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The newly established human hepatocyte cell line: application for the bioartificial liver

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***Background/Aims*: Human hepatocyte cell lines are reported to lose many of their biochemical functions in a hybrid artificial liver support system (HALSS). Differentiation therapy is useful to up-regulate liver function.**

***Methods*: The human hepatoblastoma cell line HepG2 was transfected with HSV/tk gene. Albumin synthesis and ammonia removal activity were evaluated when HepG2/tk was cultured with histone deacetylase inhibitor (FR228) and peroxisome proliferator activated receptor-gamma ligand (pioglitazone). To investigate the function of HepG2/tk in vivo, cell transplantation for 90% hepatectonized rats was conducted.**

***Results*: We established stable cell lines which expressed HSV/tk and were sensitive to gancyclovir in vitro and in vivo. Both albumin synthesis rate and ammonia removal rate improved for HepG2/tk incubated with FR228 and pioglitazone for 3 days, which induced nuclear transport of p21. Rats with intrasplenic injection of HepG2/tk precultured for 3 days with FR228 and pioglitazone survived significantly longer than the control rats. The ammonia and total bilirubin concentrations were significantly lower in the test group than in the control group. The injection of gancyclovir inhibited the prolonged survival of the rats with precultured HepG2/tk.**

***Conclusions*: HepG2/tk is safe as well as enhancing high levels of liver function. It will be a potential cell source for HALLS in the future.**

***Keywords*: HepG2; Hepatoblastoma; Differentiation; Bioartificial liver; Histone deacetylase inhibitor; Peroxisome proliferator activated receptor-gamma ligand**

1. Introduction

Because it is estimated that 80% of patients with acute liver failure die while on the waiting list for liver transplantation [1], the development of liver support system is urgently needed for use as a bridge to transplantation [2–8]. Fulminant hepatic failure patients might benefit from temporary liver support techniques and this type of system might also allow the native liver to regenerate, thus avoiding the transplantation [9,10]. To date, various hybrid artificial liver support systems (HALSS) using porcine hepatocyte have been submitted for clinical trials [11,12], but Zoonosis issues have become more visible after the recent discovery that the pig genome harbours retroviruses that can infect human cells in vitro [13,14] and NOD/SCID mice in vivo [15]. Although there is no evidence of PERV infection in 160 patients who received porcine living cell therapy [16], the Public Health Service has strictly revised their guidelines regarding xenotransplantation, including HALSS [17]. Primary human hepatocytes are an ideal cell source for HALSS. Gerlach et al. [18,19] developed HALSS using human hepatocytes combined with albumin dialysis and hemodialysis, but the shortage of human livers limits this modality.

Human hepatocyte cell lines established from hepatocellular carcinomas can replicate well in vitro, and they are resistant to cryopreservation. It has also been reported that such cells lose many biochemical functions of hepatocytes, such as ammonia removal activity [20]. However, it has been reported that HepG2 can maintain certain biochemical

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functions of hepatocytes [21,22]. Sussman et al. [23] reported the efficacy of HALLS utilizing C3A (derived from HepG2) in hollow fiber, but they failed to demonstrate the efficacy in recent clinical trial [24]. We previously reported that HepG2 has high liver function and is useful for HALLS [25,26], but safety is needed for clinical use because they are cancer cell lines. Although hepatocytes were separated from the patient's blood in HALLS, there is the possibility of cell leakage from the membrane. To approve the safety for clinical use, induction of suicide gene such as herpes-simplex-virus thymidine-kinase (HSV/tk) that provides negative selection with gancyclovir (GCV) is often used [27].

Histone acetylation provides an enzymatic mechanism to regulate transcription by affecting the interaction between DNA and histones. Inhibitors of histone deacetylase (HDAC) have the capacity to inhibit cell growth while increasing gene expression [28]. Several HDAC inhibitors were previously studied as differentiating agents. We previously analysed the effects of trichostatin A (TSA), specific HDAC inhibitor, on human hepatoma cell lines such as HepG2 [29]. TSA induced hepatocyte differentiation in HepG2. In addition, FR228 is also HDAC inhibitor and is now in clinical use. Peroxisome proliferator activated receptor (PPAR) gamma is a member of the nuclear hormone receptor group. PPAR-gamma is highly expressed in adipose tissue and is important in adipocyte differentiation. Pioglitazone, one of the PPAR-gamma ligands, is an antidiabetic agent that improves hyperglycemia in obese and diabetic patients in clinical use. This ligand also inhibited cell proliferation in vitro and differentiation in cancer cells [30]. Our previous study demonstrated that low dose pioglitazone and FR228 (HDAC inhibitor) can result in HepG2 having more profound growth arrest and differentiation than either drug alone in vitro.

For the cell source of HALSS, HepG2 can grow well in vitro, which is convenient for sudden demand, but safety and liver function is not sufficient because of the hepatoma cell line. In this study, we established the human hepatoma cell line expressing HSV/tk and examined the effect of FR228 and pioglitazone on hepatocyte differentiation in vitro. Additionally, this drug-induced differentiated cell line was injected into the spleen of rat liver failure model and then we demonstrated the hepatocyte function in vivo.

2. Materials and methods

2.1. Cell culture and reagents

The hepatoma cell line HepG2 from Riken Cell Bank (Tokyo, Japan) was maintained in DMEM supplemented with 10% fetal bovine serum (JRH BIOSCIENCES, Australia), and 500 IU/ml penicillin-streptomycin. DMEM, Williams' E medium and penicillin-streptomycin were purchased from Gibco Co. Ltd (NY, USA). FR228 and FK506 were kindly provided by Fujisawa Pharmaceutical Co. (Osaka, Japan), and pioglitazone was kindly provided by Takeda Chemical Industries (Osaka, Japan). For the reverse transcriptase-polymerase chain reaction (RT-PCR), primer of HSV/tk and beta-actin were used in previous report [31]. For immunostaining, antibodies against alpha-fetoprotein (AFP) and albumin was purchased from DakoCytomation Co. Ltd (Glostrup, Denmark) and antibody against p21 was purchased from Cell Signalling Technology, Inc. (Massachusetts, USA).

2.2. Stable transfectant

The plasmid including HSV-tk gene which Dr Hasegawa [32] provided was transfected to HepG2 by lipofection (Invitrogen Co., CA, USA), following selection with G418 (800 μg/ml) for 2 weeks. Each of colonies was checked for HSV/tk expression using RT-PCR and specific cytotoxity to GCV. Among some colonies expressing HSV/tk mRNA, we selected one colony with the highest liver function in regard to the ammonia removal and albumin synthesis rate.

2.3. Cell proliferation assay

To analyze the effects of FR228 and pioglitazone, HepG2/tk was inoculated at a density of 3.0×10^5 viable cells per petri dish (diameter, 35 mm) without both drugs for the first 3 days in 2 ml of Williams' E medium containing 10% FBS. For the next 3 days, the medium with both drugs was changed every day. After 7 days, the medium without both drugs was changed every day. The concentration referred to the previous report (unpublished data).

2.4. Ammonia removal and albumin synthesis rate

To investigate ammonia removal activity, we added 1 mM NH_4Cl to Williams' E medium containing 10% FBS in all three petri dishes on culture days 1, 2 and 3. After the samples were cultured for 24 h, the medium was collected and ammonia in the medium was measured using Ammonia test Wako kit (Wako). The amount of albumin was also measured using ALBWELL II (EXOCELL, Inc, Philadelphia, USA). The cell concentration of each dish was determined by counting the nuclei using the method described by van Wezel [33].

2.5. Immunofluorescent and immunohistochemical staining procedure

For immunofluorescent staining, cultured cells on culture slide (BD Bioscience, Bedford, USA) were washed with PBS, fixed in 4% paraformaldehyde, then washed in PBS containing 0.1% Triton-X. The sections were incubated with primary antibodies, p21 and albumin. Alexsa488-labeled anti-rabbit IgG and Alexsa594-labeled anti-mouse IgG (Molecular Probes, Inc., Eugene, USA) were used as secondary antibody. Cells were viewed under confocal laser-scanning microscope Radiance2000 (Bio-Rad Laboratories, Inc., Tokyo, Japan). The avidin–biotin–peroxidase complex technique was used for immunohistochemical staining, described in the previous report [34].

2.6. Rat liver failure model with 90% hepatectomy

SD rats, between 180 and 220 g in body weight, were purchased from Charles River Japan, Inc. (Yokohama, Japan). For intrasplenic hepatocyte transplantation, small incision was made in the flank, and the spleen was exposed [35]. HepG2/tk (5×10^7) suspended in 1 ml of DMEM was injected into the spleen of rats using a 25-gauge needle. The blood flow in the splenic artery and vein was occluded for 5 min. We divided the rats into three groups according to the type of injection, only DMEM (group1), HepG2/tk (group 2) and HepG2/tk were precultured for 3 days with FR228 (1 ng/ml) and pioglitazone (10 μM) (group 3). After the injection, the site of the injection was ligated using 1-0 silk. All animals underwent 90% hepatectomy 1 day after transplantation and received daily intramuscular administration of FK506 (1 mg/kg) [35]. Ninety percent hepatectomy was described in the previous report [36]. After surgery, animals were injected with 10 ml 5% glucose subcutaneously. All in vivo experiments were performed under protocols approved by the University of Kyushu Animal Care and Use Committee.