Video Article

Intrasplenic Transplantation of Hepatocytes After Partial Hepatectomy in NOD.SCID Mice

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Abstract

Partial hepatectomy is a versatile and reproducible method to study liver regeneration and the effect of cell based therapeutics in various pathological conditions. Partial hepatectomy also facilitates the increased engraftment and proliferation of transplanted cells by accelerating neovascularization and cell migration towards the liver. Here, we describe a simple protocol for performing 30% hepatectomy and transplantation of cells in the spleen of a non-obese diabetic/severe combined immunodeficient NOD.SCID (NOD.CB17-*Prkdc*^{scid}/J) mouse.

In this procedure, two small incisions are made. The first incision is to expose and resect the left lobe of the liver, and another small incision is made to expose the spleen for the intrasplenic transplantation of cells. This procedure does not require any specialized surgical skills, and it can be completed in 5-7 minutes with less stress and pain, faster recovery, and better survival. We have demonstrated the transplantation of hepatocytes isolated from a green fluorescent protein (GFP) expressing mouse (Transgenic C57BL/6-Tg (UBC-GFP) 30Scha/J), as well as hepatocyte like cells of human origin (NeoHep) in partially hepatectomized NOD.SCID mice.

Video Link

The video component of this article can be found at https://www.jove.com/video/56018/

Introduction

Currently, hepatocyte transplantation is proposed as an alternative to whole organ transplantation for treating patients having severe liver disorders. It is believed that it can bridge patients to whole organ transplantation[1]. In addition to the allogenic hepatocytes[2], xenogenic hepatocytes[3] and hepatocytes derived from stem cells[4] are also being investigated in animal models. In this context, the homing and engraftment potential of the transplanted cells in the recipient is an important criterion for cell based therapy in acute hepatic failure (AHF).

For investigating the transplantation of hepatocytes or hepatocyte-like cells[5], AHF is created in an animal model either by surgical[6] or pharmacological[7] procedures, followed by transplanting cells. To make an AHF animal model by pharmacological reagents, many hepatotoxins such as d-galactosamine[8], acetaminophen[9], carbon tetrachloride[10], thioacetamide[11], Concanavalin A[12], lipopolysaccharide[13], *etc.*, have been used. From this list, every reagent generates a unique set of features for AHF, but unfortunately no single reagent mimics the human AHF. Moreover, the AHF induced by hepatotoxins takes a long time, which puts animals under chronic stress, and reproducible results are difficult to obtain.

On the other hand, the surgical procedure of partial hepatectomy (PHx) is skill dependent, and reproducible results are easy to obtain after developing required skills. To induce AHF by surgical intervention alone, resection of more than 70% of the liver is required; however, less than a 70% hepatectomy can still be utilized to study engraftment and proliferation of transplanted cells in the liver for analyzing their therapeutic capacity during liver damage[14]. The transplantation of hepatocytes have been performed post hepatectomy through the peritoneum[15], tail vein[16], hepatic vein[17], or the spleen[18]. Currently, hepatic vein infusion and intrasplenic transplantation of hepatocytes are the preferred procedures, as they are easier to reproduce.

In this paper, we have described a procedure for a 30% partial hepatectomy in NOD.SCID (NOD.CB17-Prkdcscid/J) mice in which the left lobe of the liver is excised. It is followed by transplantation of 0.2 million GFP expressing mouse (C57BL/6-Tg (UBC-GFP) 30Scha/J) hepatocytes as well as human origin NeoHep[19] in the spleen. This procedure leads to engraftment of the transplanted cells in the liver. This procedure is the least invasive and a minimally painful technique.

Protocol

Procedures presented in this protocol have been approved by the Institutional Animal Ethics Committee of the National Institute of Immunology, New Delhi. The serial reference number of the approval is IAEC#319/13.

Note: There are excellent resources on general surgery procedures[20] and specific protocols for rodent surgery[21]. For those doing animal surgery for the first time, it is recommended to extensively practice surgical procedures on dummies before operating on animals.

1. Preparation

1. Prior to experiment, keep sterile phosphate buffered saline (PBS) or saline ready.
2. Assemble a surgery kit containing scissors, serrated forceps, tissue forceps, cotton, cotton buds, nylon threads, and different micro needle holders. Autoclave the surgery kit. Special care should be taken if an immune-compromised NOD.SCID mouse is included in the protocol.
3. Perform the complete experimental procedure, from the preparation to the end of surgery, in the bio-safety class I cabinet.
4. Weigh a NOD.SCID mouse of 6-8 weeks old prior to the surgery. Mice weighing between 14-18 g are used in this study.
5. Shave the upper central and hypochondriac abdominal region of the mouse with the hair trimmer. Apply hair removing cream evenly throughout the region with a spatula to completely remove the trimmed hairs. Remove the hairs gently with the help of a piece of wet sterile cotton after 2-5 min.
6. Place the mouse in the isoflurane chamber and unlock the valve of the oxygen cylinder. Maintain the oxygen flow at the rate of 4 L/min and the isoflurane vaporization at 4% to induce anesthesia.
    1. Ensure that the mouse has been anesthetized properly by gentle toe pinching.
7. Place a surgery board inside a biosafety cabinet. Place the animal on the surgery board, such that the ventral portion of the mouse is facing up and the anterior portion of the mouse is placed inside the nose cone connected to the isoflurane and oxygen supply.
8. Reduce the isoflurane vaporization to 2%, and maintain it throughout the surgical procedure.
9. Disinfect the skin of the mouse and sterilize it by wiping with 70% ethanol soaked sterile cotton.

2. Surgical Procedure

1. **Partial Hepatectomy**
    1. Make a transverse incision of around 1 cm in the skin just beneath the sternum, perpendicular to the xiphoid process and parallel to the ribcage, with the help of straight operating scissors.
    2. Gently separate the skin attached with the abdominal muscle layer in the vicinity of the incised area with forceps or sterile moistened cotton tips to distinguish between skin and abdominal muscle layer. Soak the intradermal region with PBS using sterile cotton tips to evade desiccation.
    3. Expose the area of the left lobe smoothly by making a transverse incision through the peritoneal layer just beneath the xiphoid. Use two moistened cotton tips to expose and lift the left lobe of the liver.
    4. Place one of the cotton tips on the abdominal side of the cut, and place another cotton tip on the diaphragm side. Gently press the tip placed towards the diaphragm, and give a sliding push by the other tip to lift the left lobe of the liver.
    5. Slip a nylon thread with a loop through the lifted left lobe, and slide the loop towards the base of the left lobe close to the hilum with the help of the micro-forceps or cotton tips. Gently push down the nylon thread loop to the base of the left lobe.
    6. Tie two ends of the nylon thread over the top of the left lobe using a microsurgery needle holder and the micro forceps. Make two additional knots on the other side.
    7. Dissect out the tied lobe with the help of scissors. Do not attempt to cut very close to the thread. In case the procedure lasts more than 5 min, keep the peritoneal cavity and organs moist with sterile PBS to avoid desiccation due to fluid loss.
    8. Sew the peritoneum by continuous suturing using a 4-0 Catgut suture. Subsequently, close the skin by discontinuous suturing as quickly as possible.

2. **Cell Transplantation**
    Note: The GFP expressing hepatocytes were isolated from transgenic GFP mice (C57BL/6-Tg (UBC-GFP) 30 Scha/J), according to the procedure described by Lee *et al.*[22] and Shen *et al.*[23] Hepatocyte-like cells (NeoHep) of human origin differentiated from monocytes[24] were also used for transplantation. However, cells derived from any other sources may also be used in the protocol.
    1. Suspend 0.2 million viable cells in around 50 µL of Iscove's Modified Dulbecco's Medium (IMDM), and aspirate it in a 1 mL insulin syringe capped with 30G needle. Keep the syringe cold by placing it on ice.
    2. Place the mouse in a way that the left lateral portion faces up toward the person performing surgery. Identify the splenic area and transversely dissect the skin near the hypochondriac region, followed by a short incision through the peritoneal layer just to expose the spleen.
    3. Gently lift the spleen and hold it outside the cavity with the help of two PBS moistened cotton tips.
    4. Hold the spleen carefully with two cotton tips in one hand, and place the needle of the syringe exactly vertical to the spleen. Gently pierce the spleen and push the needle very slowly inside; the needle should not get deeper than 2 mm.
    5. Push down the piston of the syringe slowly to inject the cells into the spleen. After transplantation, keep the needle of the syringe stable and remove it slowly from the spleen to avoid bleeding or loss of cells.
    6. After placing the spleen back into the peritoneal cavity with the cotton tips, close the peritoneal layer by continuous suturing with a 4-0 Catgut suture. Sew the skin discontinuously with the same suture. Avoid using wound clips for closing skin; instead, close it by a 4-0 suture. The wound clips restrict the natural movement of the mouse, and often clips become loose and come out quickly.

3. Post-Operative Care

1. After closing the skin, wipe the surroundings of both the suture sites with iodine solution (betadine) using a sterile cotton tip.
2. Inject a dose of antibiotic cefotaxime as 600 mg/kg body weight (typically 12 mg cefotaxime in 100 µL of saline/mouse)intraperitoneally using 1 mL syringe.
3. Give daily doses of analgesic Meloxicam as 1 mg/kg body weight (typically 12 µg Meloxicam in 100 µL of saline/mouse) to the animal intraperitoneally, for up to three days after the surgery.
4. After completion of the surgery, stop the flow of isoflurane gas and place the mouse back in the individually ventilated cage.

4. Euthanization and Characterizations

1. After the experimental end-points (1 day and 10 days post-surgery), euthanize the mice according to the institutional animal ethics guidelines.
2. Collect blood by terminally bleeding the animals, puncturing the ocular terminal plexus.
3. Isolate serum from the blood.

Representative Results

Hepatocyte proliferation after 30% partial hepatectomy: The proliferation of hepatocytes in the remaining liver after 30% hepatectomy was examined by immunohistochemical (IHC) staining for a cell proliferation marker, Ki-67. One-day post hepatectomy, the mice were euthanized, the remaining liver lobes were excised, and paraffin sections were obtained. The sections were stained with Ki-67 antibody, followed by labelling with horseradish peroxidase (HRP) conjugated secondary antibody. Di-Amino Benzidine (DAB) was used as substrate for HRP for the development of brown color to identify stained cells. The nucleus was counter stained with hematoxylin and viewed under a 20X objective microscope. **Figure 1** shows a representative IHC image of a liver section. Around 13% of cells (13.66 ± 0.317, N=3) were Ki-67 positive, which confirmed that a 30% hepatectomy facilitates proliferation of hepatocytes in the mouse liver.

Anatomical Study: A representative image of the liver of a NOD.SCID mouse, 10 days post hepatectomy is shown in **Figure 2**. This image confirmed that the remaining liver was healthy with no visible abnormalities.

Presence of transplanted cells during first hour of surgery: The homing of the transplanted cells was confirmed with flow cytometric analysis by examining the presence of GFP positive hepatocytes 2 hours after transplantation.

A single cell suspension was obtained from the spleen and liver of the host mouse after enzymatic digestion of the excised tissue post 2 hours of GFP-hepatocytes transplantation. The percentage of transplanted GFP positive cells was estimated using a flow cytometer. A scatter gate was selected from the corresponding FSC-SSC dot plot to eliminate the debris and doublet cells. The quadrant gate of the plot was created by the background fluorochrome intensity of cell suspensions obtained from the spleen and liver of a control mouse in which no cells were transplanted. Around 1.7% GFP positive hepatocytes were found in the spleen, and no GFP hepatocytes were found in the liver 2 hours after transplantation. Representative data are shown in **Figure 3**.

Immunohistochemistry: Ten days post-surgery, the mice were euthanized, and the right lateral lobe, the right medial lobe, and the left medial lobes of the liver were excised and cryo-sectioning was performed to obtain 5 µm sections. These sections were then examined for the homing and engraftment of the transplanted cells. **Figure 4** shows the representative images of immunohistochemical (IHC) staining against anti-GFP (red) to identify the GFP expressing mice hepatocytes (panel **A**, **B**, and **C**); and against anti-human albumin (red), as well as anti-human connexin 32 (green), to identify hepatocyte-like cells (NeoHep) of human origin (panel **D**, **E**, **F**, and **G**). The nucleus was counterstained with 4', 6-diamidino-2-phenylindole (DAPI, blue) in both the cases. In these image panels, a few engrafted GFP expressing hepatocytes (panel **A**, **B**, and **C**) and NeoHep (panel **D**, **E**, **F**, and **G**) are clearly visible.

Biochemical analysis of liver secreted enzymes: In order to check the functionality of the liver after the surgical procedure, biochemical analysis of different liver secreted enzymes was performed. The bar graphs in **Figure 5** represent the mean value of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALK-PHOS) in the serum of healthy NOD.SCID mice, and different groups of partially hepatectomized mice. It is clear from the graph that after 1 day of hepatectomy, levels of AST, ALT, and ALK-PHOS enzymes increased significantly, when compared with the healthy mice.

The ALT and AST enzyme levels were restored to normal, while ALK-PHOS levels remained high post ten days surgery in non-transplanted mice. However, the levels of all three enzymes dropped to normal in transplanted mice after 10 days.

Liver histological study: The liver samples of the hepatectomized and transplanted mice were further processed for histological analysis to study the anatomical changes post-surgery. **Figure 6** shows the representative bright field images of hematoxylin and eosin (H and E) stained liver sections of healthy mice without surgery, 1 day post partial hepatectomy and 10 days post partial hepatectomy and NeoHep transplantation. In these images, liver damage due to the mild peri-biliary fibrosis or connective tissue proliferation was observed after 1 day of partial hepatectomy, and no abnormalities were observed ten days post-surgery.

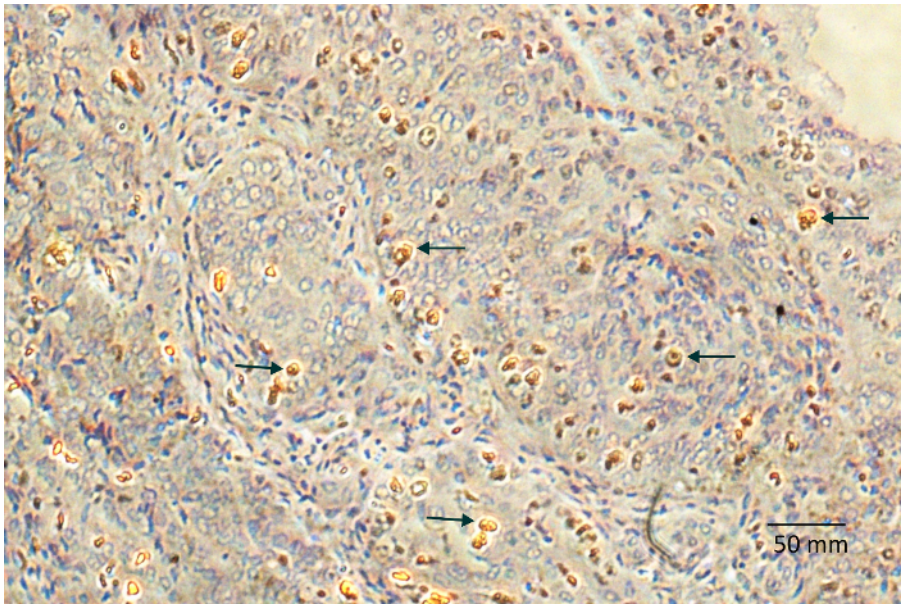

Figure 1: A representative image of Ki-67 staining of NOD.SCID liver section after 1 day of 30% hepatectomy. Section was stained with Ki-67 antibody, horseradish peroxidase (HRP) conjugated secondary antibody, and DAB (brown) was used as HRP substrate, and nuclei were counterstained by hematoxylin (blue). The indicative arrowheads point out some of the Ki-67 positive (brown) cells. Please click here to view a larger version of this figure.

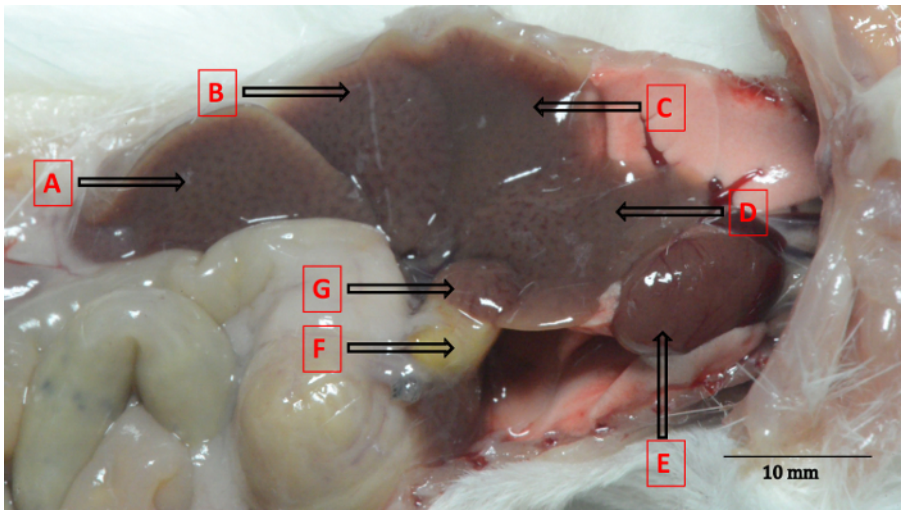

Figure 2: Regenerated liver lobes of a NOD.SCID mouse 10 days post-surgery. A: caudate lobe (Caudate process), B: right lateral lobe, C: right medial lobe, D: left medial lobe, E: heart, F: remaining of left lateral lobe after hepatectomy, and G: caudate lobe (Papillary process). Please click here to view a larger version of this figure.

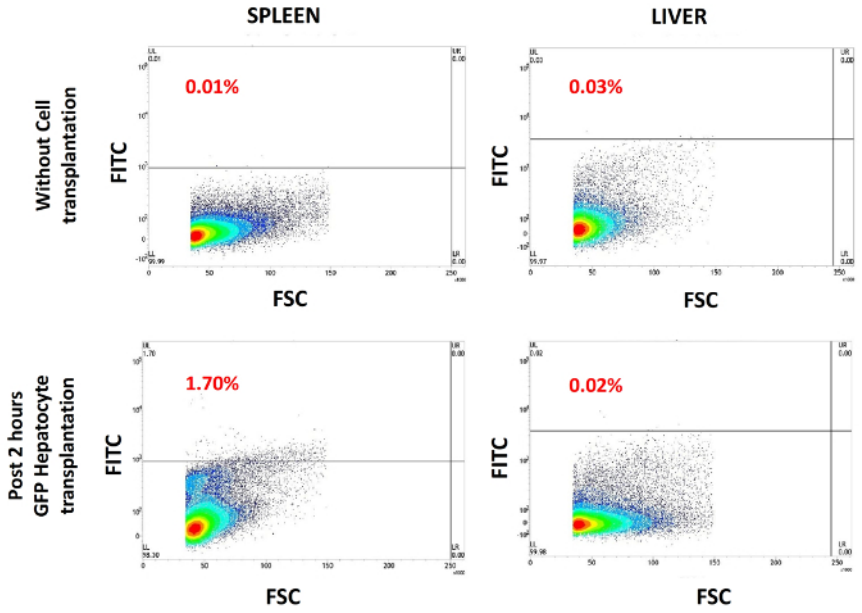

Figure 3: Percentage of GFP positive mouse heptatocytes in the spleen and liver of an NOD.SCID mouse. The upper panels show the cell profile of an age matched control mouse in which no cells were transplanted. Lower panels show the cell profile after 2 hours of GFP hepatocyte transplantation in a hepatectomized mouse. The y axis of the plots denotes the fluorescence intensity of GFP (FITC channel) measured in arbitrary units on a log scale, and the x axis denotes the forward scatter (FSC) in arbitrary units on a linear scale. Please click here to view a larger version of this figure.

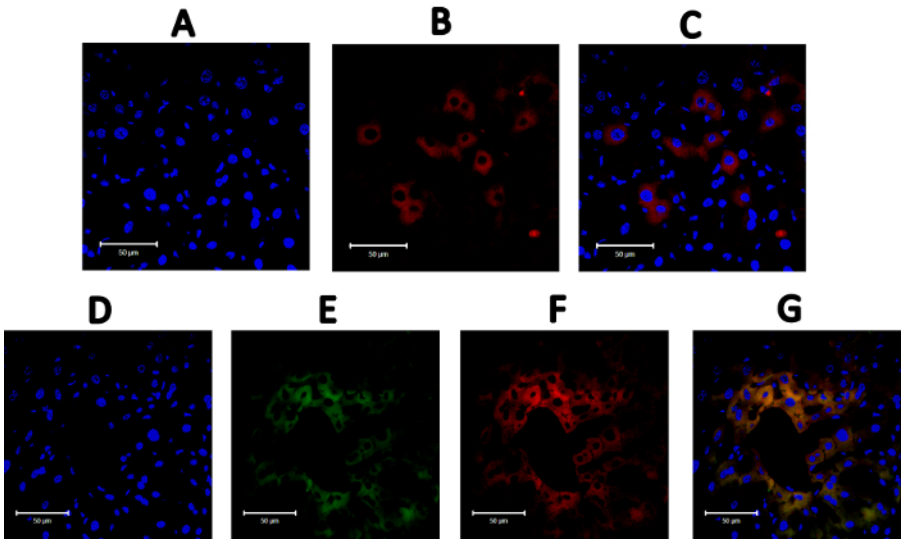

Figure 4: The homing of transplanted GFP expressing mouse hepatocytes and NeoHep in the regenerated host liver. Representative images of liver sections of a partially hepatectomized NOD.SCID mouse transplanted with GFP positive mouse hepatocytes (A-C), showing cell engraftment and homing. Panel A: Nucleus (blue: DAPI), Panel B: anti-GFP (Red: Alexa Fluor 594), Panel C: Merged image. Panels (D-G) show liver section of the partially hepatectomized NOD.SCID mouse in which NeoHep were transplanted. Panel D: Nucleus (blue: DAPI), Panel E: human anti-Connexin 32 (Green: Alexa Fluor 488), Panel F: human anti-Albumin (Red: Alexa Fluor 594) and Panel G: Merged image. Please click here to view a larger version of this figure.

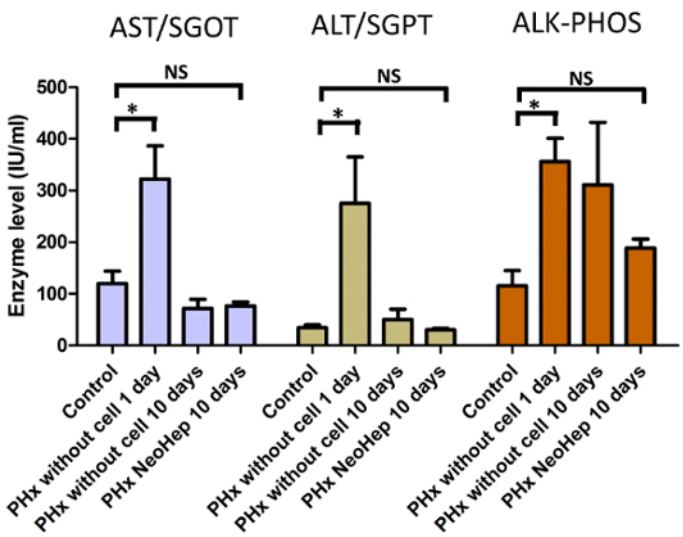

Figure 5: Biochemical analysis of different liver secreted enzymes in mice serum. Column bars represent the mean values of different enzymes (AST/SGOT, ALT/SGPT, ALK-PHOS). The time point of study post-surgery is shown on the X axis. In the control group, there were age matched NOD.SCID mice and no surgery was performed. Error bars signify the standard error of the mean, N=3 and * indicates $p<0.05$, NS indicates non-significance at $p>0.05$. Please click here to view a larger version of this figure.

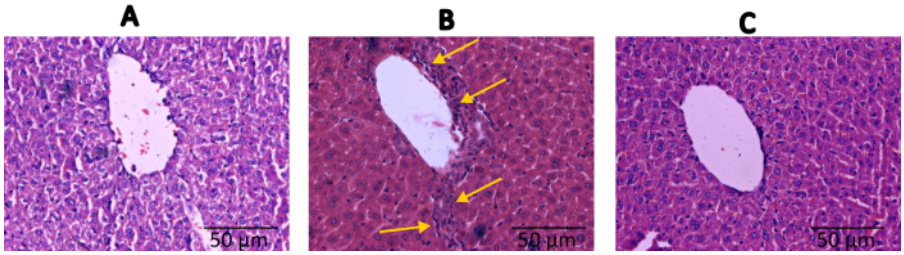

Figure 6: Histopathological study of mice liver tissue sections. Representative bright field microscopy images of hematoxylin and eosin (H and E) stained liver tissue sections of NOD.SCID mice under 20X objective lens. Panel A shows a liver section from a healthy NOD.SCID mouse of the same age without any surgery. Panel B shows a liver section 1 day post partial hepatectomy. In this image, arrow heads indicate the mild peri-billiary fibrosis or connective tissue proliferation region. Panel C shows a liver section ten days post partial hepatectomy and cell transplantation. Please click here to view a larger version of this figure.

Discussion

Partial hepatectomy is an established technique for investigating liver regeneration, and excessive hepatectomy is reported to mimic the AHF model. Among animal models of AHF, rodents, particularly mice, are the most researched model. To obtain a liver injury model in mice, up to a 70% hepatectomy has been reported with a good survival rate[25,26]. However in nude and other immunodeficient mouse, a 70% hepatectomy was reported as fatal and animals died within 24 hours[27].

Mitchell and Willenbring[28] demonstrated a reproducible and well tolerated method for 2/3 partial hepatectomy in mice. For NOD.SCID mice, we obtained a 100% survival rate when the hepatectomy was restricted up to the resection of the left liver lobe, which is close to 30% of the total liver mass. In line with the observations on nude mice[27], we observed that any further increase in the percentage of hepatectomy in NOD.SCID mouse leads to a dramatic lowering of the survival rate. Moreover, proliferation of hepatocytes in the remaining liver lobes post 1 day of 30% partial hepatectomy confirmed the utility of this procedure in transplantation and engraftment studies.

In a more recent paper, Ahmed S.U. *et al.*[29] have demonstrated a procedure for intrahepatic hepatocellular carcinoma xenografts in immunodeficient mice. They have shown a procedure for transplantation of tumor cells in various organs, including the spleen, and performed hepatectomy to facilitate intrahepatic engraftment.

In the procedures reported by Mitchell[28] and Ahmed[29], there is an opportunity for refinements by making much smaller incisions, as smaller incisions are generally preferred in surgery. There is evidence[30] that a smaller length surgical incision leads to less secretion of stress hormones, such as cortisol and catecholamine. Additionally, we found that procedures involving one larger incision require a higher level of skills, and are more difficult to reproduce, compared to procedures having two smaller incisions.

In this paper, we have described a procedure in which minimum incision is required to expose the left lobe of the liver and, after performing a 30% hepatectomy, another small incision was made to expose the spleen where cells were implanted. This procedure does not require any specialized surgical skills and can be completed in 5-7 min. Moreover, we found no evidence of any morphological or anatomical abnormality in the remaining liver mass, as evidenced by histological studies. Furthermore, there was an absence of ischemia or necrosis during the surgical procedure of the 6-8 week-old immune compromised mice. The induction of liver injury after partial hepatectomy is confirmed by the elevated

levels of liver enzymes AST, ALT, and ALK-PHOSin mice serum. An intrasplenic route was chosen for cell transplantation over other venous routes, because the portal venous system has higher accessibility into the liver cortex than other venous systems. We have demonstrated the transplantation of hepatocytes isolated from a transgenic GFP mouse and NeoHep which are differentiated hepatocyte-like cells of human origin, in partially hepatectomized NOD.SCID animals. The procedure does not restrict the type of cells used for transplantation.

However, this procedure does not create the AHF condition in a mouse, as only a 30% partial hepatectomy is performed. This limited hepatectomy provides a proliferative potential to hepatocytes present in the remaining liver, which thereby provides additional opportunities for transplanted hepatocytes for engraftment. It only demonstrates the migration and engraftment of hepatocytes, and NeoHep from the spleen to the liver, and no additional damage to the liver is done by the transplantation procedure.

Over all, this procedure is simple, and can be practiced and mastered easily to obtain reproducible results. The regenerative potential of several cellular sources (stem cells or hepatocyte-like cells) in the context of liver injury, or the liver regeneration study, can easily be evaluated with this surgical procedure.

Disclosures

The authors have nothing to disclose.

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