PURE POPULATIONS OF PROLIFERATING ENDOTHELIAL CELLS DERIVED AND EXPANDED *IN VITRO* FROM EMBRYONIC STEM CELLS

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ABSTRACT

Because embryonic stem cells exhibit long-term proliferation, they could potentially provide an unlimited supply of tissue for meeting human transplantation needs. Our lab is particularly interested in the endothelial cell lineage. In the present study, we described a methodology for isolating pure populations of actively proliferating endothelial cell populations from murine ES-D3 cells using a 2-D differentiation system combined with two rigorous cell sorting steps. The unique portions of this protocol included sorting Flk-1+ cells and a second manual selection step based on cell morphology that led to further purification of the endothelial-like cell outgrowths. Using this methodology, we have been able to make significant progress in the ability to derive endothelial cells from ES cells, and have repeatedly obtained uniform populations of proliferating endothelial cells that could be expanded at least 20 to 25 population doublings each. In addition to expressing several common endothelial cell markers, these ES-derived endothelial cells are also able to form vascular structures and reorganize their cytoskeleton in response to laminar shear stress. These cells may now be used to further examine and characterize *in vitro* cell maturation and test for *in vivo* integration and proliferation potential.

INTRODUCTION

Vascular endothelial, or endothelial progenitor cells, derived from stem cells could potentially lead to a variety of clinically relevant applications. These cells could be used to in therapeutic vascularization to repair and revascularize ischemic tissue in patients exhibiting vascular defects[1]. In addition, it has been shown that endothelial cells are able to transdifferentiate into cardiomyoctes and may potentially be used to repair cardiac function.[2] Since it is well known that endothelial cells inhibit platelet adhesion and clotting, endothelial cells are needed for lining the lumen of a synthetic or tissue-engineered vascular graft. Moreover, because endothelial cells line the lumen of blood vessels and can release proteins directly into the blood stream, they are ideal candidates to be used as vehicles of gene therapy. Endothelial cells may additionally be used for vascularizing tissue-engineered materials prior to implantation and for investigating mechanisms of angiogenesis and vasculogenesis.

One potential source for these therapeutic endothelial cells is the embryonic stem (ES) cell. These ES cells boast unlimited *in vitro* expansion potential.[3] ES cells have been shown to differentiate into a variety of cell types using 3-D structures called embryoid bodies that at least partially mimic the spatial organization of the embryo. Endothelial cells have been derived from human embryonic stem cells by isolating the differentiating endothelium from an embryoid body. [4] Although the embryoid body system enables investigation of vasculogenesis virtually as it occurs in the embryo[5-8], the multiple cell-cell contacts and cell lineages make it difficult to study and control the behavior of the maturing endothelial cell in detail. However, it has been shown that the 3-D structure is not requisite for endothelial maturation from ES cells.[9] Endothelial, hematopoietic, and smooth muscle cells have been derived from Flk-1+/E-cadherin- outgrowths from murine ES cells grown on type-IV collagen coated surfaces.[9] This 2-D technique of endothelial differentiation allows one to more closely control the *in vitro* maturation of endothelial cell derivation.[9-10]

METHODS

Cell Culture

ES-D3 embryonic stem cells (American Type Culture Collection, Manassas, VA) were initially maintained on irradiated embryonic fibroblast feeder layers in Knockout Dulbecco's modified eagle medium containing 15% ES Cell Qualified Fetal Bovine Serum (Gibco), 5% Knockout Serum Replacement, 1,000 units per ml of leukemia inhibitory factor (LIF) and $5x10^{-5}$ M β-mercaptoethanol. Cells were then cultured on 0.1% gelatin (no feeders) for one week before switching to differentiation conditions.

Initiating differentiation, 30,000 ES cells were transferred to collagen type IV-coated dishes and cultured for 4 days without LIF in α-Minimal Essential Medium, 15% Fetal Bovine Serum, and β-mercaptoethanol. On day 4, the cells were stained for Flk-1, FACS sorted, and re-plated on collagen type IV in differentiation medium supplemented with 50 ng per ml of recombinant human $VEGF_{165}$.

After culturing for approximately one week, cells exhibiting predominantly two different phenotypes emerged. These included cells with a cobblestone morphology, very similar to endothelial cells, and more striated smooth muscle-like cell populations (Fig. 1)

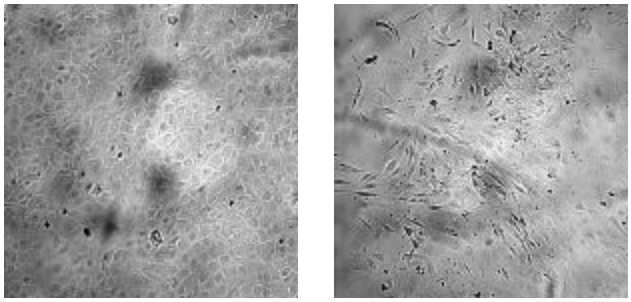

Fig 1. Outgrowths of Flk-1 positive cells consisted of primarily two cell populations: endothelial-like cells exhibiting a cobblestone-like morphology (left), and striated smooth muscle-like cells (right).

Manual selection of endothelial-like cells

Prior to isolation of the endothelial-like Flk-1 outgrowths, the cells were first washed with PBS and incubated in cell dissociation solution for 5 minutes. The cells that exhibited endothelial-like morphologies were manually selected using a flame-pulled pasteur pipet connected to an aspirator assembly fitted with a syringe filter. Based on cell morphology alone, 5-10 cells were excised and replated in each well of a collagen type-IV coated 12-well plate. At this stage, the cells were fed endothelial cell EGM-2 media supplemented with 10ml FBS, 0.2 ml hydrocortisone, 2ml hFGF-B, 0.5ml VEGF, 0.5ml R3-IGF-1, 0.5 ml ascorbic acid, 0.5ml hEGF, 0.5 ml GA-1000, 0.5 ml heparin (EGM-2 Bullet Kit, Clonetics), $5x10^{-5}$ M β-mercaptoethanol, and an extra 50 ng per ml of recombinant human $VEGF_{165}$. These cells were then expanded up to 25 population doublings on mouse collagen type-IV, 0.1% gelatin, or fibronectin. At each passage, the concentration of $VEGF_{165}$ was decreased until the only VEGF remaining was a proprietary amount provided in the EGM-2 Bullet Kit.

RESULTS

Consistent with the literature,[10] the outgrowths from the isolated Flk-1 positive cells exhibited predominantly two morphologies: a cobblestone-like morphology, very similar to endothelial cells, and a striated smooth muscle-like morphology (Fig 1). The striated cells were positive for α-smooth muscle actin and negative for endothelial marker expression. Each of the manually isolated endothelial-like populations, consisting of 5 to 20 cells each, were expanded to 20 to 25 population doublings. To our knowledge, these ES-derived endothelial cells are the most pure and actively proliferating ES-derived cells obtained without the aid of genetic manipulations.

Since these ES-derived endothelial cells were expanded virtually from clonal isolations, the cell behavior and morphology sometimes differed slightly between the cell populations from different isolations. Some of the cell populations were observed exhibiting a behavior that seemed to be mimicking 2-D vasculogenesis (Fig. 2), while others maintained a cobblestone-like morphology not unlike the cells depicted in Fig. 1. The cells that consistently formed 2-D tube-like networks maintained this behavior for many passages and still continued after the cells had been frozen and thawed several months later.

In addition to careful observation of cell morphology, the ES-derived endothelial cells exhibited many common endothelial cell markers. The cells stained positive for Flk-1, CD34, Flt-1, VE-cadherin, PECAM-1, and CD105, organized into 3-D networks when embedded in collagen type-I gels, and elongated and aligned in the proper direction when exposed to 15 dynes/cm^2 of shear stress for 24 hours.

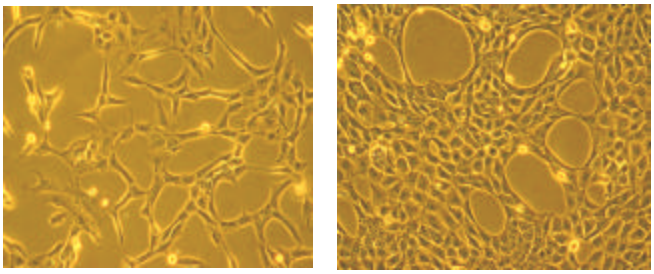

Fig 2. Some ES-derived endothelial cells were observed mimicking vasculogenesis for several cell passages. Note that at confluence, the 2-D tube-like structures are maintained and the cells will not proliferate further until reseeded at low density.

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