Development and characterization of pig xenograft model for human hepatocellular carcinoma

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Abstract: The lack of large animal transplantable tumor models has limited the study of novel therapeutic strategies for the treatment of liver cancer. Pig is an ideal animal for applying clinical research as it has similar anatomical and physiological characteristics to humans. However, there is no human hepatocellular carcinoma (HCC) model available in pigs. The aim of our study is to develop a large-animal human HCC xenograft model in pigs. Under laparoscopic guidance, HepG2-GFP cells were delivered into the liver of fifteen three-month-old Bama mini pigs and saline was injected into two pigs as control. Peripheral blood was harvested from the recipients for human-specific protein tests, liver function test and analysis of immune cells at different time points. At the sacrifice, liver tissues were completely excised for histological and immunohistochemistry analysis. Human-specific AFP was increased in porcine serum in the first week after implantation, and was still detectable at the end of experiment. Immunohistochemical staining showed that a few GFP-positive HepG2 cells were scattered as individual cells or nodules containing 2-5 cells in porcine liver, and the repopulation rate was significant higher in PH+HepG2+IA group than that in other groups (P<0.05). There was a rise in the levels of hepatic enzymes and bilirubin at first day after treatment, and the levels recovered in 2 weeks. A pig model of HCC can be rapidly achieved by orthotopic implantation of human hepatoma cells after partial hepatectomy.

Keywords: Hepatocellular carcinoma, partial hepatectomy, laparoscopy, pigs

Introduction

Hepatocellular carcinoma (HCC) is one of the most common primary tumor, the third leading cause of cancer-related death, and represents a challenge for clinicians [1]. Because of low survival rate, better preventive, diagnostic and therapeutic approaches are urgently needed. Cancer animal models play an important role in better understanding the pathophysiology, tumor characteristic and drug treatment [2]. Over the last few years a broad number of in vivo models of HCC have been developed [3]. Several chemicals were used to damage the liver and induce the development of tumors [4]. However, chemicals induced hepatocarcinogenesis shows a few limits such as long-time of the administration, animal strain-related efficacy and high mortality.

The rodent model of HCC is commonly used in cancer studies. Tumor xenograft models induced by injection human cancer cells into immune deficient mice are applied for the study of drug treatment and cancer cell proliferation [5]. Numerous investigations have established subcutaneous [6] and orthotopic [7] xenograft model of human hepatoma cells in severe combined immunodeficiency mice. However, it is limited when considering preclinical applications, including minimally invasive laparoscopic surgery, ablative treatment and transarterial chemoembolization [8]. Thus, alternative animal models are required to overcome this size limitation. Mini pigs as ideal animals are used...
Liver tumor model in pigs

to establish a xenograft model for investigation of human liver cancer [9], on account of their anatomical and physiological characteristics similar to humans. Unfortunately, few researchers have focused on the engraftment of human hepatoma cells in pigs [10, 11].

Several relatively recent reports created different tumor models in large animal by orthotopic implantation of human tumor cells [12]. High grade glioma have been presented in fifteen pigs by implanted in the parietal lobe with human glioblastoma cell lineage under a chemical immunosuppression [13]. In terms of liver anatomy and physiology, pigs represent a fairly close to humans. A current study was carried out to create hepatocellular carcinoma in the pig. Human hepatoma cell line was delivered in two immunosuppressed pigs by portal vein injection. Although serum alpha-fetoprotein (AFP) level was increased in the first week, there was no evidence of liver tumor at the end of experiment [11].

The present study describes a pig xenograft model of HCC. We performed orthotopic implantation of human hepatoma cells by laparoscopic technique at the time of partial hepatectomy. Engrafted human hepatoma cells were discovered in porcine liver, and human-specific albumin (ALB) and AFP were detected in the serum. In addition, our data indicated that using of combined immunosuppressive agents could reduce immune rejection and prolongate survival of engrafted human hepatoma cells in the pig.

Material and methods

Animals

Seventeen healthy male Bama mini pigs three-month old and weighing 16.8–25.5 kg were used for this study. They were obtained from the laboratory animal center of Harbin veterinary research institute (number of animal license SYXK 2011-0039). All experimental procedures and animal care were performed in accordance with the national animal research guidelines (Approved by the State Council on October 31, 1988 and promulgated by Decree No. 2 of the State Science and Technology Commission on November 14, 1988) and were approved by the Experimental Animal Ethics Committee of Northeast Agricultural University and Harbin Medical University Ethics Committees, China. The pigs were housed individually, and were fed a standard piglet diet (Shenzhen Jinxinnong Feed, China) along with tap water ad libitum.

The animals were randomly divided into three groups. In SHAM group (control group), left partial hepatectomy (40%) was performed without any treatment in two pigs. In PH+HepG2 group, pigs received HepG2 cells after partial hepatectomy (PH). In PH+HepG2+IA Group, pigs received both HepG2 cells and immunosuppressive agents after surgery.

Cell culture and preparation

Human hepatoma cell line HepG2 expressing GFP (Shanghai SBO medical biotechnology co., LTD), was maintained at 37°C with 95%/5% air/CO₂ in Dulbecco’s modified eagle medium (DMEM, Life Technologies, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Biological Industries, Israel), 100 U/ml penicillin, 100 U/ml streptomycin (P/S, Life Technologies, USA), 2 mM L-Glutamine (Life Technologies, USA) and 2 μg/ml puromycin (Life Technologies, USA).

Prior to implantation, cells were harvested with a 0.25% solution of trypsin/EDTA (Life Technologies, USA) and resuspended at a final concentration of 300 million cells in 100 μl PBS (Life Technologies, USA).

Establishment of partially hepatectomized pig model

Pigs were pre-medicated with an intramuscular injection of atropine sulfate (0.04 mg/kg) along with intramuscular injection of 1 mg/kg xylazine and 10 mg/kg ketamine hydrochloride. Once unconscious, orotracheal intubation was performed and sedation was maintained by a continuous inhalation of isoflurane 2%. All surgical procedures were performed under aseptic conditions. The PH model which was left hepatectomy by laparoscopy in pigs was applied as described by Zhang [14]. Liver attachments were freed, and left hepatic lobes were dissected and removed after ligating hepatic hilum. The weights of removed lobes were recorded, and tissue specimens were taken for further investigations. After surgery, the animal was monitored in a recovery room. A 5 mg fentanyl
Liver tumor model in pigs

Table 1. The summary of Bama miniature pigs in this study

<table>
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<tr>
<th>Pig number</th>
<th>Age (month)</th>
<th>Weight (kg)</th>
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<th>Injected cell number</th>
<th>PH graft (days)</th>
<th>Duration of graft (days)</th>
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Histology and immunohistochemistry

Livers removed from the recipients were cut into several pieces containing injection spots. Tissue specimens fixed by immersion in 4% paraformaldehyde for 24 hours, then dehydrated and embedded in paraffin. After slicing into 4 μm thick sections, some of them were stained with haematoxylin and eosin (H&E), and the others were examined by immunohistochemistry (IHC) staining. After epitope retrieval, sections were incubated with primary antibodies (AFP, MBL, 1:1000; human-specific AFP, Abcam, 1:500) overnight at 4°C. After rinsing in PBS/Triton X-100, secondary antibody conjugated with HRP (anti-rabbit and anti-mouse, BOSTER) was incubated for 1-2 hours at room temperature. DAB substrate solution (BOSTER) was applied to the sections, and the color development was allowed for 1 minute. All the sections were counterstained with hematoxylin solution (Sigma). The percent of positive areas in samples were determined by counting the positive stained areas in ten randomly selected sections on slide and normalized for the total area. Quantification of GFP-positive areas was performed using Photoshop 5.0 and Image-Pro Plus 6.0 software.

Human-specific AFP and ALB secretion analysis

Human AFP ELISA kits (RayBiotech, USA) and human ALB ELISA kits (RayBiotech, USA) were used to measure human-specific AFP and ALB protein serum levels in accordance with the manufacturer’s instructions. Briefly, the standards and samples were added into the antibody coated 96 well plate, incubated at 4°C for 16 hours; after repeated washes, the substrate was incubated in each well at 37°C for 20 minutes. Absorbance was measured immediately at 450 nm using a SpectraMax M5e (Molecular Devices, USA).

Biochemical analysis of liver metabolic function

The serum was separated from blood by centrifugation of 3000 rpm at 4°C for 10 minutes, patch was given every 3 days for 6 days, and ampicillin (20 mg/kg; Shandong Lukang Record Pharmaceutical, China) was administered intra-muscularly every 8 hours for 3 days.

Cell transplantation

Under laparoscopic guidance, freshly prepared cells were implanted into liver parenchyma at the time of PH surgery. The cells were slowly injected into right anterior lobe at ten spots via trocar-cannula unit at the dose of 1×10^8 or 3×10^8. Animals were kept in recovery room after surgery, and the pigs were transferred to a 25°C hog house until they could stand on their own. At the different time points, animals were sacrificed under general anesthesia and the liver was completely excised. Removed livers were flushed with saline solution and weighed after gently drying. The liver tissues were taken for further investigations.

Immunosuppressant treatment

To prevent rejection of the grafted cells, combined immunosuppressive agents were given at 12 hours after transplantation. Mycophenolate mofetil (0.25 g; Shanghai Roche Ltd), methylprednisolone (1 mg/kg; Pfizer Italia Srl) and tacrolimus (0.3 mg/kg; Astellas Ireland Co., Ltd) were administered orally twice a day.
Liver tumor model in pigs

and then stored at -80°C for further analysis. Hepatic enzymes (aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase and alkaline phosphatase), proteins (total protein, ALB, and globulin) and bilirubin (total bilirubin and direct bilirubin) were determined as previously described [15].

Statistical analyses

Data between different groups were analyzed by Student’s t-test, and were expressed as the mean ± standard deviation (SD). A value of P<0.05 was considered significant.

Results

Engraftment of human hepatoma cells into pig liver

We at first investigated if human hepatoma cells could survive and function in pigs. It has been shown that the growth of engrafted cells could be enhanced in partially hepatectomized rats, in which regenerating liver constitutes a highly active anabolic focus [16-18]. As shown in Table 1, HepG2-GFP cells under growth status (Figure S1) were injected into 2 pigs (No.1/No.3) after PH, and 2 pigs (No.2/No.4) received HepG2-GFP cells only. Two pigs (No.1/No.2) sacrificed at 4 months after transplantation, and the other pigs (No.3/No.4) were sacrificed 3 months and 3 weeks after surgery respectively. Engrafted HepG2-GFP cells were detected by IHC staining using antibodies specifically against GFP. GFP-positive HepG2 cells were hardly observed in pigs which obtained only the cells, while a few engrafted surviving HepG2 cells were discovered in two recipients which treated by both cell implantation and PH. A few GFP-positive HepG2 cells were found as scattered individual cells or nodules containing 2-5 cells in liver, and human-specific AFP were detectable in the serum of the two recipients (Figure 1A).

Improving long-term graft survival by combined immunosuppressive agents

Although AFP serum levels had been increased at the first week, there was subsequent decrease in AFP level until the end of experiment (Figure 1B). These results indicated engrafted tumor cells may have been rejected by porcine immune system. Immunosuppressive agents are considered capable of inhibiting proliferation of T- and B-cells as well as prevention of allograft rejection [19]. We wondered whether...
immunosuppressive agents could prolong xenograft survival. Therefore, 13 pigs were divided into three groups and the PH were performed on the recipients. Two pigs in the first group did not receive any treatment during the experiment (SHAM group). Six pigs in the second group received HepG2 cells (PH+HepG2 group). In the last group, 5 pigs received HepG2 cells with combined immunosuppressive agents (PH+HepG2+IA group). To analyze the survival of grafted cells, a cohort of recipients (SHAM group, n = 1; PH+HepG2 group, n = 5; PH+HepG2+IA group, n = 3) was assessed by histologic analysis 28 days after transplantation. At sacrifice, the recipients in PH+HepG2 group and PH+HepG2+IA group showed GFP-positive of engrafted cells. These data indicated that using of combined immunosuppressive agents could prolong survival of engrafted human hepatoma cells in pigs.

**Human-specific AFP and ALB secretion analysis**

To determine whether HepG2-GFP cells in chimeric livers were functional, serial section with IHC staining of GFP and human-specific AFP was carried out. AFP-positive cells were observed in GFP-positive liver tissues (Figure 3A). However, no positive signal was found in SHAM group. This was further confirmed by detection of human-specific AFP and ALB secretion of engrafted cells in the serum. Human-specific ALB and AFP were detectable in the serum of the recipients until the end of experiments. As shown in Figure 3B, the serum of the recipients in PH+HepG2+IA group contained 39.53±20.28 ng/ml human-specific AFP in 2 weeks after transplantation, and level decreased over the next few weeks. Notably, PH+HepG2+IA group showed higher level of AFP than PH+HepG2 group at the different time points over 12 weeks after transplantation. There was no significant difference in serum level of human-specific ALB between the two groups (Figure 3C). These results showed that grafted HepG2 cells were proliferating and expressing functional proteins in porcine liver.

**Histologic characterization of liver tissue**

Normal liver histology was confirmed by H&E staining before treatment (Figure 4A). Hepatic tissue of SHAM group was basically normal after operation and no rejection feature was seen under microscope (Figure 4B). Shown in Figure 4C, trabecular pattern composed of tumor cells were much wider than the normal liver plate that is two cells thick, and abnormal cells with prominent nucleoli have high N/C ratio in PH+HepG2+IA group. There was no discernable normal lobular architecture, though vascular structures were present. Necrosis of hepatocytes and hepatic steatosis were observed in one recipient (No. 6) in PH+HepG2 group (Figure 4D). All the animals in this study did not have any severe complications including abdominal abscess. Compared to PH+HepG2+IA group, severe inflammatory cell infiltration and fibrosis which are rejection fea-
Liver tumor model in pigs

Figures were found in a number of the recipients in PH+HepG2 group after transplantation (Figure 4E and 4F).

Evaluation of liver function after transplantation

Blood biochemical measurements indicated that there was an increase in alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and total bilirubin levels on the first day after PH performance in all three groups. The decline tendency of these levels were already evident on day 3 and all the recipients recovered in 14 days after the PH (Figure 5A). We also found that serum level of alkaline phosphatase, gamma-glutamyltransferase, direct bilirubin and indirect bilirubin were in the same trend. Serum concentrations of total protein, ALB and globulin were measured by liver function test. There was a slight rise in ALB level in 14 days after transplantation. The levels of total protein and globulin decreased in three days after transplantation. Moreover, globulin level dramatically decreased in PH+HepG2+IA group compared to PH+HepG2 group in three days after transplantation (Figure 5B).

Discussion

Xenograft animal models are widely used to improve our knowledge of HCC, in particular the rodent model [20]. Although such models help to understand the development of HCC and the therapeutic approaches, no rodent models was ideal for developing surgical procedures and devices, due to their small body size relative to human [21]. The pig model is one of the best candidates, as anatomical and physiological characteristics of pigs are similar to those of humans. In particular, larger animal models that might be developed by minimally invasive image-guided interventions seem a step closer to the human scale [22]. In this paper, we described an HCC model in large animals, induced by implantation of human hepatoma cells.

Several relatively recent reports have developed porcine liver tumors, and these models mimic the human condition of HCC [23-25].
Liver tumor model in pigs

Dosing of diethylnitrosamine was administrated every week for 3 months, and cirrhosis and tumor were developed over the course of 12-15 months [23]. The main limit of this drug-induced model is the long duration of experiments for HCC development. Moreover, this model is characterized by numerous small tumors, which is uncommon in humans. As many human malignant cell types implanted at different anatomical sites have yielded human tumors in immunodeficient mice [26], it should be possible to generate human liver tumors in porcine liver by suppressing the immune response. A current study have created genetically engineered pig model of tumorigenesis which was produced by orthotopically injected cells which are expressing proteins known to cause human cancers. Highly undifferentiated tumors were formed in mammary gland of immunosuppressed pigs at day 15 post-injection. The main advantage of xenograft model is related to the short time span occurring between injection and tumour development. This study demonstrated that human tumor model could be produced rapidly by injection of human malignant cells in immunosuppressed pigs.

Previous studies employed various methods to develop rabbit HCC model, such as injection of a VX2 tumor cell suspensions directly into the liver parenchyma [27-29]. VX2 cellular foci centered around the interlobular region and the tumor cells proliferated. Solitary cancer cells were dispersed in the sinusoids, which had regions of infiltration. In our study, engrafted tumor cells were found in hepatic lobule, and interdigitated with normal porcine hepatocytes. H&E staining showed tumor cells arranged in broad trabeculae and the malignant cells of HCC were composed of liver cords that are much wider than the normal liver plate. Moreover, abnormal cells with high N/C ratio and fatty change which were important diagnostic features of HCC were observed in PH+HepG2+IA group. In addition, a number of small inflammatory cells fibrosis occurred in a number of the recipients in PH+HepG2 group after transplantation. Scale bars = 50 μm.

Figure 4. Representative histopathology of liver tissue at the transplantation spot. A. Normal liver histology was shown by H&E staining before transplantation. B. Liver tissue that was harvested from the recipients showed normal liver histology. C. Abnormal cells with prominent nucleoli showed high N/C ratio and were smaller than normal in PH+HepG2+IA group. There was no discernable normal lobular architecture, though vascular structures were present. D. Necrosis of hepatocytes and hepatic steatosis were observed in one recipient (No. 6) in PH+HepG2 group. E. The recipients in PH+HepG2+IA group showed slight inflammatory cell infiltration after transplantation. F. Severe inflammatory cell infiltration and fibrosis occurred in a number of the recipients in PH+HepG2 group after transplantation. Scale bars = 50 μm.
Liver tumor model in pigs

Figure 5. Biochemical analysis of metabolic and synthetic functions after transplantation. A. The levels of serum enzyme ALT, AST, GGT and ALP were increased on first day after PH in all groups, and the levels decreased three days after PH and recovered in 14 days. TBIL and DBIL followed similar trends. B. Serum concentrations of TP, ALB and GLOB were measured by liver function test. There was a slight rise in ALB level in 14 days after transplantation. The levels of TP and GLOB decreased in three days after transplantation. Moreover, GLOB level dramatically decreased in PH+HepG2+IA group compared to PH+HepG2 group in three days after transplantation (*P<0.05).
Liver tumor model in pigs

in serum AFP level in the first week. Unfortunately, tumor cells did not implant in porcine livers at the end of six weeks. Our results were in general agreement with this study. In this study, human-specific AFP which are known to be secreted by human hepatoma cells was up to 39.5±20.28 ng/ml, and then the levels decreased slightly. AFP were detectable at the end of experiments even at very low level in serum. In view of raised AFP level at the first week, the fact of repopulation of engrafted cells in porcine livers is evident. Moreover, GFP-positive cells were found as individual cells or nodules in porcine liver until the end of experiments. In our study, 10/15 pigs showed evidence of tumor growth. The reason of our higher success rate might be a larger volume of hepG2 suspension cells.

Laparoscopic surgery has shown advantages to open surgery, including decreased hospital stays, less postoperative discomfort and complications. A previous study has shown that laparoscopic surgery could be safely used for islet cell transplantation [30]. It can be inferred that laparoscopic surgery may be applicable for other cell transplantation procedures. Our study indicates that laparoscopic surgery should be appropriate to implant human hepatoma cells in pigs.

Subsequent decrease in AFP value was probably related to rejection of tumor cells by porcine immune system, and this was confirmed by histology characteristics of liver tissues. Severe inflammatory cell infiltration which is a rejection feature was found in a number of the recipients in PH+HepG2 group after transplantation. Previously study showed that cell-mediated immunity played an important role in the rejection of xenografts [31]. Immunosuppressive agents are not only used in allograft rejection prevention, but also in pig-to-nonhuman primate xenotransplantation models [32]. Therefore, combined immunosuppressive agents were used to prolong survival of xenografts [33]. In this study, inflammatory cell infiltration was hardly observed in PH+HepG2+IA group after transplantation. IHC staining of GFP exhibited that HepG2 cells repopulated up to 2.79±1.17% of the liver parenchyma in PH+HepG2+IA group, which was significant higher than PH+HepG2 group. Moreover, PH+HepG2+IA group showed higher level of AFP than PH+HepG2 group in 12 weeks after transplantation. These results demonstrate that using of combined immunosuppressive agents could attenuate inflammation and prolongate survival of engrafted human hepatoma cells in porcine liver.

In summary, we have developed a large-animal human liver tumor xenograft model in the pig. Orthotopic implantation of human hepatoma cells using immunosuppressive agents leads to engraftment of tumor cells in porcine liver. Although we found that engrafted cells were still detectable in the recipient 126 days after transplantation, there was no macroscopic solid tumor at the end of experiment. Our study could be applied for the study of tumor cell proliferation and drug treatment. For successfully introducing pig liver models into the clinic, further works on multiple genetic modifications pigs (to overcome innate and adaptive immune responses) and more effective immunosuppressants are required.

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Disclosure of conflict of interest

None.

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References

Liver tumor model in pigs


Liver tumor model in pigs


Figure S1. Characterization of HepG2-GFP cells.