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Liver Cell Transplantation – Novel Animal Model for Human Hepatic Viral Infections

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Aim. To generate a model of human hepatitis B infection (HBV) in immunocompetent rats with chimeric human liver.

Results. Normal rats were tolerized to human hepatocytes by exposure to human hepatocytes at day 17 of gestation. We transplanted human hepatocytes and inoculated HBV into the rats after birth. Mixed lymphocytes assay, a measure for tolerance, indicated that animals fetally exposed to human hepatocytes developed tolerance to human hepatocytes. Spleen lymphocytes from tolerized animals did not proliferate when challenged with donor human hepatocytes. In contrast, control animals given saline fetally developed no tolerance to human hepatocytes. Tolerant animals with transplanted human hepatocytes were susceptible to HBV infection. Western blot analysis and immuno-histochemistry of liver sections from tolerized, HBV infected animals with transplanted human hepatocytes showed the presence of functioning human hepatocytes that synthesized human albumin, of which 30% were also positive for HB surface antigen and HBV DNA. The presence of covalent closed circular HBV DNA in the liver indicated active HBV viral replication.

Conclusion. Tolerized rats with chimeric human livers can be infected with HBV and used as an animal model for HBV infection. Tolerized rats with chimeric human livers can also be used for generating models of other human hepatic viral diseases.

Key words: *cell transplantation; hepatitis B surface antigens; hepatitis B virus; liver; models, animal; transplantation tolerance*

The study of many human diseases is hampered by the lack of useful animal models. This is especially true for viral infections of the liver that result in acute and chronic hepatitis. Persistent hepatitis B (HBV) (1) and chronic hepatitis C (HCV) (2) viral infections are major health problems worldwide. HBV and HCV are highly tissue- (liver) and species-selective. HBV animal models based on homologous viruses in non-primates, such as woodchuck (3), or HBV in immunodeficient (4) or transgenic animals (5), while providing useful information lack many aspects of human disease. Beside the chimpanzee, there are no animal models for HCV (6). Research on transgenic animals expressing individual HCV gene products has yielded conflicting results where the function of the proteins is concerned. Until there is an experimental model system that supports HCV replication and infection, the role of each individual protein in the pathogenic outcome of HCV infection can not be clearly delineated.

In recent years, transplantation of normal or genetically altered syngeneic and allogeneic hepatocytes has been used successfully for gene therapy of different diseases in animal models (7,8) and humans (9,10). Hepatocyte transplantation experiments have also provided useful information on the localization and vascular and tissue requirements for the maintenance of transplanted cells (11), liver gene regulation (12), and regulation of liver growth. In most allogeneic transplantation experiments, immunosuppression has been necessary to sustain functioning transplanted cells. In developing animal models for human HBV or HCV viral diseases, it is important that they have intact and normal immune system because the host immune system plays an important role in the pathogenesis of the viral infections (13). Our hypothesis was that tolerizing rats with immature immune system to human hepatocytes would result in rats that can host transplanted human hepatocytes. Tolerant rats with chimeric human livers could be infected with HBV or HCV. Here we review our data that supported this hypothesis.

Generation of Chimeric Human Liver in Normal Rats

Induction of Tolerance to Human Hepatocytes

The major obstacle in establishing chimeric human liver in a normal rat is the rejection of transplanted xenogenic human cells by the host immune system. Therefore, our first step was to induce donor-specific tolerance towards human cells in the host before the transplantation.

Earlier, Medawar and co-workers (14) showed that "actively acquired tolerance" to foreign cells could be achieved by exposing fetal animals to the foreign cell. This method of tolerization was used by Kline et al (15) for significant prolongation of cardiac allograft survival. To induce tolerance, human hepatocytes were injected into intraperitoneal cavity of fetal rats at 15-17 days of gestation, a time frame when T-cells are educated to distinguish self from foreign antigens. Mixed lymphocyte assay, a measure of tolerance, indicated that spleen lymphocytes from control saline-treated animals were stimulated to proliferate when mixed with human hepatocytes, as indicated by the increased ^{3}[H]-thymidine uptake (Fig. 1, column 3). In contrast, lymphocytes from the animals that were fetally exposed to human hepatocytes were not stimulated to proliferate when mixed with donor human hepatocytes (Fig. 1, column 4). This indicated that the animals became tolerant to human hepatocytes. Similarly, lymphocytes from the animals that were fetally exposed to and subsequently transplanted with human hepatocytes were not stimulated to proliferate when mixed with donor human hepatocytes (Fig. 1, column 5). The degree of ^{3}[H]-thymidine uptake in lymphocytes from fetally tolerized rats when exposed to donor human hepatocytes was similar to that found in lymphocytes alone (Fig. 1, column 1) or in human hepatocytes alone (Fig. 1, column 2).

In animals, intrathymic injection of the donor cells or antigens (16) or oral gavaging of large concentrations of foreign antigen (17) can also induce tolerance to foreign antigen and donor cells. We tested which of the three methods would be optimal in inducing tolerance to human hepatocytes in the normal rat (Fig. 2). Although intrathymic injection (Fig. 2, column 3) and oral ingestion (Fig. 2, column 4) can induce tolerance, exposure of fetal rats to human hepatocytes (Fig. 2, column 2) produced the greatest degree of tolerance. Thus, rats with normal immune system can be made tolerant to human hepatocytes by fetal exposure to the human cells.

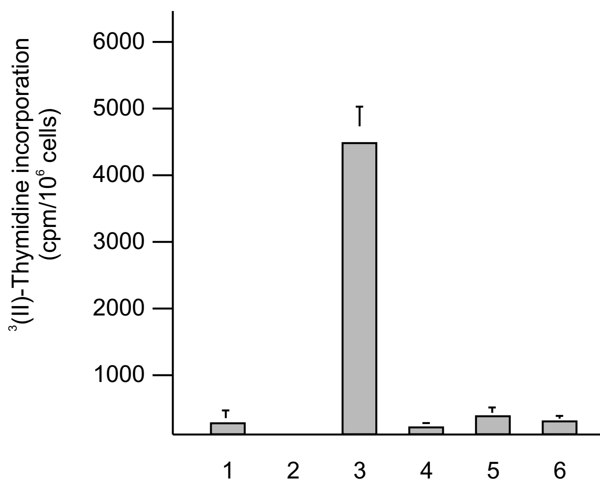

Figure 1. Mixed lymphocyte assay for tolerance. Responder cells: rat spleen lymphocytes. Stimulator cells: -radiation-inactivated donor human hepatocytes. Column 1: spleen lymphocytes from rat with fetal saline injection. Column 2: spleen lymphocytes from saline treated rat stimulated with irradiated lymphocytes from the same animal. Column 3: spleen lymphocytes from saline treated rat stimulated with irradiated donor human hepatocytes. Column 4: spleen lymphocytes from rat with fetal exposure to human hepatocytes and stimulated with irradiated donor human hepatocytes. Column 5: spleen lymphocytes from rat with fetal exposure and transplanted human hepatocytes, and stimulated with irradiated donor human hepatocytes. Column 6: irradiated donor human hepatocytes alone.

Transplantation of Human Hepatocytes into Tolerized Rats

Can rats tolerant to human hepatocytes be successful hosts to transplanted human hepatocytes?

To answer this question, human hepatocytes were transplanted to rats fetally tolerized with human hepatocytes at 17 day of gestation within 24 h after their birth. To determine the success of human hepatocyte transplantation, serum and liver samples were collected. Western blot analysis using an affinity purified anti-human albumin antibody indicated that human albumin could be detected in the serum of tolerized rats that received transplanted hepatocytes (Fig. 3). The antibody was specific for human albumin (Fig. 3, column 1) and could not detect standard rat albumin (Fig. 3, column 2). In tolerized rats that received transplanted column hepatocytes, human albumin could be detected in serum at least up to 6 weeks after transplantation (Fig. 3, columns 3-7). Rats tolerized and transplanted with a human fibroblast cell line, IMR 90, did not produce human serum albumin (Fig. 3, column 8), which indicated that albumin production is specific to human liver cells only.

Human albumin production by transplanted human liver cells was also confirmed by immunofluorescence of fresh frozen liver tissues. Figure 4 is a representative immunofluorescence of liver section taken from rat 3 weeks after the transplantation. In control animals, which fetally received injection of saline and

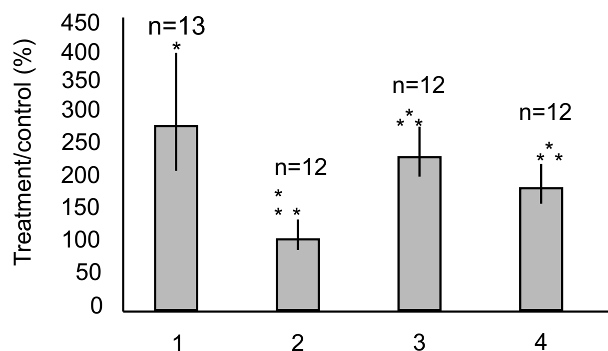

Figure 2. Mixed lymphocyte assay of spleen lymphocytes from fetal, intra-thymic and oral tolerization. Column 1: spleen lymphocytes cells from non-tolerized, saline-treated control rat stimulated with irradiated human hepatocytes. Column 2: spleen lymphocytes from fetally tolerized rat stimulated with irradiated donor human hepatocytes. Column 3: spleen lymphocytes from rat after intra-thymic tolerization, stimulated with irradiated donor human hepatocytes. Column 4: spleen lymphocytes from orally tolerized animal stimulated with irradiated donor human hepatocytes. N – number of rats. One asterisk indicates $p<0.05$ between column 1 and columns 2, 3, or 4. Two asterisks indicates $p<0.05$ between column 2 and columns 3 and 4.

were not transplanted with human hepatocytes, no immunofluorescence for human albumin could be detected (Fig. 4, panel A). Three weeks after human hepatocytes transplantation, tolerized rat was positive for human albumin, as indicated by the positive fluorescent cells (Fig. 4, panel B), whereas tolerized control animals were not positive for human albumin (Fig. 4, panel C).

Generation of a Rat Model of HBV Infection in Tolerized Rats with Chimeric Human Liver

To generate HBV infection, tolerized rats to which human hepatocytes were transplanted 24 h after birth were inoculated with HBV virus one week after the transplantation. Serum samples were collected weekly. Serial liver biopsies were performed at timed intervals after infection. Immuno-histochemical analysis (Fig. 5) revealed that human hepatocytes containing human albumin and producing HBV surface antigen were present in rat livers at 15 weeks after HBV infection (Fig. 5, panels A and B). In addition, 30% of the cells positive for human albumin were also positive for HBV surface antigen. Livers from tolerized animals that received transplanted hepatocytes but had no HBV infection were positive for human albumin (Fig. 5, panel C), but did not have HBV surface antigen staining (Fig. 5, panel D). When inoculated with HBV, these tolerized animals were negative for both human albumin and HBV surface antigen (Fig. 5, panels E and F). To determine that HBV surface antigen in the human liver cells did indeed come from viruses, the presence of HBV viral DNA (HBV DNA) was detected by *in situ* hybridization with a digoxigenin-HBV DNA probe (Fig. 6). HBV DNA positive cells were present only in the liver from tolerized animals that received transplanted hepatocytes and were infected with HBV (Fig. 6, panel A). Animals that did not receive transplanted human hepatocytes but were infected with HBV were not positive for HBV-DNA (Fig. 6, panel C). Similarly, livers from tolerized control animals (Fig. 6, panel D) and animals tolerized

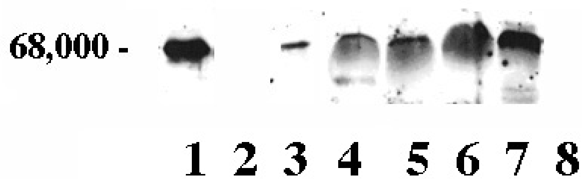

Figure 3. Western blot of rat serum, developed with anti-human albumin antibody and horseradish peroxidase-labeled secondary antibody. Lane 1: 10 ng standard human albumin. Lane 2: 10 ng standard rat albumin. Lane 3-7: serum from tolerized rat with transplanted human hepatocytes. Lane 3: two days after transplantation. Lane 4: two weeks after transplantation. Lane 5: three weeks after transplantation. Lane 6: five weeks after transplantation. Lane 7: six weeks after transplantation. Lane 8: serum from a rat tolerized and transplanted with human fibroblast cell line IMR-90.

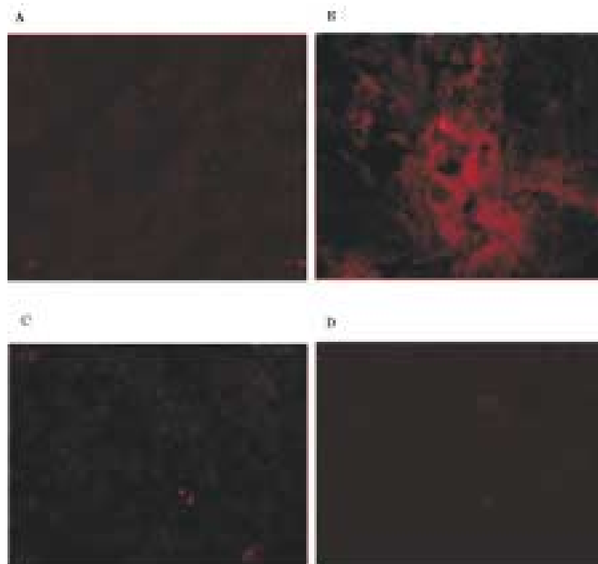

Figure 4. Immunofluorescence of fresh frozen liver tissue using anti-human antibody and Texas-red labeled secondary antibody. **Panel A:** liver from control fetally treated rat without transplanted human hepatocytes. **Panel B:** liver from fetally tolerized rat 3 weeks after human hepatocytes transplantation. **Panel C:** liver from fetally tolerized rat without transplanted cells. **Panel D:** same as panel B, but without secondary antibody.

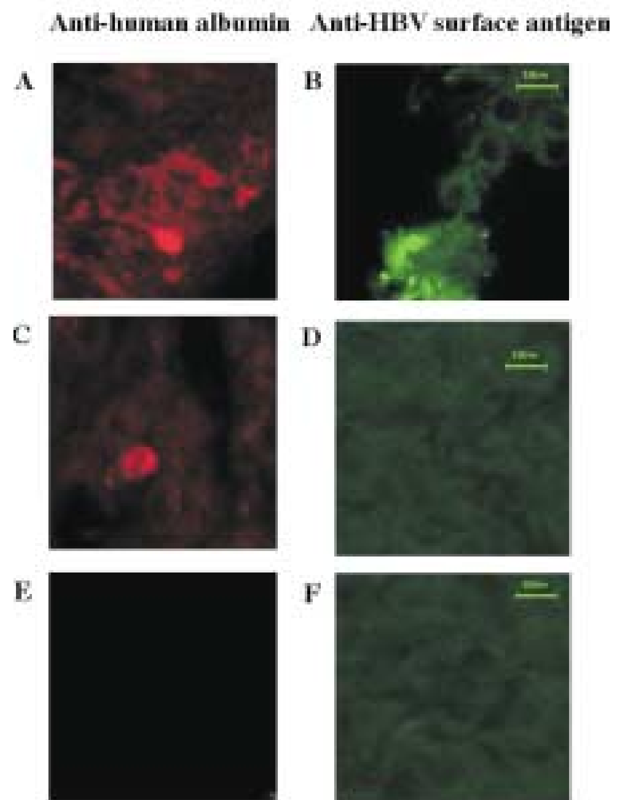

Figure 5. Immunofluorescence of fresh frozen liver tissue using anti-human antibody developed with Texas-red labeled secondary antibody and anti-HBV surface antigen developed with FITC-labeled secondary antibody. **Panels A and B:** liver from tolerized rat with transplanted human hepatocytes, and infected with HBV at 15 weeks after infection. **Panels C and D:** liver from tolerized rat with transplanted human hepatocytes but without HBV infection. **Panels E and F:** liver from tolerized rat without transplanted human hepatocytes but inoculated with HBV.

with human liver cell transplantation (Fig. 6, panel B) were negative for HBV DNA. If infection had taken place and viruses were replicating, DNA from the viruses should be present in sera of infected animals. Serum HBV DNA was confirmed by the presence of an expected 355-bp fragment of HBV genome spanning nt 2079-2434 (Fig. 7). Serum HBV DNA was detectable at 1 week after the infection (Fig. 7, lanes 4 and 7) and remained detectable over 15 weeks (the duration of the experiment) after HBV inoculation (Fig. 7, lanes 6 and 9). Serum HBV DNA was not detected in tolerized rats that received transplanted hepatocytes and were not inoculated with HBV (Fig. 7, lanes 10 and 11), nor was it found in tolerized rats that did not receive transplanted hepatocytes but were inoculated with HBV (lanes 12-17). At 15 weeks after HBV infection, there were 5,000 copies of HBV genome/mL serum in tolerized rats transplanted with human liver cells and inoculated with HBV. Finally,

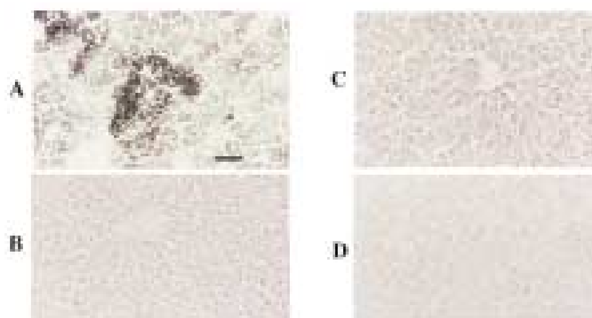

Figure 6. *In situ* hybridization (digoxigenin-HBV DNA probe) of frozen liver sections for HBV DNA, detected with diaminobenzedine. **Panel A:** liver from a tolerized rat that received transplanted human hepatocytes and was infected with HBV. **Panel B:** liver from a tolerized rat that received transplanted human hepatocytes but was not infected with HBV. **Panel C:** liver from a tolerized rat without transplantation but inoculated with HBV. **Panel D:** liver from a tolerized rat without transplantation or HBV inoculation. (Permission to reprint this figure was obtained from Blackwell Publishers/ Blackwell Science Ltd/ Polity Press, Osney Mead, Oxford OX2 OEL, UK.)

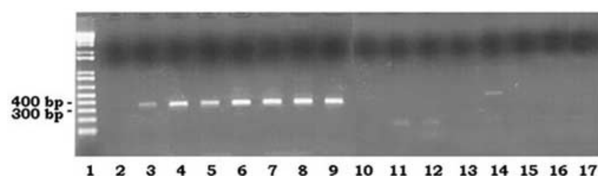

Figure 7. PCR detection of serum HBV DNA. Lane 1: DNA molecular weight markers. Lane 2: serum from control untreated rat. Lane 3: positive control DNA from HBV producing HepG2.2.15 cells. Lanes 4-6 and 7-9: serum from two representative tolerized rats that received transplanted human hepatocytes and were inoculated with HBV, at 1, 5, and 15 weeks, respectively, after inoculation. Lanes 10-11: serum from tolerized rats that received transplanted human hepatocytes but were not inoculated with HBV. Lanes 12-14 and 15-17: serum from tolerized rats that did not receive transplantated human hepatocytes but were inoculated with HBV, at 1, 5, and 15 weeks, respectively, after inoculation. (Permission to reprint this figure was obtained from Blackwell Publishers/ Blackwell Science Ltd/ Polity Press, Osney Mead, Oxford OX2 OEL, UK.)

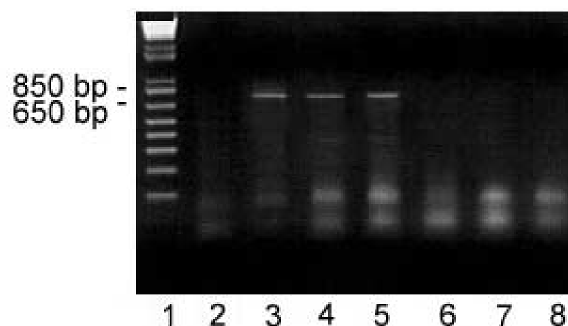

Figure 8. PCR detection of covalent closed circular HBV DNA in liver tissues. Lane 1: DNA molecular weight markers. Lane 2: control untreated rat. Lane 3: DNA from HBV producing HepG2.2.2.15 cells. Lanes 4 and 5: DNA from two tolerized rats transplanted with human hepatocytes and inoculated with HBV at 15 weeks after inoculation. Lane 6: liver from tolerized rat with human hepatocytes transplantation but without HBV inoculation at 15 weeks. Lanes 7 and 8: livers from two tolerized rats without transplantation but with HBV inoculation at 15 weeks after inoculation.

HBV replication could be demonstrated in livers by the presence of covalent closed circular HBV DNA (Fig. 8). It was detected by polymerase chain reaction primers specific to the region of HBV genome that is incomplete in the plus strand of the viral particle, but is covalently closed during HBV replication (Fig. 8, lanes 4 and 5). The same 698-bp band of cDNA could not be detected in livers of tolerized animals that received transplanted cells but were not infected by virus (Fig. 8, lanes 7 and 8) or in livers of tolerized animals that did not receive the transplant but were infected with HBV (Fig. 8, lane 6).

Conclusion

Rats that were fetally tolerized to human hepatocytes could host human hepatocytes in the liver and were susceptible to HBV infection. Tolerized animals with chimeric human livers could also be used to develop a rat model for HCV.

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