Cell-based endothelialization approaches for emergent/emergency coronary artery bypass surgery

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Cardiovascular disease is the leading global cause of death, with coronary artery disease as the largest contributor to that death toll. Coronary artery bypass graft surgery (CABG) using small-diameter (< 6 mm) vascular grafts is the leading treatment for coronary artery disease. Native vessels are commonly used for CABG but have limited availability in some patients. Synthetic grafts fail due to thrombosis typically. This makes biological grafts on which a non-thrombogenic luminal endothelial layer is created from autologous nonimmunogenic cells prior to implantation very attractive solutions for CAB grafts. There are several sources of endothelial cells being explored currently, but few are suitable for the case of emergency CABG. This review summarizes the cell sources used in recent efforts for *in vitro* endothelialization of CAB grafts and analyzes their suitability for use in emergency CABG. The conclusion is that only adipose-derived endothelial cells provide a suitable source for emergency CABG.

Introduction

The World Health Organization reports that cardiovascular disease (CVD) is the leading global cause of death with ~17.9 million CVD-deaths (32% of all deaths) in 2019. Of those deaths, 85% resulted from myocardial infarction and stroke[1]. In the United States, CVD is the leading cause of death regardless of sex and most racial and ethnic backgrounds with ~679 thousand CVD deaths in 2020, 20% of all 3,358,814 deaths. Coronary artery disease (CAD) is the leading cause of CVD-related mortality with a death toll of 382,820 (11% of all deaths, 56% of CVD deaths) in the US in 2020 alone. Of people 20 years old and older, 20.1 million suffer from CAD. About 2 in every 10 CAD deaths occur in people aged less than 65 years[2,3].

Coronary artery bypass (CAB) grafts are one of the leading treatments for CAD. In 2016, there were 201,840 coronary artery bypass grafting surgeries (CABG) performed in the United States[4]. In most cases, CABG involves the “bypassing” of an occluded coronary artery with a new vessel anastomosed to the blocked coronary artery upstream and downstream of the blockage. This requires the use of a small diameter (< 6 mm) vascular graft, typically 3-4 mm diameter. About 63% of CABG cases (~127 thousand) are non-elective, 49% of patients (~99 thousand) have diabetes, and 31% (~42 thousand) have already undergone percutaneous coronary intervention[5], with US annual cases shown parenthetically based on the 2016 total.

Emergency coronary artery bypass grafting surgeries

CABG with urgent planning (an approximately 54-hour time frame[6] due to the mortality risk is considered an "emergency" (eCABG) *versus* "elective." Those most likely to benefit from and receive eCABG are those with more advanced disease that cannot be managed with catheter-based therapies. eCABG was performed on ~1 million acute myocardial infarction (AMI) patients between 2000 and 2017. That equates to 9.2% of all AMI admissions during that period for an average of ~59 thousand eCABG cases for AMI patients alone annually. There is a trend of increasing lengths of hospital stays and hospitalization costs over that time even though the absolute number of CABG cases is decreasing[7]. Though the trends are decreasing, the number of patients requiring CABG for survival remains quite large considering the prevalence of CAD. In isolated CABG cases (those not associated with AMI) between 1993 and 2019, ~2.95% of cases were emergent, again with a decreasing trend over time, reaching ~1.7% in 2016 and ~2.1% in 2019[7]. In 2016, that would have been ~3,400 cases, far less than CABG associated with AMI.

Autologous vessels for use as coronary artery bypass grafts

Autologous vessels are the current gold standard for CABG[8] because they are available immediately, and as living tissue, they can remodel and adapt to their new location after implantation. The saphenous veins, internal thoracic arteries, radial arteries, and internal mammary arteries are the most common native vessels used as CAB grafts. The left internal mammary artery performs by far the best with > 90% 10-year patency. However, the saphenous vein is used in 80-90% of CABG surgeries worldwide, making it by far the most used option. When saphenous vein grafts (SVG) are placed into arterial circulation, they spontaneously remodel to adapt to the increased stresses of the arterial system: smooth muscle cells appear and produce more extracellular matrix leading to wall thickening. This ability to "arterialize" is a benefit of using a living graft that can be replicated to a certain extent *in vitro*[9], though complications from this remodeling are a significant source of SVG failure[8]. Notably, 10-25% of SVG occlude within the first year after implantation (usually due to intimal hyperplasia), and 40-50% occlude within 10 years (usually due to atherosclerosis)[8].

Need for tissue-engineered coronary artery bypass grafts

It was reported that up to 30% of patients did not have suitable saphenous veins for CABG based on venography[10]. This could be due to prior usage or a patient's disease state. As another example, transradial catheterizations cause damage in the radial artery that persists up to 12 months[5]. This makes the prospect of small-diameter vascular grafts very attractive for general use in CABG. Such grafts would eliminate the reliance upon existing suitable native vessels for graft material and reduce comorbidities associated with vessel harvest. The failure of any synthetic material to perform as a CAB graft, usually due to occlusive clotting, led to the concept of a tissue-engineered vascular graft (TEVG), that is, a graft produced by living cells[11]. The ideal TEVG would be available "off-the-shelf" (acellular),

non-immunogenic, anti-thrombogenic, and regenerative (*i.e.*, able to be transformed into native tissue via recellularization post-implantation). Using a TEVG possessing a functional endothelium might be the best way to me*et all* these requirements, although such a graft cannot then be strictly "off-the-shelf," as further considered below.

The importance of the anti-thrombogenic activity of the endothelial cell (EC) cannot be overstated. Small diameter (< 6 mm) grafts lacking this endothelial layer are at high risk of occlusion due to thrombosis within minutes after implantation in the absence of anticoagulation. Examples include grafts made from synthetic polymers such as polyethylene terephthalate or expanded polytetrafluoroethylene [12] that typically perform well for diameters > 6 mm.

Numerous efforts to endothelialize small-diameter grafts *in vitro* have shown some promising results [13], but methods to pre-endothelialize vascular grafts vary in complexity and culture time required. In most culturing techniques, the goal is to replicate the in-situ mechanical environment for the graft. The most important mechanical stimuli include luminal shear stress and wall stress. These forces regulate the alignment and phenotype of endothelial cells both *in vivo* (regulating the arterialization process for transplanted venous grafts) and *in vitro*. Physiological shear stress, in particular, induces EC to exhibit a non-thrombogenic phenotype [14] and adhere more strongly to graft surfaces [15].

Some recent reviews have examined common cell sources for *in vitro* pre-endothelialization of vascular grafts in general (*e.g.*, [16]) or how graft surface and mechanical properties affect endothelialization (*e.g.*, [13]), but few have considered the specific requirements of eCABG requirements (*e.g.*, [17]). This review thus focuses on cell sources and techniques commonly used for *in vitro* endothelialization of TEVG and analyzes their suitability for eCABG.

Common cell types and tissue sources for *in vitro* endothelialization of vascular grafts

A typical CAB graft with a 4 mm diameter and 10 cm length has a luminal surface area of ~12.5 cm^2. EC in an endothelium range 235 – 1,178 μm^2 in surface area per cell [18], which means 1.0 million – 5.3 million cells are needed to form an endothelial monolayer on a single CAB graft.

Adipose-derived stromal vascular fraction

Subcutaneous adipose tissue can be conveniently collected in large quantities as a lipoaspirate from a liposuction procedure. This adipose tissue is then minced and digested with an enzyme like collagenase, dispase, trypsin, etc. Because adipocytes are more buoyant than other cells, they can be removed from the resulting slurry through centrifugation. The resulting heterogeneous mixture of cells is commonly referred to as the stromal vascular fraction (SVF) and is composed of stem/stromal cells commonly referred to as adipose-derived stem cells (ASC), immune cells, EC, pericytes, and other cells associated with the microvascular niche.

Recently, it has been found that subcutaneous adipose tissue is 74% adipocytes, 14.9% stem/stromal cells (including both ASCs and endothelial progenitor cells (EPC)), 7.4% immune cells, and 3.7% other cells by cell number [19]. There are 146,000 or 231,000 cells per gram of fat for male or female humans respectively, with respective viabilities of 89.5% and 91.6% [20]. A typical lipoaspirate is 2.14 +/- 1.8 L [21].

A typical liposuction with ~2:1 ratio of tumescent solution to adipose aspirate[22] would therefore produce ~700 grams of tissue assuming a density of 1 g/mL for tissue. That gives ~91 million or ~148 million viable cells for a male or female, respectively. Others have reported the proportion of cells in only the SVF (lipoaspirate after removal of adipocytes)[23]. Using these studies, one can estimate the number of each cell type derived from a typical lipoaspirate assuming 100% yields, summarized in Table 1. Due to these high cell yields, SVF may be the most promising source of seed cells for TEVG.

Table 1: Estimated cell yields from adipose. EC: endothelial cells; SVF: stromal vascular fraction.

Cell type present in lipoaspirate	**Cell proportion[19]**	**Approximate total yield of cells[20]**	
		Male	**Female**
Total cells		91 million	148 million
Adipocytes	74.0%	67 million	109 million
Stromal cells	14.9%	13 million	22 million
Other	3.7%	3 million	5.4 million
Cell type present in SVF	**Cell proportion[23]**	**Approximate total yield of cells[20]**	
		Male	**Female**
Total cells	26.0% of lipoaspirate	23 million	38 million
ECs	10%-20% of SVF	2.3–4.7 million	3.8–7.7 million
Stromal cells	15%-30% of SVF	3.5–7.1 million	5.7–11.5 million

Adipose-derived microvascular endothelial cells

Autologous, primary EC may seem to be the most obvious source of EC for *in vitro* endothelialization, especially since their use avoids the need for any long, complicated differentiation and/or culture steps. When isolated in large enough quantities, native EC may be seeded directly onto any cell-adhering graft to grow into a confluent monolayer.

EC isolation from large vessels is well established and relatively simple; however, over 95% of endothelium in humans is present in the arterioles, capillaries, and venules. These small vessels are collectively referred to as the "microvasculature." Adipose and brain tissue have very dense microvasculature. Table 1 and Figure 1 show a very rough estimate of the expected EC yield from a typical lipoaspiration. The use of Adipose-derived microvascular endothelial cells (AMEC) is preferable to other sources of EC because they are available in such large quantities with little risk of other associated morbidities.

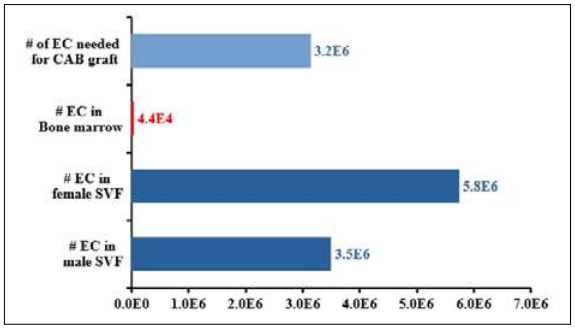

Figure 1: Estimated endothelial cells yields for bone marrow endothelial cells relative to endothelial cells required for a coronary artery bypass graft monolayer.

AMEC isolation protocols are well established and continue to be used today[24]. The development of superparamagnetic immunobeads (commonly Dynabeads ®) has led to much more efficient and higher-yield isolations than before. These beads can be ligated to platelet-EC adhesion molecule-1 (PECAM-1, a.k.a. CD31) to bind almost uniquely to EC. This method of isolation has made possible isolation of AMEC from other tissue sources including lung, stomach, and large vessels like the HUV[25].

Briefly, the SVF is isolated from a lipoaspirate according to the methods described above. The enzymatic digestion is allowed to singularize most cells before the adipocytes are removed. At this point, the Dynabeads with bound anti-CD31 antibodies are incubated with the cell suspension for about 20 minutes. The suspension is then exposed to a magnet, the buffer is removed, then the beads and the cells that adhered to them are resuspended in new buffer, repeatedly. The beads will be removed once the cells are trypsinized[26].

Endothelial progenitor cells

EPC refers generally to the cell type in the bone marrow, peripheral blood, and vessel wall that differentiates into EC and normally contributes to neovascularization and vessel remodeling in the body. The nomenclature used to describe EPC in the literature is inconsistent and sometimes conflicting, but EPC can be isolated from peripheral or cord blood or bone marrow and expanded and differentiated *in vitro* into EC. They are a subset of vascular progenitor cells and are commonly used to synthesize TEVG[27]. EPC derived from cord blood are more stable and likely superior for the purpose of *in vitro* endothelialization[16], but are severely immunogenic if allogenic. EPC can also be captured from circulation *in vivo* using grafts modified with capture molecules, as discussed below.

Capture of endothelial progenitor cells from circulating blood

Efforts have been made to modify grafts to capture EPC from circulation to augment the natural endothelialization process. One group[28] modified the surface of fixed, allogenic rat arterial grafts with MSC-derived exosomes containing the protein klotho to promote EPC attachment, and siRNA for adenosine kinase (ADK) to inhibit ADK production after exosome endocytosis. They found that exosomes alone increased EPC recruitment 9.1 times *in vitro* (which supports the idea that MSC accelerate endothelialization through paracrine action), and klotho/ADK modified exosomes increased recruitment 5.3 times relative to exosomes alone (for a total increase of ~48 times). The presence of modified exosomes further increased EPC proliferation by 72.7%. *In vivo*, the klotho/ADK-exosome-modified grafts showed 90% patency at 30 days and good endothelialization, while the group with klotho-exosomes alone had a 30% patency rate with high levels of intimal hyperplasia, and all unmodified grafts were occluded.

Endothelial colony forming cells

Endothelial colony forming cells (ECFC), as defined by[29], are a rare type of EPC found in bone marrow, the vessel wall, and peripheral blood with a high proliferative capacity. When isolated from peripheral blood and cultured *in vitro*, ECFC give rise to “blood outgrowth endothelial cells” (BOEC) whose uses are described below. Recent authors often do not distinguish between ECFC and BOEC (*e.g.*,[30]), though others consider ECFC and BOEC to be distinct populations[31].

Blood outgrowth endothelial cells

BOEC derived from ECFC, as defined in[29], have a transitional phenotype between human umbilical vein endothelial cells (HUVEC) and coronary artery endothelial cells[30], and are, therefore, fully differentiated EC.

One recent protocol study[31] reports the appearance of BOEC after 7-10 days and yields as high as 1 million BOEC after 10-14 days of culture from the buffy coat of 100 mL of pig blood, and 6-10 million cells at the first passage. Other established protocols exist in the literature[32], including those that use only the peripheral blood mononuclear cells to produce what may be called "late-outgrowth" BOEC[31].

Endothelial Cells via Differentiation from adipose-derived stem cells

Adipose-derived stem cells (ASC) are isolated from the SVF[19] and resemble classical mesenchymal stromal/stem cells (MSC) in every way, except they are $CD34^{+}$[33]. For this reason, ASC are functionally SVF-derived MSC.

MSC are commonly isolated from bone marrow or adipose tissue, but the SVF is the most easily accessible and abundant source. ASCs can be isolated using various methods[34]. Briefly, the SVF is isolated, then the adherent cell fraction is allowed to proliferate through multiple passages until a sufficient number of cells is acquired.

ASC are already widely used for wound healing applications, and are reported to differentiate into adipocytes, osteoblasts, EC, chondroblasts, SMC, skeletal muscle cells, and cardiomyocytes through various established culture methods[35].

EC differentiation of ASC can be achieved in ~12 days using commercially available EC differentiation medium or using recent serum free methods[36]. Important cytokines in ASC-to-EC differentiation include basic fibroblast growth factor (bFGF), VEGF, hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGF).

One group[37] recently induced mouse ASC to increase their expression of endothelial markers CD31, vWF and Factor VIII through culture on fibronectin-coated plastic in endothelial growth medium with VEGF. Apparently, this differentiation will not occur in simple 2D culture without the coating of matrix proteins[38]. The group[37] identified the long non-coding RNA called "maternally expressed 3" (MEG3) to be essential for EC differentiation of ASC. Other groups have identified tumor necrosis factor alpha (TNF-α) promotes EC differentiation by inducing VEGF receptor 2 (VEGFR2) in the mouse tumor microenvironment[39], and that purinergic 2 (P2) receptors can regulate early vascular commitment in human ASC[40]. Culture conditions also play a role in EC-differentiation of ASC. For example, culturing ASC in 3D hydrogels instead of on 2D TCP increases their CD31 expression potential[38].

Other sources of primary endothelial cells

Excluding the SVF, EC are generally not available in sufficient number to extract from the body without co-morbidity. Large blood vessels in the body would be the most obvious immediate source of autologous EC, but if such vessels were available for harvest, they would be preferentially used for CABG.

Further, there are significant differences between EC from different organs (*e.g.*, liver *vs.* bone marrow) and from different types of vessels (*e.g.*, veins *vs.* capillaries *vs.* arteries)[41]. The ability of venous EC to adapt to arterial conditions when used in CABG may indicate that the source of vascular EC is not of significant import for TEVG seeding, but further investigation is required for verification. Finally, the success of primary EC expansion depends heavily upon culture conditions and culture medium content.

Human umbilical vein endothelial cells

Human umbilical veins (HUV) can be automatically and precisely isolated from the umbilical cord by freezing the umbilical cord and using a lathe to remove the frozen tissue surrounding the luminal EC layer[42]. HUVEC can be reliably isolated and cultured from HUV for up to 5 months and are thus frequently used as the EC source for *in vitro* graft endothelialization studies. For examples, one recent study[43]

cultured decellularized HUV seeded with SMC on the ablumenal surface followed by passage-3 or 4 HUVEC seeded on the luminal surface. The SMC were cultured for 45 days under dynamic shear before HUVEC were seeded. The grafts were shear-conditioned for 7 days using either constant frequency pulsatility or physiologically-modeled pulsatility. HUVEC under physiological pulsatility showed greater coverage and alignment than those under a constant frequency.

However, HUVEC (like all EC) express HLA, ABO[44], and ABH antigens[45] indicating that autologous or immune-matched HUVEC must be used for a pre-endothelialized graft. Even with HLA matching, however, immune suppression will be required due to other minor-antigen mismatch[46]. For the large-scale use of immune-matched HUVEC, cell banking technology[47] and techniques must first reach maturity although the large number of HLA combinations (there are 59 different HLA-A proteins, 118 different HLA-B and 124 different HLA-DR) will likely make this approach infeasible. Since HUVEC are a convenient source of *bona fide* EC, these *in vitro* studies are expected to be applicable to other sources of autologous EC that are not immunogenic.

Bone marrow endothelial cells

Isolation of bone marrow endothelial cells (BMEC) using Dynabeads is similar to isolation of AMEC, but one group first isolated and expanded the TCP-adherent cell fraction from mouse bone marrow, then performed Dynabead separation with a CD31 antibody. They reported that BMEC make up less than 2% of bone marrow cells[48], though the absolute number available from a typical bone marrow harvest is difficult to determine. From a typical bone marrow harvest of ~ 70 mL[49], 2% by volume would be 1.4 mL which is the volume of ~ 44,000 EC[18].

The native BMEC population is heterogeneous but can be broadly identified as the population of $CD45^-$, $Ter119^-$, $CD31^+$, and $CD202b^+$ cells[50]. Interestingly, this group used negative selection (CD45 and Ter119) in addition to CD31 positive selection to purify their BMEC. Further, they found that dispase II in the place of collagenase D increased the purity of BMEC isolation.

Considerations for pre-endothelialization of vascular grafts for eCABG

For any endothelialization technique to be clinically relevant, the manipulation of cells should be minimized to simplify equipment and staff requirements and to minimize regulatory barriers to translation. For the particular case of eCABG, modifications of the graft should take place within hours or days rather than weeks. For most AMI patients, graft endothelialization should be complete to allow for CABG within 54 hours of hospital admission[6].

Cell sources used in recent efforts to pre-endothelialize CAB grafts suffer from several serious problems. 1) Non-autologous cell sources (*e.g.*, HUVEC) are severely immunogenic. 2) Cells require significant amounts of manipulation and/or modification over weeks of culture time before implantation (*e.g.*, BOEC, ASC). Autologous cell sources utilized are not readily available in sufficient quantities to create an endothelial monolayer on the luminal surface of the graft without prior lengthy expansion. This limits the options to AMEC and ASC, which can both be readily isolated from the SVF in large quantities, although purified AMEC require Dynabead separation, and ASC still require expansion *in vitro* and/or selection before seeding on grafts as well as time for EC differentiation if that is the approach used. Table 2 summarizes the pros and cons of the common cell sources for TEVG endothelialization *in vitro*. Importantly, Figure 1 illustrates the proportion of a typical CAB graft the contemplated EC sources could cover using the values of Table 1.

Table 2: Pros and cons of endothelial cells sources for pre-endothelialization of grafts for coronary artery bypass grafting surgeries. AMEC: Adipose-derived microvascular endothelial cells; ASC: Adipose-derived stem cells; BEMEC: bone marrow endothelial cells; BOEC: blood outgrowth endothelial cells; EC: endothelial cells; ECFC: Endothelial colony forming cells; EPC: endothelial progenitor cells; HUVEC: Adipose-derived stem cells; SVF: stromal vascular fraction.

EC source	Pros	Cons	References
AMEC	• Primary EC • Enough available to immediately coat graft (roughly 2.3–4.7 million in males, and 3.8–7.7 million in females from a typical lipoaspiration) • Liposuction is minimally invasive • Feasible to isolate from SVF	• Require Dynabead separation • Dynabeads remain in cell culture indefinitely; may pose barriers to clinical translation.	24,25
EPC	• Captured from circulation *in vivo*	• Not available for immediate use for *in vitro* endothelialization	27,28
Late-outgrowth BOEC (a.k.a. ECFC by some authors)	• Mature, autologous EC • Isolated from peripheral blood mononuclear fraction • Can be expanded to large numbers in-vitro from small volumes of blood	• Long expansion time: first colonies of BOEC visible after 22-27 days	29–32
BOEC	• Mature, autologous EC Isolated from peripheral blood • Can be expanded to large numbers in-vitro (~ 1 million after 10-14 days) • Shorter expansion time than late-outgrowth BOEC	• Long expansion time: 7-10 days for BOEC to appear, 10-14 days to achieve ~1 million cells from 100 mL of porcine blood	29,31
EC via ASC differentiation	• Autologous ASC available in large quantities • Liposuction is minimally invasive • Feasible to isolate from SVF • Can be differentiated with serum-free medium • Presence of ASC accelerates native endothelialization	• Long culture time for EC differentiation: ~12 days for stable expression of EC markers • Increased burden of differentiation protocol	34-40
HUVEC	• Mature EC • Automatic and precise isolation procedure • Good for *in vitro* studies	• Non-autologous thus highly immunogenic	42-45,47
BMEC	• Mature, autologous EC	• Isolated from bone marrow • Require Dynabead separation and/or long expansion time • EC are a low proportion of bone marrow cells	48,50

Conclusion

Only adipose-derived endothelial cells provide a suitable autologous feasible source in terms of accessibility and abundance to create an endothelial monolayer on a CAB graft for eCABG, and to achieve shear-resistant endothelial adhesion, this would likely need to be a TEVG.

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