Introduction

Endothelial colony-forming cells (ECFCs) have been identified as endothelial progenitor cells with robust proliferative potential in vitro and for their ability to form new blood vessels in vivo when transplanted in immunodeficient mice (1)(2)(3). Thus, ECFCs have emerged as a favorable target for vascular regenerative therapies for a variety of diseases, including ischemia, diabetic retinopathy, bone repair and cancer. However, the therapeutic study of ECFCs is compromised by low frequency in both peripheral blood (PB) and umbilical cord blood (UCB) and therefore samples must be expanded in vitro prior to use. Current formulations for expanding ECFCs in vitro contain fetal bovine serum (FBS), and as such exhibit additional variability. Furthermore, animal proteins or serum in the medium raise concern when ECFCs are considered for therapeutic applications. To minimize these risks associated with exposure to animal serum, new culture media are required. Here we characterized ECFCs derived and expanded in EC Cult™-XF ECFC Medium, a novel xeno-free (XF) medium and matrix system to isolate and expand ECFCs from primary CB without the use of FBS. Our data shows that EC Cult™-XF ECFC Medium is highly efficient at supporting cell attachment, clonogenic growth and long-term expansion of ECFCs directly from CB under xeno-free culture conditions without any serum requirement.

Materials & Methods

Derivation of ECFC Colonies

Primary UCB was processed by Lymphoprep® density separation and mononuclear cells (MNCs) were isolated. For outgrowth of ECFC colonies, MNCs were either resuspended in EC Cult™-XF ECFC or serum-containing (SC) media. MNCs (2.5 - 5 x 10^5/well) were seeded onto 6-well pre-coated plates with animal component-free (ACF) attachment substrate. The frequency of ECFC colonies was determined by measuring the total number of colonies in the primary culture on day 10.

Expansion of ECFCs

ECCFs were expanded by passing at 10,000 cells/cm^2 in the medium in which they were derived. The number of population doublings (PD) occurring between passages (P) was calculated as log2(# viable cells at harvest/# cells seeded). The population doubling time (PDT) was determined using the time interval between cell seeding and harvesting divided by the number of PDs for that passage.

Single Cell Clonogenic Assays

Early passaged (1 - 2) ECFC-derived EOs were plated at one cell per well in 96-well plates pre-coated with animal component-free (ACF) attachment substrate. Cells were cultured in either EC Cult™-XF ECFC or SC media for 14 days. Cells were fixed with 4% paraformaldehyde, washed and stained with 1.5 µg/mL DAPI, and examined for the growth of ECFC colonies.

Immunophenotyping of ECFCs

Early passaged ECFCs (p4-p5) cultured in EC Cult™-XF ECFC or SC media, were analyzed by flow cytometry for expression of endothelial markers CD31, CD144 and hematopoietic marker CD45. To assess the ability of endothelial cells to incorporate Dil-acetylated low-density lipoprotein (Di-Ac-LDL), attached cells were incubated with 10 µg/mL Di-Ac-LDL for 4 hours at 37°C. Cells were washed 3 times and stained with 1.5 µg/mL of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma). Cells were examined for uptake of DiAc-LDL using a Zeiss microscope.

In Vitro Functional Assay

In vitro vascular network formation was tested by seeding 5,000, 7,500, 10,000 and 20,000 cells ECFCs on Matrigel® coated wells of a 96-well plate. The vascular network formation was documented by acquiring images at 2 hour intervals covering a time period of 24 hours using an Olympus IX81 HD.

In Vivo Functional Assay

To evaluate vascular repair ECFCs derived in EC Cult™-XF ECFC or SC media were used in a mouse model of oxygen-induced retinopathy (OIR). Oxygen-induced retinopathy was induced in C57BL6 wild-type mice, as previously described(5). Briefly, postnatal day (P) 7 newborn mice received a 0.5 µL intratretinal injection containing EC Cult™-XF ECFC or SC derived ECFCs and were exposed to 75% oxygen for 5 days. DiPBS was used as vehicle and injected in the left eye of each pup as a control. At P12 they were transferred back to normal air. All pups were euthanized at P17 and retinal flat mounts were stained with isocitron 4B-594. Stained retinas were visualized and imaged using a confocal microscope. Obliteration area and neovascularization were quantified using Adobe Photoshop as described.

Results

Table 1: Pheno-tipic characterisation of human CB ECFCs derived and expanded in SC and EC Cult™-XF ECFC Media. Immunophenotyping of the endothelial monolayer revealed that ECFCs cultured in EC Cult™-XF ECFC medium expressed endothelial cell surface antigens CD31 and CD144, similar with those cultured in SC medium. Most importantly, ECFC colonies cultured in EC Cult™-XF ECFC medium did not express the hematopoietic cell surface antigen CD45.

Figure 1: Isolation of ECFCs and number of colonies obtained in SC and EC Cult™-XF ECFC media. A) Colonies derived in either media displayed a cobblestone appearance with variations in size, consistent with heterogeneous proliferative rates previously reported. B) No difference was observed in the frequency of ECFCs recovered on day 10 under EC Cult™-XF ECFC and SC media (n = 3 donors).

Figure 2: ECFC expansion in SC and EC Cult™-XF ECFC Media. A) Average cell expansion of cultured ECFCs from P4 to P9 in EC Cult™-XF ECFC and SC media. B) To quantify and compare the proliferative kinetics of ECFCs expanded in both media cumulative PDs were calculated. There was no significant difference in the PD and PDT of EC Cult™-XF ECFC derived ECFC compared with SC derived ones.

Figure 3: Clonogenic ability is maintained in human CB ECFCs derived and expanded in SC and EC Cult™-XF ECFC Media. A) A significantly higher number of colonies formed from ECFCs incubated in EC Cult™-XF ECFC than in SC medium (37.7 ± 3.5 versus 25.3 ± 6.4, mean ± SEM; p<0.05, paired t test). B) The entire hierarchy of ECFCs, composed of high proliferative potential (HPP), low proliferative potential (LPP) ECFC, endothelial-cluster, and non-dividing mature ECs was derived from ECFCs cultured in both EC Cult™-XF ECFC or SC media. Remarkably 40% of single-plated EC Cult™-XF ECFC derived ECFCs that divided formed well-circumscribed colonies containing from 2,000 to 10,000 cells in the 14-day culture period.

Figure 4: Phenotypic characterisation of human CB ECFCs derived and expanded in SC and EC Cult™-XF ECFC Media. Immunophenotyping of the endothelial monolayer revealed that ECFCs cultured in EC Cult™-XF ECFC medium expressed endothelial cell surface antigens CD31 and CD144, similar with those cultured in SC medium. Most importantly, ECFC colonies cultured in EC Cult™-XF ECFC medium did not express the hematopoietic cell surface antigen CD45.

Figure 5: Single Cell Clonogenic Assays

- SC Cult™-XF ECFC
- SC ECFC
- EC Cult™-XF ECFC

Figure 6: EC Cult™-XF ECFC and SC derived ECFCs form tube-like structures in vitro. Representative images of vascular network formation from 2 donors of ECFCs cultured in SC and EC Cult™-XF ECFC on Matrigel®. Representative pictures are taken at 18 hrs. ECFCs grown EC Cult™-XF ECFC form much bigger tubes than ECFCs grown in SC media (p<0.0001, “Incyte” software).

References


Summary

- EC Cult™-XF ECFC is a new medium able to efficiently support attachment, isolation, clonal growth and expansion of ECFCs directly from primary human CB.
- ECFCs cultured in EC Cult™-XF ECFC Medium showed similar expansion to ECFCs cultured in serum control medium.
- For the first time, it is possible to derive and expand ECFCs from human CB in a defined xeno-free culture system facilitating their use in translational research.