Sustained survival of human hepatocytes in mice: A model for *in vivo* infection with human hepatitis B and hepatitis delta viruses

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Persistence of hepatocytes transplanted into the same or related species has been established[1-8]. The long-term engraftment of human hepatocytes into rodents would be useful for the study of human viral hepatitis, where it might allow the species, technical and size limitations of the current animal models to be overcome. Although transgenic mice expressing the hepatitis B virus (HBV) genome produce infectious virus in their serum, the viral life cycle is not complete, in that the early stages of viral binding and entry into hepatocytes and production of an episomal transcriptional DNA template do not occur[9,10]. As for hepatitis delta virus (HDV), another cause of liver disease[11,12], no effective therapy exists to eradicate infection, and it remains resistant even to recent regimens that have considerably changed the treatment of HBV (ref. 13). Here, we demonstrate long-term engraftment of primary human hepatocytes transplanted in a matrix under the kidney capsule of mice with administration of an agonistic antibody against c-Met. These mice were susceptible to HBV infection and completion of the viral life cycle. In addition, we demonstrate super-infection of the HBV-infected mice with HDV. Our results describe a new xenotransplant model that allows study of multiple aspects of human hepatitis viral infections, and may enhance studies of human liver diseases.

We first attempted to find a suitable anatomic space to maintain the human hepatocytes in mice. We tested the kidney capsule, portal vein and subcutaneous space on the dorsal surface of non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice as potential sites for human hepatocyte transplantation (Fig. 1). Isolated human hepatocytes used in ectopic transplantation were mixed with Matrigel before being transplanted. We determined the viability and maintenance of the transplanted hepatocytes *in vivo* by periodically measuring a hepatocyte-specific human serum marker, alpha-1-antitrypsin (hAAT), as used for transplanted transgenic mouse hepatocytes that express this human transgene[4].

The human hepatocytes delivered by intraportal infusion were rapidly lost (a decrease to less than 1%) over a period of a week, whereas the numbers of hepatocytes transplanted subcutaneously and in the kidney capsule slowly decreased over a period of 3–6 months (Fig. 1). Because the kidney capsule transplants seemed to have the longest period of survival, we used this method in further studies.

We determined whether the loss of the human transplanted hepatocytes was due at least in part to the absence of an essential growth factor such as human hepatocyte growth factor (HGF). HGF signal transduction occurs by phosphorylation of c-Met (the HGF receptor) and is essential in the development and regeneration of the liver[14]. Because HGF has a half-life of minutes, we used an agonistic antibody against human c-Met mouse IgG1 (called c-Met antibody here) that phosphorylates human but not murine c-Met (not shown), in combination with the transplantation procedure. We administered 50 μg c-Met antibody intravenously every 2 weeks for the first 57 days. c-Met antibody did stabilize hepatocytes, as shown by steady-state serum hAAT concentrations (Fig. 2*a* and *b*). The 35–40% decrease in hAAT levels at 5 months may have been due to the lack of c-Met antibody administration after day 57.

To further establish that the hepatocytes within the transplant were viable, we did histological analyses at 3 and 24 weeks after transplantation (Fig. 2*c* and *d*). Cells with the characteristic hepatocyte morphology were visible and their identity as human hepatocytes was confirmed by immunostaining for hAAT (Fig. 2e).

To establish whether or not the transplanted hepatocytes could serve as an *in vivo* model for infection with human viruses, we transplanted mice with human hepatocytes freshly isolated (Fig. 3*a*) or cultured for 2 days before transplantation (Fig. 3*b*), and infused them 3 days later with 0.25×10^8 or 1.4×10^8 hepatitis B virus (HBV) genome equivalents by injection of equal amounts into the transplant and intravenously. We periodically monitored serum samples for hepatitis B virus surface antigen (HBsAg) and HBV DNA titers (Fig. 3*a* and *b*). There was a transient peak of HBsAg between days 3 and 7 in all mice because of 'carry over' from the original injection, but HBsAg was undetectable at later times in control mice that did not receive human hepatocytes (Fig. 3*a* and *b*). However, in six of six mice transplanted with human hepatocytes and given c-Met antibody, the amount of HBsAg and HBV DNA slowly increased, starting at 2 weeks, and persisted. The peak HBV titers ranged from 1.5×10^5

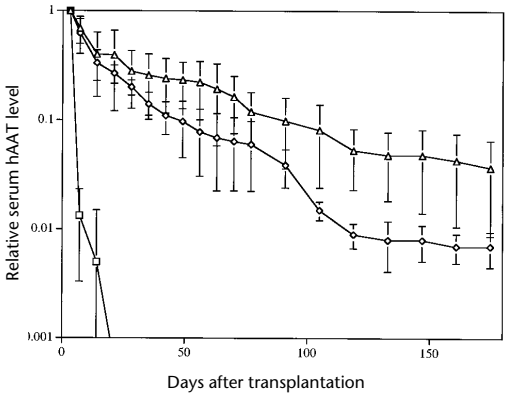

Fig. 1 Persistence of human hepatocytes transplanted in NOD/SCID mice. Human hepatocytes were transplanted into the subcutaneous space (6×10^6 cells; ◇; $n = 8$), kidney capsule (4×10^6 cells; △; $n = 10$) or liver by intraportal infusion (2×10^6 cells; □; $n = 6$). The data are a combined set of three different experiments from different adult hepatocyte donors. The viability of hepatocytes were 90, 75 and 55% by trypan blue exclusion, and 90, 60 and 40% by plating efficiency, respectively, for subcutaneous, kidney capsule and intraportal sites. Serum was collected and assayed for the serum marker hAAT protein by ELISA as a measure of hepatocyte survival. Because of the differences in viability of each liver isolate, the relative level of hAAT was compared with the value obtained 3 d after transplantation. The values represent the average relative concentration with the standard deviation. The hAAT concentrations on day 3 ranged from 500 to 10,000 ng/ml.

to 2.2×10^5 viral genome equivalents per ml in different mice. In three mice monitored long-term, there was an eventual decrease in HBsAg and a decrease in HBV DNA titers of 30% 5 months after infusion that may have been due to the lack of c-Met antibody. HBV infection did not affect the viability of the hepatocytes (Fig. 2*a*).

To further establish the presence of HBV production, we evaluated the implanted hepatocytes within the renal capsule by immunohistochemical staining at 24 weeks after infection. Serial sections showed cellular co-localization of both hepatitis B core antigen (HBcAg)(Fig. 3*c*), which was consistent with virus replication[15], and HBsAg (Fig. 3*d*) in more than half of the engrafted human hepatocytes, mainly in the cytoplasm, but HBcAg was also present in the nuclei (Fig. 3*c*). In the same mice, we did not detect staining for HBcAg (Fig. 3*e*) or HBsAg (not shown) in the mouse liver parenchyma. Likewise, there was no HBcAg (Fig. 3*f*) or HBsAg (not shown) staining in transplanted human hepatocytes that did not receive HBV. HBV replicative DNA forms that included relaxed circular, double-stranded linear and single-stranded DNA were all detectable in HBV-infected grafts by Southern blot analysis (Fig. 3*i*) (ref. 10). As expected, no DNA signals were detected in the livers of the HBV-positive mice or in kidney samples of the human-hepatocyte-transplanted mice that did not receive HBV inoculation (Fig. 3*i*).

To confirm that completion of the viral life cycle occurred and that the virions produced in the mouse were indeed infectious, we inoculated a human-hepatocyte-transplanted mouse with 200 µl HBV-infected mouse serum containing 3×10^4 HBV DNA genome equivalents obtained after 10 weeks from the original inoculation, a time when there was no detectable residual HBV inoculum. Immunohistological examination of the samples at 15 weeks after the serial inoculation showed positive staining of both HBsAg and HBcAg only in the human hepatocytes (Fig. 3*g* and *h*), indicating that HBV virions produced in the original mouse were indeed infectious.

Hepatits delta virus (HDV) can accompany HBV infections in humans, often exacerbating the course of liver disease. HDV is an enveloped virus, yet it does not make its own envelope proteins. Instead, HDV uses HBsAg provided by a coexisting HBV infection for this. Replication of the HDV is otherwise independent of HBV (ref. 12). Although the mouse is not a natural host of HDV, experimental transmission of HDV into immunodeficient mice has been reported[16]. Such infections, however, occur at a very low efficiency (a maximum of 0.5% of hepatocytes), are rapidly cleared (with a peak of infection at 5–10 days), and there is no evidence of release of progeny virus into the serum. This may well reflect the lack of a natural receptor for HDV on mouse hepatocytes and the absence of a source of HBsAg to permit assembly of new HDV virions. Both of these conditions can be overcome using the mouse model described here, because we transplanted human hepatocytes and they in fact become infected by HBV. Thus, the transplanted hepatocytes might also

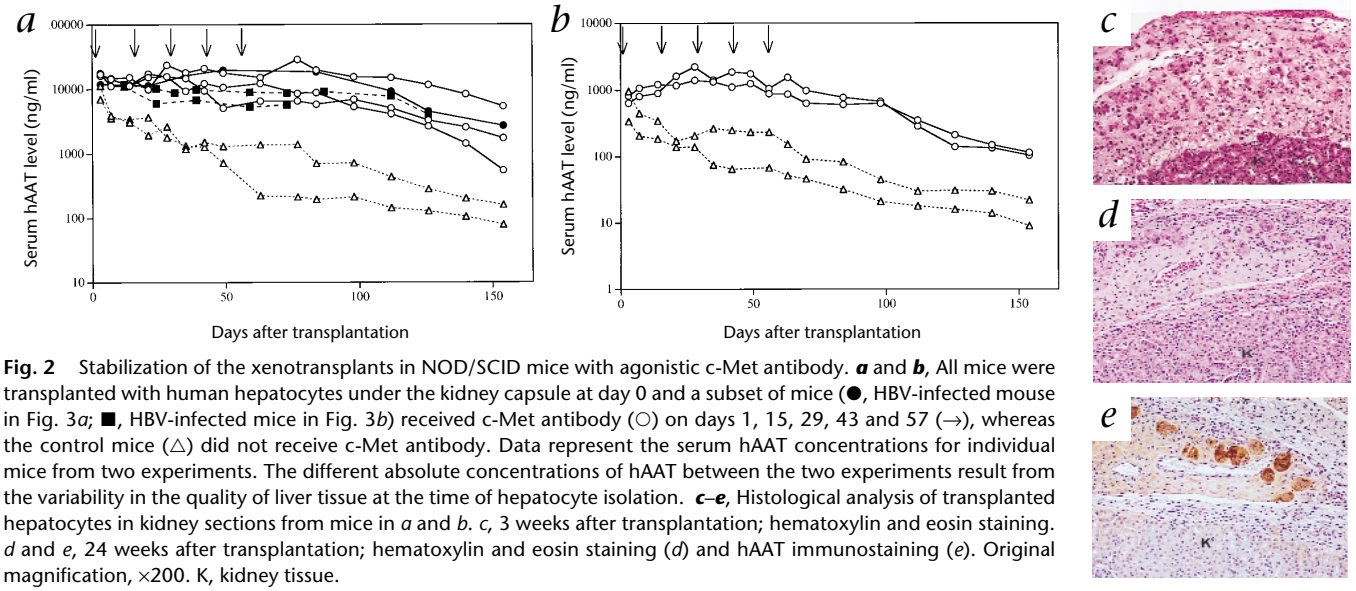

Fig. 2 Stabilization of the xenotransplants in NOD/SCID mice with agonistic c-Met antibody. ***a*** and ***b***, All mice were transplanted with human hepatocytes under the kidney capsule at day 0 and a subset of mice (●, HBV-infected mouse in Fig. 3*a*; ■, HBV-infected mice in Fig. 3*b*) received c-Met antibody (○) on days 1, 15, 29, 43 and 57 (→), whereas the control mice (△) did not receive c-Met antibody. Data represent the serum hAAT concentrations for individual mice from two experiments. The different absolute concentrations of hAAT between the two experiments result from the variability in the quality of liver tissue at the time of hepatocyte isolation. ***c***–***e***, Histological analysis of transplanted hepatocytes in kidney sections from mice in *a* and *b*. *c*, 3 weeks after transplantation; hematoxylin and eosin staining. *d* and *e*, 24 weeks after transplantation; hematoxylin and eosin staining (*d*) and hAAT immunostaining (*e*). Original magnification, ×200. K, kidney tissue.

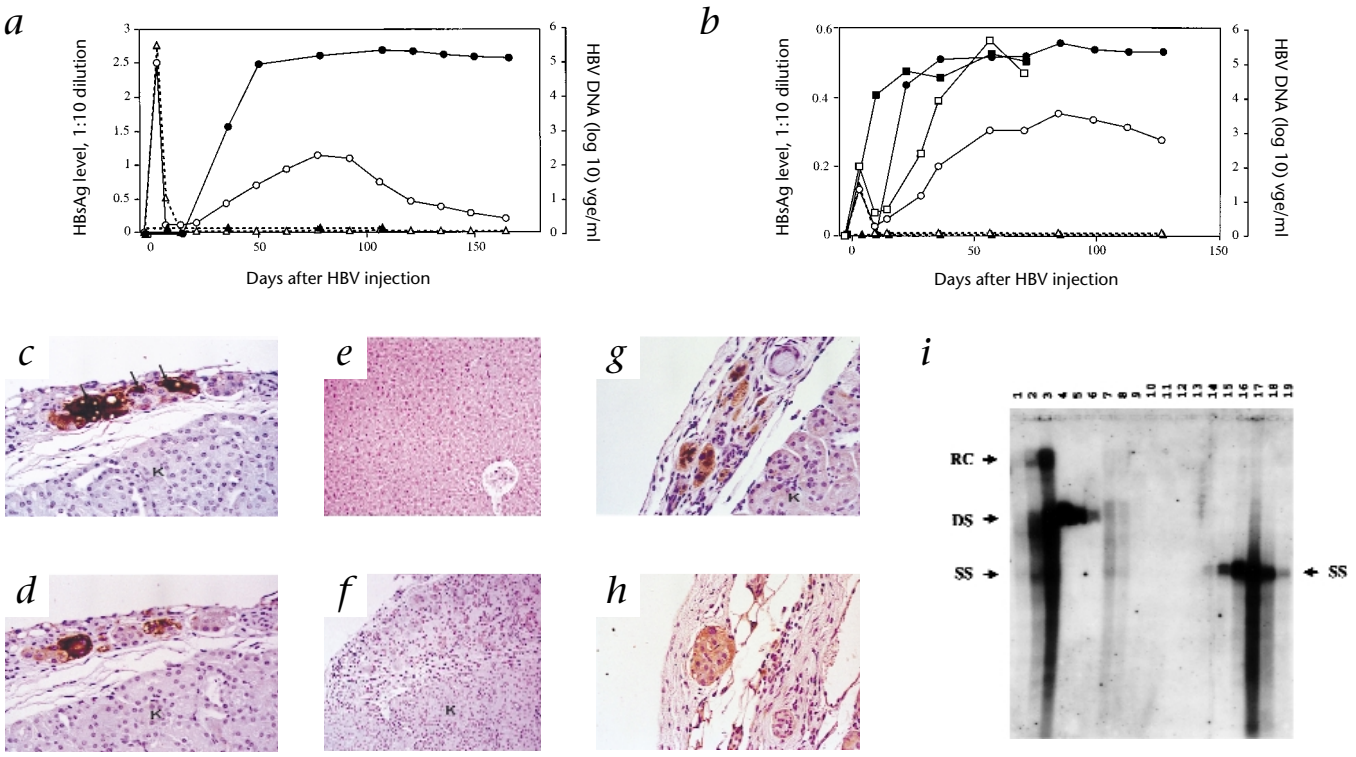

Fig. 3 HBV infection in mice. Mice were transplanted with human hepatocytes on day –3 and c-Met antibody on days –2, 12, 26, 40 and 54. Mice were inoculated with 200 µl HBV-infected single human serum containing 2.5×10^7 HBV DNA equivalents on day 0 (***a***) or 100 µl 'pooled' donor human and chimpanzee sera containing 1.4×10^8 HBV DNA equivalents (***b***) in a split dose administered 1:1 through the kidney capsule and intravenously. *b*, Mice were transplanted with hepatocytes cultured for 48 h before transplantation. Original viability of the hepatocytes: 90% by trypan blue exclusion and plating efficiency (*a*) and 85% and 55% by trypan blue exclusion and plating efficiency, respectively (*b*). Triangles, control mice that received HBV but no hepatocytes; circles and squares, mice that received hepatocytes and HBV; filled symbols, serum HBV DNA titers (vge/ml indicates DNA molecules/ml); open symbols, serum HBsAg concentrations. *a* and *b* represent two separate experiments. OD490, absorbance at 490 nm ***c***–***h***, Histolological analysis of kidney (K, kidney tissue) or mouse liver sections for transplanted human hepatocytes and HBV-infected hepatocytes. *c* and *d*, Serial sections of transplanted hepatocytes under the kidney capsule 24 weeks after HBV inoculation with immunostaining for HBcAg (*c*) or HBsAg (*d*). Arrows, hepatocytes with both nuclear and cytoplasmic staining. *e*, HBcAg immunostaining of mouse liver injected with HBV. *f*, HBcAg immunostaining of transplanted human hepatocytes in an uninfected mouse. *g* and *h*, Kidney section of a mouse that received HBV-positive mouse serum, with HBsAg (*g*) and HBcAg (*h*) immunostaining of the transplanted cells. Original magnifications, ×400 (*c*,*d*,*g*,*h*), ×100 (*e*) and ×200 (*f*). ***i***, Southern blot analysis of transplanted human hepatocytes in a mouse inoculated with HBV 18 weeks before being killed. Lanes 1–3 and 17–19, naive mouse genomic DNA added to DNase I-resistant cytoplasmic HBV DNA extracted from 2.2.15 cells (4%, 0.8% and 0.16% of extracted DNA); lanes 4–6 and 14–16, naive mouse genomic DNA 'spiked' with pGEM ayw.2x digested with *Eco*RI (3, 15 and 75 pg); lanes 7 and 8, human hepatocytes transplanted under the right and left kidney capsules from the HBV-inoculated mouse, respectively; lane 9, liver from the HBV-inoculated mouse; lane 10, transplanted human hepatocytes under the kidney capsule that did not receive HBV inoculation; lane 11, liver tissue from a mouse that did not receive HBV inoculation; lane 12, naive mouse kidney; lane 13, naive mouse liver. HBV DNA forms: RC, relaxed circular; DS, double-stranded linear; SS, single-stranded.

support an HDV infection.

To test this, we inoculated two mice with HBV and injected them 60 days later with HDV-positive serum obtained from an infected chimpanzee. HDV genomic RNA became detectable in the serum by day 10 and remained so through at least 4 weeks after inoculation (Fig. 4*a*). That this RNA was the result of an established infection and not simply residual inoculum, as indicated by the lack of detectable HDV RNA at day 2 after inoculation. In addition, no HDV RNA could be detected at corresponding times in control mice that received HBV and HDV without prior hepatocyte transplantation, hepatocytes and HBV but no HDV, or hepatocytes but no HBV or HDV. To confirm that the HDV RNAs detected in the serum reflected infection of the transplanted hepatocytes, we did immunohistochemistry with an antibody against hepatitis delta antigen (HDAg) on tissue sections. Serial sections showed co-localization of HBcAg and a strong nuclear staining pattern for HDAg, characteristic of HDV infection[16,17] (Fig. 4*b* and *c*). This staining pattern was readily apparent among the transplanted human hepatocytes from mice inoculated with HBV and HDV, but not in any of the other control mice (not shown).

In our xenotransplantation mouse model, HBV infection occurs by infusion of the virus *in vivo*. The ability to infect these mice with HBV represents a more biologically relevant system than a recently published mouse model[18]. In that model, immunodeficient mice undergo irradiation sufficient to kill the mice without subsequent transplantation and 'radioprotection' with a SCID mouse bone marrow transplant before the implantation of liver slices[18]. Moreover for HBV infection, the virus is infected onto the liver slices in an *ex vivo* manner before transplantation, and their infected grafts only persist for a 10- to 30-day period[18].

The studies described here demonstrate that in principle human hepatocytes can be engrafted long-term in mice and

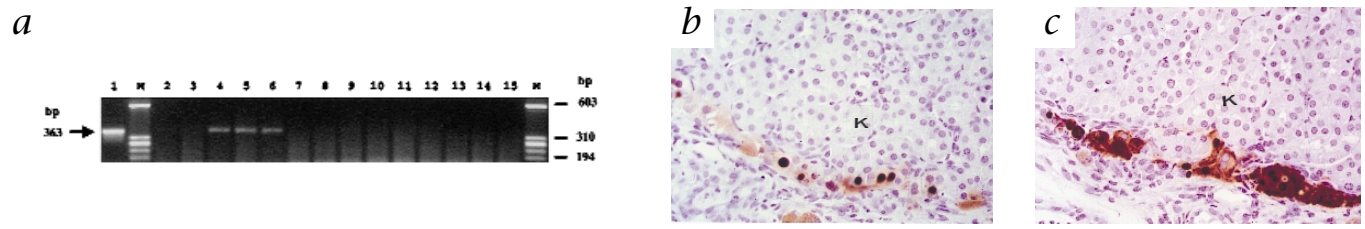

Fig. 4 Establishment of HDV Infection. Two mice developed HBV infection and were inoculated with 4×10^{10} genome equivalents of HDV under the kidney capsule 60 d later. Two naive mice that did not receive hepatocyte transplantation but received HBV and the same dose of HDV served as controls. ***a***, Serum analyzed by RT–PCR for HDV RNA. Data represent one mouse from each treatment group. Lane 1, infected chimpanzee serum. Lanes 2–6, HDV infusion in transplanted and HBV-infected mouse; lanes 7–11, HDV infusion in non-transplanted, HBV-injected mouse (time relative to HDV infusion): lanes 2 and 7, before; lanes 3 and 8, 2 d after; lanes 4 and 9, 10 d after; lanes 5 and 10, 3 weeks after; lanes 6 and 11, 4 weeks after. Lanes 12–13, transplanted and HBV-infected mice without HDV infusion (time relative to HBV inoculation): lane 12, 8 weeks after; lane 13, 11 weeks after. Lanes 14–15, mice with human hepatocytes but no infection of HBV nor HDV (time relative to transplantation): lane 14, 8 weeks after; lane 15, 11 weeks after. Markers (M), ϕx174-*Hae*III; sizes, left and right margins. ***b*** and ***c***, Serial sections of transplanted hepatocytes 10 weeks after HDV inoculation, with staining for HDAg (*b*) and HbcAg (*c*). K, kidney tissue. Original magnification, ×400.

serve as a model for human diseases such as HBV and HDV infection. Another important feature of the model is that primary human hepatocytes can be cultured for short periods of time (at least 48 hours) before being transplanted and still be used for HBV infection *in vivo* (Fig. 3*b*). Our results show that the transplanted human hepatocytes seem not only to be susceptible to HBV and HDV infection, but also to be able to support the replication and release of these viruses back into the serum. Thus, these essential aspects of the HDV life cycle should now be amenable to study in our small-animal model. Moreover, this model provides an excellent system in which to test proposed new antiviral strategies directed against HDV (ref. 19). Finally, it should prove useful in the study of other human hepatotropic viruses, such as hepatitis C virus, for which no small-animal models now exist.

Methods

Hepatocyte isolation. Hepatocyte isolation was done as described with a minor modifications[20]. An apical piece of liver 3 cm by 3 cm by 2 cm in size was perfused with 100 ml pre-perfusion solution (Earle's balanced salt solution without calcium, with 0.7 mM EGTA) for 20 min, followed by perfusion for 20 min with 80 ml collagenase D (0.5 mg/ml; Boehringer). Cells were filtered and hepatocytes were separated from non-parenchymal cells by three rounds of low-speed centrifugation. Cells were stored at 4 °C before transplantation. All hepatocytes were obtained from donors that were negative for hepatitis B, C and delta virus and human immunodeficiency virus.

Transplantation procedures. All animal studies used the institutional guidelines set forth by the Standard University Animal Care Committee. In all studies here, NOD/SCID mice were used, because in our preliminary experiments this strain of mouse seemed to allow for greater persistence of the human hepatocytes (data not shown). For the portal vein transplantation study, the collected hepatocytes were resuspended in cold Williams E Medium without serum and injected intraportally with 0.3 ml of fluid containing 2×10^6 hepatocytes. For the kidney capsule and subcutaneous transplantations, the hepatocytes were resuspended in cold media as described above with an equal volume of cold liquid Matrigel (Becton Dickinson, Franklin Lakes, New Jersey). A total of 4×10^6 hepatocytes in 0.4 ml suspension were transplanted by dividing the dose between the kidney capsule spaces, and 6×10^6 hepatocytes in 0.6 ml suspension were transplanted in subcutaneous space between the scapulae. Because Matrigel quickly polymerizes into a three-dimensional gel at room temperature, all the procedures involving Matrigel were done at 4 °C.

Production of antibody against cMet. The monoclonal antibody 3D6 is a mouse IgG1 that binds and activates human but not mouse c-Met. It was derived from a mouse that was immunized with a recombinant form of the extracellular domain of human c-Met. It induces tyrosinephosphorylation of c-Met in MDA-MB-435 mammary carcinoma cells and in A549 lung adenocarcinoma cells and results in hepatocyte proliferation of cultured ferret hepatocytes but does not cross-react with mouse c-Met. The antibody is a non-commercial reagent that was obtained from Genetech (South San Francisco, California).

Enyme-linked immunosorbent assay (ELISA). Serum HBsAg concentrations were determined using the Auszyme Kit (Abbott Laboratories, Abott Park, Illinois) at a serum dilution of 1:10 (linear range), and hAAT concentrations were assayed as described[4]. The antibodies against hAAT (Dia Sorin, Stillwater,Minnesota) do not cross-react with the mouse protein.

Southern blot analysis of HBV DNA. DNA was isolated from human hepatocytes and adjacent kidney tissue obtained from a mouse 18 weeks after HBV inoculation, a mouse that received human hepatocytes (18 weeks after transplantation) but no HBV, mouse liver tissue or kidney from a naive mouse. Of the total DNA extracted from these samples, 20 µg were digested with *Hin*dIII, separated by 2.0% agarose gel electrophoresis, and assessed by Southern blot analysis. Molecular markers representing relaxed circular, double-stranded linear, and single-stranded HBV DNA forms were prepared as follows: DNase I-resistant cytoplasmic HBV DNA was extracted as described from 2.2.15 cells, an HBV-producing cell line[21]. This DNA preparation contained relaxed circular, double-stranded and single-stranded HBV DNA forms. Naive NOD/SCID mouse liver DNA (20 µg) was added to 4%, 0.8%, and 0.16% of the cytoplasmic HBV DNA extracted from an 80%-confluent, 10-cm tissue culture dish, and then digested with *Hin*dIII, and separated by electrophoresis immediately or after being denatured at 100 °C for 5 min in 50% formamide. pGEMayw.2x plasmid DNA containing two head-to-tail tandem copies of HBVayw genomic DNA (ref. 22), representing the double-stranded HBV form, was digested with *Eco*RI and 3, 15 and 75 pg were also added to 20 µg naive liver DNA and treated in the same manner, resulting in about 2, 10 and 50 pg of HBV DNA. The separated DNA was transferred to a nylon membrane and probed with ^{32}P-labeled, whole HBV genomic DNA.

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections of liver and kidney were stained immunohistochemically with a polyclonal rabbit antibody against hAAT (Boehringer) as a primary antibody at a dilution of 1:500. The primary antibody was detected by avidin–biotin complex immunoperoxidase technique using an ABC Elite kit (Vector Laboratories, Burlingame, California). Sections were developed using DAB (3, 3′-diaminobenzine tetrahydrochloride). Experimental samples without the addition of the primary antibody did not stain positively. Human liver served as a positive control, and mouse liver, as a negative control.
For hepatitis B viral antigens, a polyclonal goat antibody against HBsAg and polyclonal rabbit antibody against HBcAg (both from Dako,

Carpinteria, California) were used for primary antibody at dilutions of 1:10,000 and 1:2,000, respectively. For the detection of HDAg, human antibody against HDAg (ref. 23) was used at a dilution of 1:10,000. After incubation of samples, the binding of biotinylated primary antibody against goat, rabbit or human IgG was detected using a Vectastain ABC kit (Vector Laboratories, Burlingame, California) following the manufacturer's directions. Treated sections were developed with DAB, and hematoxylin was used as a counterstain. HBV-infected human liver was used as positive control tissue. Liver and kidney from an uninfected mouse that had been implanted with human hepatocytes served as a negative control. Kidney sections including human hepatocytes HBV- or HDV-infected mice did not stain positive for HBsAg, HBcAg or HDAg when the primary antibody was omitted from reaction mixture (not shown).

HBV DNA determinations. HBV DNA was quantified by a quantitative HBV DNA PCR ELISA (P.L.M. and M.A. Winters, unpublished). Mouse serum was diluted in PBS containing 10% fetal bovine serum and denatured with an equal volume of 0.2 N sodium hydroxide. After being incubated at 60 °C, the mixture was neutralized and added to a PCR 'master mix' containing buffer, 2.5 mM magnesium chloride, 200 μM dNTP, 2.5 units Taq DNA polymerase (Life Technologies), and 30 pmol each of primers HBV-1 (5'–GGAGTGTGGATTCGCACT–3') and biotinylated HBV-2 (5'–TGAGATCTTCTGCGACGC–3')[24]. Serial 0.5-log dilutions of plasmid containing hepatitis B genome were amplified in parallel, along with mouse serum standards prepared in the lab. The PCR product was then quantified using binding to avidin-coated plates, then probing with a digoxigenin-labeled HBV oligonucleotide (HBV-3, 5'–TAGAAGAAGAACTCCCTCGCCTCGCAGACG–3') that was detected by a peroxidase-labeled antibody against digoxigenin (Boehringer) reacted with the appropriate substrate. Absorbances were measured relative to a curve generated using known amounts of HBV DNA. The assay gives results equivalent to those using the Roche Amplicor system (data not shown). The mean variability ranges from 150% in the same assay to 180% in samples tested in different assays.

PCR for HDV detection. Mouse serum (20 μl; or 5 μl HDV-positive chimpanzee serum used for mouse inoculum) was extracted using the QIAamp viral RNA mini kit (Qiagen, Valencia, California) and used for subsequent detection of HDV genomic RNA by RT–PCR. A 'cocktail' containing the Superscript™ One-Step™ RT–PCR system (Life Technologies) and primers AG1279 (5'–CCCTCGAGAACAAGAAGAAGCAGCTATCGG–3', complementary to positions 1,250–1,279 of HDV genomic RNA) and G917 (5'–CGCGAGACGCAAACCTGTGAGTGGAAACCC–3', corresponding to positions 917–946 of the HDV genome[25]) (synthesized at the Stanford PAN DNA Synthesis Facility, Stanford, California) was added to aliquots of the extracted sera solutions, and the reactions were processed according to the manufacturer's instructions using a RoboCyler 40 (Stratagene, La Jolla, California). After reverse transcription at 42 °C for 30 min and reverse transcriptase inactivation at 94 °C for 5 min, the samples underwent 50 cycles of 1 min at 94 °C, 1 min at 42 °C and 1 min at 72 °C before a final extension step of 8 min at 72 °C. Fractions of the reactions were then separated by 1.6% agarose gel electrophoresis and detected by ethidium bromide fluorescence. This set of primers amplifies a 363-base-pair region of HDV.

Acknowledgments

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