Video Article

Seven Steps to Stellate Cells

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Abstract

Hepatic stellate cells are liver-resident cells of star-like morphology and are located in the space of Disse between liver sinusoidal endothelial cells and hepatocytes[1,2]. Stellate cells are derived from bone marrow precursors and store up to 80% of the total body vitamin A[1, 2]. Upon activation, stellate cells differentiate into myofibroblasts to produce extracellular matrix, thus contributing to liver fibrosis[3]. Based on their ability to contract, myofibroblastic stellate cells can regulate the vascular tone associated with portal hypertension[4]. Recently, we demonstrated that hepatic stellate cells are potent antigen presenting cells and can activate NKT cells as well as conventional T lymphocytes[5].

Here we present a method for the efficient preparation of hepatic stellate cells from mouse liver. Due to their perisinusoidal localization, the isolation of hepatic stellate cells is a multi-step process. In order to render stellate cells accessible to isolation from the space of Disse, mouse livers are perfused *in situ* with the digestive enzymes Pronase E and Collagenase P. Following perfusion, the liver tissue is subjected to additional enzymatic treatment with Pronase E and Collagenase P *in vitro*. Subsequently, the method takes advantage of the massive amount of vitamin A-storing lipid droplets in hepatic stellate cells. This feature allows the separation of stellate cells from other hepatic cell types by centrifugation on an 8% Nycodenz gradient. The protocol described here yields a highly pure and homogenous population of stellate cells. Purity of preparations can be assessed by staining for the marker molecule glial fibrillary acidic protein (GFAP), prior to analysis by fluorescence microscopy or flow cytometry. Further, light microscopy reveals the unique appearance of star-shaped hepatic stellate cells that harbor high amounts of lipid droplets.

Taken together, we present a detailed protocol for the efficient isolation of hepatic stellate cells, including representative images of their morphological appearance and GFAP expression that help to define the stellate cell entity.

Video Link

The video component of this article can be found at https://www.jove.com/video/2710/

Protocol

C57BL/6 mice should be used at ~20 weeks of age or older. The use of male mice weighing 25-30g is recommended. The yield of stellate cells can be increased by feeding mice a vitamin A-enriched diet for 2 months prior to stellate cell isolation. Approximately $2x10^5$ hepatic stellate cells can be purified from the liver of one C57BL/6 mouse, whereas the yield of stellate cells from Balb/c mice is considerably higher. The following protocol is adjusted to 5 mice. Animal care and experimentation were performed in accordance with approved Institutional Animal Care and Use Committee protocols.

1. *In situ* perfusion of mouse livers with digestive enzymes

1. Warm the SC1 buffer and enzyme perfusion solutions in a 37°C water bath.
2. Mount a winged infusion set onto the silicone tube of a peristaltic pump.
3. Calibrate the pump with PBS to obtain a laminar flow of 6.5ml/min, which is the flow rate that will be used for the *in situ* perfusion of the liver. Equilibrate the silicone tube with SC1 solution.
4. Anesthetize the mouse by intraperitoneal injection of Ketamine (90 mg/kg) and Xylazine (10 mg/kg) solution and test for loss of reflexes to ensure deep narcotization.
5. Fix the mouse in supine position on a suitable base.
6. Use scissors and forceps to perform a longitudinal incision in the abdominal skin and expose the peritoneum.
7. Carefully open the peritoneum and move the intestines out of the abdominal cavity to the left side of the animal in order to expose the portal vein.
8. Insert the cannula of the infusion set into the portal vein. For this step, the use of a stereomicroscope is recommended.
9. Start the perfusion of the liver with 30ml SC1 solution. Open the vena cava inferior immediately after initiating the flow. Successful flushing of the liver is indicated by a loss of color of the liver tissue.
10. Perfuse the liver with 30ml Pronase E solution. Upon successful digestion, the liver lobes will swell, and the lobules will appear distinct through the capsule.

11. Perfuse the liver with 30ml Collagenase P solution. The liver at this point has lost its shape and looks atonic and amorphous.
12. Carefully separate the liver from the diaphragm and surrounding organs and store it in 70ml SC2 solution on ice.
13. Repeat the procedure for additional mice.

2. In vitro digestion of mouse livers

All further working steps should be carried out under sterile conditions in a laminar flow hood.

1. Cut the livers into pieces of approximately 2x2x2mm^3 using sharp scissors.
2. Combine the suspension of 70ml SC2 including the liver pieces with 50ml of Pronase E-Collagenase P solution and add 1ml of DNase I solution.
3. Digest the livers for 20min at 37°C while stirring.

3. Density gradient centrifugation

1. Filter the cell suspension through 70µm cell strainers into 6 50ml Falcon tubes and add up to 50ml with SC2 buffer. Centrifuge for 10min at 600g and 4°C.
2. Carefully aspirate 40ml of the supernatant, then add 150µl DNase I solution to each tube and resuspend the cells.
3. Pool the cell suspensions into 4 50ml Falcon tubes and wash with GBSS-B, centrifuge for 10min at 600g and 4°C.
4. Carefully aspirate as much of the supernatant as possible without disturbing the pellet and add 150µl DNase I solution to each tube. Resuspend the cell pellets in 10ml GBSS-B per tube.
5. Pool the cells into 2 50ml Falcon tubes and add GBSS-B to a total volume of 36ml per tube. Add 14ml Nycodenz solution to each tube and mix well.
6. Transfer 10ml of the cell suspension into one 12ml gradient centrifugation tube, resulting in 10 tubes in total. Gently overlay the cell suspension with 1.5ml GBSS-B per tube.
7. Centrifuge the gradients for 15min at 1500g and 4°C without brake. Subsequently, hepatocytes will be pelleted at the bottom of the tube whereas stellate cells are found in the interphase as a white ring.
8. Carefully harvest the interphase containing the stellate cells and wash them with GBSS-B; centrifuge for 10min at 600g and 4°C.
9. Aspirate the supernatant and resuspend the cells in 20ml DMEM supplemented with 10% heat-inactivated FBS, 1% Penicillin/Streptomycin, 2mM L-Glutamine, 1mM sodium pyruvate, and 10mM HEPES. Transfer the cells into tissue culture flasks with a concentration of 2x10^4 cells/cm^2. Incubate the cells at 37°C and 5% CO_2.
10. Change the media as soon as the hepatic stellate cells are adherent (approximately 2h after the preparation) to wash off dead cells and cell debris.
11. On the following day, the stellate cells should develop their characteristic star-shaped morphology with perinuclear vitamin A-storing lipid vesicles.
12. For subsequent experiments, hepatic stellate cells can easily be detached from non-coated plastic surfaces using mild enzymatic solutions such as Accutase, e.g.

4. Representative Results:

Following the preparation of hepatic stellate cells using the protocol provided, the purity of the isolated population can be tested considering three major characteristics of this cell type, such as star-like shape, perinuclear lipid droplets, and expression of glial fibrillary acidic protein (GFAP). Representative pictures for the characteristic appearance of hepatic stellate cells 2h after cell isolation, as well as on day 1 and 3 of *in vitro* culture are depicted in Figure 1. Figure 2 shows a representative immunofluorescence staining for GFAP in hepatic stellate cells, which have been cultured for 3 days following cell isolation. Hepatic stellate cells differentiate into myofibroblastic cells during *in vitro* culture. A characteristic hallmark of those activated stellate cells is the expression of alpha smooth muscle actin (αSMA). Figure 3 shows immunofluorescence staining for aSMA and myosin IIA in activated stellate cells on day 7 of *in vitro* culture.

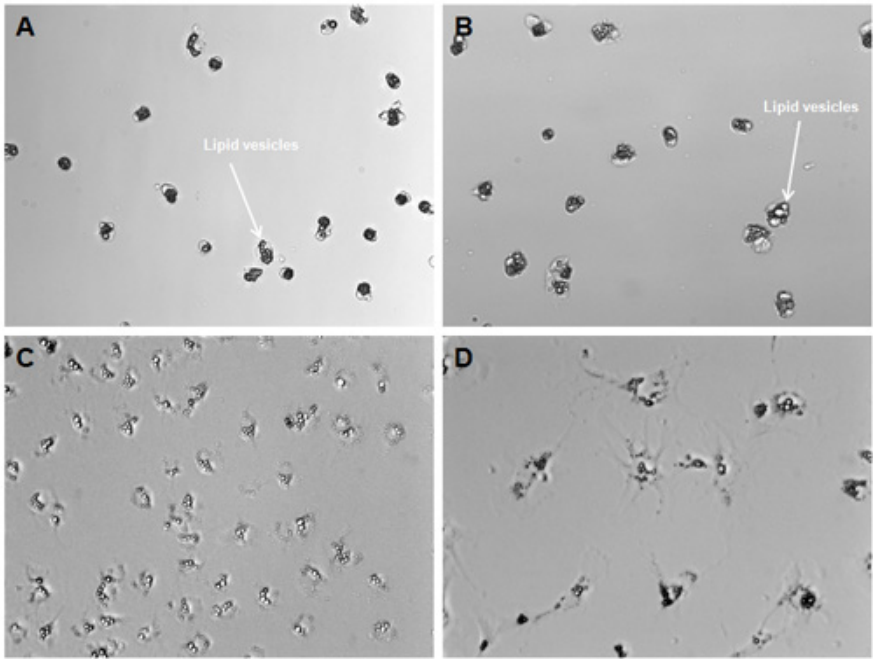

Figure 1. Characteristic morphology of hepatic stellate cells. Stellate cells were isolated from mouse livers using the protocol provided. The images depict stellate cells 2h after cell isolation (a, b), as well as on day 1 (c) and day 3 (d) of *in vitro* culture. Hepatic stellate cells exhibit high amounts of lipid vesicles at perinuclear sites and acquire their distinctive astral-like morphology during the first days of *in vitro* culture (Magnification 200x).

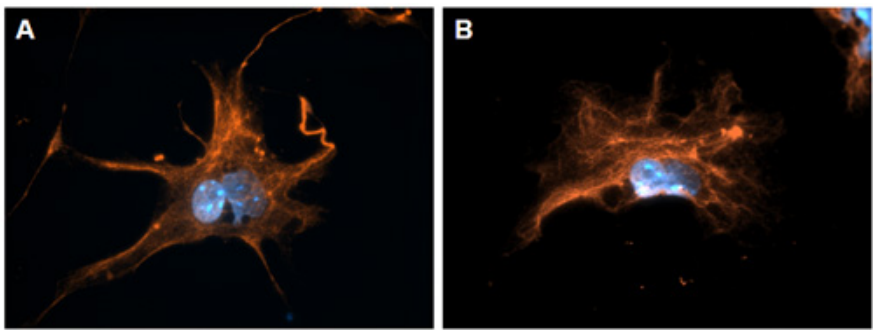

Figure 2. Hepatic stellate cells specifically express GFAP in the liver. Stellate cells were isolated and immunofluorescently stained for GFAP (red) on day 3 of *in vitro* culture. Cell nuclei are depicted in blue (Hoechst stain).

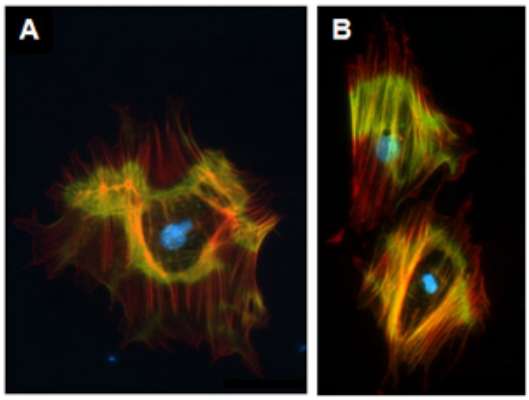

Figure 3. Liver stellate cells differentiate into myofibroblasts. Hepatic stellate cells isolated from C57BL/6 mice were cultured for 7 days. Subsequently, they were transferred to chamber slides and stained for aSMA (shown in red) and myosin IIA (depicted in green). The cell nuclei were counterstained with Hoechst (blue).

SC1 Buffer	
EGTA	190mg

Glucose	900mg
HEPES	10 ml of 1M stock solution
KCl	400mg
Na_2HPO_4 x 2 H_2O	151mg
NaCl	8g
NaH_2PO_4 x H_2O	78mg
$NaHCO_3$	350mg
Phenol Red	6mg
dH_2O	fill up to 1l
SC2 Buffer	
$CaCl_2$ x $2H_2O$	560mg
HEPES	10ml of 1M stock solution
KCl	400mg
Na_2HPO_4 x 2 H_2O	151mg
NaCl	8g
NaH_2PO_4 x H_2O	78mg
$NaHCO_3$	350mg
Phenol Red	6mg
dH_2O	fill up to 1l
GBSS-A Buffer	
KCl	370mg
$CaCl_2$ x $2H_2O$	225mg
Glucose	991mg
KH_2PO_4	30mg
$MgCl_2$ x 6 H_2O	210mg
$MgSO_4$ x 7 H_2O	70mg
Na_2HPO_4 x 2 H_2O	75mg
$NaHCO_3$	227mg
Phenol Red	6mg
dH_2O	fill up to 1l
GBSS-B Buffer	
$CaCl_2$ x $2H_2O$	225mg
Glucose	991mg
KCl	370mg
KH_2PO_4	30mg
$MgCl_2$ x 6 H_2O	210mg
$MgSO_4$ x 7 H_2O	70mg
Na_2HPO_4 x 2 H_2O	75mg
NaCl	8g
$NaHCO_3$	227mg
Phenol Red	6mg
dH_2O	fill up to 1l
Pronase E Perfusion Solution	
Pronase E	100mg (4000 PU/mg min)

SC2 Buffer	200ml
Collagenase P Perfusion Solution	
Collagenase P	85mg (1.78 U/mg lyo)
SC2 Buffer	200ml
Pronase E-Collagenase P Solution	
Pronase E	50mg (4000 PU/mg min)
Collagenase P	85mg (1.78 U/mg lyo)
SC2 Buffer	50ml
DNase I Solution	
DNase I	6mg (ca. 2000U/mg)
GBSS-B	3ml
Nycodenz Solution	
Nycodenz	8g
GBSS-A	28ml

Table 1. Buffers and enzyme solutions required for isolation of hepatic stellate cells. The pH of all buffers should be adjusted to 7.3-7.4. Furthermore, sterile filtration of all buffers is recommended. It is important to adapt the amount of enzyme used according to the given enzymatic activity.

Discussion

Hepatic stellate cells regulate essential physiological and pathophysiological processes in the liver. Moreover, stellate cells possess antigen presenting properties, rendering them an important component of the hepatic immune response. Although hepatic stellate cells comprise 10-15% of the total cell number in the liver, isolation of these cells is challenging due to their localization in the perisinusoidal space of Disse.

Here we present a straightforward method to isolate hepatic stellate cells from mouse livers by *in situ* digestion and subsequent gradient centrifugation. This protocol permits the isolation of highly pure stellate cells suitable for immunologic assays.

The entity of the isolated cells can be controlled considering three major characteristics of hepatic stellate cells, including star-like shape, perinuclear vitamin A-storing lipid vesicles, and expression of GFAP. According to these criteria, stellate cells obtained using the described protocol are routinely ~99% pure as assessed by immunofluorescent staining and flow cytometry. Potential contaminants of stellate cell isolations are Kupffer cells and liver dendritic cells (DCs). Therefore, we analyzed stellate cell cultures by flow cytometry staining for the surface molecules F4/80 (Kupffer cells) and CD11c (DCs). Accordingly, absence of F4/80+ and CD11c+ cells excluded contamination of stellate cell preparations by Kupffer cells or DCs. Thus, no further enrichment or sorting methods are required, rendering the described protocol for hepatic stellate cell isolation a convenient and fast procedure.

Concerning the entity of hepatic stellate cells, it is important to keep in mind that *in vitro* cultured cells become activated and differentiate into myofibroblasts that are fundamentally different from quiescent stellate cells. During this metamorphosis, hepatic stellate cells loose expression of GFAP and upregulate αSMA, which can be readily detected on day 7 of *in vitro* culture. Notably, activation of stellate cells *in vitro* strongly resembles their activation pattern *in vivo*[6]. This is reflected by their involvement in liver fibrosis, e.g., albeit other collagen-producing myofibroblast populations contribute to disease development[7]. To conclude, hepatic stellate cells obtained by the described protocol provide a model for quiescent stellate cells as well as for activated liver myofibroblasts depending on their differentiation stage.

Disclosures

No conflicts of interest declared.

Acknowledgements

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