Is Endothelium the Origin of Endothelial Progenitor Cells?

Mervin C. Yoder

Abstract—Endothelial cells provide the dynamic lining of blood vessels throughout the body and provide many tissue-specific functions, in addition to providing a nonthrombogenic surface for blood cells and conduit for oxygen and nutrient delivery. As might be expected, some endothelial cells are injured or become senescent and are sloughed into the bloodstream, and most circulating endothelial cells display evidence of undergoing apoptosis or necrosis. However, there are rare viable circulating endothelial cells that display properties consistent with those of a progenitor cell for the endothelial lineage. This article reviews historical and current literature to present some evidence that the endothelial lining of blood vessels may serve as a source for rare endothelial colony–forming cells that display clonal proliferative potential, self-renewal, and in vivo vessel forming ability. The article also discusses the current gaps in our knowledge to prove whether the colony-forming cells are in fact derived from vascular endothelium. (Arterioscler Thromb Vasc Biol. 2010;30:1094-1103.)

Key Words: angiogenesis ■ endothelial function ■ endothelium ■ thrombosis ■ endothelial progenitor cells ■ vascular endothelial cells

Many articles have been published in the past decade attempting to clarify the definition, origin, and function of endothelial progenitor cells (EPC). Over that time, it has become apparent that many different cell types play a role in vessel repair and regeneration and that these cells may display a variety of cell surface markers in common. At present, no specific markers can prospectively identify an EPC, and thus, the origin of the cell cannot be clearly defined. The present article does not attempt to provide an overview of all the various EPC definitions but rather asks whether there is sufficient published evidence that some resident vascular endothelial cells or the rare viable circulating endothelial cells (CEC) display functional attributes consistent with those that one would envision for an EPC. We also discuss some of the current impediments to proving the origin of the CEC with proliferative potential. Although this review discusses both animal and human systems, it is weighted toward representing ability. The article also discusses the current gaps in our knowledge to prove whether the colony-forming cells are in fact derived from vascular endothelium.

Endogenous Endothelial Cell Turnover

Endothelial cells form a nearly continuous lining of the blood vascular system that serves to prevent thrombosis, deliver blood-borne nutrients into tissues, and serve tissue and organ-specific functional requirements for necessary levels of oxygen delivery and waste removal. Vascular endothelial cells also regulate arterial reactivity to various stresses through synthesis and release of numerous vasoactive molecules. The endothelial cells lining large conduit vessels are exposed to a variety of biomechanical forces related to bolus delivery of cardiac output at rest and during periods of cardiovascular stress. Such stressors may lead to endothelial injury requiring repair or replacement. Evidence from numerous laboratories has demonstrated that the overall basal rate of endothelial cell replication in normal blood vessels is negligible; however, even at homeostasis, there are certain regions of the vascular endothelium that demonstrate healthy replicative rates.1–7 Endothelial replication is also influenced by the age of the subject, hyperlipidemia, hypertension, and endotoxemia.8–11 Of interest, the proliferative behavior of the endothelial cells is not evenly distributed among all the intimal cells. When 3-month-old female rats were administered 3 doses of tritiated thymidine, maps of the aortic endothelium revealed clustered foci of replication that reached as high as 60% in individual areas. Although these areas could represent sites where endothelial cells were being lost and replaced, it was also likely that the areas of proliferation could represent areas of vessel enlargement or growth. Thus, endothelial proliferative potential is not equivalent among all the endothelial cells comprising the aorta in the growing rodent; some areas display essentially no replicative potential, whereas other areas display high replicative potential.3 In addition, all current assumptions implicate the proliferative endothelial cells to be retained and stationary within the intima, but it is possible that these cells may become highly migratory and even mobilized on activation.4

The exact stimulus for inducing endothelial replication in an intact vessel at homeostasis remains largely unknown but may represent a mechanism to replace injured or senescent cells without exposing the underlying basement membrane.12 Experimental denudation of arterial and venous vessels in experimen-
tional animals has suggested that removal of small strips of endothelium can be repaired without immediate endothelial proliferation, although larger areas of injury induce local proliferation. In one study, endothelial wounding in the rat femoral artery via insertion of a stainless steel wire led to a reproducible denuded area of approximately 6 to 8 endothelial cells wide and 3 cm in length. Endothelial integrity was largely restored by 24 hours after the injury via spreading and migration of viable endothelial cells from the edge of the wound, though proliferation was induced in cells scattered throughout the uninjured cells bordering the edge of the original injury. Over the next 24 hours, endothelial replication appeared within nearly all the cells along and within the original wound border, and by 96 hours, the cell density within the resolving wound area was >2-fold greater than the nonwounded endothelium in the same vessel. Increased endothelial density remained in the area of the healed wound for as long as 16 weeks after the original injury. Thus, endothelial loss via injury and the resulting loss of contact inhibition (for the remaining viable endothelium) may be one stimulus for inducing endothelial migration and replication within the viable endothelial cells within a vessel in vivo. Alternatively, the damaged and wounded cells may emit some form of message to the surrounding healthy cells. How this message is conferred to the viable resident cells outside the boundaries of the injury is unknown, but cells as far away as 240 μm may be recruited to proliferate as a response to the injury. These data raise additional questions as to the mechanism whereby endothelial cells induced to replicate may contribute to the healing wound; do they migrate (crawling along in contact with the denuded basement membrane), do they mobilize (released into the circulating blood losing all contact with the endothelial surface or denuded basement membrane), or do all the endothelial cells simply shift position?

Whether all the proliferating cells (of the healing wound) emerge from within the endothelium of the vessel of injury or are recruited via the bloodstream from circulating endothelial or endothelial precursor cells has not been clarified. Many of the early studies of the repair of denuded arteries and veins in experimental animals used light, scanning, or transmission electron microscopy; Hauden preparations (removal of fixed sheets of endothelial cells that retained the original distribution of the cells within the vessel); and radiolabeling of nuclei in dividing cells, and these techniques, although informative, were at times inadequate to provide definitive analysis of the cell types present within the injured vessel during repair. By all measures, platelet deposition on the denuded basement membrane was an early and consistent feature of many kinds of injury in rats or rabbits. If the injury was small, endothelial cells appeared to be able to close the wound within several days. If the wound was large, then in some cases, full endothelial covering took several weeks to a month in the rat. Before full endothelial closure, a variety of hematopoietic and cells of indeterminate origin of flattened appearance were noted to attach to the denuded membrane. In most experimentally induced injuries in rats, the denuded membrane was completely replaced with endothelium arising from proliferating cells emigrating from the arterial branches. However, in the rabbit, a species prone to atherosclerosis, very large denuded areas often failed to completely re-endothelialize, and numerous smooth muscle cells were reported to line the nonendothelial covered areas. These data suggested that some species differences may exist with respect to the degree of endothelial responsiveness (and independence from other supportive cells) to healing a denudation injury.

In contrast to the proliferative behavior observed in systemic blood vessels in vivo, the endothelial cells in some tissues do not divide to maintain tissue homeostasis. Corneal endothelial cells form a fragile monolayer of cells along the posterior edge of the mammalian cornea that is responsible for regulating the transparency of the cornea. Water and nutrients from the aqueous humor permeate the corneal endothelium and move into the collagenous corneal stroma, where the hydration status is regulated by the corneal endothelium via the use of potent barrier properties and ionic pump functions. Human corneal endothelial cells do not proliferate during the normal adult life span, and thus, the density of cells diminishes with age. Rather than proliferate to replace cells lost to injury, disease, or senescence, the corneal endothelial cells simply spread out and migrate toward each other to cover the area previously occupied by the lost cell. Thus, the morphology of corneal endothelial cells in elderly patients typically reveals larger cells with less density in the central region of the cornea with smaller more densely populated cells along the corneal periphery. Not surprisingly, corneal transparency can be lost when the endothelial density falls below a threshold level, and patients may require corneal transplant or Descemet membrane endothelial keratoplasty to replace the dysfunctional endothelial monolayer.

**Evidence for Circulating Viable Endothelial Cells in Animal Model Systems**

Reports of putative CEC seeding materials implanted within large arteries and veins in experimental animals systems have been widely disseminated for nearly 50 years. Examination of implanted aortic grafts derived from artificial biomaterials or formalized porcine heterografts in experimental dogs led to the report that none of the graft surfaces were lined by a complete endothelial lining. In the area of the anastomosis of the graft with host, one could identify a host endothelial intima; however, no other endothelial cells were found except in some dispersed islands near the center of the grafts. These and other reports suggest that the origin of such endothelium in implanted nylon vascular grafts may result from regeneration and extension of host endothelium or from capillary ingrowth. Stump et al constructed intraprosthetic Dacron hubs that were implanted in the aorta of growing pigs and demonstrated that islands of endothelium formed on the Dacron directly from cells circulating in the bloodstream by 2 weeks after insertion (the hubs were not seeded by contiguous endothelium from the aorta). Dacron grafts made impermeable to transmural capillary ingrowth have also been implanted in the canine descending aorta or inferior vena cava. None of the implants were noted to be clotted, and all showed evidence of scattered islands of endothelial cells, providing further proof of “fallout endothelialization” of Dacron vascular grafts in both arterial and venous systems. The degree of fallout endothelialization of the implanted Dacron grafts was inversely correlated with the shear force of blood flow in these
grafts, as endothelial cell coverage was highest on the grafts implanted in the inferior vena cava and lowest in the descending thoracic aorta. Despite clear evidence of the phenomenon, the origin of the cells seeding the grafts was not evident. A canine bone marrow transplantation assay in which donor and host DNA could be clearly distinguished by a sensitive and specific microsatellite assay was used to determine whether the endothelial cells lining an implanted impervious Dacron graft were derived from the transplanted bone marrow or from within the host. Whole marrow cells were infused into the conditioned recipient animals, and donor origin circulating blood cells were evident as long as 6 months later. Examination of grafts implanted within the thoracic aorta of the transplant recipient dogs revealed islands of endothelium that only expressed the DNA alleles of the donor marrow cells. Some of these endothelial cells expressed the CD34 antigen. In the same report, human marrow CD34+ cells were demonstrated to give rise to endothelial colonies in vitro at a frequency of 4 to 10 per 100,000 CD34+ cells plated. These data were interpreted to support a role of CD34+ bone marrow hematopoietic stem cells to give rise to circulating endothelial precursors that seed implanted Dacron graft material within blood vessels and form an endothelial neointima. However, no evidence was presented that coexpression of canine donor marrow alleles and the CD34 antigen was present in any of the canine endothelial cells that coated the implanted Dacron material, thus leaving some unresolved questions. Indeed, recent data obtained from human peripheral blood and umbilical cord blood support the derivation of circulating outgrowth endothelial cells from a small subset of CD34+ cells; however, these endothelial cells with proliferative potential do not appear to be derivatives of the hematopoietic system or from a hemangioblastic precursor cell.

### Evidence of Viable CEC in Human Subjects

Whereas the experimental animal model systems provide evidence of CEC that can seed implanted materials intravascularly, evidence for a similar phenomenon occurring in human subjects is less robust. One approach to search for CEC in human patients has been to examine the blood-membrane barrier of implanted left ventricular assist devices (LVAD) used to treat patients with myocardial failure. Among all LVAD, the first generation device, Heartmate I, is unique because the surface of the blood-pumping mechanism is comprised of titanium microspheres with a fibrillar textured inner surface. This unique surface chemistry permits formation of a circulating blood-derived “pseudointima” that is antithrombogenic. Whereas all other LVAD require patients to receive warfarin therapy, patients bearing the Heartmate I require only aspirin for anticoagulation. Extended implantation of an LVAD in patients results in formation of a collagenous covering, presumably produced by resident myofibroblasts within the pseudointima. Rarely, one may also find islands of endothelial cells attached to the pseudointima. These cells may be deposited via blood-borne endothelium or endothelial precursors, but they may also represent pieces of tissue dislodged at implantation. Other reports suggest that some endothelial cells may originate from ingrowth of native endothelial cells invading the pseudointima as transmural capillaries.

However, a detailed analysis of the cellular composition of the pseudointima formed in the implanted Heartmate I device awaited studies conducted by Rafii et al. This group characterized the cellular composition of the pseudointima formed on the surface of an implanted LVAD in patients with myocardial failure as a bridge to transplantation. After the device was removed, the neointima was removed and sectioned, and some of the tissue was fixed and stained for morphological examination. Most of the neointima was digested into a cellular suspension using collagenase, and the isolated mononuclear cells obtained were analyzed for expression of various hematopoietic cell surface proteins using monoclonal antibodies and flow cytometry. More than 90% of the mononuclear cells were myeloid hematopoietic cells, and some were hematopoietic progenitor cells, with a minor fraction of fibroblast and myofibroblast cells. Incubation and expansion of the hematopoietic progenitor cells in vitro led to proliferation of a large number of nonadherent hematopoietic cells, including mostly CD14-positive mononuclear cells. Thus, circulating blood monocytes and monocyte-derived macrophages are the primary source of cells recruited to the fibrillar meshwork lining the Heartmate I, and these cells serve to set up a nonthrombogenic surface via recruitment of circulating mesenchymal cells. Alternatively, the monocytes may differentiate into collagen-synthesizing fibrocytes, a known differentiation pathway involved in wound healing.

Prosthetic vascular grafts comprised of expanded polytetrafluoroethylene, Dacron, and polyurethane have been used for vascular repair in human subjects for nearly half a century. One of the most remarkable features of implanted large diameter grafts is the fact that nearly all fail to become covered with an endothelial intima even after functioning for decades in vivo. Thus, many of the animal models in which “spontaneous” endothelialization of implanted materials was routinely observed failed to provide predictive outcomes for eventual human implant studies. At least 4 factors appear to influence the process of graft endothelialization: host species, age of host, anatomic dimensions of the graft, and graft surface composition. Surprisingly, dogs and juvenile nonhuman primates display significantly greater endothelialization of an implant than adult human subjects. Younger nonhuman primates demonstrate much more rapid rates of endothelial coverage of an implant than older animals. Because of size constraints, dogs cannot be implanted as puppies, whereas young sheep, calves, and pigs accommodate appropriately sized implants, and thus, many implant studies have been conducted in dogs with relatively senescent endothelium. The animal model also causes constraints in implant size (ie, rats and rabbits versus pigs and sheep) that do not permit direct comparisons of the extent of endothelial covering. Finally, the types of biomaterials implanted clearly determine differences in the rate, extent, and composition of the ingrowth of tissue. These data point out the limitations in understanding human vascular reparative mechanisms when using current animal models.

A variety of cell surface antigens that are expressed on endothelial cells have been targeted with tagged monoclonal antibodies in attempts to enumerate the concentration of CEC in the bloodstream of healthy and ill individuals. Table 1 lists some of the antigens that are most commonly used for...
Table 1. Cell Surface Antigens (CD Designation) Expressed by Human CEC

<table>
<thead>
<tr>
<th>CD Antigen</th>
<th>Other Names</th>
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<tbody>
<tr>
<td>CD31</td>
<td>PECAM-1</td>
</tr>
<tr>
<td>CD34</td>
<td>Mucosialin</td>
</tr>
<tr>
<td>CD105</td>
<td>Endoglin</td>
</tr>
<tr>
<td>CD144</td>
<td>Vascular endothelial cadherin</td>
</tr>
<tr>
<td>CD146</td>
<td>Muc18</td>
</tr>
<tr>
<td>CD202b</td>
<td>Tie2</td>
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<tr>
<td>CD309</td>
<td>Vascular endothelial growth factor receptor 2</td>
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Reviewed in detail in Timmermans et al.79

CEC detection. Because there is no single marker that can definitively identify the CEC, many approaches combine the use of several antigens to improve sensitivity and specificity. In general, there are 3 technical approaches applied to CEC determination: manual immunomagnetic, automated immunomagnetic, and flow cytometric selection systems.

Perhaps the most widely used approach relies on the use of antibodies to the CD146 antigen conjugated to paramagnetic beads. This approach was first described in 199250 and has been improved and validated in more recent publications.51 Briefly, the procedure enriches the CEC from the blood via CD146-coated magnetic microbeads, followed by cell counting using fluorescent microscopy following cell staining with acridine orange or with a fluorochrome-labeled lectin (Ulex europaeus agglutinin 1). By a consensus agreement, CEC are thought to represent mature sloughed endothelial cells of heterogeneous size (10 to 50 μm).52 Woywodt et al53 have delineated the potential pitfalls in use of this approach to count CEC. This method permits direct visualization of the CEC and thus permits discrimination of the CEC from contaminating platelets, microparticles, and leukocytes.54 More recent protocols using polychromatic flow cytometry have more accurately defined the CEC, and the concentration of circulating elements is much nearer those values identified using the manual or automated immunomagnetic bead method.62,64

Despite the multiplicity of methods to identify and count CEC present in the bloodstream in normal and diseased human subjects, few of these methods permit prospective identification of the viable CEC with proliferative potential. As noted above, the consensus opinion is that CEC are mature sloughed cells devoid of proliferative potential. The rarity of the CEC in the peripheral blood of a normal healthy adult subject69,51 makes it difficult to enrich for these rare events, as there are currently no specific antigens for the CEC (see above). In actuality, the concentration of the viable endothelium with proliferative potential is considerably less than the CEC concentration, with only 1 proliferative endothelial cell present in 10^n to 10^6 peripheral blood mononuclear cells.65 Nonetheless, Case et al29 and Timmermans et al50 reported that adult and cord blood endothelial cells with proliferative potential can be enriched using immunomagnetic beads conjugated to CD34 following a CD45 immunodepletion step with a significant enrichment of nearly 300-fold.

In Vitro Assays of CEC With Proliferative Potential

Although the presence of CEC had been widely reported in human subjects, the origin of these cells was not known, leading Lin et al69 to devise a clever approach to address this question. Peripheral blood was drawn from 4 patients who had undergone a sex-mismatched bone marrow transplant (for clinically relevant reasons) and had consented to participate in a study to permit an analysis of whether CEC grown from the blood carried donor or recipient Y-chromosome specific DNA. More than 95% of the CEC in fresh blood samples displayed the host genotype. However, outgrowth endothelium demonstrated a gradual change from host to donor genotype over 4 weeks of culture in vitro. Indeed, calculation of the cellular expansion suggested that host outgrowth endothelium increased 17-fold, whereas the donor genotype displayed more than 1000-fold expansion. These results led Lin et al69 to conclude that most of the endothelial cells that circulate in the bloodstream are vessel derived; however, in the context of a bone marrow transplant recipient, those circulating rare cells with exuberant proliferative potential are engrafted circulating angioblasts. Although this was an exciting
hypothesis, the authors concluded that proof of the existence of a narrow angioblast would require identification of a novel marker restricted to this cell and not other mature endothelium and a single-cell culture method to permit accurate determination of the starting clone and its specific progeny.

We have developed a specific method to isolate and culture outgrowth endothelial cells in a clonal fashion.56 By plating cells under the defined conditions at a single-cell level, we found that a complete hierarchy of endothelial colony–forming cells (ECFC) could be identified from adult peripheral blood or umbilical cord blood outgrowth endothelial cells. Although the majority of the single plated cells displayed no proliferative potential, some endothelial clones with high proliferative potential gave rise to more than 10,000 progeny in the 2-week culture period. The cell surface antigen expression of the various ECFC populations (high proliferation versus no proliferation) was not distinguishable from one another or from endothelial cells isolated from the cord blood umbilical vein. The high proliferative cells displayed self-renewal activity in addition to the capacity to generate numerous progeny with lower levels of proliferative potential and even mature nondividing endothelium. The high proliferative potential-ECFC (HPP-ECFC) displayed higher levels of telomerase activity than the cells with low proliferative potential. The frequency of ECFC was higher in umbilical cord blood samples than in adult peripheral blood. Subsequently, we31 and others70–74 demonstrated that cord blood ECFC display robust in vivo human blood vessel formation on implantation in immunodeficient mice. Not only do the ECFC form a complex 3D capillary plexus, but these vessels insulate with the murine vessels to become part of the murine systemic blood vascular system. Thus, use of this clonal system for ECFC analysis permits one to determine that the resident and circulating ECFC display clonal proliferative potential, the HPP-ECFC display self-renewal and secondary endothelial colony–forming potential, and ECFC readily form human blood vessels in vivo de novo in immunodeficient mice. Are these properties consistent with those of an EPC?

Although EPC definitions are not the focus of this particular review, they are varied and controversial, ranging from use of certain cell surface antigen profiles (Table 2) to a variety of colony assays of adherent cells.42,75–79 A review of the published evidence for the various roles of circulating cells from human subjects in the process of neovascularization suggests that there are numerous hematopoietic cell subsets that play important roles in promoting and regulating the process of vascular repair and regeneration, although these cells play only transient roles as vessel lining cells.75–79 It is not unexpected that hematopoietic cells migrating through tissues would attach to residual basement membrane proteins from regressing blood vessels (where the endothelial cells are dead and gone but the membrane remains) in an area of tissue damage or high cellular turnover (as in a tumor microenvironment), because hematopoietic cells are some of the earliest invading cells involved in wound resolution and angiogenesis induction.80–84 However, the only cells that display in vivo vasculogenic activity (formation of a primitive capillary plexus in the complete absence of any prior vessel presence) are the rare clonogenic circulating ECFC and ECFC present in the endothelial lining of human blood vessels.34 Perhaps one feature that may assist in the definition of an EPC is the ability of the recruited cell to synthesize and deposit an endothelial basement membrane during the new vessel formation.

One interesting observation about the ability to isolate and culture endothelial cells with clonal proliferative potential is that the ability to isolate ECFC varies from one animal system to another. Thus, ECFC, which are numerous in the bloodstream of nonhuman primates,85 human subjects,29,31,65,86 pigs,87 dogs,88 and cows,89 are so rare in mice that the entire blood volume of 5 or more 8-week-old mice was required to identify endothelial colonies in 11% of attempts to isolate at least 1 endothelial colony.90 These data suggest that species differences may exist with respect to the presence of CEC with clonal proliferative potential, and this is an interesting and important area for future comparative investigation.

### In Vitro Assays of Resident Endothelial Cell Proliferative Potential

As noted above, the vascular endothelium demonstrates a low level of replication at homeostasis. However, it is a well-known fact that robust cultures of proliferating endothelium can be derived from nearly any human and most animal vessels. In fact, cultures of human umbilical vein endothelium have long served as a fundamental culture system to examine nearly every aspect of endothelial biology. Where does all the replicative potential reside? Are all the cultured endothelial cells equally capable of proliferation? These specific questions can only be addressed using a single cell (clonal) analysis of endothelial cell proliferative potential.

We have reported that the complete hierarchy of ECFC demonstrated in umbilical cord blood also resides in primary cultures of human umbilical vein and adult aortic endothelial cells.86,91 Similar to cord blood ECFC, umbilical vein and aortic endothelial cells with the highest proliferative potential displayed the highest telomerase activity. Thus, the replicative potential is not uniform throughout the vascular endothelium; some cells display high proliferative potential,

<table>
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<th>Immunophenotype of Human Endothelial Progenitor Cells (Not Inclusive)</th>
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<tr>
<td><strong>Immunophenotype</strong></td>
</tr>
<tr>
<td>CD31 CD34+</td>
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<tr>
<td>CD34+ CD62L+</td>
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<tr>
<td>CD34+ CD133+</td>
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<td>CD34+ CD11b+</td>
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<td>CD34+ FGFR1+</td>
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<tr>
<td>CD14+ CD34+</td>
</tr>
<tr>
<td>ALDH+PI+</td>
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<td>CD133+ CD45−</td>
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whereas the majority of cells fail to divide in the clonal assay. Of interest, these results support the observations of Schwartz and Benditt, who described focal clusters (perhaps clonal progeny) of proliferative endothelium mixed with areas of nondividing cells in the developing rodent aorta.

More recently, a direct comparison of the proliferative potential residing in the endothelium of the rat pulmonary microvasculature and pulmonary artery was reported. Numerous differences in cell surface markers and physiological responsiveness had previously been reported between rat pulmonary microvascular endothelial cells (PMVEC) and pulmonary arterial endothelium (PAEC), including the higher replicative potential always noted in the cultured microvascular endothelium. When plated in the single cell assay, rat PMVEC were noted to have a completely different distribution of ECFC, with significantly more HPP-ECFC than were found in the individually plated PAEC. This observation could explain the fact that PMVEC as a population are known to display a proliferative rate 2 times that of PAEC in vitro. This difference in growth rate was maintained even when both populations were cultured in low-serum (0.1%) conditions, as nearly one third of the PMVEC remained in S phase. One potential molecular explanation was the finding that cyclin D1 expression was significantly higher in PMVEC than PAEC even in low-serum conditions. Although the reduced serum concentration failed to growth arrest the PMVEC in vitro, contact inhibition eventually did inhibit PMVEC growth, and this was associated with a significant increase in the Cdk inhibitor p27Kip. At present, the upstream signaling pathways regulating the enhanced cyclin D1 expression in the PMVEC remain undetermined. When the highly proliferative PMVEC-derived HPP-ECFC were collected and pooled, these progenitors continued to display tighter barrier function in vitro than the PAEC, similar to the freshly isolated lung microvascular endothelium. Thus, despite displaying high proliferative potential, these PMVEC-derived cells retained some of the unique functional features previously reported for the resident microvascular endothelium.

Although human corneal endothelial cells fail to sufficiently divide during the normal lifespan of adult human subjects, these cells retain replicative capacity that can be displayed rarely in vivo and reproducibly in vitro. Laing et al reported that in one individual who developed a rejection reaction to an implanted cornea, mitotic figures were detected by specular microscopy. Serial measurements over an 8-month period documented the appearance of multiple clusters of small endothelial cells gradually enlarging and replacing the much larger original corneal endothelium. Thus, at least in this patient, corneal endothelial cells may display clonal replicative potential as a response to an immune-mediated injury to the endothelium. Evidence of corneal endothelial proliferation may also be observed in corneas cultured intact ex vivo following a scratch wound or as a response to calcium chelation that releases the endothelium from contact inhibition. Human corneal endothelial cells can also be isolated, cultured, and expanded in vitro for a limited number of passages. Of interest, age-related differences in expression of negative regulators of the cell cycle (p16INK4a and p21WAF1/Cip1) in human corneal endothelial cells have been reported that may account in part for reduced proliferative responses displayed by corneal endothelium in elderly subjects.

In contrast to the limited growth of human corneal endothelial cells, bovine corneal endothelium is well known to proliferate extensively in vitro. Surprisingly little has been reported concerning the clonal proliferative properties of cultured corneal endothelial cells. We have recently discovered that the clonal proliferative potential of bovine corneal endothelium is similar to that displayed by endothelium isolated from bovine aorta or coronary arteries. A complete hierarchy of ECFC, including HPP-ECFC, was detectable in the corneal endothelial cultures performed in clonal fashion, pointing out the fact that replicative potential is a property that is heterogeneously displayed by the cultured cells. In sum, these studies suggest that ECFC reside as primary endothelium in some endothelial monolayers removed from mammalian tissues.

**Markers to Define ECFC**

To date, there are no known specific and restricted molecules that permit discrimination of human ECFC from nondividing mature endothelium, whether the endothelium is resident in a vessel or present within the circulation. However, because the majority of human CEC are senescent or apoptotic, one may simply search for those CEC that are viable to find some cells with proliferative potential. ECFC also differ from other putative EPC within the blood by not expressing the common leukocyte antigen CD45 or the myeloid antigen CD14 or CD115. Cultured ECFC express CD34, CD105, CD31, CD146, and CD144 (Table 1), and on interleukin-1 stimulation, they upregulate vascular endothelial cell adhesion molecule-1 expression. Thus, some molecules have been defined that permit enrichment of ECFC but not definitive prospective isolation or determination of the cells. Indeed, there are no distinct antigens yet detectable that permit discrimination of ECFC from resident vascular endothelial cells or viable CEC that lack proliferative potential. Furthermore, ECFC are difficult to isolate via flow cytometric sorting (despite the use of the large macrosort nozzle, diminished sheath pressure, and use of antioxidants in the cells sorting culture medium) and have largely been enriched using immunomagnetic bead approaches, which complicates approaches to obtain highly purified populations. Finally, the challenge of identifying ECFC within resident vascular endothelium will require novel reagents that permit determination of those cells that possess proliferative potential, even if they largely exist in a dormant state integrated among the more numerous nonreplicative endothelial cells.

**Preclinical Studies Supporting a Role for Proliferative Endothelium as a Cell Therapy**

The majority of preclinical and clinical trials of human cell therapy for various forms of ischemic vascular disease have relied on autologous bone marrow–derived cells (fresh, cultured, or enriched for CD34 expression) that have displayed modest regenerative or reparative roles that have not been as efficacious as predicted from preclinical studies performed in rodent models. In addition to modifying the current protocols to improve outcomes, alternative sources of cell therapy have been examined. For example, direct injection of early outgrowth EPC combined with late outgrowth endothelial cells (contains ECFC) resulted in significantly greater vascular regeneration and rescue.
of limb function following an ischemic injury in mice than infusion of either cell population alone. When tissue-engineered human skin substitutes containing human keratinocytes were seeded with a variety of endothelial precursors, the cord blood–derived ECFC exhibited greater human blood vessel density in the viable human grafts (implanted in immunodeficient mice) than in grafts seeded with adult human peripheral blood ECFC or human umbilical vein endothelial cells. As noted above, although many authors have reported that cord blood and adult peripheral blood ECFC spontaneously form blood vessels on implantation in immunodeficient mice, the application of this finding has been limited to the few preclinical studies cited above. Thus, the actual clinical potential of using ECFC to repair vascular damage in human subjects remains exciting and full of potential but unproven, and it requires additional investigation.

Summary

The evidence supporting endothelial proliferation within mammalian blood vessels is without question. Whether this proliferation is focally distributed (arising in clusters from a progenitor in situ) in the endothelium is a question that has been addressed in only a few species (rats and a human subject) under a few experimental variables. Future studies focused on investigating the normal turnover of endothelial cells in systemic vessels in multiple mammalian species (across the range of short-lived rodents to long-lived and much larger species) may prove whether the endogenous proliferation of resident endothelium correlates with the recent in vitro clonal studies demonstrating a hierarchy of proliferative potential residing within individual vascular endothelial cells. Some of these ECFC are also found to circulate within the bloodstream of several mammalian species, including dogs, pigs, cows, monkeys, and human subjects, although they are exceedingly rare in mice. Future studies focused on identifying whether these rare circulating cells are derived from the resident endothelium are warranted and will require some novel imaging and lineage-restricted reporter systems to provide unambiguous results. Although these inducible lineage restricted reporter systems are best developed in the mouse, the limited proliferative potential of the vascular endothelium (and general lack of CEC) in this species will make it challenging to extrapolate findings to larger and more long-lived species. It is hoped that continued advances in the generation of transgenic rats and pigs will permit such comparative analyses to be performed. The properties displayed by the ECFC are those that meet many of the qualities of an EPC as proposed by some investigators.

Determination of unique markers for circulating and resident ECFC would simply clarify their role in normal and abnormal vessel formation and function. Novel approaches to the isolation of viable CEC that permit documentation of the clonal properties of the cells may be required because current flow cytometric approaches have proven detrimental to ECFC survival in vitro. Finally, development of in vitro and in vivo assays to evaluate the molecular interactions among the proangiogenic hematopoietic cells and the in vivo vessel forming ECFC (with respect to vasculogenesis, inosculation, and vessel remodeling) may permit the ability of investigators to isolate these cell populations from patients with various diseases and document the functional deficiencies that may exist but that are currently undetectable.

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Disclosures

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