Reversible immortalization of human hepatocytes mediated by retroviral transfer and site-specific recombination

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Abstract

AIM: To establish a method for the reversible immortalization of human hepatocytes, which may offer a good and safe source of hepatocytes for practical applications.

METHODS: We successfully isolated primary human hepatocytes from surgically resected liver tissue taken from a patient with liver hemangiomas. The freshly isolated cells were then immortalized with retroviral vector SSR#69 expressing simian virus 40 large T antigen (SV40T) and hygromycin-resistance genes flanked by paired loxP recombination targets.

RESULTS: The freshly isolated hepatocytes with high viability (85%) were successfully immortalized using retroviral gene transfer of SV40T. SV40T in the immortalized cells was then excised by Cre/loxP site-specific recombination. This cell population exhibited the characteristics of differentiated hepatocytes.

CONCLUSION: We successfully established reversibly immortalized human hepatocytes, which will provide an unlimited supply of cells for practical applications.

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Key words: Hepatocyte; Primary human hepatocytes; Reversible immortalization; Hepatocyte isolation; SV40T

Core tip: It is meaningful to establish reversibly immortalized human hepatocytes which can be economically grown in tissue culture. Toward this goal, we successfully established a method for the reversible immortalization of human hepatocytes using Cre/loxP site-specific recombination, which may offer a good and safe source of hepatocytes for the bioartificial liver (BAL) system in the near future. If a sufficient number of human hepatocytes can be used, these extracorporeal devices will serve as successful “bridge-to-transplant” therapies. With the progress made in bioreactor development, the next-generation BAL system could reach the level of artificial kidney and save more patients.

Acute liver failure is associated with high mortality. The
current standard treatment for acute liver failure is orthotopic liver transplantation\[1,3,4\]. However, with the increasing shortage of organ donors, a considerable number of the patients die while still on the transplant waiting list. Among the liver assist therapies, bioartificial liver (BAL) therapy is considered the most promising solution to bridge them to liver regeneration or to liver transplantation\[5,6\]. Several BAL systems have been shown to result in significant improvements in survival in clinical applications\[7\]. In developing BAL therapy, normal human cells are an ideal cell source. However, the utility of primary cells is hampered by difficulties in timely obtaining the cells that have a limited lifespan in vitro. An attractive alternative source would be immortalized hepatocytes which would exhibit the characteristics of differentiated hepatocytes and make unlimited supplies of cells feasible\[8]. Simian virus 40 large T antigen (SV40T) is a typical tool used for cellular immortalization. However, it is dangerous to use cells possessing oncogenes in the clinical setting. SV40T in immortalized cells would expose patients to tumorigenic risk. An attractive solution to such risk would be the use of the novel reversible immortalization strategy by using Cre/loxP site-specific recombination\[9\]. Cells derived from such a procedure would have the advantages of freedom from infectious pathogens, uniformity, and unlimited availability.

We have made significant efforts to establish liver cell lines for cell therapies\[8-10\]. We described a novel strategy for establishing porcine reversibly immortalized hepatocyte lines. However, when using xenogenic porcine cells, there would be a concern that species-specific pathogens may be transmitted to recipients (such as infection by endogenous retroviruses). Therefore, it is meaningful to establish reversibly immortalized human cells that can make unlimited supplies in tissue culture using the techniques of gene transfer. Toward this goal, we have focused on human hepatocyte reversible immortalization by using Cre/loxP site-specific recombination. We hope to establish reversible immortalization of human hepatocytes which may offer a good and safe source of hepatocytes for the BAL system in the near future.

MATERIALS AND METHODS

Primary human hepatocyte isolation

Following ethical and institutional guidelines and after obtaining informed consent from the tissue donor, a sample was collected from a patient undergoing liver surgery. The patient was suffering from liver hemangiomata and required partial hepatectomy. We successfully obtained 32 g of liver tissue (Figure 1A). Primary hepatocytes were isolated under sterile conditions using a modified four-step retrograde perfusion technique as previously reported\[8\]. Briefly, the resected sample was first cannulated using a suitable pipette in a visible blood vessel on the cut surface (Figure 1A). Then the selected liver tissue was continuously perfused with a pre-warmed (37 °C) digestion buffer solution (8.4 g/L Dispase II and 0.5 g/L Collagenase IV, Sigma, St. Louis, MO, USA). Following sufficient digestion, the liver capsule was mechanically disrupted (Figure 1B) and the emerging cell suspension was filtered through a 250 μm nylon mesh and centrifuged at 50 g and 4 °C for 2 min (Figure 1C-D). The resulting suspension then underwent several wash steps. The resulting cell clumps were finally suspended in culture medium (William’s medium E, supplemented with 100 ml/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum). Hepatocyte yield and viability were determined using the traditional standard trypan blue exclusion technique. Finally, the freshly isolated hepatocytes were seeded at a concentration of 4-5 × 10⁶/mL in culture flasks. The medium was changed every 24 h. The morphology of the cultured hepatocytes was assessed using a Nikon Diaphot inverted microscope.

Immunofluorescent analysis of SV40T in immortalized human hepatocytes

Indirect immunofluorescent staining was performed to detect SV40Tag, using mouse monoclonal immunoglobulin G antibody to SV40Tag (Santa Cruz, CA, United States; Santa Cruz Biotechnology) and a secondary antibody, rhodamine (TRITC)-conjugated sheep anti-mouse IgG (Sigma, St. Louis, MO, United States). In order to stain nuclei (double-stranded DNA), DAPI (4',6-diamidino-2'-phenylindole dihydrochloride, Roche, Cat. No. 10236276001) blue-fluorescent dye was used.

Expression of liver-specific genes associated with liver function in immortalized human hepatocytes

Total RNA was extracted from immortalized human cells. According to the manufacturer’s protocol, reverse transcription-polymerase chain reaction (RT-PCR) was performed. Primers used were as follows: human albumin (576 bp), AAACCTCTTTGTAAGAGGACC (5’ primer) and CAAAGCAGGTCTCCTTATCG (3’ primer); human ASGR (495 bp), TAGGAGCCAAAGTGAGAAA (5’ primer), ACCTGCAGGCAGAAGT (3’ primer) (Table 1); SV40T (422 bp), CAG-
RESULTS

Successful isolation of primary human hepatocytes resulted in a cell yield of $6.56 \times 10^6$ cells/g liver tissue. The viability of cells immediately after isolation was 85%, as revealed by the trypan blue exclusion technique. The freshly isolated primary human cells attached to the plates showed the typical morphological appearance with granular cytoplasm, a polygonal shape and one or more nuclei (Figure 3).

After 100 μg/mL hygromycin selection within 2 wk, several clones (Figure 4B-C) grew steadily in the culture medium. These selected clones were then isolated by cloning rings. The selected cultured clones displayed the morphological characteristics of human cells featuring a large round nucleus with a few nucleoli and multiple granules in the cytoplasm. Treatment with Cre recombinase resulted in loss of proliferation of the expanded hepatocytes and they reverted to their pre-immortalized state. The SV40Tag in the nuclei of all immortalized cells and reverted cells was examined by immunofluorescent staining (Figure 5). Similar to normal primary human cells, immortalized and reverted human hepatocytes expressed the albumin gene, as shown by RT-PCR (Figure 6).

DISCUSSION

Liver transplantation is the optimal therapy for end-stage hepatic failure. However, due to donor shortage, liver transplantation is not available for a great number of patients with liver failure[11]. Recently, there has been a need to develop extracorporeal liver assist systems that can be used as a bridge to liver transplantation or for curative treatment of acute liver failure[2,13], which would represent a significant alternative to resolving the donor shortage[10,11]. Among these liver assist therapies[14], BAL therapy is the most promising technique[15-19]. The treatment efficacy depends on the bioactivity of the hepatocytes in the bioreactor[20]. Bioactive mass and cell
Table 1  Primers used in reverse transcription-polymerase chain reaction in this study

| Gene             | Forward (Tm)                                | Reverse (Tm)                               | Product size |
|------------------|---------------------------------------------|--------------------------------------------|--------------|
| SV40T            | CAGGCATAGAGTGTCTGC                         | CAACAGCTGTGGCATATG                         | 422 bp       |
| Human ASGPR      | TAGGAGCAGACAGCTGGAGAAGA                    | ACCTGCAGAGAAGAAGATCATC                     | 495 bp       |
| Human GST-Ⅱ      | GCTCTACACGTGGGTCTATT                       | GGCTAGGAACCTATGGATCA                       | 496 bp       |
| Human GS         | ATGGTGAGACATGCAAGATGGG                    | TCATTGAAAGACAGTGCC                        | 535 bp       |
| Human Albumin    | AATCTTGTGGGAAGAGCC                        | CAAAGACGTCCTCTTTATCC                      | 576 bp       |
| Human HBCF-X     | GTGCAATGGAAGAGAACCTGT                      | GAAGTCAACAGGTCAAGG                        | 493 bp       |
| Human β-actin    | TGACGGGGTCACCCACACTGTGCCCTATCTA           | AGAAGCATTTCGCTGACGATGGAGGG                | 610 bp       |

Human ASGPR: Human asialoglycoprotein receptor; GST-Ⅱ: Glutathione-S-transferase Ⅱ; GS: Glutamine synthetase; HBCF-X: Human blood coagulation factor X.

source or cell type play a key role in BAL treatment\cite{21}. In order to be used in patients, the BAL system should have adequate hepatocytes to provide sufficient bioactive support. The BAL device consists of 5 to 20 × 10^9 hepatocytes.

About 20% of healthy liver mass would be needed, known from partial hepatectomy studies. It contains about 20 × 10^9 (approximately 200 g) hepatocytes. Thus, theoretically, approximately 20 × 10^9 well-functioning hepatocytes are needed to keep a patient alive\cite{20}. In developing BAL therapy, normal human cells are an ideal cell source. However, the utility of cultured primary human hepatocytes is hampered by difficulties in timely obtaining populations which have a limited lifespan when cultured in vitro. Alternative sources of cells have been sought to overcome the limitation of primary human hepatocytes for BAL application.

To fulfill the requirements stated above, we attempted to establish easy-to-use human hepatocytes for BAL therapy. Theoretically, the ideal cells for a BAL system are normal human hepatocytes. However, there is a severe shortage of donated human livers for hepatocyte isolation. Furthermore, the cultured primary hepatocyte utility is hampered by difficulties in timely obtaining populations which cannot be expanded in vitro. Previously, we established an efficient procedure for primary porcine hepatocyte isolation using a modified four-step retrograde perfusion technique, which resulted in an im-
provement in cell viability and yield. We then expanded the method to primary human hepatocyte isolation. The majority of previous researchers used tissue from surgically resected liver specimens of malignant tumors for human hepatocyte isolation. We explored the possibility of isolating human hepatocytes from resected normal liver tissue.

With regard to hepatocyte cryopreservation, several cryopreservation methods for hepatocytes have been reported. Successful cryopreservation can be used for timely availability of hepatocytes. The availability and logistics of BAL systems would be significantly improved if methods for the long-term hepatocyte preservation, without loss of cellular activity, were developed. As reported previously, we developed a modification with a pre-incubation step prior to density gradient centrifugation. The modification allows rapid separation of viable hepatocytes from dead cells, but with less viable cell loss. However, hepatocytes are very sensitive to freezing damage. Hepatocyte functional activities after thawing are still unsatisfactory. Another alternative is primary porcine hepatocytes. Porcine livers are easily obtained and can be available on demand. However, porcine hepatocytes produce xenogeneic proteins, making this technique controversial.

Another attractive alternative source of cells for BAL.
The primary hepatocytes were immortalized by transfer of SV40Tag. After expansion of the immortalized cells, Cre/loxP recombination was performed to remove the oncogene (SV40T) and cells reverted to their preimmortalized state. The reversible immortalization procedure was devised by an oncogene transfer which can be subsequently excised effectively (Figure 7)\(^7\). As reported previously\(^8\), we established reversibly immortalized porcine hepatocytes by transfection with the retroviral vector SSR#69 (Figure 8).
With the progress in bioreactor development, next-generation BAL systems could reach the level of the artificial kidney and save more patients.

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Meng FY et al. Reversible immortalization of human hepatocytes

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