digestion, respectively, isolated and purified using QIA quick gel extraction kit (Qiagen), and then microinjected into the pronuclei of one cell-stage fertilized embryos. The DNA injected fertilized eggs were implanted into the oviducts of 12 pseudopregnant recipient mice. All together 9 positive albumin-rtTA transgenic mice and 5 tetO-uPA positive ones were confirmed by PCR. One upstream pair and one downstream pair of primers, which were designed to amplify the sequences between vector and inserted fragment were designed for albumin-rtTA as follows, 1-up-F: 5’-GTGCAGCTTGGCTTGACTGTC-3’; 1-up-R: 5’-GAGATGGGGCTATCTTAAACATCTC-3’; 1-down-F: 5’-GACCGCAGCTAGAAGATTTCTGATCTGC-3’; 1-down-R: 5’-ACCTTGCAAGATAGACGGTGTTGC-3’. By the same way, one upstream pair and one downstream pair of primers were designed for tetO-uPA as follows 2-up-F: 5’-GTTTATGTAACCGTCAGATCGCCTG-3’; 2-up-R: 5’-CTAGGCTAATAGCATCAGGCTGTCTGA-3’; 2-down-F: 5’-GTTGAGCTTGAGGATAGACACT-3’; 2-down-R: 5’-GCAATGTTGGTCAAAGAGTAC-3’. The PCR conditions were the same for each of the primer pairs: 34 cycles of 94°C for 30 s, 42°C for 30 s. Genomic DNA from wild-type mice was amplified as negative control. The PCR positive mice were the transgenic founders.

Mouse propagation and PCR analysis
At 6-8 wk of age, founder mice were backcrossed with wild-type C57BL/6J mice to generate F1. Genomic DNA were isolated from tail biopsy samples of F1 mice at 4 wk and analyzed by PCR, for which the protocols were mentioned above.

rtTA expression in different tissues of albumin-rtTA F1 transgenic mice
The isolation of total RNA from different tissues of 6-8 wk old F1 PCR-positive and negative offspring of the founders was performed using the RNeasy Mini Kit (Qiagen) following the instructions. Purified RNA was eluted in 40 μL DNA-free water. 400 ng of total RNA reverse transcribed with the Takara RNA LA PCR Kit (AMV)Ver1.1 (TaKaRa), the reaction condition was 30°C for 10 min, 42°C for 30 min, 99°C for 5 min, 5°C for 5 min. The oligonucleotide primers used for RT-PCR were rtTA-F: 5’-GACCGCAGCTAGAAGATTTCTGATCTG-3’; rtTA-R: 5’-ACCTTGCAAGATAGACGGTGTTGC-3’, the PCR reaction condition was 34 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control, the primers were GAPDH-F: 5’-TTTCCACCATGAGAAGGC-3’ and GAPDH-R: 5’-CCTCAGTGGTAGCCCAAGATGC-3’, PCR reaction condition was 34 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 30 s.

The total protein was isolated from different tissues of 6-8 wk old F1 PCR-positive and negative offspring of the founders by using the Tissue Lysis Buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.2 mmol/L sodium orthovanadate, 1% Triton X-100, 1% sodium deoxycholate, 1% sodium dodecyl sulfate) supplemented with aprotinin (2 μg/mL), pepstatin A (0.7 μg/mL), leupeptin (0.5 μg/mL), phenylmethylsulfonyl fluoride (PMSF) (1 mmol/L). Aprotinin, Pepstatin A, Leupeptin, PMSF were purchased from Amresco. For Western blotting analysis, 25 μg of the total protein was used for each loading; the primary antibody for rtTA was TetR monoclonal antibody (Clontech) (used in 1:1000 dilution), and the primary antibody for GAPDH was an anti-GAPDH polyclonal antibody (Sigma) (1:10000 dilution); and the second antibodies were HRP-labeled goat anti-mouse IgG and goat anti-rabbit IgG (both in 1:5000 dilution), respectively. For imaging results, the SuperSignal WestDura Trial Kit (Pierce) was used following the instructions.

Generation of double transgenic mice in-alb-uPA and Doxycycline administration
Double transgenic in-alb-uPA and wild type female offspring were generated from a cross between the albumin-rtTA F1 transgenic positive mice and the tetO-uPA F1 positive mice. 20 d after born, these mice were given two intramuscular injection of 2 mg Dox in 0.2 mL 0.9% NaCl-solution each week for a period of 3 wk. Another group of each type of mice was maintained off doxycycline administration.

Hydrodynamic transfection of AAV-HBV and histological analysis
After 3 weeks’ induction, a 20 μg pAAV-HBV1.3 DNA was injected hydrodynamically into the tail veins of the in-alb-uPA mice within 5 seconds. A control group of in-alb-uPA mice was injected with pAAV-internal ribosome entry site (IRES). At 20 d post transfection, mice were sacrificed and the livers were fixed with 4% (v/v) phosphate-buffered formalin, and paraffin-embedded liver sections were prepared and stained with hematoxylin and eosin. Semi-quantitative assessment of liver injury in each group was evaluated by the area of liver necrosis on the whole slide in each group. NP for no necrosis; P1 for < 10% area of necrosis; P2 for 10%-30% area of necrosis; P3 for > 30% area of necrosis. All the evaluation of liver damage was conducted by two independent observers. The average score of three mice in each group was taken as score for that group.

For uPA and HBV antigens detection, the expression of uPA protein and hepatitis B core antigen (HBcAg) were identified by double-staining with a polyclonal rabbit anti-roden t urokinase (uPA) antibody (American diagnostica Inc) and monoclonal anti-HBcAg antibody (Thermo Scientific). Diamino-benzidine and alkaline phosphatase substance (ZhongShan Goldenbridge biotech, Beijing, China) were used to visualize the uPA and HBV antigens.
Enzyme linked immunosorbent assay for HBV antigens and cytokine production

At 10 d and 20 d post transfection, mouse serum samples from different groups were harvested. The HBeAg and HBsAg enzyme linked immunosorbent assay (ELISA) kit (Wantai Biotech, Beijing) were used for the detection of the serum HBV antigens respectively. And interleukin (IL)-6 and tumor necrosis factor (TNF)-α ELISA kit (Dakewei Biotech, Beijing) were used for the quantitation of the serum cytokines. Serum ALT were measured with an Olympus Model 640 automated analyzer.

qRT-PCR analysis of α-fetoprotein mRNA expression in the livers of AAV-HBV transfected in-alb-uPA mice

The isolation of total RNA from livers of the AAV-HBV transfected in-alb-uPA mice was performed using the RNAasy Mini Kit (Qiagen) following the instructions. Purified RNA was eluted in 40 μL DNA-free water and 400 ng of total RNA in a 10 μL reaction mixture was reverse transcribed with the Takara RNA LA PCR Kit (AMV) Ver1.1. GAPDH serves as a control and the primers were GAPDH-real-F: 5’-TCACACCATTGGAGAAAGCC-3’ and GAPDH-real-R: 5’-GC-TAACGAGTTGTTGGTGC-3’. The amplification conditions included initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, extension at 68 °C for 30 s.

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was performed using Student’s t test.

RESULTS

Construction and identification of pTet-on-albumin and pTRE-uPA

For inserting the albumin enhancer and promoter sequence into pTet-on in place of the CMV promoter, a linker as designed and the following restriction sites were introduced: SpeI, EcoRV, KpnI, ApaI, NotI, and BamHI. pTet-on-link and pTet-on-Alb were digested by SpeI and ApaI respectively (Figure 1A), and results showed that the linker was introduced into pTet-on. pTet-on-Alb was identified by BamHI digestion (Figure 1B), the expected 5022 bp, 2689 bp and 1284 bp fragment can be observed. pTRE-uPA was identified by ppu II and SalI respectively (Figure 1C). In addition, DNA sequence analysis of the albumin enhancer/promoter and uPA sequence shows complete accordance with those in the National Center for Biotechnology Information database (data not shown).

Generation of the albumin-rtTA and tetO-uPA transgenic mice and PCR analysis

The albumin-rtTA expression unit contains the mouse albumin enhancer/promoter, rtTA coding sequence and uPA cDNA and uPA exon11 (Figure 2A). In the end, 9 albumin-rtTA transgenic founder mice and 5 tetO-uPA transgenic founder mice were confirmed positive by PCR for both the upstream and downstream primers (Figure 2B).

Specific expression of rtTA in the livers of albumin-rtTA and in-alb-uPA transgenic mice

To identify the liver-specific expression of uPA in the livers of transgenic mice, RT-PCR and Western blotting analysis was performed. The data showed that rtTA mRNA expressed specifically in the livers of F1 albumin-rtTA transgenic positive mice (Figure 3A, right image), while there was no rtTA mRNA expression in all the tissues of the albumin-rtTA transgenic negative mouse (Figure 3A, left image). GAPDH mRNA was expressed equally in different tissues of these mice (Figure 3A). Results from Western blotting analysis (Figure 3B) were in accordance with those from RT-PCR analysis. By Western blotting analysis, rtTA expression was also confirmed specifically in the livers of in-alb-uPA transgenic mice and albumin-rtTA transgenic mice (Figure 3C). The cell...
Figure 2 Establishment of albumin-tetracycline reverse transcriptional activator and tetO-urokinase plasminogen activator transgenic mice. A: The albumin-tetracycline reverse transcriptional activator (rtTA) unit contains the mouse albumin enhancer/promoter, rtTA coding sequence, and SV40 polyA. The tetO-urokinase plasminogen activator (uPA) unit contains the TRE\(^{-2}\)-P\(_{\text{minCMV}}\), uPA cDNA, uPA exon11. Arrowheads depict the positions and directions of the polymerase chain reaction (PCR) primers; B: PCR identification of the transgenic founders. 1-9, PCR identification for the nine albumin-rtTA transgenic founder mice; 1-5, PCR identification for the five tetO-uPA transgenic founder mice. CMV: Cytomegalovirus; M: Marker; NC: Negative control; PC: Positive control.

Figure 3 The specific expression of tetracycline reverse transcriptional activator in the livers of albumin-tetracycline reverse transcriptional activator transgenic mice and in-alb-urokinase plasminogen activator transgenic mice. A, B: Reverse transcription polymerase chain reaction (A) and Western blotting (B) analysis of tetracycline reverse transcriptional activator (rtTA) and glyceraldehyde-3-phosphate dehydrogenase (GADPH) expression in different tissues of the 6-8 wk old F1 albumin-rtTA PCR-negative (left for A, B) or positive (right for A, B) transgenic mice. 1: Liver; 2: Brain; 3: Thymus; 4: Heart; 5: Lung; 6: Kidney; 7: Spleen; C: Western blotting analysis of rtTA and GADPH expression in the liver extracts of mice with different genotypes. 1: In-alb-uPA mice group; 2: Liver extracts of wild type mice group; 3: tetO-uPA mice group; 4: Albumin-rtTA mice group. pTet-on transfected Huh7 cell extracts were used as positive control (+).
extract from Huh7 transfected with pTet-on was used as positive control while tetO-uPA and WT mice served as negative control.

**Histological change of liver in in-alb-uPA transgenic mice after Dox induced uPA expression**

To confirm the expression of uPA in liver and its role on the hepatocytes, uPA expression and the histological changes of liver were analyzed with immunohistochemistry and HE staining respectively. The results showed light degeneration of hepatocytes and mild inflammation in the livers with in-alb-uPA double transgenic mice after 3 wk of Dox induction when compared to that of double transgenic mice without Dox or the control group mice (Figure 4A-C), which was coincident with that of uPA expression with immunohistochemistry in the livers of in-alb-uPA double transgenic mice after Dox induction while almost no expression of uPA detected in double transgenic mice without Dox. The data showed that the specific expression of uPA after Dox induction induced slight histological changes in the liver of this in-alb-uPA double transgenic mice.

**Synergistic liver injury in in-alb-uPA transgenic mice after AAV-1.3HBV transfection**

Although uPA plays critical role in hepatic repair via proteolysis of matrix elements and clearance of cellular debris from the field of injury, clinical data showed that the levels of uPA and uPAR in patients with acute and chronic hepatitis B significantly higher than that in healthy controls, which indicated that uPA level was closely related to the degree and period of inflammation and liver injury[13]. To confirm if the coexisting uPA expression and HBV replication induced serious acute liver injury, the in-alb-uPA transgenic mice were transfected with pAAV-1.3HBV, a plasmid which could mediated the production of replicative HBV virus in vivo. Large area necrosis was observed 20 d later in the liver of Dox-induced in-alb-uPA double transgenic mice that were transfected with pAAV-1.3HBV (Figure 5C), compared with that of non-induced in-alb-uPA mice transfected with pAAV-1.3HBV (Figure 5B) or with the control plasmid pAAV-IRES (Figure 5A). Double-staining immunohistochemical analysis confirmed both uPA expression (in brown) and HBcAg expression (in red) in the AAV-HBV transfected Dox-induced in-alb-uPA mice (Figure 5F). Interestingly, the coexpression of uPA and HBcAg existed in the most of the necrosis areas and the hepatocytes with HBcAg expression alone were morphological intact. The severe liver damage in the mice after HBV transfection indicated that the expression of uPA accelerated the liver injury. The result was confirmed by a statistics analysis that about 86.7% of the AAV-HBV transfected Dox-induced in-alb-uPA mice experienced severe liver pathogenic changes compared with the 20% AAV-HBV transfected non-induced in-alb-uPA mice, which could be explained by the leaky expression of uPA due to the tet-on inducible system. And about 40% of the AAV-HBV transfected Dox-induced in-alb-uPA mice experienced severe liver injury (Table 1).

**Comparison of the serum HBV antigens and cytokines produced in mice from different groups**

Previous reports have shown that HBV infection is associated with the production of a broad range of pro-inflammatory cytokines and chemokines such as IL-1β,
IL-6, IL-8, IL-12, TNF-α and IFN-γ [15-17], among which IL-6 and TNF-α are important components of the early signaling pathway that lead to liver regeneration [18]. In this study, results also confirmed the elevation of serum IL-6 and TNF-α levels in the AAV-HBV transfected in-alb-uPA mice (Figure 6). 10 d and 20 d after AAV-HBV transfection, the serum IL-6 level for the Dox-induced in-alb-uPA mice was 47.28 ± 0.57 and 96.97 ± 2.91 (pg/mL), while the level for the non-induced in-alb-uPA mice was 18.32 ± 2.38 (pg/mL) and 45.83 ± 1.50 (pg/mL) (P < 0.01) (Figure 6D). The serum TNF-α level for the Dox-induced in-alb-uPA mice was 50.55 ± 2.01 (pg/mL) and 46.72 ± 2.01 (pg/mL), while the level for the non-induced in-alb-uPA mice was 14.58 ± 3.05 (pg/mL) and 18.17 ± 3.63 (pg/mL) (P < 0.01) (Figure 6E). Compared with the non-induced in-alb-uPA mice, the average serum HBeAg level of the Dox-induced in-alb-uPA mice was significantly higher both at 10 d and 20 d after AAV-HBV transfection (P < 0.01)) (Figure 6A), while there was no significant difference between the average serum HBsAg level of the Dox-induced and non-induced mice (Figure 6B). Compared with the non-induced in-alb-uPA mice, the average serum ALT level of the Dox-induced in-alb-uPA mice was slightly higher at 20 d after AAV-HBV transfection (P < 0.01)) (Figure 6C).

Relative quantitative analysis of AFP mRNA expression in the livers of AAV-HBV transfected in-alb-uPA mice

It has been reported that AFP level in vivo decreases abruptly soon after birth and remains at a low level throughout life. And reactivation of AFP production occurs during liver regeneration [19]. In this study, we found that, compared with the non-induced in-alb-uPA mice that were transfected by AAV-HBV, the AFP mRNA level for the Dox-induced in-alb-uPA mice that were transfected by AAV-HBV was greatly higher. 10 d after AAV-HBV transfection, the average level of the AFP mRNA for those induced in-alb-uPA mice increased about 21.8 times, while the fold change further increased to 142.1 times at 20 d after AAV-HBV transfection (Figure 6F). The data further confirmed our hypothesis that uPA expression and HBV...
infection have close relations and HBV infection further accelerated liver injury and regeneration when uPA was overexpressed.

DISCUSSION

Tet-inducible expression system is one of the most suitable inducible systems which could be used to investigate the function of a given gene in vivo, including the tTA (Tet-off) system\(^\text{21}\) and rtTA (Tet-on) system\(^\text{21}\), and facilitates not only the understanding of gene function in development and pathogenesis, but also in transgenic mouse modeling\(^\text{22-24}\). On the other hand, tissue-specific expression of a target gene relies on tissue-specific promoters. Tissue-specific expression is vital for gene function research in organism development and can reduce immunological response and side effects in gene therapy applications. Many liver-specific promoters have been
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identified so far, such as the AFP promoter,[25] the albumin promoter, mouse major urinary protein promoter,[26] hSAP promoter (human serum amyloid P component promoter)[27], and apoE promoter (human apolipoprotein E promoter)[28], which have been applied in liver-specific expression of target genes. In addition, elements like enhancers influence the transcriptional activity of the these tissue-specific promoters[29].

The urokinase plasminogen activator (uPA) is a serine protease that can activate the plasminogen into plasmin, and perform multiple functions in fibrinolysis, immunity and pathology[30]. Previous studies showed uPA diverse functions in tissue remodeling, angiogenesis,wound healing and protective effects in liver diseases[31]. The levels of uPA have been found to be increased in tissues, plasma and other body fluids of cancer patients and to be markers of cancer development and metastasis. And in human immunodeficiency virus (HIV)-infected patients, the serum levels of uPA have been found to be increased[32]. Also, the abnormal levels of plasma uPA in the patients with acute or chronic hepatitis B were observed, and it seems that the plasma levels of uPA are closely related to the degree and period of inflammation for these patients[33]. Although the clinical significance of uPA in viral chronic hepatitis B, hepatitis induced liver cirrhosis and HCC has been evaluated, the role of uPA in the process is less well understood, especially in the early stage.

In 1990, an Alb-uPA transgenic mouse which carried the mouse uPA gene under the control of the mouse albumin enhancer/promoter, was developed by Dr. Brinster's team to study the pathophysiology of plasminogen hyperactivation[34]. The over-expression of the uPA gene in the liver resulted in high plasma uPA levels and hypofibrinogenemia, which led to severe and sometimes abdominal bleeding soon after birth. And the high mortality also increases the difficulty for the generation of human liver chimeric mice[35,36] and the study of hepatitis C virus infections in vivo[37,38]. In this study, we established an uPA inducible double transgenic mouse in alb-uPA, in which uPA can be expressed specifically in the liver only after Dox induction. Hypofibrinogenemia and neonatal hemorrhaging were not observed in the Dox-induced in-alb-uPA mice, which greatly brought down the mortality rate. Also the inducible expression of uPA makes it possible for us to study and illuminate the relations of uPA over-expression and HBV infection clinically.

Hydrodynamic transfection method was suitable for the AAV-mediated delivery of HBV genome in vivo[39]. To investigate the risk of HBV induced liver injury in the case of uPA over-expression, the hydrodynamic transfection of pAAV-HBV1.3, which could mediated the production of replicative HBV virus in vivo, was performed. In the Dox-induced in-alb-uPA mice that were hydrodynamically transfected by AAV-1.3HBV, severe liver histological changes were observed in the liver (Figure 5). Also uPA over-expression in the liver resulted in higher HBV antigen expression, higher IL-6 and TNF-α production and slight elevation of serum ALT level (Figure 6). Our results also found a significant increase of the AFP mRNA level in the AAV-HBV transfected Dox-induced in-alb-uPA mice (Figure 6). Produced by the embryonic yolk sac and tetal liver, the AFP level decreases abruptly soon after birth and remains at a low level throughout life. And reactivation of AFP production occurs during liver regeneration[19]. As IL-6 and TNF-α are proinflammatory cytokines that lead to liver regeneration, we came to the conclusion that the uPA over-expression in AAV-HBV transfected mice increased the liver necrosis injury, inflammation and liver regeneration, which reflects a process that may eventually lead to hepatocellular carcinoma.

It is generally considered that cell-mediated immunity and inflammation are the main mediators of the hepatic pathology induced by HBV infection. In this study, we found that HBV infection further accelerated liver injury and regeneration when uPA was overexpressed, indicating a close relation between uPA expression and HBV infection. Also as clinical data showed that the increased level of uPA in HIV infected patients, this study may in part explain the increased risk of liver disease during HIV and HBV coinfection.

**COMMENTS**

**Background**

Hepatitis B virus (HBV) infection causes a high risk of developing liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC). The immune response to HBV-encoded antigens is responsible both for viral clearance and for disease pathogenesis during HBV infection. The urokinase plasminogen activator (uPA) is a serine protease that can activate the plasminogen into plasmin, and perform multiple functions in fibrinolysis, immunity and pathology. However, the roles of uPA/uPA's receptor (uPAR) systems as important inflammatory mediators have not yet been well investigated in acute and chronic hepatitis B, a common inflammatory disease in China. Clinical studies have focused on the correlation of uPA levels with the liver disease severity in hepatitis B patients. And the role of uPA in the HBV-induced liver injury, especially in the early stage, is less investigated.

**Research frontiers**

Various researchers have found the levels of uPA to be increased in tissues, plasma and other body fluids of cancer patients and to be markers of cancer development and metastasis. And in human immunodeficiency virus (HIV)-infected patients, the serum levels of uPA have been found to be increased. Also, the abnormal levels of plasma uPA in the patients with acute or chronic hepatitis B were observed, and it seems that the plasma levels of uPA are closely related to the degree and period of inflammation for these patients. Although the clinical significance of uPA in viral chronic hepatitis B, hepatitis induced liver cirrhosis and HCC has been evaluated, the role of uPA in the process is less well understood, especially in the early stage.

**Innovations and breakthroughs**

In this study, an inducible liver-specific uPA transgenic mouse model was developed. Plasmid adeno-associated virus-1.3HBV transfection in doxycycline (Dox)-induced transgenic mice resulted in severe liver injury, higher HBV antigen and cytokine expression compared to the control group. These data further indicated for the first time in mice that the over-expression of uPA may have accelerative role in the development of liver injury, inflammation and liver regeneration during acute HBV infection. Also as clinical data showed that the increased level of uPA in HIV infected patients, this study may in part explain the increased risk of liver disease during HIV and HBV coinfection.

**Applications**

This study deepens our knowledge of uPA function in HBV-induced liver diseases, which may not only facilitate the elucidation of the molecular mechanism
of HBV pathogenesis, but also provide a basis for the uPA-targeted anti-HBV therapies.

**Terminology**

uPA is one kind of plasminogen activator that catalyzes the conversion of plasminogen to plasmin. Together with uPAR, uPA participate in fibrinolysis, innate and adaptive immunity, and pathology. Tetracycline (Tet)-inducible expression system consists of two parts: the ligand-dependent transactivator rTA as the effector and a tetO-CMV minimal promoter cassette regulating the expression of the transgene as the responder. When Dox is present, rTA binds to the tetO-sequence and induces expression of the target gene. Together with a tissue-specific promoter, it can result in transgene expression in a temporally and spatially defined fashion.

**Peer review**

The authors studied the role of uPA and found that the over-expression of uPA may have a synergistic role in the development of liver injury, inflammation and liver regeneration during acute HBV infection. They added as such new information to the field on the knowledge about uPA function in HBV-induced liver diseases.

**REFERENCES**

1. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu Rev Immunol 1995; 13: 29-60
2. Sidenius N, Sier CF, Ullum H, Pedersen BK, Lepri AC, Blasi F, Eugen-Olsen J. Serum level of soluble urokinase-type plasminogen activator receptor is a strong and independent predictor of survival in human immunodeficiency virus infection. Blood 2000; 96: 4091-4095
3. Mondino A, Blasi F, uPA and uPAR in fibrinolysis, immunity and pathology. Trends Immunol 2004; 25: 450-455
4. Chapman HA. Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. Curr Opin Cell Biol 1997; 9: 714-724
5. Ossowski L, Chunie G, Masucci MT, Blasi F. In vivo paracrine interaction between urokinase and its receptor: effect on tumor cell invasion. J Cell Biol 1991; 115: 1107-1112
6. Land AH, Turner G, Trubetskoy A, Verhoeven E, Wientjens R, Vaheri A. Soluble urokinase receptor levels correlate with poor survival in patients with colorectal cancer: relationship to tumor cell invasion. Blood 2000; 96: 4091-4095
7. Stephens RW, Nielsen HJ, Christensen JI, Thorlacius-Ussing O, Sørensen S, Dana K, Brünner N. Plasma urokinase receptor levels in patients with colorectal cancer: relationship to prognosis. J Natl Cancer Inst 1999; 91: 869-874
8. Mustjoki S, Sidenius N, Sier CF, Blasi F, Elonen E, Altalot R, Vaheri A. Soluble urokinase receptor levels correlate with number of circulating tumor cells in acute myeloid leukemia and decrease rapidly during chemotherapy. Cancer Res 2000; 60: 7126-7132
9. Dobrovolsky VN, Hefflich RH. On the use of the T-REx tetracycline-inducible gene expression system in vivo. Biotechnol Bioproc Eng 2007; 98: 719-723
10. Steiger K, Bellbela B, Le Guiner C, Moullier P, Rolling F. In vivo gene regulation using tetracycline-regulatable systems. Adv Drug Deliv Rev 2009; 61: 527-541
11. Kistner A, Gossen M, Zimmermann F, Jerecic J, Ullmer C, Lübbert H, Bujard H. Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. Proc Natl Acad Sci USA 1996; 93: 10933-10938
12. Lamartina S, Roscilli G, Rinaudo CD, Sporeno E, Silvi L, Hilgen J, Bujard H, Cortese R, Ciliberto G, Toniatti C. Stringent control of gene expression in vivo by using novel doxycycline-dependent trans-activators. Hum Gene Ther 2002; 13: 199-210
13. Huang LR. Wu HL, Chen PJ, Chen DS. An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection. Proc Natl Acad Sci USA 2006; 103: 17862-17867
14. Bidgoli SA, Zavarheir MD, Mohagheghial MH, Yazdanmehr B, Daryani NE, Ardalan FA. Differential expression of uPA in chronic hepatitis B and C, liver cirrhosis and hepatocellular carcinoma: comparison with normal liver tissues and liver metastatic tumors. Int J Cancer Res 2007; 3: 25-32
15. Zhou H, Wu X, Lu X, Chen G, Ye X, Huang J. Evaluation of plasma urokinase-type plasminogen activator and urokinase-type plasminogen-activator receptor in patients with acute and chronic hepatitis B. Thromb Res 2009; 123: 537-542
16. Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rintef A, Addison WR, Fischer KP, Churchill TA, Lakey JR, Tyyrell DL, Kneteman NM. Hepatitis C virus replication in mice with chimera human livers. Nat Med 2001; 7: 927-933
17. Barth H, Robinet E, Liang Tj, Baumert TF. Mouse models for the study of HCV infection and virus-host interactions. J Hepatol 2008; 49: 134-142
18. Ernst E, Schöng K, Bugert J, Bläker H, Pfaff E, Stremmel W, Encke J. Generation of inductible hepatitis C virus transgenic mouse lines. J Med Virol 2007; 79: 1103-1112
19. Katoch M, Tateno C, Yoshizato K, Yokoi T. Chimeric mice with humanized liver. Toxicology 2008; 246: 9-17
20. Sandgren EP, Palmeri RD, Heckel JL, Daugherty CC, Brins ter RL, Degen JL. Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene. Cell 1991; 66: 245-256
21. Suemizu H, Hasegawa M, Kawai K, Taniguchi K, Monnai M, Waku M, Suematsu M, Itó M, Pelz G, Nakamura M. Establishment of a humanized model of liver using NOG/Shi/scid II.2Rgnull mice. Biochem Biophys Res Commun 2008; 377: 248-252
22. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 1992; 89: 5547-5551
23. Gossen M, Freundlieb S, Bender G, Müller G, Hillen W, Bu jard H. Transcriptional activation by tetracyclines in mammalian cells. Science 1995; 266: 1766-1769
24. Alphonse C, Hult J, Sakamaki T, Petrell RG. Recent advances in inducible expression in transgenic mice. Semin Cell Dev Biol 2002; 13: 129-141
25. Bockamp E, Maringer M, Spangenberg C, Fees S, Fraser S, Eshkind L, Oesch F, Zabel B. Of mice and models: improved animal models for biomedical research. Physiol Genomics 2002; 11: 115-132
26. van der Weiden L, Adams DJ, Bradley A. Tools for targeted manipulation of the mouse genome. Physiol Genomics 2002; 11: 133-164
27. Hirano T, Kaneko S, Kaneda Y, Saito I, Tamaoki T, Furu yama J, Tamaoki T, Kobayashi K, Ueki T, Fujimoto K. HVJ liposome-mediated transduction of HSVtk gene driven by AFP promoter inhibits hepatic tumor growth of hepatocellular carcinoma in SCID mice. Gene Ther 2001; 8: 80-83
28. Shi Y, Son HJ, Shahan K, Rodriguez M, Costantini F, Der man E. Silent genes in the mouse major urinary protein gene family. Proc Natl Acad Sci USA 1989; 86: 4584-4588
29. Zhao X, Araki K, Miyazaki J, Yamamura K. Developmental and liver-specific expression directed by the serum amyloid P component promoter in transgenic mice. J Biochem 1992; 111: 736-738
30. Thome J, Gewirtz JC, Sakai N, Zachariou V, Retz-Junginger P, Retz W, Duman RS, Rössler M. Polymorphisms of the human apolipoprotein E promoter and its role in Alzheimer's disease? Neurosci Lett 1999; 274: 37-40
31. Pinkert CA, Ornitz DM, Brinster RL, Palmeri RD. An albu min enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. Genes Dev 1987; 1: 268-276
32. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther 1999; 6: 1258-1266
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33 Takikawa Y, Suzuki K. Is AFP a new reliable marker of liver regeneration in acute hepatic failure? J Gastroenterol 2002; 37: 681-682
34 al-Wabel A, al-Janadi M, Raziuddin S. Cytokine profile of viral and autoimmune chronic active hepatitis. J Allergy Clin Immunol 1993; 92: 902-908
35 Geneva-Popova M, Murdjeva M. Study on proinflammatory cytokines (IL-1 beta, IL-6, TNF-alpha) and IL-2 in patients with acute hepatitis B. Folia Med (Plovdiv) 1999; 41: 78-81
36 Hsu HY, Chang MH, Ni YH, Lee PI. Cytokine release of peripheral blood mononuclear cells in children with chronic hepatitis B virus infection. J Pediatr Gastroenterol Nutr 1999; 29: 540-545
37 Diao J, Garces R, Richardson CD. X protein of hepatitis B virus modulates cytokine and growth factor related signal transduction pathways during the course of viral infections and hepatocarcinogenesis. Cytokine Growth Factor Rev 2001; 12: 189-205

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