NOTE
Clinical Pathology

Human placental hydrolysate promotes the long-term culture of hepatocyte-like cells derived from canine bone marrow

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ABSTRACT. Long-term culture of canine artificial hepatocytes has not been established. We hypothesized that human placental hydrolysate (hPH) may support the long-term culture of differentiated hepatocyte-like cells. Canine bone marrow cells were cultured using modified hepatocyte growth medium supplemented with hPH. Quantitative reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemical analysis for albumin, qualitative RT-PCR for cytochrome P450 1A1 (CYP1A1), hepatocyte growth factor (HGF), Cytokeratin 7 (CK7), CD90, CD44, and CD34, and functional analyses of CYP450 activity and low-density lipoprotein (LDL) uptake were performed. Cultured hepatocyte-like cells were able to maintain hepatocyte characteristics, including morphology, albumin synthesis, CYP450 activity, and LDL uptake for 80 days. Thus, hPH may be a potential facilitator for the long-term culture of hepatocyte-like cells. Clinicopathologically, this culture protocol of artificial hepatocytes will contribute to liver function evaluation.

KEY WORDS: bone marrow, dog, hepatocyte, long-term culture, placenta

Clinicopathologically, the evaluation of the toxic effect of liver function is highly dependent on serum hepatic enzymes, such as alanine aminotransferase (ALT), or serum bile acid test, but these are not specific markers [7]. Therefore, new parameters to

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The loss of liver function due to drug toxicity or viral infection related to acute or chronic hepatitis or cirrhosis is a major cause of death in humans and dogs [1, 29]. Canine liver failure is common and is caused by hepatitis, portosystemic shunts, and hepatic cirrhosis, whose mechanisms are similar to those in humans, at a molecular and cellular level [6, 9, 17, 29]. Liver transplant is the gold standard therapy for liver failure in humans; however, the shortage of liver organ donors and the adverse effects increase the need for alternatives. Hepatocyte transplantation using differentiated hepatocytes from induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs), or mesenchymal stem cells (MSCs), as well as liver bud transplantation have been proposed for humans and dogs [10, 21, 27]. However, an efficient and long-term cell culture of primary and artificial hepatocytes for practical use and transplantation has not been established in humans or dogs.

Long-term hepatocyte culture techniques are also required to establish artificial hepatocytes for pharmaceutical toxicity screening [25]. Primary hepatocyte cultures use is generally considered the gold standard for liver-based in vitro toxicity testing [30]. However, the long-term culture of primary hepatocytes is largely impeded by the progressive loss of the hepatocyte-specific morphological and functional phenotype [3, 18, 22]. Multiple culture conditions have been investigated to improve the long-term survival of hepatocytes or artificial hepatocytes, maintaining an appropriate liver function. Zhang et al. developed a defined medium using human liver isolation medium with no Rspo1, Noggin, and forskolin, to expand a large quantity of functional primary human hepatocytes for more than a few months [31]. Mouse ESCs and iPSCs were cultured with supplements, including cytokines and sodium butyrate, and efficiently differentiated into functional hepatocytes in vitro, after 20 days [32]. However, the long-term hepatocyte culture methods are limited and far from practical use in transplantation and pharmaceutical toxicity studies.

Placenta is a rich source of many biological components, including hormones, cytokines, chemokines, and growth factors required for liver regeneration [11, 12]. Previously, we found that human placental hydrolysate (hPH) may be an effective inducer of hepatic differentiation of canine bone marrow cells (cBMCs) [19]. The complex mixture of amino acids, peptides, DNA bases, carbohydrates, and cytokines in hPH may contain potential cofactors to induce the differentiation of cBMCs into hepatocytes. In fact, placental growth factor upregulation, which is observed in regenerating livers, is correlated to liver growth [28]. Furthermore, hPH contains HGF, epidermal growth factor (EGF), and fibroblast growth factor, and has been shown to induce liver regeneration in rats, HepG2 cells, and human primary hepatocytes [15].

Clinicopathologically, the evaluation of the toxic effect of liver function is highly dependent on serum hepatic enzymes, such as alanine aminotransferase (ALT), or serum bile acid test, but these are not specific markers [7]. Therefore, new parameters to
evaluate the toxic damage or function of hepatocytes need to be established. Long-term survival of functional cBMCs-derived hepatocyte-like cells using hPH may be able to support in vitro pharmacologic testing of new drugs, as well as the detection of novel biomarkers for canine liver disease.

We hypothesized that hPH may support the long-term culture of differentiated hepatocytes. To confirm our hypothesis, we cultured cBMC-derived hepatocyte-like cells for 80 days in hepatocyte growth medium (HGM) containing hPH and assessed their morphology, gene and protein expressions, and hepatic function.

cBMCs were collected from clinically healthy dogs (2–5-year-old beagles, n=4) under sedation with butorphanol tartrate (0.2 mg/kg IM) and medetomidine hydrochloride (0.04 mg/kg IM). Animal care and handling were in accordance with the Azabu University Animal Experiment Guidelines. All experiments were reviewed and approved by the Ethics Committee of Azabu University (approval number: 180220-1). The marrow cells were separated using Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) and inoculated at a density of 1 × 10^6/cm². The marrow cells were separated using Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) and inoculated at a density of 1 × 10^6/cm². The marrow cells were separated using Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) and inoculated at a density of 1 × 10^6/cm². The marrow cells were separated using Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) and inoculated at a density of 1 × 10^6/cm². The marrow cells were separated using Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) and inoculated at a density of 1 × 10^6/cm².

For the qualitative and quantitative RT-PCR, total RNA was extracted from cultured cBMCs and hepatocytes using the RiboPure kit (Applied Biosystems, Foster City, CA, USA) and 100 ng of total RNA was converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems). Synthesized cDNA was amplified using RT-PCR. The primer sequences and amplification conditions are listed in Table 1

| Accession number | Sequence (5′–3′) | Product size (bp) | Amplification conditions of qualitative RT-PCR |
|------------------|------------------|------------------|-----------------------------------------------|
| Alb              | F:gtt ctc ggg caa gtc ttg tga | 278              | 3 min at 95°C, followed by 36 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and 5 min at 72°C |
| HGF              | F:gtt gct ata ctc ggc acc cat g | 286              | 3 min at 95°C, followed by 36 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and 5 min at 72°C |
| CYP1A1           | F:ccct gcc caa ctc ata aat ccc c | 90               | 5 min at 95°C, followed by 36 cycles at 95°C for 20 sec, 60°C for 30 sec, and 30 sec at 60°C |
| CK7              | F:gcct ggg gac ccc tga aca | 109              | 5 min at 95°C, followed by 36 cycles at 95°C for 20 sec, 55°C for 30 sec, and 30 sec at 60°C |
| CD90             | F:cag gct ggc cct gga gaa | 134              | 3 min at 95°C, followed by 36 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and 5 min at 72°C |
| CD44             | F:tcc caa act cca act cc | 177              | 5 min at 95°C, followed by 40 cycles at 94°C for 1 min, 59.5°C for 2 min, 72°C for 1 min, and 5 min at 72°C |
| CD54             | F:gcg cgg cgg cgg cgg | 550              | 3 min at 95°C, followed by 36 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and 5 min at 72°C |
| β-actin          | F:ctc acc atc acc ccc tgg | 143              | 3 min at 95°C, followed by 36 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and 5 min at 72°C |

F, forward primer; R, reverse primer.

The morphologies of cultured cBMC-derived hepatocyte-like cells were imaged using confocal microscopy with a Zeiss LSM 880 microscope (Zeiss, Jena, Germany). In addition, the expression of albumin, CYP1A1, and β-actin was confirmed using Western blotting with rabbit polyclonal antibodies against dog albumin (Bethyl Laboratory Inc., Montgomery, TX, USA), dog CYP1A1 (Abcam, Cambridge, MA, USA), and canine β-actin (Sigma Aldrich, St. Louis, MO, USA), respectively. The cells were cultured at 37°C and 5% CO₂ in 0.2 ml/ml of UltraPower DNA Safedye (Gellex International Co., Ltd., Tokyo, Japan). Canine β-actin was used as an internal control.

Canine β-actin was used as an internal control. Data were analyzed using the 2−ΔΔCT method. Statistical analysis was performed using the Mann-Whitney test. P values <0.05 were considered statistically significant and all calculations were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

immunocytochemical staining was performed on cBMCs cultured for 80 days. Cells were fixed for 30 min with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C. Next, antigen activation with PBS containing 0.1% Triton X-100 was performed for 4 min at room temperature. To block internal peroxidase, slides were incubated in methanol with 3% H₂O₂ for 15 min at room temperature. After blocking with 10% skim milk containing PBS at room temperature for 1 hr, the slides were incubated at 4°C overnight with goat anti-dog albumin polyclonal antibody (Bethyl Laboratory Inc., Montgomery, TX, USA) as the primary antibody [dilution of 1:200 with Dako REAL antibody diluent (Dako, Glostrup, Denmark)]. Then, slides were incubated in 0.2 M glycin-containing PBS at room temperature for 30 min before incubation with the secondary antibody, goat MAX PO
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Histofine; Nichirei, Tokyo, Japan), at room temperature for 30 min. Between each step, the slides were washed with PBS. The slides were visualized with 3,3′-diaminobenzidine (DAB; Nichirei) and counterstained with Dako REAL hematoxylin (Dako).

To test hepatocyte function, pentoxyresorufin was used to determine cytochrome P450 (CYP450) activity and the low-density lipoprotein (LDL) uptake was measured as previously described [19] in hepatocyte-like cells cultured for 80 days. The number of albumin- and F-actin-positive cells per field of cultured cBMCs was counted at × 400 magnification, and the positive cells: total cells ratio was averaged from 10 different fields.

Here, cBMCs from a total of 4 dogs cultured in hPH for over 80 days were able to maintain hepatocyte characteristics, including morphology (Fig. 1), whereas without hPH in the culture medium, cells had a fibroblast-like morphology, which did not change, and they did not express albumin mRNA during culture (data not shown). Cultured cBMCs attached to the bottom of the flask had a spindle-like shape on day 7 and had become polygonal (pentagonal)-shaped, resembling mature hepatocytes, on day 21. They retained their polygonal shape until day 80 (Fig. 1). F-actin was detected cortically within the cell membrane (approximately 85% of cells) in hepatocyte-like cells on day 21 (Fig. 2). Immunocytochemical staining of albumin revealed the presence of albumin within the cytoplasm of 10–15% of the hepatocyte-like cells on day 80 (Fig. 2).

Gene expression analysis of the cultured cBMCs using qualitative RT-PCR analysis revealed the expression of albumin, CYP1A1, and CK7 in cultured hepatocyte-like cells on days 21 and 80. Albumin expression on day 21 showed slight variation, for example, 3 of 4 samples were positive and only one sample was negative. HGF, CD90, and CD44 were detected in cBMCs, as well as in hepatocyte-like cells on days 21 and 80. CD34 was only detected in cBMCs. Hepatocytes from adult canine liver expressed albumin, HGF, CYP1A1, and CK7 (Fig. 3A).

Quantitative RT-PCR revealed that the mRNA expression of albumin was increased in hepatocyte-like cells on days 14, 21, 28, and 80, compared to cBMCs; statistically significant differences were observed between cBMCs and day 28 hepatocyte-like cells, and cBMCs and day 80 hepatocyte-like cells, with approximately 3 times the albumin mRNA level, compared to that of day 14 cultured cBMCs (P=0.02; Fig. 3B).

Pentoxyresorufin is a nonfluorescent compound that is O-dealkylated by CYP450 into resorufin and emits red fluorescence. Hepatocyte-like cells showed a markedly increased fluorescence within the cytoplasm (69% of cells) on day 80 (Fig. 4B). LDL uptake is represented by the red fluorescent dye, indicating Dil, in which acetylated low density lipoprotein, labeled with 1,1-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL) is taken into the cell and metabolized, and was

Fig. 1. The morphology of cultured canine bone marrow cells (cBMCs) on days 7 (A), 21 (B), 60 (C), and 80 (D). cBMCs attached to the bottom of the flask were spindle-like shaped on day 7, and became polygonal (pentagonal), adult hepatocyte-like cells, on days 21, 60, and 80. Scale bars represent 20 μm.
detected as a moderate red fluorescence within the cytoplasm of hepatocyte-like cells (20% of cells) on day 80 (Fig. 4D). No fluorescence was detected in cultured cBMCs on day 80 (Fig. 4A, 4C).

In the present study, hepatocyte-like cells differentiated from cBMCs were cultured for a long period of 80 days using hPH. On day 21, cultured cBMCs were polygonal-shaped, resembled hepatocytes, and maintained morphology throughout the 80 days of culture. F-actin was detected within the cellular membrane on day 21, which further confirmed that the hepatocyte-like cells had epithelial characteristics.

Interestingly, the albumin mRNA and protein were consistently expressed in hepatocyte-like cells until day 80, and hepatocyte function, including LDL synthesis and CYP function, were detected on day 80. Albumin is a protein synthesized by hepatocytes and is a well-known hepatocyte marker [25]. Hepatocyte markers that reflect hepatic function include those involved in drug metabolism, such as CYP450 and LDL uptake [8, 19]. The presence of these markers indicates that hepatocyte-like cells have some characteristics of canine hepatocytes and can maintain their features in the long term. Albumin mRNA expression was significantly detected as a moderate red fluorescence within the cytoplasm of hepatocyte-like cells (20% of cells) on day 80 (Fig. 4D). No fluorescence was detected in cultured cBMCs on day 80 (Fig. 4A, 4C).

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increased in hepatocyte-like cells compared to cBMCs and did not change significantly from day 21 to day 80. The main challenges regarding culture of primary hepatocytes or artificial hepatocytes are the short survival and the loss of an appropriate hepatic function. A recent study showed that mouse primary hepatocytes lost the ability to synthesize albumin after 7 days of culture [14]. The activity of CYPs (CYP1A2 and CYP3A4) expressed by ESC-derived hepatocytes was significantly lower than that of primary hepatocytes [33] and a number of liver-essential functions were progressively lost with time, in cultured primary hepatocytes [4]. Additionally, directed differentiation of mouse bone marrow cells into hepatocyte-like cells was reported; however, the culture time was 21 days [23]. CYP activity in human hepatocyte-like cells derived from bone marrow cells was observed on days 21 and 28; however, evaluation of CYP activity has not been performed in a long-term culture [24]. Here, we proposed that hPH culture conditions would allow differentiated hepatocyte-like cells to survive longer, while maintaining hepatic function.

HGF, CK7, CD44, and CD90 are highly expressed in hepatic progenitor cells and liver tissues and, here, they were expressed in differentiated hepatocyte-like cells on day 80 in a long-term culture. HGF is essential growth factor for liver regeneration and is synthesized by hepatocytes and mesenchymal cells. Here, HGF was not supplemented, and hPH contains only a low amount of HGF, which may not be enough for hepatocyte-like cells to regenerate. HGF is synthesized by bone marrow stromal cells and stimulates liver regeneration in an autocrine and paracrine manner [10, 20, 26]. Here, HGF produced by cBMCs may have contributed to the regeneration and maintenance of hepatocyte characteristics for a long period of 80 days. CK7 mRNA was not expressed in cBMCs and was only detected in differentiated hepatocyte-like cells from day 21 to day 80. CK7 expression is observed in bile duct epithelial cells in the liver and in other epithelial cells, such as the acini and ductal epithelial cells of the salivary gland and anal sac gland, and the transitional cells of the urinary bladder; however, it is not expressed in hepatocytes [5]. These findings indicate that cBMCs can differentiate not only into hepatocyte-like cells, but also other epithelial cells. CD44 and CD90 are surface markers expressed in mesenchymal stroma cells derived from cBMCs [16] and are also liver progenitor markers [13]. Here, CD44 and CD90 were observed in cBMCs and in differentiated hepatocyte-like cells until day 80. Persistent expression of CD44 and CD90, which are stem cell markers, may contribute to the long-term survival of hepatocyte-like cells. The progenitor cell population expressing CD44 and CD90 might be the source for the production of mature hepatocytes.

Although it was not clear in the present study, there are a few possible reasons why hPH may contribute to the prolonged period of hepatocyte-like cell culture. hPH contains HGF, EGF, stem cell factor, amino acids, and interleukins (IL1 and IL6) [15]. hPH promotes liver regeneration by activating cytokines and growth factors associated with liver regeneration and eliminating oxidative stress.
stress [15]. Additionally, hPH exhibits a protective role in hepatocyte apoptosis by inhibiting oxidative stress and maintaining cell homeostasis [2]. Collectively, these factors may affect the long-term culture of hepatocyte-like cells. However, the precise level of each component in the hPH used here is unknown, since some of the factors in hPH were not measured correctly because of the low concentration of these components.

There are some limitations of this study: the albumin expression level in our cultured hepatocyte-like cells was only 1/10^5–1/10^7, much lower than in hepatocytes (data not shown), which emphasized the necessity for further improvement of the culture conditions for the hepatocyte-like cells to have an appropriate hepatic function. Moreover, additional effective components necessary for the long-term culture of hepatocyte-like cells in hPH should be elucidated. The next step for the effective long-term culture of hepatocytes or hepatocyte-like cells would be to use 3D cultures and iPSC or directly reprogrammed cells as the cell source.

Here, cBMC-derived hepatocyte-like cells were able to maintain hepatocyte characteristics for a long period, and the potential contribution of hPH was indicated. Clinico-pathologically, the establishment of this long-term culture protocol for cBMC-derived hepatocyte-like cells will significantly contribute to establish new parameters to evaluate liver function. Further studies are warranted to investigate the individual hPH components and mechanisms of action to establish the optimal culture conditions for hepatocytes or hepatocyte-like cells.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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