Efficient replacing of fetal bovine serum with human platelet releasate during propagation and differentiation of human bone marrow-derived mesenchymal stem cells to functional hepatocytes–like cells

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Objectives The aim of this study was to find out substitution effect of fetal bovine serum (FBS) with human platelet releasate (HPR) as a major growth factor source during expansion and differentiation of human bone marrow–derived mesenchymal stem cells (hBMSC) into hepatocytes.

Methods Propagation and differentiation potential of hBMSCs into hepatocyte–like cells in a medium fortified with HPR instead of FBS were investigated with morphological, cytochemical and molecular experiments.

Results Multiplex analysis showed that HPR was more efficient than FBS in supporting hBMSC outgrowth. The proliferation rate of MSC in presence of HPR (derived from $10^9$ platelets/ml) was about threefold greater than that of FBS ($P < 0.001$). Despite the differences in MTT value, hBMSCs–driven HPR or FBS did not differ in terms of gross morphology, immunophenotype and osteogenic differentiation potential. Hepatic differentiation of hBMSCs was successfully performed in the media enriched with HPR. Immunoreactivity of cells with monoclonal antibodies against for albumin and α-fetoprotein (AFP) was even more positive in hepatocytes differentiated in presence of HPR as compared to that of FBS. The gene expression of albumin, AFP and cytokeratin–18 at mRNA levels in differentiated cells attest to supporting role of HPR in hepatic differentiation media. These findings were further confirmed with greater urea production (approximately twofold) in the culture media of cells differentiated under HPR compared to that in FBS ($P < 0.001$).

Conclusion Human platelet releasate is an efficient and safe substitute for FBS in culture media used for expansion and differentiation of hBMSCs to hepatocyte.

Key words: differentiation, fetal bovine serum, hepatocytes, mesenchymal stem cells, platelet releasate.

Introduction

The studies in stem cell biology have shown that mesenchymal stem cells (MSC) can differentiate into hepatocytes [1–3].

However, the challenge remains to develop robust protocols to generate hepatocytes from bone marrow MSCs for the treatment of liver diseases. Traditionally, fetal bovine serum (FBS) has been used for expansion and differentiation of MSCs to different lineages. However, certain risks associated with the use of FBS have stimulated a search for alternatives.

The transmission of variant Creutzfeld–Jacob disease through ingestion of bovine material created awareness of
the possibility of FBS carrying prions or other zoonotic agents, but the risk of transmission of diseases via FBS is considered to be small [4]. A greater risk associated with the use of MSC cultivated in FBS seems to be the immunogenicity of FBS proteins [5]. Indeed, MSC transplantation failure has been noted as a consequence of immune attack on FBS proteins carried by transplanted cells expanded in FBS [6,7]. Taking into consideration the platelet secretory factors [8–10], it appears that substitution of FBS with human source during human stem cell manipulation would be advantageous. Johansson and co-workers demonstrated that platelet lysate (PL) medium supported cell growth and maintained viabilities comparable or superior to FBS [11]. Indeed, studies using MSCs and platelet-derived products were often dependent on FBS-supplemented culture [5,12–15], but most recently, two studies have proposed using of human PL as an FBS replacement for clinical-scale expansion of MSCs [16,17]. These studies and the report by Doucet and co-workers [18] demonstrated that MSCs cultured in the presence of PL maintain their osteogenic, chondrogenic and adipogenic differentiation potential. Despite the recent progress in replacement of FBS with PL, the effect of human platelet products in development of MSCs to different lineages, such as hepatocytes, has not been investigated. In this study, the substitution effect of FBS with human platelet releasate (HPR) on differentiation potential of human bone marrow MSCs (hBMSCs) to active hepatocytes has been investigated. Cellular and molecular characteristics of the differentiated cells were used to check the performance of cells derived from hBMSCs.

Materials and methods

Chemicals and reagents

Dulbecco’s modified Eagle’s medium (DMEM), FBS, glutamine, antibiotics, 0·25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution were obtained from Gibco BioCult (Paisley, Scotland, UK). Enzyme-linked immunosorbent assay (ELISA) quantification kits for growth factors were purchased from R&D systems (Minneapolis, MN, USA). Antibodies for flow cytometric assay were purchased from Dako (Glostrup, Denmark) and eBioscience (San Diego, CA, USA). RNA extraction kit, cDNA Synthesis Kit and materials for polymerase chain reaction (PCR) amplification were from Qiagen ( Valencia, CA, USA) and Fermentas (Hanover, MD, USA). Quantitative kits for urea and alkaline phosphatase (ALP) activity were obtained from Pars Azmun (Iran). Hepatocyte growth factor (HGF), dexamethasone (DEX), oncostatin M (OSM), mouse antihuman antibodies for albumin and α-fetoprotein (AFP), goat antimouse fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G (IgG), Alizarin red staining kit, cell growth determination kit (MTT kit) and other reagents were the products of Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of human platelet releasate

Approximately 450 ml whole blood was collected from 10 donors who had provided informed consent. Tests for sterility (bacterial or fungal contamination) and routine testing of blood donors were performed in Iranian Blood Transfusion Organization. Then, bloods were centrifuged at 1000 g for 15 min at 20°C to separate platelet rich plasma. The plasma was centrifuged at 3000 g for 10 min at 20°C to obtain platelet pellet. The platelet concentrate was dissolved in phosphate buffer saline (PBS), pooled and incubated at room temperature for 30 min on a rotating platform to eliminate platelet agglomerates. Platelets were counted using Sysmex K-1000 (Kobe, Japan). Then autologous thrombin was prepared as per the method of Lucarelli et al. [15]. In this step, 330 μl of calcium gluconate (100 mg/ml) was added to 10 ml of plasma. Then, 1 ml of thrombin preparation was added to 4 ml of platelet concentrate and incubated for 1 h at room temperature to facilitate growth factors release. The platelet releasate was centrifuged at 4000 g for 5 min to reduce the presence of platelet membrane fragments. The supernatant was filtered with a 0.22-μm pore filter, divided into aliquots and frozen at −70°C for further use.

Isolation and primary culture of human bone marrow mesenchymal stem cells

Bone marrow aspirates (10 ml) were obtained from iliac crests of human donors (n = 4) within the age range of 19–32 years. The samples were collected at the Bone Marrow Transplantation Center, Shariati Hospital, Tehran, Iran, after the informed consent was obtained according to the guidelines of the Medical Ethics Committee, Ministry of Health, I.R. Iran. Based on the previous reports [19,20], mesenchymal stem cells were isolated from bone marrow as described below. Briefly, the aspirates were diluted with PBS. The mononuclear cell layer was isolated by Ficoll–Hypaque (D = 1.077 g/ml), washed in PBS, resuspended in growth medium containing DMEM—low glucose supplemented with 15% FBS, 2 μg/ml glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin, and plated in 75-cm² plastic cell culture flasks. The cell cultures were maintained at 37°C in a humidified 5% CO₂ incubator. Following 3- or 4-day incubation, the non-adherent cells were washed away leaving behind the adherent cell population that was growing as fibroblastic cells in clusters and the medium replaced with a fresh medium. When cells reached 70–90% confluence, cultures were harvested with 0·25% trypsin-EDTA solution, plated in 25-cm² plastic cell culture flasks at the density of 10⁴ cells/cm² and cultured in the medium supplemented with either FBS or HPR.
Multiplex growth factor analysis and cell proliferation

The hBMSCs at passage 1 were seeded at a concentration of 1 × 10^4 cells per well in 96-well plates and cultivated in different concentrations of HPR- or FBS-supplemented DMEM-low glucose. For all the experiments, the media was replaced every 3 days and 9 days after cells treated with different concentration of HPR, cell proliferation was analysed by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test based on cell growth determination protocol [21]. ELISA kits were used to estimate the concentration of epidermal growth factor, platelet-derived growth factor-AA, basic fibroblast growth factor, transforming growth factor-β, and tumour necrosis factor-α in HPR obtained from 10^9 platelets/ml.

Immunophenotyping of human BMSCs by flow cytometry

Human BMSCs cultured in HPR- or FBS-supplemented DMEM-low glucose were detached from the tissue culture flasks after three passages with trypsin-EDTA and centrifuged at 200 g for 5 min at room temperature. The pellet was resuspended in human serum and incubated for 30 min at room temperature. After centrifugation at 200 g for 5 min, pellet was suspended in 3% human serum albumin (HSA) in PBS and incubated with appropriate antibodies including FITC-conjugated mouse antihuman CD44 (H-CAM), CD105 (Endoglin or SH2), CD34 and phycocerythrin (PE)-conjugated CD166 (ALCAM), CD45 (leucocyte common antigen) for 1 h on ice, washed twice in PBS, and centrifuged for 5 min. The cells were resuspended in 100 μl of PBS and studied by a Coulter Epics-XL flow cytometer (Beckman Coulter, CA, USA). An isotype control with FITC- or PE-labelled was included in each experiment, and specific staining was measured from the cross-point of the isotype with specific antibody graph. Creation of histograms was performed with Win MDI 2.8 software (Scripps Institute, CA, USA).

Osteogenic differentiation assay

Osteogenic differentiation was performed with MSC derived from FBS and HPR-supplemented cultures after three passages [22,23] and was evaluated with alizarin red staining and processed for measuring of ALP activity. The activity was assayed using p-nitrophenyl-phosphate as the substrate according to manufacturer’s recipes. The absorption at 405 nm was measured using spectrophotometer and converted to U/l.

Hepatic differentiation protocol

Hepatic differentiation was performed as described in our recent publication [24]. Briefly, 1 × 10^4 cells at passage 3 were seeded per well in 24-well plates and hepatic differentiation was processed using a two-step protocol employing the HGF, DEX and OSM. In the first step, which lasted for 7 days, the cells were differentiated on a culture medium consisting of DMEM supplemented with 15% FBS or 20% HPR, 20 ng/ml HGF, and 10^{-7} mol/l DEX. In the second phase, OSM (10 ng/ml) was added to the culture medium and continued until day 21. The culture media was changed twice weekly and different assays were used to evaluate hepatic differentiation.

Immunocytochemistry

After 21 days of differentiation, the cultured cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30–45 min at room temperature, then permeabilized with 0.4% Triton-X-100 for 20 min. The cells were washed thrice with PBS and then incubated overnight at 4°C with primary monoclonal mouse antihuman antibodies against albumin (1 : 1000) and AFP (1 : 500). Subsequently, the cells were washed with PBS three times and incubated with fluorescence-labelled secondary antibody, FITC-labelled goat antimouse IgG at 37°C for 3 h in dark room. The cells were then rinsed thrice with PBS and incubated with DAPI (4',6-diamidino-2-phenylindole; 1 : 1000) for nuclear staining. The cells were visualized by a fluorescence microscope (Nikon, TE-2000, Tokyo, Japan). The ratio of immunopositive cells to the total number of cell nuclei labelled with DAPI was recorded. Human hepG2 hepatoma cells cultured in 24-well plates were simultaneously stained for albumin and AFP considered as positive control.

Total RNA isolation and reverse transcription–polymerase chain reaction

Expression of albumin, AFP and cytokeratin-18 (CK-18) at mRNA levels was assessed by reverse transcription (RT)-PCR. Briefly, total RNA was isolated from cells using RNA extraction kit. Prior to RT reaction, the RNA samples were digested with DNase 1 to remove contaminating genomic DNA. Standard RT was performed using Reverse RevertAid™ first strand cDNA synthesis kit (Fermentas, Hanover, MD, USA) and 2 μg of total RNA, 0.5 μg oligo (dt)_{18} per reaction, according to the manufacture’s instructions. PCRs were conducted using approximately 200 ng cDNA to amplify a number of hepatocyte marker genes listed in Table 1. After initial denaturation at 95°C for 1 min, PCR amplification was continued at 95°C for 40 second, annealing temperature for 40 second, and 72°C for 1 min for a total 30 cycles, and final extension at 72°C for 5 min. The amplified DNA fragments were electrophoresed on 2% agarose gel. The gels were stained with ethidium bromide (10 μg/ml) and photographed on an ultraviolet transilluminator (Uvidoc, Cambridge, UK).
Urea production

On day 21 of differentiation, the cells were incubated with medium containing 5 mM NH₄Cl for 24 h in 5% CO₂ at 37°C. Following incubation, the supernatant from 5 × 10⁴ cells per well was collected and urea concentration was measured using a colourimetric assay kit. Undifferentiated hBMSCs were similarly treated and considered as negative control group.

Statistical analysis

Data are presented as mean ± SEM. The results were analysed by Student’s t-test. *P < 0·05 was considered statistically significant.

Results

Proliferation rate of MSCs in presence of HPR or FBS

The proliferation of hBMSCs was relatively more susceptible to HPR as compared to that of FBS concentration in culture milieu. MTT value was increased depending on the HPR concentration added to the culture media (Fig. 1). The MTT value for MSCs cultivated in culture medium containing 20% v/v HPR derived from 10⁹ platelets/ml was approximately threefold greater than that grown in presence of 20% v/v FBS (*P < 0·001). The cell proliferation in NO-stimulated platelets-driven culture was significantly lower than in FBS (Fig. 1). The concentration of selected growth factors in HPR obtained from 10⁹ platelets/ml is shown in Table 2.

Table 1 Primers and annealing temperatures used for reverse transcription–polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>F 5'-CCA GAG ATT TCC CAA AGC TG-3'</td>
<td>208</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>R 5'-TGG GAT TTT TCC AAC AGA GG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFP</td>
<td>F 5'-AGC TGG GTG GTG GAT GAA AC-3'</td>
<td>252</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>R 5'-CCC TCT TCA GCA AAG CAG AC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK-18</td>
<td>F 5'-CCC GCT ACG CCC TAC AGAT-3'</td>
<td>271</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>R 5'-ACC ACT TIG CCA TCC ATCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>F 5'-GTC CTC TCC CAA GTC CAC AC-3'</td>
<td>198</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>R 5'-GGA AGA CCA CCA AAA GGC TCC AT-3'</td>
<td></td>
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</tbody>
</table>

Table 2 Mean growth factors concentration of human platelet releasate. The platelet preparations (10⁶ platelets/ml) obtained from 10 individuals. The results presented are mean ± SEM of 10 separate samples

<table>
<thead>
<tr>
<th>Growth factors</th>
<th>Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>450 ± 46</td>
</tr>
<tr>
<td>PDGF-AA</td>
<td>1000 ± 82</td>
</tr>
<tr>
<td>bFGF</td>
<td>98 ± 9</td>
</tr>
<tr>
<td>TGF-β</td>
<td>9000 ± 94</td>
</tr>
<tr>
<td>TNF-α</td>
<td>25 ± 6</td>
</tr>
</tbody>
</table>

EGF, epidermal growth factor; PDGF-AA, platelet-derived growth factor AA; bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α.
Immunophenotypic profile of MSCs expanded in HPR-and FBS-driven culture

In the flow cytometric analysis, BMSCs were found to be positive for the following adhesion molecules: CD44, CD166, and CD105, which together were considered as markers for MSCs [25]. As shown in Fig. 2, the MSCs were negative for haematopoietic lineage markers, namely, CD34, and CD45. There was no significant difference between bone marrow cells cultivated with HPR and FBS, suggesting that immunophenotypic

<table>
<thead>
<tr>
<th></th>
<th>CD105</th>
<th>CD166</th>
<th>CD44</th>
<th>CD45</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPR</td>
<td>75 ± 2%</td>
<td>93 ± 2.5%</td>
<td>97 ± 1.2%</td>
<td>3.8 ± 2%</td>
</tr>
<tr>
<td>FBS</td>
<td>87 ± 4%</td>
<td>95 ± 1.6%</td>
<td>91 ± 3%</td>
<td>3.8 ± 1.2%</td>
</tr>
</tbody>
</table>

Fig. 2 Immunophenotypic analysis of human bone marrow mesenchymal stem cells (hBMSC). Flow cytometric analysis was performed for MSC-specific markers and haematopoietic markers. The shaded area shows the profile of the negative control. The figures are representative of three independent experiments. Data in table are the ratio of positive cells to the total number of cells (%). The results are mean ± SEM of three samples.
properties of MSCs were maintained during cells expansion in HPR-driven culture (Fig. 2).

Osteogenic differentiation

The efficiency of HPR in differentiation potential of hBMSCs to osteoblastic lineage was examined. The ALP activity was found to be $805 \pm 90$ and $12 \pm 5$ U/l in differentiated cells and undifferentiated cells, respectively, in HPR-driven culture. As shown in Fig. 3, unlike hBMSCs (negative control), nodule-like structures were observed in induced cells in both conditions, which were strongly positive for calcium staining as judged by Alizarin red staining. These data show that the hBMSCs cultured in HPR culture medium pose their differentiation potential to osteoblasts.

Morphology of hBMSCs-derived hepatocyte-like cells

Gross morphology of the cells differentiated in presence of HPR or FBS was assayed by phase contrast microscope (Nikon) at early and late stages of differentiation. Cells before differentiation exhibited a fibroblast-like morphology (Fig. 4c,d). Cell morphology under both conditions did not significantly differ during differentiation step 1, but, hepatocyte morphology was displayed when cells exposed to media containing OSM. The hBMSCs-derived cells in presence of HPR had larger nuclei and more polyhedral contours as compared to those differentiated in FBS (Fig. 4a,b).

Immunocytochemical staining

Albumin was weakly expressed in the hBMSCs cultured in HPR-supplemented media (Fig. 5d) and was not detectable in undifferentiated hBMSCs cultured in presence of FBS (Fig. 5b). The undifferentiated hBMSCs did not express AFP protein in media containing FBS or HPR (Fig. 6d,b), whereas after 21 days, there was a considerable expression of albumin (Fig. 5a,c) and AFP (Fig. 6a,c) in the cells differentiated in presence of HPR or FBS. The percentage of albumin- and AFP-positive cells was higher when cells differentiated in HPR ($58 \pm 4$ and $31 \pm 5\%$) than in FBS ($30 \pm 4$ and $22 \pm 3\%$). Binucleated cells (as an indicator of mature hepatocyte) were observed in some albumin-positive cells cultured in the culture media supplemented with either HPR or FBS.

Hepatocyte-specific gene expression

The expression of a panel of hepatocyte-specific markers such as albumin, AFP and CK-18 measured at mRNA levels in cells on days 0 and 21 of differentiation clearly shows the development of hBMSCs into hepatocyte-like cells in presence of the media containing FBS or HPR. As shown in Fig. 7, hBMSCs expressed extremely low levels of mRNA of hepatocyte lineage genes, but, following treatment, the mRNA expression of the studied genes was overexpressed in induced cells on day 21 of differentiation. The expression of $\beta$-actin, used as an internal control, was the same in undifferentiated and differentiated cells.
Urea production

The level of urea excretion into the culture media from the differentiated cells under the influence of HPR was twofold greater \( (P < 0.001) \) than that in FBS-driven medium on day 21 \((29 \pm 2.5 \text{ mg/dl in HPR-driven cells and } 14 \pm 2 \text{ mg/dl in FBS-driven cells}) \). Besides, the level of urea in culture media derived from undifferentiated hBMSCs was negligible.
Dependence of MSCs expansion to FBS has limited the use of these cells for clinical applications. Hence, attempts have been made to replace FBS with human factors during human stem cell manipulation. More recently, FBS was successfully replaced with human platelet lysate for clinical scale of MSCs production [16,17]. While these studies were in progress, we have been investigating the efficiency of HPR as the key culture media substitute for MSCs expansion and differentiation to different lineages, particularly hepatocytes. Data presented in this article clearly show that the growth promoting activity of HPR is comparable with or even superior to mitogenic stimulation by conventional cell culture medium supplemented with FBS (Fig. 1). The superiority of HPR over FBS that was reflected in MTT value could be attributed to the differences in the composition of growth factors. In this connection, we demonstrated that HPR prepared after activation of platelets possess major growth factors necessary for cell proliferation and differentiation (Table 2).

No differences in immunophenotyping profile of MSCs derived from HPR- and FBS-supplemented cultures (Fig. 2) and their differentiation potential to osteogenic lineage are important evidences to show the efficiency of HPR as a human growth factor source. This part of our study is in accordance with the data recently reported by others [16–18]. These findings were accomplished by processing the differentiation process of hBMSCs to active hepatocyte-like cells [24]. Differentiation of hBMSCs into hepatocytes was used as an approach to approve the efficiency of HPR as a safe source of growth factor for MSC differentiation. In this

Discussion

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study, we demonstrated that the HPR-cultured hBMSCs are capable of giving rise to hepatic transdifferentiation, assessed by an examination of morphology and hepatocyte-specific markers, such as albumin, AFP and CK-18 [26,27]. The morphological changes of fibroblast-like shape to polygonal morphology (characteristics of hepatocyte maturation) were more prominent in hBMSCs differentiated under the influence of HPR as compared to cells-driven FBS (Fig. 4). Binucleation as an indicator of terminal differentiation of hepatocytes was observed in some differentiated cells in both the media used. Although the cellular mechanism(s) that governs the passage from mononucleated MSCs to binucleated hepatocyte-like cells remains obscure, recent studies suggest that the hepatocyte binucleation could be as a result of the cell exit from mitosis because of absence or abortion of cytokinosis [28,29].

Morphological observations were corroborated with the extent of albumin- and AFP-positive cells in differentiated cells cultured in presence of HPR as compared to those cultured in FBS (Figs 5 and 6). The differentiation potential of HPR-cultured hBMSCs towards hepatocyte-like cells was further confirmed by the expression of albumin, AFP and CK-18 at mRNA levels (Fig. 7). Further quantitative assays can discriminate the possible differences in liver gene expression in hBMSCs induced in presence of HPR or FBS.

Urea production by hepatocyte-like cells was used as an additional marker of the cellular function, which revealed that the cells differentiated in presence of HPR produced higher levels of urea approximately twofold greater ($P < 0.001$) when compared to those obtained in FBS-supplemented medium. Replacement of FBS with HPR in the differentiation medium was found to be in favor of specific differentiation to hepatocyte lineage. The inducing capacity of HPR in hepatocyte differentiation could be due to the differences in the growth factor composition of HPR and FBS.

Overall results presented here show that the efficient propagation and differentiation of human MSCs with HPR not only eliminate the risk of bovine pathogen contamination and xenoimmunization, this would represent a major novel step towards safe stem cell therapy. Further investigations especially in vivo studies are required to better understand the features and functionality of differentiated cells in FBS-free medium fortified with HPR.

Acknowledgement

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