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Liver sinusoidal endothelial cells promote lymphohematopoietic differentiation from murine embryonic stem cells: Role of soluble factors

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ABSTRACT

Liver sinusoid endothelial cells (LSEC) constitute an *in vitro* and *in vivo* microenvironment for the proliferation and differentiation of hematopoietic stem cells (HSC). Previously, we have shown that LSEC support the survival and growth of murine embryonic stem cells (ESC). In this study, we investigated the capacity of LSEC to promote hematopoietic differentiation from the murine ESC cell line, CGR8. Undifferentiated ESC were cultured on LSEC monolayers in the absence of exogenous cytokines. After 10 and 20 days, cells were harvested and examined by their morphology, phenotype and capacity of hematopoietic colony formation. Microscopic observation of LSEC/ESC cocultures showed the presence of cobblestone areas formation, which indicates active hematopoiesis. Morphological analysis of cell from these foci showed the presence of hematopoietic cells at different stages of differentiation. Cells expressing B lymphoid markers (B220 and CD19) were detected by flow cytometry, and clonogenic assays showed the formation of CFU-pre B colonies. Similar results were observed when ESC were cultured with LSEC conditioned media. Myeloid precursors were also detected by the presence of CFU-GM colonies and cells expressing myeloid markers. These results indicate that LSEC provided an *in vitro* microenvironment mainly for B cell development, but also myeloid differentiation from ESC. Coculture of ESC with LSEC may constitute a very powerful tool to study the mechanisms involved in B cell generation from ESC.

1. Introduction

Embryonic stem cells (ESC) are pluripotent cells with the ability to proliferate indefinitely and with the potential to give rise to all cell types of an organism when cultured in appropriate culture conditions [1,2]. Regarding differentiation of these cells, there is great interest to generate hematopoietic cells from ESC, *in vitro*. Numerous reports have shown the capacity of ESC to differentiate into hematopoietic lineage cells [3–7]. Three different methods have been used to induce ESC to differentiate into hematopoietic cells: (1) from embryoid bodies (EBs) [8]; (2) by culturing ESC in the presence of cytokines [9]; and (3) by co-culturing ESC on stromal cell monolayers [6].

There is evidence showing that ESC can be induced to differentiate into hematopoietic lineage cells when cocultured with appropriate stromal cell lines [4]. Stromal cell lines used to induce ESC differentiation are generally derived from hematopoietic microenvironments. These include stromal cells from mouse bone marrow, fetal liver-derived stromal cells, and stromal cells derived from the aorta-gonad-mesonephros (AGM) [4–7].

We have shown the existence of a specific vascular niche for hematopoiesis at the liver, which is constituted by liver sinusoidal endothelial cells (LSEC) [10–13]. *In vitro*, LSEC support the survival, proliferation and differentiation of hematopoietic stem cells (HSC), *in vivo* as well as *in vitro* [11,14]. The ability of LSEC to support hematopoiesis, *in vivo* and *in vitro*, may be explained by their capacity to secrete numerous hematopoietic cytokines, and to express cell adhesion molecules associated with the retention of HSC [11,15]. Based on this evidence, we investigated whether LSEC might support the differentiation of ESC into hematopoietic cells. We show that LSEC promote the differentiation of ESC into B-lymphoid and myeloid cells.

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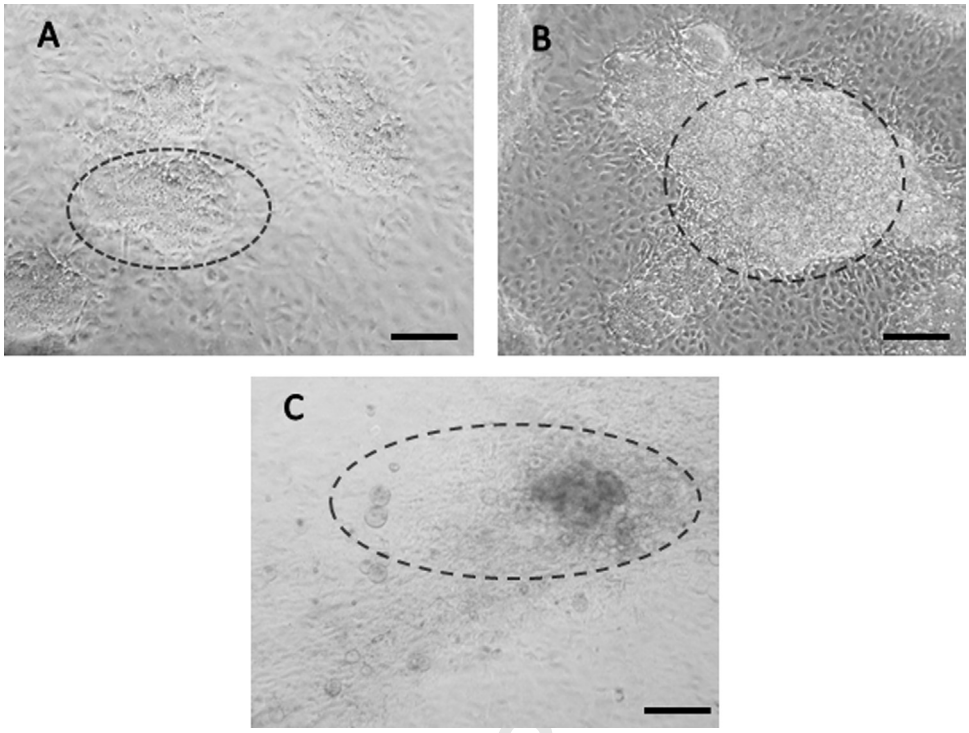

Fig. 1. Photomicrographs of ESC colonies growing on LSEC monolayers. (A) Undifferentiated ESC colony (circle) at day 3. (B) Differentiated ESC colony at day 20. (C) ESC colony grew in LSEC-CM. Bar = 200 μm.

2. Methods

2.1. Cell lines

LSEC were previously isolated, cloned and characterized [11]. LSEC were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL, NY, USA) with 10% fetal bovine serum (FBS) (Sigma, St Louis, MO, USA). They were used after reached 90% of confluence. The murine ESC cell line CGR8 (ECACC, Salisbury, Wiltshire, UK) was kindly donated by Dr Terzic (Mayo Clínic, USA). ESC were seeded on gelatin (0.1%) coated culture dishes, and maintained at 37 °C in humidified air with 5% CO_2 in standard propagation medium (PM) [1]. Propagation medium contained Glasgow's modified Eagle's medium (GMEM, BioWhittaker, Walkersville, MD) supplemented with 1 mM sodium piruvate, 1% nonessential amino acids, 1 mM L-glutamine, 0.1 mM b-mercaptoethanol, 1% penicillin–streptomycin, 7.5% FBS (all from Sigma), and 100 U/mL murine leukemia inhibitory factor (LIF) (Chemicon, USA). ESC were maintained with passaging every 3 days [1].

2.2. Coculture of ESC with LSEC

To evaluate hematopoietic differentiation from ESC, these cells were seeded (1×10^4 cells/well) onto a confluent monolayer of LSEC in 24-well plates, in IMDM media containing 10% FBS without LIF. Half of the coculture medium was collected and replaced with fresh medium every 2 days [16]. In some experiments we investigated the possible role of factors secreted by LSEC in inducing hematopoietic differentiation of ESC. For this purpose, LSEC were grown to 90% confluence, and culture medium was replaced by fresh medium (IMDM with 10% FBS), and incubated for 48 hours. After incubation, the conditioned media (LSEC-CM) was removed, centrifuged at 2000 × *g* for 15 min, and passed through a 0.2 mm filter before use. ESC were grown on gelatin-coated plates in propagation medium without LIF, but supplemented with 20% LSEC-CM.

2.3. Flow cytometry analysis

Following culture, putative hematopoietic cells were identified by flow cytometric analysis from a single-cell suspension obtained from both culture conditions. After 10 and 20 days, culture cells were collected after trypsin treatment, and cell viability was determined by trypan blue staining [17]. Cells were then incubated with a panel of 10 antibodies as follows: B220 and CD19 (for B lymphocytes); CD3, TCR, CD4 and CD8 (for T lymphocytes); Nk1.1 (for NK cells); Ter119 (for erythrocytes); and CD11b (myeloid cells). Appropriate isotype controls were performed for each flow cytometric analysis. All antibodies were purchased from Becton Dickinson (San Jose, CA). Data collection and analysis of fluorescence intensity was carried out using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Ten thousand events were acquired and analyzed using the CELLQuest software program [18].

2.4. Hematopoietic colony forming cell assays

We examined the ability of cells obtained from LSEC/ESC cocultures to form hematopoietic colonies [19]. For this purpose, these cells were assayed for CFU-pre B (colony-forming unit-B) and CFU-GM (colony-forming unit-granulocyte/macrophage) progenitors by using methylcellulose colony-forming assays (Methocult IL-7 and MethoCult GF; respectively; Stem Cell Technologies). Colonies were scored after 7 days of culture using standard morphological criteria. Single colonies were collected and spread on a glass slide using a cyto-centrifuge and stained with May Grünwald-Giemsa to observe cell morphology, under a microscope (Zeiss-Axiovert, Germany) [20].

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