Original Article

Three-dimensional spheroid culture of canine hepatocyte-like cells derived from bone marrow mesenchymal stem cells

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Introduction: Primary cultured hepatocytes are an important model for early safety evaluations of newly developed drugs. Many factors, however, hinder the wider applications of this technology, especially the difficulty to maintain these cells in long-term culture. To date, creating a stable supply of human or animal hepatocytes with proper hepatic function in vitro has not been achieved. Furthermore, frequently harvesting hepatocytes from living donors for use in culture is highly invasive and simply not feasible. We have previously reported that canine bone marrow-derived mesenchymal stem cells (cBMSCs) can be effectively converted into induced hepatocyte-like cells (iHep cells); however, these cells had reduced function in comparison to mature hepatocytes. In recent studies, spheroid formation-based three-dimensional (3D) culture has been noted to greatly increase hepatocyte function; nevertheless, no reports have described the use of this technology for culturing canine hepatocytes. Therefore, in this study, we aimed to establish a 3D spheroid culture using converted canine iHep cells to investigate their function as hepatocytes.

Methods: The iHep cells were prepared by introducing two genes, namely, the Forkhead box A1 (Foxa1) and hepatocyte nuclear factor 4 homeobox alpha (Hnf4a), into cBMSCs seeded onto an ultra-low attachment microplate to induce spheroid formation. Thereafter, the hepatic functions of these spheroids were evaluated using immunocytochemistry, as well as qualitative and quantitative PCR.

Results: Notably, albumin was observed in the iHep spheroids and the expression of hepatic genes, such as albumin and drug metabolism CYP genes, could also be detected. Another interesting finding was evident upon further comparing the quantified albumin gene and CYP2E1 gene expressions in the two-dimensional and three-dimensional culture systems; notably, a 100- to 200-fold increase in gene expression levels was observed in the three-dimensional spheroids when compared to those in conventional monolayers.

Conclusions: Upon incorporating three-dimensional technology, we managed to achieve iHep spheroids that are closer in gene expression to living liver tissue compared to conventional monolayer cultures. Thus, we are one step closer to creating a sustainable in vitro hepatocyte model. Furthermore, we believe that this system is capable of maintaining the stable drug metabolizing capacity of canine hepatocytes in vitro, which might be useful in improving current drug assessment studies.

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1. Introduction

Various diseases, including hepatic portal system shunts, hepatitis, and cirrhosis, commonly occur in dogs and other canines, and often require veterinary intervention, especially for the smaller animals [1]. However, an effective therapeutic regimen for these illnesses is crucial in the veterinary setting, as current treatment approaches unfortunately yield subpar results. Although the liver is very regenerative, this in itself is insufficient to maintain the expression levels of hepatic function-related genes including CDH, ALB, and CYP, which are decreased during long-term culture [2]. In fact, in humans and mice alike, drug enzyme activity and albumin expression were not observed in hepatocyte monolayer cultures. Indeed, several recent studies have demonstrated that three-dimensional cultures are capable of sufficiently promoting these liver functions and the expression levels of their associated genes, as well as maintaining them for a long time. A spheroid refers to an aggregate which consists of hundreds to thousands of cells. Hepatocyte spheroids are superior to their monolayer counterparts in terms of high cell density and cell–cell interactions and can perform complex activities such as albumin synthesis and drug metabolism (P–450) in vitro. Moreover, these spheroids can be maintained for a relatively long time in culture [5]. Previous studies have shown that 3D culture conditions are suitable for maintaining the hepatic properties of cultured hepatocytes because they mimic the in vivo environment [5]. Therefore, the function of canine liver tissue should be assessed in three-dimensional structures. Here, we aimed to establish a three-dimensional spheroid culture system to assess basic hepatic function, including ALB, CDH, and CYP expression, in canine iHep cells and hopefully provide a better means for drug-safety assessment.

2. Materials and methods

2.1. Animals

Four healthy Beagle dogs (mean age, 5.0 ± 2.7 years) were involved in this study. All animal experiments were conducted based on the laboratory dynamic experiment guidelines of Azabu University (No 160706–1). The animals were housed at a small animal breeding facility in the Azabu University Veterinary Clinical Center. The dogs were clinically healthy, without a medical history, and were not administered any drugs within one month of the start of the experiments.

2.2. Preparation of cBMSCs

The animals were sedated by administering butorphanol tartrate (0.02 mg/kg IM) and medetomidine hydrochloride (0.04 mg/kg IM), following which bone marrow specimens were collected from their femurs and ilia using a bone marrow aspiration needle.

The bone marrow specimens were filtered through a 100-μm cell strainer (BD, Franklin Lakes, NJ, USA) to remove any clots. A hemolytic agent, comprising 83 g/L NH₄Cl, 8.4 g/L KHCO₃, and 3.7 g/L EDTA · 2 Na, was dissolved in 10 × distilled sterilized water. After adding the hemolytic agent to the filtered specimens, the mixture was allowed to stand at room temperature for 5 min and separated by centrifugation at 300g for 5 min. Next, the supernatant was removed and the cell sediment was mixed with phosphate-buffered saline (PBS; Gibco, MA, USA), followed by two additional centrifugations (300 g at 5 min each). The cell sediment was finally suspended in 1 mL of PBS and the number of viable cells was measured by using trypan blue staining.

The bone marrow cells were seeded in a 25-cm² flask (1.0 × 10⁷ cells) and cultured in Dulbecco’s modified Eagle medium (DMEM; Sigma–Aldrich, MO, USA) supplemented with 10% foetal bovine serum (PBS; Gibco, MA, USA) and 1% penicillin–streptomycin solution (Fujifilm, Wako, Osaka, Japan). These primary cells were cultured in basal medium supplemented with 2 mM L-glutamic acid (Fujifilm, Wako, Osaka, Japan) and incubated at 37°C and 5% CO₂.

The medium was initially changed four days after seeding the cBMSCs and was thereafter replaced with fresh medium every three days. Adherent cells established on the bottom of the flask were
designated as cBMSCs. Moreover, passaging was carried out when these cells reached a confluence of approximately 80–90%. After counting the number of viable cells using trypan blue staining, we mixed the cells with a cryopreservation solution and stored them in a cryogenic bunker (Nippon Zenyaku Kogyo, Fukusimuma, Japan) according to the manufacturer’s protocol. This cell cryopreservation solution was used for seeding in the differentiation experiments.

2.3. Preparation of the retroviral vector

The Foxa1 and Hnf4a genes were cloned into the retroviral vectors pGCDNsam–Foxa1 and pGCDNsam–Hnf4a (Addgene, Watertettort, MA, USA), respectively, which were then transfected into the Platinum-A retroviral packaging cell line (Cell Biolabs, Inc, Watertown, MA, USA), which were then transfected into the Platinum-A retroviral packaging cell line (Cell Biolabs, Inc, San Diego, CA, USA) to prepare the required retroviral solutions for cBMSC transfection.

2.4. Induction of differentiation from cBMSCs to hepatocyte-like cells

P0 to P2 cBMSCs were seeded in collagen I-coated 12-well plates (Corning, New York, USA) at a density of 5 × 10^4 cells/well and cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C and 5% CO2. On the following day, a viral mixture, obtained by adding protamine sulphate (5 μg/mL) (Nacalai Tesque, Kyoto, Japan) to the prepared Foxa1 and Hnf4a viral solutions, was added to the culture medium. Next, the solution of each plate was spun at 700 g for 10 min at room temperature and then incubated at 37 °C and 5% CO2. After 8 h, the culture supernatant was removed by aspiration and the medium was changed. Culture and gene transfection experiments were performed three to four times (n = 3 or 4) for each canine BMSC sample.

The viral transfection process was maintained for three consecutive days. The amount of virus-containing solution used on the second and third days was 1.5 times the amount used on the first day. When replacing the medium on the final day of viral transfection, it was replaced with hepatocyte growth medium (HGM: DMEM/Nutrient Mixture F-12; Gibco, MA, USA) containing 10% FBS and 1% penicillin-streptomycin. The medium was changed on the following day as well, and incubation at 37 °C and 5% CO2 was continued for 8 h, followed by a replacement of the medium. Culture and gene transfection experiments were performed three to four times (n = 3 or 4) for each canine BMSC sample.

2.5. Spheroid culture of iHep cells

The adherent iHep cells in culture were detached from the cell culture flask with Accutase (Innovative Cell Technologies, Inc, San Diego, CA, USA) and collected. The iHep cells were then counted using a hematocytometer after adding trypan blue dye, seeded at 3000 cells/well in a sterile 96-well Black/Clear Round Bottom Ultra-Low Attachment Spheroid Microplate (Corning, New York, USA), and cultured at 37 °C under 5% CO2 for three days. During the culture, the same amount of HGM that induced iHep cells was added to the medium.

2.6. Reverse transcription (RT) polymerase chain reaction (PCR) and quantitative RT-PCR

Total RNA was extracted using a RNeasy Micro kit (Qiagen, Tokyo, Japan). RT was performed using random primers and a high capacity RNA to cDNA kit (Thermo Fisher Scientific, Waltham, MA, USA). This was followed by PCR using Takara ExTaq Hot Start polymerase (Takara Bio, Shiga, Japan) and the following cycling parameters: initial denaturation at 94 °C for 3 min followed by 35 cycles at 95 °C for 15 s, annealing at the primer-specific temperature for 30 s, and extension at 72 °C for 1 min followed by a final extension at 72 °C for 5 min. PCR products were resolved on a 2% agarose gel stained with ethidium bromide and observed using an ultraviolet transilluminator (ATTO, Tokyo, Japan).

As for the quantitative RT-PCR, total RNA was first extracted using the RiboPure RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). RT was performed using the PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). Quantitative RT-PCR was performed in duplicate using a Thermal Cycler Dice RealTime System II (Takara Bio) with a TB Green Premix Ex Taq TM II (Takara Bio), according to the manufacturer’s instructions; the used primer sequences are listed in Table 1. The following cycling parameters were considered: denaturation at 95 °C for 3 min followed by 40 cycles at 95 °C for 5 s and annealing at the primer-specific temperature (60 °C or 65 °C) for 30 s.

| Table 1 | Primer list used for quantitative PCR. |
|---------|----------------------------------------|
| Gene    | F/R | Sequence (5’→3’)                       | Product size (bp) |
| canine Alubumin | F   | GTT CCT GGG CAC GTT TTT GTA TGA        | 278               |
| canine β-actin | F   | CTT GCG GTG CTT TCG TTG TGT AAC        | 100               |
| canine CDH1  | F   | AAA TGG GCC TTT CTT GCT T | 77               |
| canine AFP   | F   | TGG GCT CAT ATT TCC CAG T | 100               |
| canine TTR   | F   | GAG TGG CAC GAG CAC AGC AG | 182               |
| canine CYPI2E1 | F   | CAG GCC AGC ATG TTC AGA CCA | 116               |

F: forward primer, R: reverse primer.
2.7. Immunocytochemistry

The medium was aspirated and the cells were washed three times with PBS to thoroughly remove any remaining medium. The cells were then fixed with 4% paraformaldehyde phosphate buffer for 10 min and washed again with PBS (three times). Next, membrane permeation treatment was carried out for 5 min using PBS containing 0.1% Triton X-100, which was followed by another three PBS washes. Blocking was then performed by adding PBS containing 5% skim milk and the cells were allowed to stand at room temperature for 60 min before they were incubated with goat polyclonal anti-dog albumin antibody (A40-113A; Bethyl Laboratories Inc., Montgomery, TX, USA) overnight at 4 °C.

The cells were then incubated with their corresponding secondary antibodies; donkey anti goat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 (A-11055; Thermo Fisher Scientific) for 30 min in the dark. The slides were observed under a confocal microscope after adding VECTA SHIELD mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA).

3. Results

3.1. Canine iHep cells were capable of forming spheroids

Spheroids were noted in almost all wells one day after cell seeding and had an average diameter of approximately 200–300 μm (Fig. 1). In addition, the spheroids formed exhibited strong cell–cell interactions and were notably clumped together; in fact, these interactions were so strong that the spheres could not be dissociated by pipetting. Therefore, iHep cells derived from gene-transfected cBMSCs are capable of forming spheroids.

3.2. Liver-related gene levels were significantly higher in iHep spheroids

According to our qualitative PCR findings, the liver-related genes ALB, alpha-1 antiproteinase (A1-AT), alphafetoprotein (AFP), tyrosine aminotransferase (TAT), transthyretin (TTR) were 2D (adhesion) and 3D (Spheroid) Expressed in cultured iHep cells. Among the cytochrome P450 member (CYP2E1 and CYP3A12) genes, CYP2E1 was expressed only in 3D cultured iHep cells. CYP3A12 was not expressed in any cells.

CYP2E1, the gene encoding a drug-metabolizing enzyme, was almost undetectable in 2D culture, contrasting our observation in the spheroids (Fig. 2). Accordingly, these results indicated that liver-specific mRNA is increased by converting iHep cells into spheroids. Our findings were further confirmed by comparative quantitative PCR results. Notably, the expression levels of CDH1, ALB, CYP2E1, AFP, and TTR increased by approximately 2 times, 75 times, 166 times, 13 times, and 5 times in 3D cultures compared to those in 2D iHep cells, respectively (Fig. 3).

3.3. Albumin levels in iHep monolayers and spheroids were comparable

Albumin is a protein that is highly expressed in the liver, and its expression was confirmed using immunocytochemistry. Evidently, albumin was observed in both iHep spheroids and monolayer iHep

![Fig. 1. Morphology of iHep cells and spheroids. Morphologies of (a, b, c) mock-infected cBMSCs and (d, e, f) iHep cells. Canine iHep cells exhibited a circular to equilateral-circular morphology and successfully formed colonies, the boxed area is a magnified image (e) The mock group, however, consistently exhibited a spindle-shape morphology throughout the culture. Day, days after transfection of cells. Scale bars (a, b, d, e) 200 μm (c, f) 7.5 μm.](image-url)
cells (Fig. 4); however, this was not the case in either of the mock groups, as albumin was not observed in iHep spheroids or monolayers.

4. Discussion

In this study, we demonstrated that iHep cells originating from cBMSCs can be successfully cultured as spheroids, and thus, these may serve as a new source of hepatocytes for toxicity evaluation systems in the drug discovery field. Notably, iHep spheroids could be easily prepared using ultra-low attachment spheroid microplates. Although liver-specific gene expression and albumin expression levels were detected in both 2D and 3D cultures, both their levels and functions were greatly increased in the spheroids, suggesting the effectiveness of this technology in drawing out the natural potential of iHep cells by more accurately mimicking their indigenous environment. We also found that iHep cells in 2D culture had the greatest albumin expression and drug-metabolizing enzyme mRNA expressions on day 10 after gene transfer, which thereafter gradually decreased with time. However, the expression levels of many other factors, including albumin, cadherin, and others, were low compared with those in mature hepatocytes. Therefore, other culture conditions such as adding growth factors or cytokines are required to optimise iHep 2D culture; efforts to investigate such culture methods are underway. As for improving the function of spheroids, many approaches may be effective such as opting for a liver maturation medium, changing the medium more frequently after mature spheroid formation, or changing the

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**Fig. 3.** Gene expression analysis of iHep cells using comparative quantitative RT-PCR. The expression levels of iHep in both 2D and 3D cultures were normalized with respect to cBMSCs (Day 0), respectively. Day, days after transfection of cells. P values were calculated based on a t-test, comparing gene expression levels of iHep cells and cBMSCs (mock) with those of cBMSCs (Day 0), and Excel Toukei (SSRI) was used as analysis software. *; Significant difference was when $p<0.05$.

**Fig. 4.** Immunostaining analysis demonstrated the differentiation of cBMSCs into iHep cells. Mock-infected cBMSCs, iHep cells, and canine adult liver cells were stained with anti-albumin antibodies (green). In contrast, nuclei were stained with DAPI (blue) (a, d) Mock infected cBMSCs (b, e) iHep cells and (c) adult canine liver. In addition (a, b) 2D cultured cBMSCs and (d, e) 3D cultured iHep cells were shown. The area enclosed by white lines was magnified at the lower left of each panel.
At this stage, iHep spheroids are inferior to normal hepatocytes; however, we believe that they hold great potential and may become suitable in future studies. The main reason behind the enhanced effect seen in 3D spheroid cultures lies in the ability of these cells to form aggregates by CDH1-mediated cell–cell adhesion, thereby activating transcriptional coupling factors through the activation of the Hippo-YAP signalling pathway. Accordingly, YAP inactivation by phosphorylation has been found to increase the levels of Hnf1α, a hepatocyte nuclear factor, and promote iHep cell maturation, thereby improving the function of these cells [13]. Therefore, it is possible that the enhanced effect seen in the canine spheroids may be also attributed to YAP phosphorylation.

In the present study, we believe that high expression of CDH1 improved the adhesion of our hepatocyte-like cells and was the main impetus behind the successful spheroid formation in canine iHep cells. Many recent studies highlighted the importance of CDH1 in hepatocyte cultures [14,15]. Moreover, Junpei et al. investigated the role of intercellular adhesion in the inactivation of YAP during iHep cell-aggregate formation to further understand the role of the ubiquitously expressed CDH1 in the intercellular regions of iHep aggregates [13]. Furthermore, intercellular adhesion of CDH1 is inhibited in EDTA-treated iHep cells, resulting in a notable inhibition of CYP1A2 and CYP7A1 expression [13]. Overall, our results in this study suggest that high expression of CDH1 contributed to the success of the 3D culture.

To date, several factors have been reported to play crucial roles during cytoskeleton formation. Spheroid formation is a complex process that involves cell migration and morphological changes, which indicate cytoskeletal element reorganization. In previous studies, treating cultured rat hepatocytes with cytochalasin D, which targets actin filaments, caused an inhibition of spheroid formation [15]. Furthermore, Junpei et al. reported that treating mouse iHep cells during cell aggregate formation with the actin polymerization inhibitor cytochalasin D (CytoD) inhibited both YAP inactivation and cell aggregate formation. In contrast, adding the actin de-polymerization inhibitor jasplakinolide (JAS) restored cell-aggregate formation; nonetheless, it did not activate YAP [13]. Moreover, both Cyto D- and JAS-treated iHep cells have been shown to exhibit markedly lower expression levels of CYP1A2 and CYP7A1 than those in control iHep cells. Considering that these factors have not been analysed in the present study, future studies should take a more mechanistic approach to better understand these factors and hopefully improve the efficiency of three-dimensional culture.

Regrettably, the current study is not free of limitations, as we could not perform important hepatic function analyses in iHep cells, such as investigating LDL and urea production, due to technical problems. In addition, it is necessary to examine how long the iHep spheroids can sustain their functions in culture, as this is necessary for long-term cytotoxicity studies. We speculate that promoting angio genesis in the spheroids by adding vascular endothelial cells may prolong spheroid survival and increase their function, and this may be also achieved by using an extracellular matrix such as Matrigel [16]. Furthermore, the homology between mature hepatocytes and 3D-cultured cells has not been analysed using microarray and will be necessary in the future.

5. Conclusions

In summary, we showed in this study that even canine iHep spheroids have significantly increased expression levels of albumin and CYP when compared with those in their 2D-cultured counterparts. Based on these findings, we believe that iHep spheroids will be useful in the drug discovery field by offering a new platform for performing toxicity evaluations with stable drug metabolism functions. However, iHep spheroids are still relatively obscure and a part of their functions and characteristics remain unknown. Thus, more studies are necessary to further understand the mechanisms underpinning these spheroids to effectively implement them in toxicity studies.

Declaration of competing interest

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