Immunology Letters 158 (2014) 52–56

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

An efficient method to isolate and culture mouse Kupffer cells

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ARTICLE INFO

Article history:
Received 11 November 2013
Received in revised form
29 November 2013
Accepted 2 December 2013
Available online 12 December 2013

Keywords:
Kupffer cells
Isolate
Culture

ABSTRACT

Kupffer cells (KCs) play an essential role in the physiological and pathological functions of the liver. Although the isolation methods of KCs have been well-described, most of them are sophisticated and time-consuming. In addition, these methods are mainly used for isolating the KCs of the human and rat. In this study, a three-step procedure was applied to isolate KCs in sufficient number and purity from mouse liver, including the techniques of enzymatic tissue treatment, gradient centrifugation, and selective adherence. F4/80 immunofluorescence and flow cytometry were used for cell identification. The combination method resulted in a satisfactorily high yield of 5–6 $\times$ 10^6 KCs per liver, over 92.0% positive for F4/80 and 98.5% viable cells. After 24 h of culturing, the KCs showed typical macrophage morphologic features such as irregular shape, transparent cytoplasm and kidney-like nucleus. The phagocytic assay showed that the isolated cells exhibited strong phagocytosis activity. The KCs we isolated were functionally intact and exhibited a concentration dependent TNF-α production induced by LPS. The method we described is an effective method to isolate mouse KCs in high purity and yield, which consuming fewer collagenase and time without altering the functional capacity of the KCs.

1. Introduction

Kupffer cells (KCs), constitute 80–90% of the tissue macrophages present in the body, are increasingly recognized as important modulators which control the liver's response to injury and repair. They play a crucial role in liver homeostasis as well as in initiation, maintenance and outcome of liver inflammation [1]. Due to residing within the lumen of the liver sinusoids, they are constantly exposed to gut-derived bacteria, microbial debris and bacterial endotoxins, which are known to could activate macrophages. Upon activation, KCs release various products, including cytokines, nitric oxide and reactive oxygen species. Therefore, KCs are intimately involved in the liver's response to infection, toxins, ischemia, resection and other stresses [2]. KCs have been implicated in the pathogenesis of various liver diseases ranging from viral hepatitis to steatohepatitis, alcoholic liver disease, intrahepatic cholestasis, activation or rejection of the liver during liver transplantation, and liver fibrosis [3,4].

Vitro study using primary culture is a valuable tool for the exploration of specific immunological functions of KCs. Most previous described methods for KCs isolation utilize dissociation of the liver cells by two step collagenase perfusion, density gradient centrifugation and centrifugal elutriation using percoll solution [5–7]. Although these approaches provide certain numbers of KCs with reasonable purity, the procedures are sophisticated and time-consuming, requiring a great quantity of collagenase. In addition, the majority of these methods are used for isolating the KCs of the human and rat. In this study, based on appropriate modification of previously reported techniques, we provide a simple and efficient method to obtain KCs in sufficient number and purity from the mouse liver.

2. Materials and methods

2.1. Sinusoidal cell isolation

Pathogen-free male C57/BL6 mice (6–8 weeks), weighing 20 ± 0.5 g, were purchased from Experimental Animal Center of Chongqing Medical University. All the animals were housed in the animal care facility and received humane care in accordance with the National Institutes of Health guidelines for animal research and the legal requirements in China. Thirty-six mice were used in the present study. A modification method of the type IV collagenase digestion in vitro was used to dissociate liver tissue [8]. In detail, animals were anaesthetized by diethyl ether inhalation. The portal vein was inserted with a plastic catheter (2 mm diameter) and the liver was perfused in situ with 10 ml PBS (3 ml/min) 37 °C in a non-recirculating fashion to drive out red cell. The liver was then excised, transferred to a 35 mm culture dish and the tissue was minced to small pieces. The liver tissues were dispersed in

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http://dx.doi.org/10.1016/j.imlet.2013.12.002

10 ml Roswell Park Memorial Institute 1640 (RPMI 1640, Hyclone) containing 0.1% type IV collagenase and bathe-watered at 37 °C for 30 min, mixed gently with graduated pipette up and down per 10 min. Following digestion, the liver homogenate was filtered through a 74 μm stainless steel wire mesh to remove undigested tissue and the cell suspension was centrifuged at 300 × *g* (Eppendorf 5810R, Germany) for 5 min at 4 °C. The top aqueous phase was discarded and the cell sediment was reserved.

2.2. KCs purification

KCs were further separated from hepatocytes and other sinusoidal cells by gradient centrifugation. In detail, cell sediments were re-suspended with 10 ml RPMI 1640 and centrifuged at 300 × *g* for 5 min at 4 °C, the top aqueous phase was discarded, and the cell sediments was reserved. And then, cell sediments were re-suspended with 10 ml RPMI 1640 and centrifuged at 50 × *g* for 3 min at 4 °C. The top aqueous phase (cleared cell suspension) was transferred into a new 10 ml centrifuge tube and centrifuged at 300 × *g* for 5 min at 4 °C, the top aqueous phase was discarded, and the cell sediments were reserved. The cell sediments mainly contained non-parenchymal cells of the liver that were KCs, sinusoidal endothelial cells and satellite cells. To purify the obtained cell population further, the method of selective adherence to plastic was used according to Blomhoff et al. [9]. The cells were then seeded into 6-well plate at a density of 1–3 × 10^7/well in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) and 100 U/ml Penicillin/Streptomycin (Sigma, USA) and incubated for 2 h in a 5% CO_2 atmosphere at 37 °C. Non-adherent cells were then removed from the dish by gently washing with PBS, the adherent cells were KCs.

2.3. Identification of KCs

KCs were identified by immunofluorescence and flow cytometry using monoclonal anti-F4/80 antibody [BM8] conjugated fluorescein isothiocyanate (FITC) (Abcam, UK) [10,11]. Immunofluorescence was performed according to the instruction. In detail, KCs were seeded in a 24-well chamber slides at a density of 2 × 10^4 cells/ml. Then the cells were fixed by 4% paraformaldehyde (PFA) for 10 min and then incubated in 1% BSA/10% normal goat serum/0.3 M glycine in 0.1% PBS-Tween for 1 h to permeabilise the cells and block non-specific protein–protein interactions. The cells were then incubated with the antibody introduced above (1 μg/ml), overnight at 4 °C. Propidium iodide (PI) was used to stain the cell nuclei (red) at a concentration of 1.43 μM. After washing with PBS, cells were covered with the mounting medium and the slides were viewed by laser scanning confocal microscopy (LSCM).

Flow cytometry was performed for detecting the percentage of F4/80 positive cells. Briefly, after cultured in 6-well plate for 2 h at 37 °C, KCs were detached by incubation with 0.25% trypsin for 5 min, and pelleted by centrifugation for 5 min at 800 r/min. Then the cells were fixed with methanol for 5 min and incubated in 1X PBS/10% normal goat serum/0.3 M glycine to block non-specific protein–protein interactions. The cells were then incubated with the antibody (1:100 dilutions) for 30 min at 22 °C. The data were collected using a flow cytometer (Becton Dickinson, USA) and analyzed with FlowJo software.

2.4. Viability assay

The viability of KCs was determined by trypan blue staining. In briefly, 100 μl of cells was aseptically transferred to a 1.5 ml clear tube and incubated for 3 min at room temperature (25 °C) with an equal volume of 0.4% (w/v) trypan blue solution (Beyotime, China). Cells were counted using a dual-chamber hemocytometer and a light microscope. Nonviable cells were stained blue and viable cells were unstained. These two types of cells were recorded separately, and the means of six independent cell counts were pooled for analysis.

2.5. Phagocytic assay

Ink (Hongxing, China) was diluted at 1:800 in the growth medium, and added to the isolated macrophage-like cells seeded in 6-well plate (1 × 10^5 cells/well). After incubating for 4 h at 37 °C, the cells were rinsed with PBS for three times to remove the non-phagocytosed ink, and then observed in light microscope.

2.6. Tumor necrosis factor-α (TNF-α) production

Cells were cultured in DMEM with 10% FBS containing 100 U/ml Penicillin/Streptomycin in a 5% CO_2 atmosphere at 37 °C for 24 h. Subsequently, the cells were cultured in the presence of various concentrations of lipopolysaccharide (LPS) from *Escherichia coli* (Sigma, Germany) for 1 h under serum-free conditions. The supernatant was collected and stored at −80 °C until measured. Basic and LPS-induced TNF-α production of cultured cells were measured by enzyme-linked immunoabsorbent assay (ELISA) (Biosource, Belgium) according to the manufacturer's instructions.

2.7. Statistical analysis

Thirty-six samples have been performed for the statistical analysis in the three experiments, including immunofluorescence, flow cytometry, and ELISA analysis, with twelve samples in each experiment. Data were shown as mean values ± standard deviations. Statistical differences were analyzed by Student's *t*-test and the ANOVA test. All the tests were performed using SPSS ver. 17.0 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Hepatic non-parenchymal cells isolation

After the third centrifugation (50 × *g*, 3 min), the cell sediments mainly consist of hepatocytes (Fig. 1), while the top aqueous phase contain hepatic non-parenchymal cells, a few red blood cells and

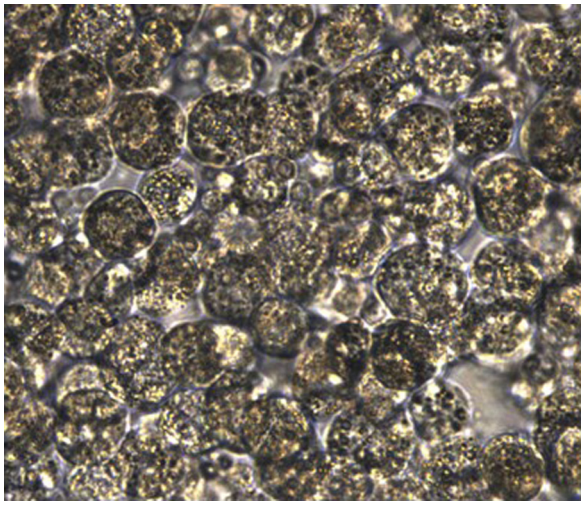

Fig. 1. The cell sediments mainly included hepatocytes after the third centrifugation. The freshly hepatocytes had a ball-like shape and contained abundant organelles, 20–30 μm in diameter when viewed under phase contrast microscope (400×).