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Research Article

Promotion of haematopoietic activity in embryonic stem cells by the aorta–gonad–mesonephros microenvironment

Anna Krassowska[a,1], *Sabrina Gordon-Keylock*[a,1], *Kay Samuel*[b], *Derek Gilchrist*[c], *Elaine Dzierzak*[d], *Robert Oostendorp*[e], *Lesley M. Forrester*[a,*], *John D. Ansell*[a]

[a]*John Hughes Bennett Laboratories, Division of Oncology, University of Edinburgh, Western General Hospital, Edinburgh, UK*
[b]*Adult Stem Cell Therapy Group, Scottish National Blood Transfusion Service, Edinburgh, UK*
[c]*Division of Immunology, Infection and Inflammation, Western Infirmary, Glasgow, UK*
[d]*Erasmus University Medical Centre, Department of Cell Biology and Genetics, Rotterdam, The Netherlands*
[e]*Medizinische Klinik und Poliklinik, Klinikum Rechts der Isar, Technical University Munich, Ismaningerstrasse 22, 81675 Munchen, Germany*

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ABSTRACT

We investigated whether the in vitro differentiation of ES cells into haematopoietic progenitors could be enhanced by exposure to the aorta–gonadal–mesonephros (AGM) microenvironment that is involved in the generation of haematopoietic stem cells (HSC) during embryonic development. We established a co-culture system that combines the requirements for primary organ culture and differentiating ES cells and showed that exposure of differentiating ES cells to the primary AGM region results in a significant increase in the number of ES-derived haematopoietic progenitors. Co-culture of ES cells on the AM20-1B4 stromal cell line derived from the AGM region also increases haematopoietic activity. We conclude that factors promoting the haematopoietic activity of differentiating ES cells present in primary AGM explants are partially retained in the AM20.1B4 stromal cell line and that these factors are likely to be different to those required for adult HSC maintenance.

Introduction

Embryonic stem (ES) cells are able to differentiate into a wide variety of mature cells in vitro [1] including a range of haematopoietic cell lineages [2–7]. A number of studies indicate that haematopoietic commitment in differentiating ES cells parallels that found in early stage embryos, making this a suitable in vitro model system of haematopoietic development [8–10]. In most of these studies, haematopoietic differentiation is achieved after the initial generation of three-dimensional embryoid bodies (EBs) and subsequent culture in classical haematopoietic progenitor colony assays. The differentiation of ES cells into haematopoietic lineages using a two-dimensional culture system has also been achieved when, for example, ES cells were differentiated directly on the OP9 stromal cell line derived from the CSF-1-deficient adult bone marrow [4]. A recent study directly comparing haematopoietic differentiation between the EB and OP9 system has indicated that optimal haematopoietic cell differentiation occurs when the three-dimensional EB system is used [11].

* *Corresponding author*. 4th Floor MRC Human Genetics Building, Western General Hospital, Crewe Road South, Edinburgh EH4 2XU, UK. Fax: +44 131 467 8450.
E-mail address: l.forrester@ed.ac.uk (L.M. Forrester).
[1] The first two authors contributed equally to the work.

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Several studies have shown that exposure of ES cells to embryonic tissue may have a significant effect on the differentiation of the cells. For example, an increase in cardiomyocyte differentiation was observed when human ES cells were cultured with visceral endoderm cells from the mouse embryo [12], and when mouse ES cells were cultured with explanted avian precardiac endoderm [13]. T lymphocytes were generated from ES cells in vitro by culture with foetal thymic rudiments [7], and pancreatic differentiation of ES cells in vitro was influenced by soluble factors produced from the developing pancreas [14]. We tested here whether exposure of differentiating ES cells to organ rudiments involved in lympho/haematopoietic development in vivo could stimulate haematopoietic differentiation of ES cells in vitro. Several embryonic organs have been implicated in haematopoietic development including the yolk sac [15], aorta–gonad–mesonephros (AGM) region [16], liver [17] and the placenta [18,19]. The AGM region plays an important role in initiating the generation of haematopoietic stem cells (HSCs) capable of long-term repopulation (LTR) of adult recipients [16,20]. In organ culture, the AGM demonstrated its autonomous capacity to expand LTR-HSCs [16,21], indicating that elements of their supporting microenvironment can be captured in vitro.

To investigate the impact of the AGM microenvironment on haematopoietic differentiation of ES cells, we established a co-culture system that combines the requirements for primary AGM organ culture with the defined conditions for ES cell embryoid body differentiation. We show that the number of haematopoietic progenitors arising from differentiating ES cells is significantly increased when they are co-cultured with the primary AGM region. We then co-cultured differentiating ES cells on stromal lines derived from the AGM and foetal liver that had previously been tested for their ability to support adult haematopoietic stem cells. Interestingly, the ability of the different stromal cell lines to promote haematopoietic activity in ES cells did not correlate with their supportive effect on adult bone marrow haematopoietic stem cells [22–24]. The UG26-1B6 and EL08-1D2 lines that had been shown to support adult haematopoietic stem cells (HSCs) did not promote ES cell haematopoietic activity whereas the number of haematopoietic progenitors generated from ES cells after co-culture on the AM20-1B4 cell line was significantly increased. As adult HSCs could not be maintained on the AM20-1B4 line, these data highlight important differences between the mechanisms involved in the induction and maintenance of HSC activity.

Materials and methods

Animals

C57Bl/6 mice were mated, the day of the vaginal plug designated as E0.5, and AGM regions were dissected from E10.5 and E11.5 embryos using fine tungsten needles [25]. Foetal liver was harvested from E13.5 embryos. All animal procedures were carried out according to the provisions of the Animals (Scientific Procedures) Act (UK) 1986.

Cell culture

The ES cell lines which express GFP either constitutively (GFP#7a) [26] or driven by the *Brachyury* promoter (Bry-201) [27] were maintained on gelatin-coated tissue culture flasks as described previously and differentiated into embryoid bodies (EBs) using the *hanging drop* method to make undifferentiated ES cell aggregates of as uniform a size as possible [28]. Stromal cell lines AM20-1B4, UG26-1B6 and EL08-1D2 were maintained as described [22] on gelatin-coated flasks in specialised stroma medium (50% MyeloCult long-term culture medium M5300 and 35% α-minimal essential medium containing 15% FCS, 4 mM L-glutamine and 10 μM 2-mercaptoethanol supplemented with 10–20% 0.2 μm-filtered supernatant from the previous passage). All stromal cultures were maintained at 33°C due to the presence of *tsA58* transgene encoding the temperature-sensitive SV40 large T antigen.

For AGM co-culture experiments, embryo tissue explants and EBs were placed in direct contact at the air/medium interface on semi-permeable hydrophilic 0.65 μm Durapore membranes supported by stainless steel grids (Fig. 1) in ES medium (in the absence of LIF) and with no additional cytokines. Co-cultures were harvested at defined time points, and the number of ES-derived (GFP^+) haematopoietic progenitors was determined using the agar-based CFU-A and/or HPP-CFC assays. In the co-cultures involving stromal cell lines, EBs were cultured directly on γ-irradiated (30 Gy) stromal cells or transparent Greiner Bio-one 24 well ThinCert-tissue culture inserts (membrane pore size of 0.4 μm and pore density of 2×10^6) were used to inhibit direct contact between the EBs and stromal cells. Cultures were harvested, digested with dispase II (1.2 U/ml) and DNAse I (7 μg/ml) in PBS for 1 h at 37°C then passed through a 23-gauge needle to generate single cell suspensions for analysis by haematopoietic colony assays and flow cytometery.

Haematopoietic colony assays

Agar-based assays

The CFU-A assay was performed as described previously [29,30] A feeder layer of 0.6% agar in a modified Eagle's medium (25% α-MEM, 20% horse serum, 0.25% sodium bicarbonate and 4 mM L-glutamine) supplemented with 10% conditioned medium from each of two cell lines, L929 and AF1-19T (a source of M-CSF and GM-CSF, respectively), was poured into 3 cm diameter tissue culture grade dishes (1 ml per dish). Cells were resuspended in 0.3% agar in Eagle's medium at a density of 3×10^4 cells/ml and plated onto the agar feeder layers in triplicate. After incubation at 37°C in a 5% O_2 and 10% CO_2 humidified atmosphere for 11 days, the colonies (>2 mm in diameter) that primarily consisted of myeloid cells were counted. The procedure for the HPP-CFC [31] assay was similar to the method for the CFU-A assay but conditioned medium from the L929 and WEHI 3b cell lines (a source of M-CSF and IL3, respectively) was added. Colonies were counted after 14 days and, like the CFU-A assay, consisted of myeloid cell types.

Methylcellulose-based assays

CFU-mix and CFU-GM assays were performed by plating 1×10^5 test cells in 35 mm plates containing 1.5 ml 1% methylcellulose