Original Article

Isolation and culture of hepatic stellate cells from mouse liver

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Hepatic stellate cells (HSCs) are the primary extracellular matrix-producing cells within the liver and have numerous vital functions. A robust protocol for the isolation and culture of HSCs is important for further investigations of cell functions and related mechanisms in liver disease. The volume of the mouse liver is much smaller than that of the rat liver, which makes it much more difficult to isolate mouse HSCs (mHSCs) than rat HSCs. At present, isolating mHSCs is still a challenge because there is no efficient, robust method to isolate and culture these cells. In the present study, C57BL/6J mice were intravenously injected with liposome-encapsulated dichloromethylene diphosphate (CL2MDP) to selectively eliminate Kupffer cells from the liver. The mouse livers were then perfused in situ, and the mHSCs were isolated with an optimized density gradient centrifugation technique. In the phosphate buffer solution (PBS)-liposome group, the yield of mHSCs was (1.37 ± 0.23) × 10⁶/g liver, the cell purity was (90.18 ± 1.61)% and the cell survival rate was (94.51 ± 1.61)%. While in the CL2MDP-liposome group, the yield of mHSCs was (1.62 ± 0.34) × 10⁶/g liver, the cell purity was (94.44 ± 1.89)% and the cell survival rate was (94.41 ± 1.50)%. Based on the yield and purity of mHSCs, the CL2MDP-liposome treatment was superior to the PBS-liposome treatment (P < 0.05, P < 0.01). This study established successfully a robust and efficient protocol for the separation and purification of mHSCs, and both a high purity and an adequate yield of mHSCs were obtained.

Keywords hepatic stellate isolation; dichloromethylene diphosphate; cell purification; cell culture; liposome

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Introduction

The liver primarily consists of five cell types: parenchymal cells (hepatocytes), Kupffer cells, endothelial cells, hepatic stellate cells (HSCs), and pit cells. HSCs are non-parenchymal liver cells that reside within the sub-endothelial space of Disse around the liver sinusoid. Their star-shaped pseudopodia surround the sinusoidal endothelial cells [1]. Although HSCs constitute <8% of the total liver cell population [2], they perform a number of vital functions. They are a storage site for vitamin A (retinol) [3−5] and the primary extracellular matrix-producing cell type within the liver. This function is essential for the maintenance of the normal perisinusoidal environment. In addition, HSCs also have been implicated as regulators of liver regeneration in normal and necrotic livers [6]. After toxicity-induced liver injury, HSCs undergo a process known as activation, in which they transit from a quiescent state into an active state. Activated HSCs are involved in hepatic epithelium cell proliferation and hepatocyte death and are associated with liver repair after liver injury as supporting cells in the stem cell niche [7]. A robust protocol for HSC isolation, purification, and culture is important for further investigations of cell functions and the related mechanisms associated with liver disease. Using enzyme perfusion combined with density gradient centrifugation, Knook et al. [8] successfully isolated rat HSCs, and since then, most researchers used this process. However, this method cannot eliminate contamination by Kupffer cells because the Kupffer cells and HSCs have similar densities. In addition, how to harvest an adequate number of cells while effectively control contamination by other cell types remains a challenge. Because the volume of the mouse liver is much smaller than that of the rat liver, the isolation of HSCs from mouse livers is more difficult. In the present study, we developed a method for the isolation of highly purified mouse HSCs (mHSCs) with adequate cell yields. The protocol includes the intravenous administration of liposome-encapsulated dichloromethylene diphosphate (CL2MDP) to selectively eliminate Kupffer cells from the liver [9], followed by perfusion of the livers in situ and an optimized density gradient centrifugation step. Primary
cultured mHSCs were identified by desmin, α-smooth muscle actin (α-SMA), and Oil Red O staining, followed by phase contrast microscopy and fluorescence microscopy.

**Materials and Methods**

**Animals**

C57BL/6J mice (8–12 weeks old) were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The feeding, maintenance, and use of the animals were performed in accordance with the guidelines of the Experimental Animal Ethics Committee of Fudan University.

**Reagent preparations**

*Solution A.* Pre-treatment perfusate: D-Hanks liquid (GeneChem Company, Shanghai, China) with 0.5 mM ethylene glycol tetraacetic acid (EGTA; Sigma-Aldrich, Poole, UK), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Gibco, New York, USA).

*Solution B.* Pronase E solution: Hanks liquid (GeneChem Company) with 1 mg/ml pronase E (Roche, Basel, Switzerland), 2 mM CaCl₂, and 20 mM HEPES (Sigma-Aldrich).

*Solution C.* Collagenase NB4G solution: Hanks liquid with 0.25 mg/ml collagenase NB4G (Serva, Heidelberg, Germany), 2 mM CaCl₂, and 20 mM HEPES.

*Solution D.* DNase I solution: Hanks liquid with 0.2 mg/ml DNase I (Roche), 2 mM CaCl₂, 100 μg/ml streptomycin, and 100 IU/ml penicillin.

*Solution E.* Centrifugation dilution solution: Leibovitz’s L-15 medium (Sigma-Aldrich) with 0.25% bovine serum albumin (Sigma-Aldrich), 20 mM HEPES, 100 μg/ml streptomycin, and 100 IU/ml penicillin.

*Solution F.* Density gradient centrifugation solution: 15% OptiPrep (Axis-shield, Oslo, Norway) and 11.5% OptiPrep prepared according to the manufacturer’s instructions.

**mHSCs culture medium**. High-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Sigma-Aldrich), 20 mM HEPES, 100 μg/ml streptomycin, and 100 IU/ml penicillin.

**The isolation and culture of mHSCs**

The procedure for the isolation and purification of mHSCs is illustrated briefly in Fig. 1, and the detailed protocol is as follows.
The pre-treatment of mice with CL2MDP-liposomes and liver enzyme perfusion in situ. C57BL/6J mice (n = 24) were randomly divided into two groups. One group was treated with CL2MDP-liposomes (10 ml/kg) by intravenous injection, and the other group was treated with phosphate buffer solution (PBS) containing liposomes (PBS-liposomes) as the control group. The liposome injection was performed 3 days before the isolation of mHSCs. Three days later, the abdominal wall of each mouse was opened after successful anesthesia, and the intestines were gently moved aside to expose the vena cava and portal vein (Fig. 2A). After placing a thread around the portal vein, a fine cannula was inserted into the vein, which was ligated and fixed to an appropriate location. All perfusion solutions were incubated at 37°C before use. An adjustable peristaltic pump was used to control the in situ liver perfusion process. After the liver swelled, the cannula in the inferior vena cava was opened to allow draining (Fig. 2B). Subsequently, the liver was perfused in turn with EGTA (Solution A), pronase E (Solution B), and collagenase NB4G (Solution C) for 8–10 min at 37°C. Finally, the liver was gently removed from the abdominal cavity and placed in a sterile dish.

Liver digestion in vitro. After 25 ml of DNase I solution (Solution D) was added to the dish, the liver capsule was opened. The liver tissue was disrupted to prepare a cell suspension and tissue pieces, which were placed in a 50 ml flask and further digested by stirring with a magnetic stirrer (200 rpm/min) at 37°C for 5 min. Then, 20 ml of cold centrifugation dilution solution (Solution E, 4°C) was added to the flask to terminate the digestion process. The obtained cell suspension was subsequently filtered through nylon mesh (70 μm; BD Falcon, New Jersey, USA).

Liver cell suspension fractionation and gradient centrifugation. The liver cell suspension was transferred to a 50 ml centrifuge tube and centrifuged at 25 g for 5 min (4°C) to remove the residual liver parenchymal cells. The supernatant was collected and centrifuged at 400 g for 10 min (4°C) to collect the non-parenchymal cells. The collected non-parenchymal cells were resuspended in 15% OptiPrep and transferred to a 15 ml centrifuge tube after sufficient mixing. Then, 5 ml of 11.5% OptiPrep and 2 ml of Gey’s Balanced Salt Solution (GBSS; Sigma-Aldrich) were individually layered carefully onto the cell suspension. The tube was centrifuged at 1400 g for 20 min (4°C, low acceleration), and the cell layer between the 11.5% OptiPrep and GBSS was carefully collected. The obtained cells were purified mHSCs. These cells were washed two times with centrifugation dilution solution (400 g for 10 min, 4°C) and seeded with culture medium.

Primary mHSC culture. The viability of the mHSCs was estimated by Trypan blue staining. mHSCs were seeded in 35 mm Petri dishes at a density of 4 × 10⁵/ml in complete medium consisting of DMEM containing 10% FBS. The cells were incubated at 37°C in a humidified atmosphere with 5% carbon dioxide, and the medium was replenished every 2 days. After being cultured for 5–7 days, the cells became confluent and required passaging.

Assessment of Kupffer cells. All mice (n = 8) were randomly divided into two groups. One group received PBS-liposomes and the other received CL2MDP-liposomes via intravenous injection (10 ml/kg). A carbon particle suspension (0.1 ml; India ink, Hannover, Germany) was injected through the tail vein 3 days later. The mice were sacrificed for the collection of liver tissue samples 30 min after the injection of this solution. Liver tissues were stained with hematoxylin–eosin (HE) to count the number of Kupffer cells, which contained carbon particles, and the difference between the two groups was analyzed. In addition, the mHSCs were isolated from both of the groups and cultured separately. After the mHSCs had
been cultured for 5 days, 2 μl of carbon particle suspension was added to the medium. Eight hours later, the difference in the number of carbon-containing Kupffer cells between the CL2MDP-liposome group and the PBS-liposome group was analyzed.

Histological and immunohistochemical analysis
For histological analysis, liver tissues were fixed overnight using 4% paraformaldehyde at 4°C, and sections (5 μm) were stained with HE to determine the number of carbon-containing Kupffer cells. To identify mHSCs by Oil Red O (Sigma-Aldrich) staining and immunostaining of cellular constituents, primary cultured mHSCs (24 h) were stained with Oil Red O and then immunostained for desmin (Abcam, Cambridge, UK) and α-SMA (Abcam) as described previously [11]. For immunohistochemistry, primary mHSCs cultured for 14 days were fixed using 2% paraformaldehyde for 10 min at 4°C. The cell membranes were disrupted with 0.5% Triton X-100 (Sangon Biotech, Shanghai, China) for 10 min. The mHSCs were incubated with mouse monoclonal anti-α-SMA (1:100) and rabbit anti-desmin (1:100) antibodies. Labeled cells were visualized with matched secondary antibodies: goat anti-mouse Alexa Fluor 488 (Invitrogen, Carlsbad, USA; 1:500) and goat anti-rabbit Alexa Fluor 488 (Invitrogen; 1:500). A DAPI working solution (Sigma-Aldrich; 1:1000) was used to visualize the nuclei. All samples were visualized with a Carl-Zeiss Axiovert 200 microscope (Carl-Zeiss, Jena, Germany) and a computer-assisted image analysis program (AxioVision Ver. 4.0; Carl-Zeiss).

Statistical analysis
All data were presented as the mean ± SD and analyzed with SPSS 17.0 software. Student’s t-test was used to assess the differences between the two groups. Values with \( P < 0.05 \) were considered to be significantly different.

Results
Clearance of Kupffer cells by CL2MDP-liposomes
in vivo
C57BL/6J mice (n = 12) were randomly divided into two groups and pretreated with PBS-liposomes or CL2MDP-liposomes (10 ml/kg) by intravenous injection. Three days later, 0.1 ml of carbon particle suspension was injected through the tail vein, and then, mice were sacrificed 30 min later for the collection of liver tissue samples. Due to the presence of phagocytized carbon particles in the cytoplasm, Kupffer cells would appear black, and these cells were observed in both the CL2MDP-liposome group and the PBS-liposome group with HE staining (Fig. 3A,B). The number of carbon-containing Kupffer cells in the CL2MDP-liposome group was \( (3.73 ± 1.82)/\text{FOV} \), compared with \( (18.57 ± 3.2)/\text{FOV} \) in the PBS-liposome group. The difference between the two groups was significant (\( P < 0.01 \)) (Fig. 3C). This result indicated that CL2MDP-liposomes can effectively eliminate Kupffer cells from mouse livers.

Assessment of the contamination of primary cultured mHSCs by Kupffer cells
C57BL/6J mice were randomly divided into two groups and pretreated with CL2MDP-liposomes (n = 6) or PBS-liposomes (n = 6). mHSCs were isolated and cultured as described above. After primary culture for 5 days, Kupffer cells showed typical characteristics, with a round or oval shape and no lipid droplets in the cytoplasm, when observed under a light microscope. A carbon particle suspension (2 μl) was added to the medium of cultured mHSCs. Eight hours later, the Kupffer cells had phagocytized carbon particles and were dyed black (Fig. 4A). mHSCs had a star-shaped appearance with multiple pseudopodia that contained highly refractive triacylglycerol-rich droplets in the cytoplasm without...
Kupffer cells in the CL2MDP-liposome group (0.88 × 10^6/g liver) contained highly reflective retinoid droplets in the cytoplasm but no carbon particles in the cytoplasm (Arrow 1). Kupffer cells contained carbon particles, making the cells appear black, but did not contain lipid droplets in the cytoplasm (Arrow 2). (A) PBS-liposome group. (B) CL2MDP-liposome group. Scale bar = 50 μm. (C) Quantification of carbon-containing Kupffer cells. The CL2MDP-liposome group had a significantly lower number of carbon-containing Kupffer cells in the primary mHSC cultures compared with the PBS-liposome group. The data are shown as the mean ± standard error of 10 random high-power fields per animal. *P < 0.05. FOV, field of view.

Table 1. The yield, purity, and survival rate of mHSCs from the PBS-liposome and CL2MDP-liposome groups

<table>
<thead>
<tr>
<th>mHSCs</th>
<th>PBS-liposome</th>
<th>CL2MDP-liposome</th>
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<tbody>
<tr>
<td>Cell yield (10^6/g liver)</td>
<td>1.37 ± 0.23</td>
<td>1.62 ± 0.34*</td>
</tr>
<tr>
<td>Cell purity (%)</td>
<td>90.18 ± 1.61</td>
<td>94.44 ± 1.89**</td>
</tr>
<tr>
<td>Cell survival rate (%)</td>
<td>94.51 ± 1.61</td>
<td>94.41 ± 1.50</td>
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C57BL/6J mice were randomly divided into two groups and treated with PBS-liposomes or CL2MDP-liposomes. The yield, purity, and survival rate of the isolated mHSCs were analyzed in both the groups. The cell yield and cell purity were significantly higher for the CL2MDP-liposome group (n = 12) than for the PBS-liposome group (n = 12). The data are shown as the mean ± standard error of the mean.

*P < 0.05, **P < 0.01 vs. PBS-liposome.

Identification of mHSCs
mHSCs were identified with several methods. (i) The cellular morphology of mHSCs was observed by phase contrast microscopy. mHSCs cultured for 24 h were in a quiescent state. A typical feature of these cells is the presence of cytoplasmic triacylglycerol-rich droplets. These lipid droplets could be easily detected by their high refraction under a light microscope (Fig. 5A). After 5 days of primary culture, mHSCs could transit from a quiescent phenotype into an active proliferation phenotype [11]. During this activation period, mHSCs developed star-shaped pseudopodia, and the density of lipid droplets in the cytoplasm decreased gradually (Fig. 4A,B). After being cultured for 14 days, mHSCs were fully activated and evolved into myofibroblast-like cells with no lipid droplets (Fig. 6A). (ii) mHSCs could be detected directly by fluorescence microscopy. The retinoid droplets in the cytoplasm exhibited a striking rapidly fading blue-green autofluorescence when excited with 328 nm light.
(iii) Quiescent mHSCs can be identified with Oil Red O [12]. The retinoid droplets in the mHSCs cytoplasm exhibited a bright red color when stained by Oil Red O and observed under a light microscope (Fig. 5C). (iv) mHSCs could be identified using the ‘gold standard’ markers of desmin and α-SMA, which were the most reliable markers of HSCs [13]. The mHSCs were cultured for 24 h, and immunofluorescence staining revealed that desmin was expressed in the mHSCs.

(Fig. 5B).
expressed in the cytoplasm but α-SMA was not (Fig. 5D). mHSCs were fully activated after being cultured for 14 days, and double immunofluorescence staining showed that desmin and α-SMA were both expressed in the cytoplasm (Fig. 6B–D).

Discussion

The isolation and culture HSCs are critical steps in the study of cell functions and cell interactions related to liver disease. However, there are few reports on the isolation and culture of HSCs from mouse livers because the mouse liver volume is much smaller than that of the rat liver, making it more difficult to manipulate. The ideal mHSCs isolation method should be simple, rapid, and inexpensive and should have both high cell purity and high cell yield. In the present study, the intravenous administration of CL2MDP was used to selectively eliminate Kupffer cells from mouse livers. This process largely reduced contamination by Kupffer cells, which usually adhered to mHSCs and contributed to lower cell yield and cell purity for mHSCs. In addition, using both the perfusion of mouse livers in situ and an optimized density gradient centrifugation technique, we successfully developed a robust and efficient method to isolate and purify mHSCs.

Conventional methods for the isolation of HSCs cannot eliminate contamination by Kupffer cells because the densities of these two cell types are similar. The average density is 1.053 g/ml for HSCs, 1.060 g/ml for Kupffer cells, and 1.080 g/ml for sinusoid endothelial cells [14]. Because the sizes and lipid droplet contents of HSCs vary in the same liver, HSCs and Kupffer cells inevitably overlap in terms of density. In addition, HSCs and Kupffer cells easily adhere to each other, and thus, the isolated HSCs are contaminated by Kupffer cells. This contamination leads to the decrease of yield and purity in the prepared HSCs [15]. CL2MDP has selective cytotoxicity against Kupffer cells. Studies have demonstrated that CL2MDP-liposomes injected through the tail vein can eliminate Kupffer cells without affecting other liver cells [16]. Yata et al. [17] reported that the rat HSCs purity could be increased to 98% when CL2MDP-liposomes were used to selectively eliminate Kupffer cells in the liver, followed by density gradient centrifugation. In our study, the intravenous injection of CL2MDP-liposomes was also effective in removing intrahepatic Kupffer cells. After the application of CL2MDP, the cell yield of mHSCs increased from (1.37 ± 0.23) × 10⁶/g liver to (1.62 ± 0.34) × 10⁶/g liver, and the cell purity increased from (90.18 ± 1.61)% to (94.44 ± 1.89)%. The cell survival rates of the two groups were similar. Furthermore, during the period of primary culture from 3 to 14 days, the growth status and activated procession of HSCs showed no significant difference between the two groups. These data indicated that pre-treatment with CL2MDP-liposomes before mHSCs isolation was safe and effective.

The concentration of the perfusion solution and the perfusion speed of the pronase E and collagenase NB4G solutions were key factors affecting the quality of hepatic perfusion and could directly affect the cell yield and cell survival rate of mHSCs. To enhance the yield and survival rate of mHSCs, livers were perfused in turn with EGTA, pronase E, and collagenase NB4G in situ. The first perfusion step used calcium-free D-Hank’s solution with 0.05 mM EGTA to remove blood cells and Ca²⁺ from the liver mesenchyme and facilitate the separation of liver cells. During the second step, pronase E solution was applied to selectively destroy hepatocytes. The destruction of these cells decreased hepatocyte contamination, improved the purity of mHSCs, eliminated adhesion between mHSCs and hepatocytes, reduced mHSCs loss, and enhanced the cell yield [10]. A previous study omitted the perfusion step with pronase E [18], and as a result, mHSCs adhered to hepatic parenchymal cells, thus decreasing the yield and purity of mHSCs.

In this study, the in situ liver perfusion was in accordance with the liver’s anatomic structure and contributed to complete digestion and uniform liver cell suspensions. Increasing the uniformity of single-cell suspensions can significantly improve the yield and purity of mHSCs. Other researchers have performed the pronase E and collagenase digestion process in vitro [19], which could reduce the survival rate of mHSCs due to overdigestion. In addition, DNase I could reduce the adherence effect of DNA released during the destruction of hepatocytes during the liver digestion process.

Density gradient centrifugation is another important step in the purification of mHSCs. HSCs are the least dense of the different cell types in the liver, and they can be purified by sedimentation onto or flotation through a suitable density barrier. Both the strategies have been used effectively, but flotation is often regarded as superior to sedimentation because HSCs tend to adhere to other cells when the cells traverse a sample/gradient interface [20]. When using sedimentation to purify cells, mHSCs are easily contaminated by other cells due to adhesion. In this study, cell flotation was used, in which 15% OptiPrep, 11.5% OptiPrep, and GBSS formed a three-layer density gradient. During the purification process, the flotation method with two different density barriers was applied to enhance the cell purity.

In order to increase the purity of HSCs, Tacke and Weiskirchen [21] established protocols to isolate HSCs by fluorescence-activated cell sorting (FACS). Zhao et al. [22] successfully isolated HSCs based on the FACS technique. These higher purity cells can address the special experiment needs, such as analysis of gene-expression profiles of HSCs or cell-surface expression studies. However, this method involved long sorting procedures and relatively low number of highly pure HSCs could be obtained. The shear force
during the operation process would influence the viability of the cells, and the cell viability would further be decreased. In addition, complex technology, expensive equipment, and more requirements limited its extensive application. We established an ideal mHSCs isolation method that is simple, rapid, and inexpensive and has both high cell purity and high cell yield. In this study, the cell purity was improved to (94.44 ± 1.89)%, and this purity could usually meet the requirements of further experiments [19,23]. In addition, based on our study, even higher purity could be obtained when combined with FACS technique, which could meet the requirements in the special fields of stellate cell biology.

In summary, we have successfully developed a robust and efficient method for the isolation and purification of mHSCs. This method can be used to investigate cell functions and the related mechanisms of liver disease.

Supplementary Data

Supplementary data are available at ABBS online.

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References