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Review Article

Experimental approaches to derive CD34+ progenitors from human and nonhuman primate embryonic stem cells

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Abstract: Traditionally, CD34 positive cells are predominantly found in the umbilical cord and bone marrow, thus are considered as hematopoietic progenitors. Increasing evidence has suggested that the CD34+ cells represent a distinct subset of cells with enhanced progenitor activity; CD34 is a general marker of progenitor cells in a variety of cell types. Because the CD34 protein shows expression early on in hematopoietic and vascular-associated tissues, CD34+ cells have enormous potential as cellular agents for research and for clinical cell transplantation. Directed differentiation of embryonic stem cells will give rise to an inexhaustible supply of CD34+ cells, creating an exciting approach for biomedical research and for regenerative medicine. Here, we review the main methods that have been published for the derivation of CD34+ cells from embryonic stem cells; specifically those approaches the human and nonhuman primate stem cells. We summarize current status of this field, compare the methods used, and evaluate the issues in translating the bench science to bedside therapy.

Keywords: CD34+ progenitor cells, embryonic stem cells, differentiation, cell therapy, nonhuman primate model, bioassay

Introduction

The molecule CD34 was found in hematopoietic stem cells in 1984 [1]. It is a mucin-like transmembrane phosphoglycoprotein with molecular weight of 115 kD; the extracellular domain contains the groups that are heavily sialylated as well as glycosylated [2]. There is increasing interest in analyzing and better understanding the function of CD34 protein, What is known so far is that CD34 protein plays an important role in mediating cell-cell adhesion [3]; for example, it mediates the attachment of stem cells to bone marrow matrix or to stromal cell surfaces. Several molecules have been identified as its natural ligands including L-selectin (CD62L) and Crk-like protein. When cell-cell interactions with signaling molecules such as CD62L occur, CD34+ cells may be mobilized and become proliferative. Therefore, it is believed that CD34-mediated adhesion regulates cell differentiation and proliferation. Adult cells expressing CD34 are quiescent under physiological conditions but become active when homeostasis is altered. Once mobilized, they propagate into lineage-specific maturity according to the nature of the signaling molecules. For the past few decades, the focus on CD34+ cells has been primarily in regard to hematopoiesis in bone marrow. Now emerging evidence shows that CD34+ progenitors exist within a variety of tissues and can be mobilized. To date, it has been demonstrated CD34 expressing cells, in addition to hematopoietic stem cells, exist in stromal, epithelial, and vascular tissues. In an intriguing recent review [4], the authors implied that CD34 is a general marker for a variety of progenitors in tissues prior to commitment to the lineage-specific differentiation.

CD34-based stem cell therapy has been under development for more than a decade. Injection of CD34+ hematopoietic stem cells selected by monoclonal antibodies reconstituted bone marrow hematopoietic capacity after lethal irradiation of baboons [5]. The development of a ther-

apy was accelerated by the collaborative effort of the research team at Fred Hutchinson Cancer Research Center and CellPro Inc. [6, 7]. Years later, the discovery of circulating endothelial progenitor cells positive for CD34 has deeply impacted the growth of this field [8, 9], extending beyond hematopoietic reconstitution to the demonstration that the progenitors in the circulation could repair the vascular damage. To date, clinical trials using CD34+ cells have been conducted to treat a variety of diseases including acute and chronic ischemic heart failure, spinal cord injury, liver cirrhosis, and peripheral vascular diseases. A recent PubMed search using the keyword "CD34 cell therapy" revealed 6,948 articles about this topic. Because of the therapeutic potential, there is a need to identify easily accessible and reliable source of CD34+ cells, which are a primary focus for future translational application. Currently autologous or allogeneic CD34+ cells from adult tissues are used in clinical trails, but this strategy is probably not practical or maximally efficient or effective for large scale future applications. With the advancement of capabilities to manipulate embryonic stem cells, the controlled/directed differentiation of pluripotent stem cells has made it possible to obtain large CD34+ populations of clinical relevance [10]. This review focuses on the experimental approaches to generate CD34+ cells from embryonic stem cells (ESCs) and discusses their properties.

CD34+ cell derivation based on recapitulation of embryological events

As a result of the ability of ESCs to undergo differentiation, many functional cell types can be generated in vitro; these cells are capable of contributing to numerous tissues in vivo [10, 11]. Many investigators have been attempting to recapitulate the embryonic events that lead to the mature cells and direct the development of embryonic stem cells along a specific pathway to acquire derivatives with particular functionalities [12]. The general strategy for this approach is to remove the factors that maintain the pluripotency of stem cells and to add factors that stimulate differentiation toward of particular developmental fate. Specifically, confluent human ESC colonies are disrupted either manually or enzymatically into small aggregates [13] or aggregates of a specified size [14]; then, they are cultured on a low adherence plate, in suspension culture, or in semisolid medium [15]. They become a virtually spheroid structure, called an "embryoid body". ESCs inside an embryoid body undergo spontaneous differentiation to some extent but exhibit rigorous changes in the presence of particular growth factors in a time-dependent manner. Although ESCs differentiate in either monolayer culture or suspension culture, ESCs within embryoid bodies may be a better model to initiate the early differentiation events and to respond to cues that direct embryonic development [10]. This differentiation process parallels normal morphogenesis and results in the formation of a micro-tissue; therefore, many investigators generate CD34 cells based on this directed differentiation approach as reviewed elsewhere [16].

Direct differentiation using co-culture system

Although ESC differentiation toward CD34+ cells can be carried out by recapitulating the developmental process, this approach might not be very efficient, because there are many unknown factors involved in this process. Several investigators have tried to enhance the efficiency of the differentiation process by co-culturing stimulating/signaling cells and ESCs, since the development of functional cells naturally relies on intercellular interactions. When human ESCs were cultured together with bone marrow stromal cell line S17 [17] or OP9 [18], higher yields and highly hematopoietic activities of CD34+ cells could be achieved. It has been reported that up to 20% CD34+ cells and 10 million cells with 95% purity could be obtained using that approach. The CD34+ cells generated in this manner expressed hematopoiesis-associated genes and retained clonogenic potential after in vitro expansion. Further culture on MS-5 stromal cells in the presence of SCF, Flt-3, and IL-7 and IL-3 gave rise to lymphoid and myeloid lineages [18]. The application of co-culture has become more sophisticated recently as researchers aimed at creating niches to enhance the generation of CD34+ progenitors. A recent example of this approach established that when ESCs were cultured with a mixture of macrophage and mesenchymal stromal/stem cells, which may be reminiscent of the bone marrow niche, CD34 cell yield was not only increased, but their engraftment potential was enhanced for a period of 16 weeks [19]. It seems reasonable that in vitro

recapitulation of an in vivo microenvironment promotes the differentiation process. A niche, a structural complex consisting of several types of cells, might give a better outcome than the environment provided by the single stromal cell type.

Growth factors essential for CD34+ cell differentiation

Studies from developmental biology show that three signaling pathways activin/Nodal, BMP, and canonical Wnt regulate the development of ESCs to blood cells [20]. Using a serum-free culture medium, a group of investigators outlined the minimal factors required for directed differentiation toward CD34+ cells [21]. They proposed that four factors, including BMP-4, activin A, bFGF and VEGF, were sufficient to specify mouse ESCs to a hematopoietic fate; but, the induction must be stepwise in order to maximize CD34 expression. When human ESCs were cultured in a fully defined serum-free medium containing BMP-4, Flt-3 ligand, SCF, thrombopoietin and VEGF, CD34+ cells were generated [13]. For baboon ESC culture, we found that those factors, together with bFGF, gave rise to CD34+ cells after a stepwise differentiation process [14, 22]. The need for bFGF was also suggested by another report [23].

It has been reported that CD34+ yield could be enhanced if some signaling pathways are inhibited. Earlier studies showed that an efficient method to generate CD34+ cells involved inhibition of MEK/ERK signaling and activation of BMP-4 signaling at the same time [24]. A more recent report demonstrated that inhibition of glycogen synthase kinase-3 (GSK-3) could increase the production of CD34+/KDR+ cells [25].

Integrated system for progenitor generation

To best mimic the embryonic development of CD34-related cells, a combined cultured system has been reported. While embryoid body formation mirrors normal development, co-culturing with stromal cells may release growth factors that enhance the generation of well-functioning progenitors. A representative of such a system was the initial embryoid body method to form a mesodermal structure for 7 days followed by co-culture with OP9 stromal cells plated on the culture dishes for another 7 days [26]. The authors that describe this approach state their protocol enabled efficient expansion of several types of CD34+ cells with long-term repopulating ability.

Hope and hype of CD34+ cells in regenerative medicine

Based on the current pace of stem cell research and the development of improved strategies for enhancing efficiency, there is hope that stem cell therapies may change the future of medical modalities [10, 16, 27]. However, embryonic derived CD34+ progenitors have not been tested in a clinical setting. By comparison to retinal pigment epithelium progenitor cells and neuronal progenitor cells that are in clinical trials [28, 29], ESC derived CD34+ cells have been left behind. Following are some of the future explorations that are required to unleash the promise of CD34+ cell therapy to become a reality.

First, we need to establish the procedure to formulate CD34+ progenitor cells as therapeutic agents. The differentiation of CD34+ cells from ESCs is an extremely spatial- and temporal-dependent event, and the signaling pathways directing lineage commitment are not entirely clear; consequently, a robust and reproducible protocol remains to be defined [30]. It is possible that different CD34+ subpopulations directed by specific differentiation methods have distinct therapeutical potentials that are currently unknown. Many progenitor cells may express this molecule, as mentioned above; thus further characterization is essential to determine their differentiation fates and the functional characteristics of their derivatives. For example, $CD34^{brignt}$/CD31+/KDR+/CD45+ may be the cells with hemato-endothelial potential [31], while CD34+/CD43-/CD45+ cells represent hematopoietic progenitors with myeloid lineage potential [32]. Therefore, it is important to know the composition of CD34+ formulated cell lines, since subpopulations positive for CD34 have the biological functions that remain to be explored.

Second, there is a strong demand to establish bioassays to verify what type of cells are required for a specific clinical need, as part of mode of action studies. For a long time in the field of ESC derivative studies, there have been more efforts focusing on gene expression pro-

filing, in vitro functionality testing, and nude mice transplantation, and less on cellular behaviors in a natural environment where the cells will need to function. Alternatively, we need a bioassay to test the functional characteristics of the derivatives in vitro or ex vivo. The bioassay needs to involve a live tissue in order to assess or standardize the activity of the agents containing CD34+ cells. Using a living section of either a tissue or an organ will enable us to track the adhesion, homing, and propagation of engrafted cells in the very site that cell replacement will be required. In our studies, we established an ex vivo bioassay to evaluate the functional reconstitution of CD34+ cells derived from baboon ESCs. We tested the ability of these cells to repair the damage induced by experimental manipulation [14, 22]. By doing so, we could characterize the therapeutic cells in an interactive environment, in which we could not only access the functional characteristics but also the suitable maturation stage. In the future, we will use this assay to standardize the protocols for vascular progenitor cells in order to determine reliably and consistently the functionalities of candidate cells and to adjust experimental protocols to meet the requirements for the future medical use of ESC-derived cells.

Third, the action of therapeutic cells needs to be tested in a relevant animal model. We all must establish that the derivatives are capable of long-term engraftment in animals with appropriate multi-lineage proliferation and differentiation to execute their therapeutic effects. However, the animals to be grafted should be physiologically close to humans in order to provide the correct information for the translational medicine. Scientists have used animals such as sheep [19, 33], and chicken [34], and mouse. for this type of research. However, increasing evidence indicates that dramatically different mechanisms regulating stem cell differentiation exist between species. Despite striking similarities exist between mouse and human development, numerous stem cell therapies developed in mice have failed when tested in humans [35, 36]. One of the reasons may be attributable to species-specific differences in signaling pathways that maintain pluripotency and affect differentiation. The need for an animal model that is more similar to humans prompted us to initiate research that uses a large nonhuman primate, the baboon, for early stage translational research to establish the feasibility of stem cell-derived cell therapy. We have developed techniques that allow us to generate endothelial progenitors from baboon ESCs whose phenotypes are found in adult vasculatures; and we have developed the baboon as a model for research on cardiovascular disease in order to test rigorously the effectiveness and safety of stem cell derived vascular progenitors in primates.

Although a plethora of CD34+ cell generation methods have been reported [37], the rationale of the differentiation approach remains unexplained in most instances. Similarly, few protocols have been validated yet. However, successful ESC-based therapies depend developing a simple and efficient protocol for the differentiation of ESCs into large number of functional CD34+ progenitors. This is a major focus of translational stem cell biology. We believed that the integration of understanding the early events of embryogenesis in culture and validating a scientifically sound primate test system for identifying useful cell type(s) will accelerate the CD34+ cell therapy for treating patients.

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