Biochemical and Biophysical Research Communications 381 (2009) 549–553

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

The isolation and *in vitro* expansion of hepatic Sca-1 progenitor cells

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ARTICLE INFO

Article history:
Received 16 February 2009
Available online 20 February 2009

Keywords:
Oval cells
Liver progenitor cells
Liver injury
Liver regeneration
Gall bladder
Sca-1
Laminin
Collagen I

ABSTRACT

The intra-hepatic population of liver progenitor cells expands during liver injury when hepatocyte proliferation is inhibited. These cells can be purified by density gradient centrifugation and cultured. Separated by size only this population contains small cells of hematopoietic, epithelial and endothelial lineages and is thought to contain liver stem cells. The identity of liver stem cells remains unknown although there is some evidence that tissue Sca1$^+$ CD45$^-$ cells display progenitor cell characteristics. We identified both intra-hepatic and gall bladder Sca1$^+$ cells following liver injury and expanded *ex vivo* Sca1 cells as part of heterogenous cell culture or as a purified population. We found significant difference between the proliferation of Sca-1 cells when plated on laminin or collagen I while proliferation of heterogenous population was not affected by the extracellular matrix indicating the necessity for culture of Sca1$^+$ cells with laminin matrix or laminin producing cells in long term liver progenitor cell cultures.

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Introduction

Non-parenchymal cells that appear in the rat liver following treatment with carcinogenic agents, ethionine, 2-acetylaminofluorine (2-AAF) and 3-methyl-4-dimethyl aminobenzene were first described by Faber and termed "oval cells" [1]. Subsequently oval cells (OCs) have been thought to have stem cell properties as their expansion coincides with expression of genes associated with hematopoietic stem cells (HSCs) (such as c-Kit or Sca-1), they express markers characteristic of both hepatocyte and billary lineages (AFP, Alb and CK-7, CK-19, respectively) and have potential to differentiate into those lineages *in vitro* and *in vivo* [2–6].

Currently the most commonly used method to purify OCs from adult liver employs a specialised matrix such as Percoll which separates cells according to density. The first report of OC enrichment using this method was by Sells and co-workers in 1981, using liver from rats treated with Choline Deficient Ethionine supplemented diet (CDE) as the source [7]. The analysis of purified OCs reveals the presence of cell surface antigens traditionally associated with hematopoietic (Flt-3, c-Kit, Sca-1, CD45, CD34,), endothelial (CD31) mesenchymal (CD44, CD90) or epithelial (E-Cadherin) cells or their progenitors, which have size and morphology comparable to those of OCs [8–12].

The composition of OC populations expanded *in vitro* remaines heterogenous and variable. Following loss of CD45 or CD133 cells in the first 2–4 weeks (when cultured in the absence of HGF and EGF) [13] OC culture reaches equilibrium and remaines stable for up to 30 passages before the high number of differentiated cells appear. Currently there are no standards for describing components of the OC culture which may have implications in the interpretation of OCs fate or potential.

Subfraction of Sca-1$^+$ cells may contain multipotent liver progenitor cells. Originally identified as an antigen upregulated on activated lymphocytes [14] Sca-1 is the routinely used, in combination with negative selection against mature markers, to enrich adult murine HSCs [15,16]. Sca-1 is also expressed by a mixture of stem, progenitor, and differentiated cell types in wide variety of tissues and organs. Sca-1$^+$ cells are found in a variety of murine tumors, consistent with the cancer stem cell theory. To date, Sca-1 expression has been identified on putative stem/progenitor cell populations within the skeletal system, skeletal muscle, mammary gland, prostate, dermis, heart and liver [17]. Additionally, foetal or normal adult liver derived Sca-1$^+$ cells were reported to have dual differentiation potential *in vitro* [18,19,20]. Therefore a subset of Sca-1$^+$ cells may contain mutipotent adult stem cell found in adult tissues and we chose to further analyze properties of this subpopulation. In injured liver Sca-1 cells are found in the regions of OC

Abbreviations: OC, oval cell; HSC, hematopoietic stem cells; CDE diet, Choline Deficient Ethionine supplemented diet; HGF, hepatocyte growth factor; EGF, epidermal growth factor; ECM, extracellular matrix; αSMA, αSmooth Muscle Actin; ESAM, Endothelial cell-Selective Adhesion Molecule

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0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.02.079

response (Fig. 2A), areas also associated with the presence of laminin and/or collagen I producing stellate cells and myofibroblasts [21–24]. However, it remains unknown which extracellular matrix (ECM) component supports expansion of specific subpopulations of liver progenitor cells. In this study we intend to test if ECMs affect OC proliferation and in particular proliferation of FACS sorted Sca-1$^+$ subfraction.

Materials and methods

Oval cell induction, purification and culture. All animal experiments were conducted as specified by Home Office Project Licence. 8 week old mice were treated with a CDE diet (100% choline deficient chow, 0.15% ethionine in the drinking water water) for 7–21 days. The liver was perfused firstly with Perfusion Medium; (GibcoBRL) supplemented with Gentamycin (Gibco BRL) at a final concentration of 50 μg/ml for 5 min, then with Digestion Medium: (L15 [Sigma] supplemented with 50 μg/ml Gentamycin, 200 mM L-glutamine, 36 ng/ml Dexamethasone (Sigma), 1 μg/ml Insulin (Sigma), 40 mM Hepes (Sigma), 50 μg/ml DNase 1 (Roche), 250 μg/ml Collagenase type IV (Sigma) for 10 min. Liver cells were disaggregated in the petri dish then re-suspended in OC Complete Medium: 45% DMEM High Glucose (PAA), 45% Ham's F10 Medium (PAA,) 10% FBS (Hyclone), 1 μg/ml Insulin, 50 μg/ml Hydrocortisone (Sigma), 50 μg/ml Gentamycin, 2.5 ml Sodium Pyruvate (PAA). The cell suspension was filtered through a 70-μm nylon mesh and hepatocytes were depleted by centrifugation for 1 min at 300 rpm. The remaining cells were underlayed with 20% and 50% Percoll (Sigma), centrifuged at 1700 g for 20 min at 4 °C, washed twice then cultured in OC Complete Medium O/N. The next day the medium was replaced to remove non-adherent cells.

Flow cytometry and cell sorting. OCs from primary culture were washed with ice cold PBS/1% BSA. Following Fc blocking (eBiosciences) cells were stained with Rat anti-mouse antibodies: CD133 PE 1:50, c-Kit PE-Cy5 1:100, Thy-1 PE 1:100, Sca-1 APC 1:1000 (all from eBiosciences), Rat anti-mouse CD45PerCP (BD) 1:250 or Rat anti-ESAM 1:1000at 4 °C for 1 h. ESAM stained cells were incubated with anti-rat AlexaFluor 488 antibody for 30 min. Cells were washed twice with PBS/1% BSA prior to analysis on FACSCalibur or isolation on FACS Vantage (BD).

Cell proliferation analysis. A total of 1.6×10^6 Sca1$^+$ CD45$^-$ was obtained from the FACS sort. 1×10^5 of the FACS sorted cells were re-suspended in OC Complete Medium then plated on 12 well plates either uncoated or pre-coated with Laminin or Collagen I (Sigma), dilution 0.5 mg/ml and placed in an 37 °C 5% CO_2 incubator for 48 h. Cells were washed in PBS, fixed with 1% PFA, overlayed with mounting medium containing DAPI then counted using fluorescent microscopy.

Immunohistochemistry and immmunocytochemistry. Liver tissue was fixed in 2% paraformaldehyde/PBS at 4 °C for 6 h then in 20% sucrose O/N at 4 °C then stored at −80 °C. Cultured cells were washed in PBS then fixed with 1% paraformaldehyde/PBS for 10 min at 4 °C. Liver sections or cells in culture dishes were blocked in 1% serum/PBS for 30 min, incubated with primary antibody for 1 h RT, washed twice in 0.1% BSA/PBS then incubated with secondary antibody for 30 min, washed twice then mounted in medium containing DAPI (Vector Laboratories). The following primary antibodies were used: Mouse anti-Cytokeratin 7 1:100 (AbCam), Mouse anti-αSMA (SIGMA) 1:2000, Rabbit anti-panKeratin (DAKO) 1:200, Rat anti-ESAM 1:500(gift from S Butz), Rat anti-c-Kit 1:100 (eBioscences), Sca-1 1:1000 (eBioscences) and Rat anti-CD45 1:200 (BD). All secondary antibodies were from range of AlexaFluor conjugates (Invitrogen). Microscopy and Image Capture Light microscopy was performed on a Zeiss (Oberkochen, Germany) Axioskop Optical microscope using a Micropublisher 3.3 RTV digital camera (Q-Imaging®, Canada) equipped with Q-CapturePro software. Fluorescent microscopy utilised a Zeiss (Oberkochen, Germany) Axioskop 2 fluorescence microscope equipped with a triple-bandpass filter and a Hamamatsu® (Hamamatsu City, Japan) ORCA ER digital camera and analyzed using OpenLab software (Improvision® Ltd, England). Confocal microscopy used a Leica TCS SP5 microscope (Leica Microsystems GmbH®, Wetzlar, Germany). Images were analyzed with the Zeiss LSM Image browser.

Results and discussion

Induction and isolation of liver progenitor cells

Hepatic and billary progenitor cells exist at very low frequency in normal liver. We used CDE diet to induce chronic liver injury that led to hepatocyte depletion followed by activation of epithelial progenitor cell compartment [25]. Liver regeneration is usually accompanied by neovascularization and therefore expansion of endothelial progenitor cells [26]. In addition to above infiltration of hematopoietic cells and activation of stellate cells is identified as clusters of small cells in the periportal areas of the liver [27,28]. Those cells were isolated by gradient centrifugation of perfused and collagenase digested livers (Fig. 1). This method enables the purification of cells of a similar density and size, with low cytoplasmic to nucleus ratio, that represent several cell lineages found in the regenerating liver.

Sca-1 positive epithelial cells

Immmunostaining of liver sections from mice fed normal or CDE diet shows presence of small number of Sca-1 positive cells in periportal area of the normal liver and expansion of this population in the injured liver (Fig. 2A). Recent findings show that Sca-1 is expressed on liver endothelial progenitor cells, we have observed varying expression of Sca1 on subpopulation of extrahepatic biliary epithelial cells within the gall bladder (Fig. 2B). The identification of abundant Sca-1$^+$ cells within the gall bladder wall is of interest as gall bladders are regularly removed from patients and may be developed as a novel and abundant source of hepatic progenitor cells. Expression of Sca-1 on liver epithelial cells can be further confirmed by partial co-staining of cultured cells with panKeratin antibody. Primary cultures contained 15–30% Sca-1 positive cells;

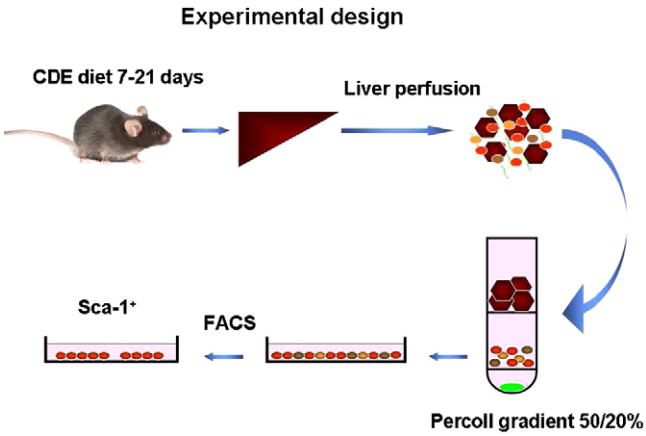

Fig. 1. Experimental design. C57B6 mice were on the CDE diet regime for 7–21 days. Following liver perfusion and digestion cells were separated using a Percoll gradient, then cultured for 4–8 weeks. A subpopulation of Sca-1$^+$ cells was isolated by FACS, plated on laminin or collagen I coated dishes and proliferation was compared to that of an unsorted population grown in similar conditions.

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