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Canine bone marrow cells differentiate into hepatocyte-like cells and placental hydrolysate is a potential inducer

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ABSTRACT

Hepatocyte growth factor (HGF) can stimulate human and rat bone marrow (BM) cells to differentiate into hepatocytes. A human placental hydrolysate (hPH) stimulates proliferation of hepatocytes, but its role as a potential inducer of BM cells to form hepatocytes is unclear. To determine if canine BM cells stimulated with HGF or hPH differentiate into hepatocyte-like cells, BM cells were cultured with HGF or hPH. The cultured cells underwent morphological examination, expression of albumin and cytokeratin 18 (CK18), hepatic function tests including uptake of low-density lipoprotein (LDL) and cytochrome P (CYP) 450 activity. Albumin mRNA and protein expression of albumin and CK18 proteins were detected in cultures with HGF and hPH. Furthermore, these cells demonstrated LDL uptake and CYP450 activity. These results indicate that canine BM cells can differentiate into hepatocyte-like cells when stimulated by both HGF and that hPH may be an effective inducer of hepatic differentiation.

1. Introduction

Canine liver failure is a common disease seen in veterinary small animal practice, and hepatitis, hepatic portal systemic shunts and hepatic fibrosis often occur in dogs (Allen et al., 1999; Rutgers et al., 1993; Watson, 2004). As treatment of these illnesses is limited, an effective therapeutic regime would be very useful. Although, the liver has marked regenerative capacity, in severe disease, its ability to regenerate is decreased. Liver transplantation is often the only therapeutic option for dogs with severe hepatic failure. However, transplantation has many problems, including the lack of donors, the stress of surgery, and tissue rejection. In particular, a lack of knowledge regarding canine leukocyte antigens prevents successful liver transplantation from a donor. Thus, an alternative therapy is necessary.

The differentiation of bone marrow (BM) cells into hepatocytes has been reported (Petersen et al., 1999; Theise et al., 2000). Moreover, transplanted BM cells have been shown to restore biochemical function in recipients with progressive liver failure, and hepatic cells differentiated from BM cells protect against or can ameliorate the fibrosis induced by CCl_4 in recipient rats (Oyagi et al., 2006; Zhao et al., 2005). These results suggest that transplantation of hepatocytes, differentiated from bone marrow cells, is a feasible treatment option for dogs with severe liver failure.

Several growth factors, including hepatocyte growth factor (HGF), epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), and fibroblast growth factor-4 (FGF-4), have been studied as potential inducers of BM cell differentiation into hepatocytes, both *in vitro* and *in vivo* (Ishikawa et al., 2006; Oh et al., 2000; Miyazaki et al., 2002; Schwartz et al., 2002; Shi et al., 2005). HGF, a natural ligand for c-Met, has mitogenic, motogenic, and morphogenic activities for various cell types and functions as an organotrophic factor for regeneration of the liver, kidney, and lungs (Borowiak et al., 2004; Panis et al., 1998). HGF induces the expression of c-Met mRNA in BM cells, indicating that it may affect the function of BM cells (Miyazaki et al., 2002; Neo et al., 2005; Oh et al., 2000). However, evidence of hepatocyte differentiation from canine BM tissue has not been clearly demonstrated.

Interestingly, human placental hydrolysate (hPH) has been shown to induce liver regeneration in rats (Nakayama et al., 1989). Several growth factors known to be important in hepatic differentiation, including HGF, EGF, and FGF, are contained in hPH. Because human placental hydrolysate contains a complex mixture of various kinds of amino-acids, peptides, DNA bases, carbohydrates, and cytokines, it may have the potential to induce the differentiation of BM cells into hepatocytes. Accordingly, we examine the effects of HGF, EGF, and hPH on the differentiation of canine BM cells into hepatocytes.

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2. Materials and methods

2.1. Bone marrow cells and cell culture conditions

BM cells were collected from humeri and femora of clinically healthy dogs (5- to 6-year-old beagles, $n = 6$) under anaesthesia. The animals received humane care complying with the guidelines for treatment of experimental animals at Azabu University. The marrow cells were inoculated at a density of $8 \times 10^5/cm^2$ in 25-cm collagen-coated plastic dishes for RNA extraction or 4-well collagen-coated chamber slides for immunochemistry. The cells were cultured in a CO_2 incubator (5% CO_2 in humidified air) at 37 °C. Modified HGM medium (Miyazaki et al., 2002) supplemented with fetal bovine serum (10%) was used as the basal medium. The cells were cultured for 7, 14, 21, or 28 days. The medium was changed every 3 days, and a fresh aliquot of EGF (Sigma, St. Louis, MO, USA) with feline recombinant (fr) HGF (kindly provided by Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan) (Miyake et al., 2004) or hPH (Laennec; kindly provided by Japan Bio Products Co., Ltd., Tokyo, Japan) was added to the HGM medium. Three culture conditions were used: HGM medium supplemented with 10% FBS and EGF (20 ng/ml); HGM medium supplemented with 10% FBS, EGF, and frHGF (1 μg/ml); and HGM medium supplemented with 10% FBS, EGF, and hPH (0.2 ml/ml).

2.2. Total RNA isolation and detection of albumin mRNA

Total RNA was extracted from cultured BM cells using an RNA isolation reagent, and 1 μg of total RNA was reverse transcribed to produce the cDNA template using the reverse primers of canine albumin cDNA and β-actin, and AVM reverse transcriptase (Takara Bio Inc., Kyoto, Japan). The cDNA template was amplified by denaturation at 94 °C for 1 min, followed by five cycles of 94 °C for 30 s, 65 °C for 1 min, and 72 °C for 1 min; then five cycles of 94 °C for 30 s, 62 °C for 45 s, and 72 °C for 45 s; and lastly, 50 cycles of 94 °C for 30 s, 57–60 °C for 45 s, and 72 °C for 45 s. Primers were designed based on canine albumin cDNA (GenBank accession number AB090854 [sense: tcttgctgaggtggaaagag, antisense: agactaaggcagcttgagca]). Canine β-actin was used as an internal control. Amplified PCR products were purified (QIAquick PCR purification kit, Qiagen, Hilden, Germany) and subcloned into a pCRII plasmid vector (Invitrogen, Carlsbad, CA, USA). DNA sequencing of plasmids was performed using an Applied Biosystem Model 310 sequencer (Applied Biosystems, Foster City, CA, USA) and the dideoxy-mediated chain-termination method.

2.3. Immunocytochemical staining

On day 28, cells cultured on collagen type I-coated chamber slides were fixed for 24 h with 10% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature. To block internal peroxidase, slides were placed in methanol with 3% H_2O_2 for 30 min at room temperature. After blocking with normal rabbit serum at room temperature for 1 h, the slides were incubated at room temperature for 4 h with a goat anti-dog albumin polyclonal antibody, at a dilution of 1:200 with PBS–BSA (BETHYL Laboratory Inc., TX, USA) or a mouse anti-human cytokeratin 18 (CK18) monoclonal antibody at a dilution of 1:100 (Progen Biotechnik, Heidelberg, Germany). Afterward, the slides were incubated at room temperature for 20 min with a biotin-labelled goat anti-rabbit IgG or biotin-labelled rabbit anti-mouse IgG, A, and M (Histofine; Nichirei, Tokyo, Japan). Then, cells were reacted with HRP-conjugated streptavidin at room temperature for 10 min. Between each of the above steps, the slides were washed with PBS. The colour was visualized with 3-3′-diaminobenzidine (DAB; Nichirei) and counterstained with Lilly–Mayer hematoxylin.

2.4. Pentoxyresorufin assay

The cytochrome P (CYP) 450 activity was estimated using a 7-pentoxyresorufin O-dealkylation (PROD) assay. The hepatocyte-like cells at $2 \times 10^4/cm^2$ were plated on collagen type I-coated chamber slides and cultured in HGM medium containing frHGF or placental hydrolysate for 28 days. On the day of the PROD assay, the culture medium was removed, HGM medium containing 50 μM pentoxyresorufin (Molecular Probes Inc., CA, USA) and 2% DMSO (v/v) was added, and the cells were incubated for 4 h at 37 °C. Then, the cells were washed three times with PBS. The nuclei were stained by DAPI (Vectorshield® with DAPI, Vector Laboratories, Burlingame, CA, USA). The fluorescence intensity caused by PROD metabolism was used to estimate CYP450 activity on day 28. Detection and assessment of resorufin *in situ* was performed using confocal microscopy. Fresh hepatocytes were obtained from the liver tissue of healthy dogs as a positive control.

2.5. LDL assay

Cells were cultured as for the pentoxyresorufin assay. To assay LDL uptake, the culture medium was removed on day 28, and HGM medium with 10 μg/ml of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil–Ac–LDL, Biochemical Technologies Inc., Stoughton, MA, USA) was added and incubated for 4 h at 37 °C. After staining with DAPI, the LDL uptake was estimated using confocal microscopy.

2.6. HGF/c-Met signal pathway inhibition

The HGF/c-Met signal pathway was inhibited by synthesized protein according to the procedure reported by Bardelli et al. (1999). The synthesized protein, which suppresses c-Met tyrosine kinase selectively, contains 35 amino-acid sequences (H–RQIKIWFQNRRHKWKKIGEHFVHVNATFVNVKCVA–OH). This sequence contains a sequence of mouse c-Met tail region (Y1349 and Y1356) with modification including tyrosine into phenylalanine. This peptide was added in group B and group C at 20 μM at every medium change.

3. Results

3.1. Morphology of differentiated BM cells

When BM cells were cultured with frHGF, small numbers of quadrilateral cells were observed among fibroblast-like cells on day 21, and the percentage of quadrilateral cells gradually increased until day 28. In control cells cultured with EGF but without HGF or hPH, fibroblast-like cells were detected on day 3 on the bottom of the flasks, and no change in cell morphology was observed by day 28 (Fig. 1A and B). In cells cultured with hPH, quadrilateral cells began to appear at the bottom of the flasks on day 10, and the percentage of polygonal cells resembling mature hepatocytes gradually increased (Fig. 1C). On day 28, polygonal cells occupied most of the bottom of the flasks.

3.2. Albumin mRNA expression

Expression of albumin mRNA was examined by RT-PCR on days 7, 14, 21, and 28 of culture. Albumin mRNA was detected on day 28 in BM cells cultured with frHGF, whereas albumin mRNA was detected on day 14 in cells cultured with hPH. A weak but clear band was detected from cells cultured with frHGF, whereas a stronger signal was detected from cells cultured with hPH. The sequence

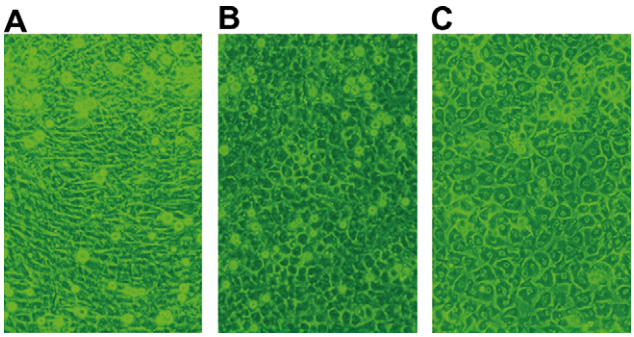

Fig. 1. Primary bone marrow cell cultures (day 28). Bone marrow cells were cultured for four weeks in different media. A: HGM medium supplemented with 10% FBS + EGF. B: HGM medium supplemented 10% FBS + EGF + feline recombinant HGF. C: HGM medium supplemented with 10% FBS + EGF + human placental hydrolysate (hPH). Quadrilateral cells and polygonal cells are numerous in B and C, respectively (magnification 200× for all pictures).

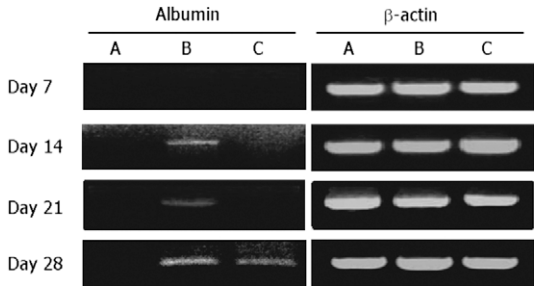

Fig. 2. Expression of albumin mRNA in BM cells cultured under various conditions. RT-PCR was performed on days 7, 14, 21, and 28. Expressions of albumin (left) and β-actin used as the internal control (right) are shown. A: HGM medium supplemented with 10% FBS + EGF. B: HGM medium supplemented with 10% FBS + EGF + hPH. C: HGM medium supplemented with 10% FBS + EGF + feline recombinant HGF. Albumin mRNA was detected on day 14 in lane B and on day 28 in lane C, but not in lane A.

of the amplicon was identified as a partial cDNA of canine albumin (data not shown). No PCR product was amplified from BM cells cultured without frHGF or hPH (Fig. 2).

3.3. Immunocytochemical characterization

The presence of albumin and CK18 on day 28 of BM cell culture was confirmed by immunocytochemical staining. Clear and strong staining for albumin were observed on day 28 in the cytoplasm of most cells treated with hPH and frHGF. The albumin expressing cell ratio of hPH and HGF is 90.5% and 30.0%, respectively. Moderate staining for CK18 was detected on day 28 in the cytoplasm of some cells treated with frHGF and in most cells treated with hPH. The CK18 expressing cell ratio of hPH and HGF is 39.8.5% and 26.8%, respectively. No staining of these proteins was observed from the BM cells cultured without frHGF or hPH (Fig. 3).

3.4. Functional characterization

Pentoxyresorufin is a nonfluorescent compound that is O-dealkylated by CYP450 into resorufin to emit a red fluorescence. At one week after cell induction, cells stimulated with frHGF and hPH both showed light to moderate fluorescence, but cultures without these factors did not produce obvious fluorescence. The fluorescence of cells induced by both frHGF and hPH was maintained until day 28, while a negative reaction was observed in cultures without these factors (Fig. 4A–C).

LDL uptake was shown as slight to moderate fluorescence in undifferentiated cells and the cells induced by frHGF and hPH from day 0 to 7 (data not shown). On day 28, significant and obvious fluorescence was detected in cells cultured with frHGF or hPH, while the simultaneous control culture showed a negative reaction (Fig. 4E–G). Normal hepatocytes showed clear fluorescence in both the LDL uptake and CYP450 assays.

3.5. HGF/c-Met signal pathway inhibition

Furthermore, we confirmed that hepatic differentiation was suppressed completely when the HFG/c-Met signal pathway was inhibited by synthesized protein. In both group B and group C, morphological hepatic differentiation was not observed under microscopy, and albumin mRNA expression was not detected during culture (days 14, 21, and 28).

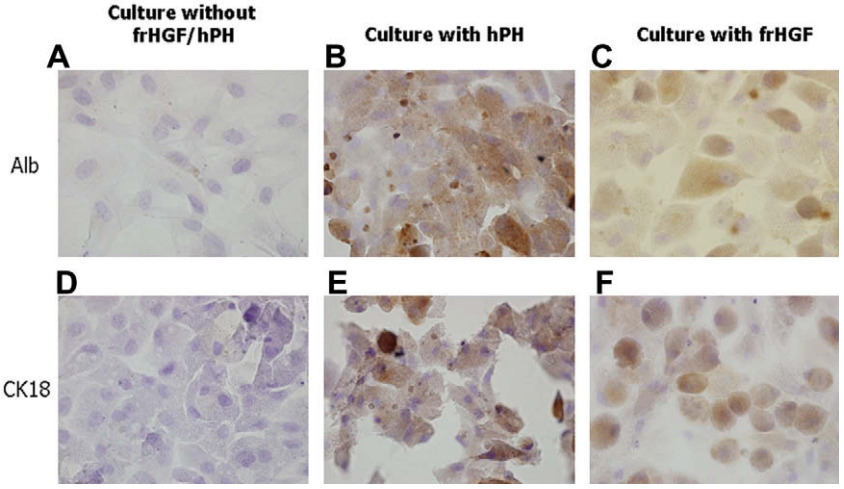

Fig. 3. Immunocytochemical staining of albumin and CK18 in cultured BM cells under various conditions. A and D: HGM medium supplemented with 10% FBS + EGF. B and E: HGM medium supplemented with 10% FBS + EGF + hPH. C and F: HGM medium supplemented 10% FBS + EGF + feline recombinant HGF. Immunofluorescent staining of BM cells with anti-human albumin polyclonal antibody (A–C) and anti-human CK18 monoclonal antibody (D–F). Albumin was strongly positive in cells cultured with hPH (B) and moderately positive in cells cultured with frHGF (C). CK18 was strongly positive in cells cultured with hPH (E) and moderately positive in cells cultured with frHGF (F) (magnification 400× for all pictures).

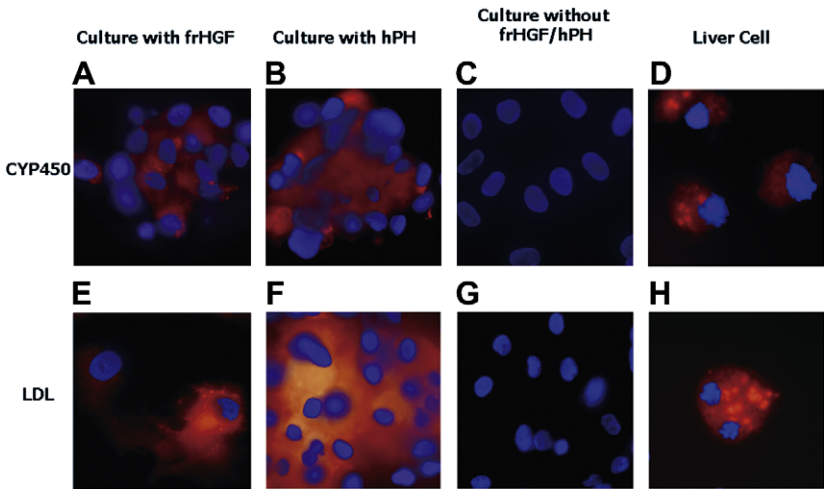

Fig. 4. *In vitro* functional characterization of hepatocyte-like cells differentiated from bone marrow cells. Uptake of LDL and activity of cytochrome P450 (CYP450), indicated by red fluorescence, were detected in differentiated cells at day 28 post-induction by frHGF (A and E) or hPH (B and F). Nuclei were stained by DAPI (blue), and the photomicrographs were overlaid with those of LDL and CYP450 fluorescence. Positive reactions of these functions were not detected in control cultures without frHGF and hPH on day 28 (C and G). Normal canine hepatocytes as a positive control showed LDL uptake and CYP450 enzyme activity (D and H) (magnification 1000× for all pictures) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

We show that canine BM cells are differentiated into hepatocyte-like cells when cultured with HGF or placental hydrolysate, *in vitro*. These cells are morphologically similar to hepatocytes, express albumin and CK18, and function as hepatocytes. Albumin is a protein synthesized in hepatocyte cells, and CK18 is an epithelial protein that is distributed as a cytoplasmic filament network and as a band just below the microfilaments surrounding the plasma membrane in hepatocytes (Van Eyken and Desmet, 1993). Therefore, albumin and CK18 are well known as markers for hepatocyte expression, and the proteins or mRNA expression levels are often used to confirm the differentiation of a hematopoietic cell lineage into hepatocytes (Lange et al., 2005; Okumoto et al., 2006; Wang et al., 2004). In this study, these proteins and albumin mRNA were expressed in cells induced by both frHGF and hPH. However, several reports have shown that human and rat mesenchymal stem cells, or multipotent progenitor cells derived from BM, expressed these markers, so the significance of these gene expressions as a hepatocyte-specific marker is controversial (Shu et al., 2004). On the other hand, no study has clarified the significance of these markers in canine BM cells and hepatocytes, and our data showed that canine BM cells did not express albumin and CK18. Thus, the expression of albumin and CK18 may be quite low or absent in the canine BM cell.

The BM-derived hepatocyte-like cells induced by frHGF or hPH in this study demonstrated hepatic functions because these cells showed LDL uptake and CYP450 activity. Some studies revealed that hepatocyte-like cells derived from human multipotent adult progenitor cells or mesenchymal stem cells stimulated with HGF showed mature hepatocyte function (Lee et al., 2004; Schwartz et al., 2002). The liver is recognized as the major site of the terminal catabolism of lipoproteins. Dil–Ac–LDL is taken into hepatocytes via LDL receptors. Dil is a fluorescent substance that is released from LDL due to the metabolism of lysosome enzymes in hepatocytes and expresses fluorescence. In this study, both the cells stimulated by HGF and hPH showed distinct fluorescence on day 28 of culture, indicating that these cells express LDL receptors like mature hepatocytes.

CYP450 plays a role in the oxidative metabolism of xenobiotics as well as many endogenous substances, such as fatty acids and steroids, principally within liver tissue. CYP450 activity was seen from day 7 of culture, and this activity was maintained for three weeks in cells cultured with HGF and hPH. Schwartz reported that CYP450 activity in human and murine cells was detected on day 28 and 18 of culture, respectively (Schwartz et al., 2002), but the CYP450 activity of cultures in our study appeared earlier. However, the cell source, composition of the medium and growth factors, and concentrations of HGF and other growth factors were different in our study, so the reason is unclear. Effective culture conditions and combinations of growth factors for efficient hepatic differentiation have not been identified, and an adequate protocol is needed (Stieger et al., 2006; Kanazawa and Verma, 2003).

Appropriate conditions that effectively induce BM cells to differentiate into hepatocytes are currently under study, including the type of medium, cell densities, and type of inducers. The HGM medium used in this study was previously shown to be an efficient medium for active growth of hepatocytes when supplemented with HGF, EGF, transforming growth factor-α, and phenobarbital (Block et al., 1996; Miyazaki et al., 1998). HGM medium contains various factors, including nicotinamide, dexamethasone and proline that are known to stimulate hepatocyte DNA synthesis and cell growth and support the survival of primary hepatocytes. In addition, hepatocyte-like cell colonies grown by differentiation of rat BM cells in HGM medium are known to express the terminal differentiation markers tryptophan oxygenase and tyrosine aminotransferase, implying that they are mature hepatocytes (Miyazaki et al., 2002). Thus, HGM culture medium is useful to induce differentiation of hepatocytes from BM cells.

In our study, canine BM cells cultured with frHGF were induced to differentiate into hepatocyte-like cells, while the cells cultured with EGF did not express hepatic markers and lacked hepatic function. These results indicate that HGF has an important role in hepatic differentiation of canine BM cells. Because HGF induced differentiation of mouse, rat and human BM cells into hepatocytes, HGF may play an important role in liver regeneration in several species (Oh et al., 2000; Miyazaki et al., 2002; Schwartz et al., 2002). Furthermore, we confirm that hepatic differentiation is suppressed completely when the HFG/c-Met signal pathway is inhibited by synthesized protein, according to the procedure reported by Bardelli et al. (1999) (data not shown). This result indicates that HGF is an essential factor in establishing hepatic differentiation in

the dog, as it does in humans and rodents. In this study, frHGF was used in the hepatic induction experiment as a stimulator instead of canine HGF because an effective recombinant canine HGF is not available and canine and feline HGF have a 97.5% amino-acid homology (Miyake et al., 2003).

We used mouse anti-human cytokeratin 18 monoclonal antibody for ICC analysis, because there is no appropriate canine CK18 antibody. Human CK18 has a 77.0% amino-acid homology compared with canine CK18. A recent report has demonstrated that CK18 was detected immunohistochemically, in the normal canine skin and cutaneous adnexal tumors, using the same antibody as in this study (Kato et al., 2007). In addition, the cross-reactivity of this cytokeratin 18 monoclonal antibody has been confirmed using canine hepatocellular carcinoma cell line in our laboratory.

Interestingly, placental hydrolysate dramatically induced hepatocytes from BM cells, such as frHGF, and the findings indicated that hPH contains a remarkable activator to differentiate hepatocytes. A commercial placental hydrolysate, Laennec, contains various factors such as FGF, NGF, IL-1, IL-6, leptin and dehydroepiandrosterone that have the capacity to promote proliferation of hepatocytes (Liu et al., 1998; Nakamura et al., 1983). Because only a small amount of HGF is present in Laennec (final concentration of 0.026 ng/ml according to the manufacturer), factors other than HGF may affect canine BM cell differentiation. Some studies revealed that members of the fibroblast growth family (FGF), such as FGF2 and FGF4, play important roles in the induction of hepatic differentiation from BM cells or mesenchymal stem cells, and Laennec contains fibroblast growth family members (Ishikawa et al., 2006; Miyazaki et al., 2002; Oh et al., 2000; Schwartz et al., 2002; Shi et al., 2005). Furthermore, Somerset suggested that the placenta contains HGF-activator (HGF-A), which is a circulating serine protease that induces trophoblast DNA (Somerset et al., 2000). Thus, hPH was considered to be an effective new agent for hepatic cell differentiation. Identifying the most important factor in hPH for induction of hepatic differentiation may provide valuable information for more efficient hepatic induction.

In conclusion, this study demonstrates the differentiation of canine BM cells into hepatocyte-like cells by addition of hPH to the culture medium. This may indicate a potential and effective source of inducers that will support liver regeneration by hepatocytes originating from BM cells. Establishment of a method for differentiation of canine BM cells into hepatocyte-like cells may provide a new practical therapy for canine patients with severe liver disease. However, further studies are required to analyze the expression of other hepatocyte-specific genes, cell phenotypes, functions and morphology using electron microscopy in order to clarify the character of these induced hepatocyte-like cells. Furthermore, such studies should help to clarify the important factors needed to promote hepatic differentiation in the placenta. This is the first report to demonstrate hepatic differentiation of canine BM cells and a significant first step in regenerative therapy of canine liver disease in small animal practice.

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