Emergence of Tissue-Engineered Human Blood Vessels by Self-Assembly as Vascular Models

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Summary

The production of small-diameter vascular grafts is one of the most exacting challenges of tissue engineering. Some of the key features of tissue-engineered blood vessels may be the imitation of native vessels' biology and functionality. One of the approaches developed in response to the quest for the ideal graft is self-assembly, a technique that refers to the self-organizing properties of cells to construct their own living environment. We believe that such grafts may be useful not only in a clinical context but also as a human model that could reduce or replace animal use in vascular research. We present our model in this short article and discuss its advantages, pitfalls, and applications, followed by a consideration of other methods of vascular tissue engineering that can also serve as vascular models.

Keywords: tissue engineering, adventitia, blood vessels, vasa vasorum, capillaries

1 Introduction

One of the most exacting challenges of tissue engineering is the production of small-diameter blood vessels as human vascular grafts, notably for coronary artery bypass surgeries. Several captivating reviews have been written specifically to address the problems (Isenberg et al., 2006; Nerem and Ensley, 2004; Mitchell and Niklason, 2003; L'Heureux et al., 2006; Desai et al., 2011). Many methodologies have been developed to answer the clinical needs of the perfect graft. The generation of a vessel that is biologically and functionally comparable to native vessels obviously is quite a challenge (Gong and Niklason, 2006). In parallel, there is a need to replace animals when studying the vascular system. As has been shown many times, results obtained from animals do not always transfer to humans. However, it is difficult to obtain available human tissue that can be dissected out in good condition (Stoclet et al., 1996). Furthermore, uncontrollable factors relating to the donor may interfere with the experiments. Therefore, we believe that both the development of the optimal vascular graft and the development of novel valuable models for the study of the vascular system can benefit from the innovations in tissue-engineered blood vessels (TEBV). The scope of this article is to present a human self-assembly TEBV made by cell-sheet technology as developed at the *Laboratoire d'Organogénèse EXpérimentale* (LOEX). This kind of model could be used to reduce, or in some cases replace, animal use in fundamental research.

2 Self-assembly and cell-sheet based technology

More than 20 years ago, Weinberg and Bell (1986) pioneered the field of biological TEBV by seeding bovine cells within isolated collagen vessel scaffolds. Those TEBV were mechanically inadequate due to a lack of organization in collagen, the main constituent of the extracellular environment of cells. Some of these prostheses had to be reinforced with a synthetic mesh, making them hybrid artificial substitutes. Some cell types such as fibroblasts, osteoblasts, and chondroblasts, as well as smooth muscle cells (SMC), have the ability to secrete collagen and other extracellular matrix proteins when placed in the right conditions. Ascorbic acid, better known as vitamin C, is an essential element of cell culture for the cellular synthesis of collagen (Murad et al., 1981). Collagen-secreting cells initially cultured on the two-dimensional surface of a flask or a Petri dish can after a few weeks prosper into their own self-organized three-dimensional environment, forming a living cellular sheet. Self-assembly refers to this self-organizing capacity of cells to construct functional living structures of prescribed shape (Auger et al., 2000).

Cell-sheet based technology (CST) is the next logical step (Matsuda et al., 2007). Cellular sheets can be handled in different ways to produce more complex tissues, for example, stacking to form skin (Auger et al., 2004), corneal (Proulx et al., 2010), and bladder tissue (Bouhout et al., 2010), and rolling to form urethras (Magnan et al., 2009) and blood vessels (L'Heureux et al., 1998, 2006). Following stacking and rolling, adjoining cell sheets fuse together followed by tissue reorganization driven by the cells themselves.

3 Method of fabrication

Our laboratory, the LOEX, developed the first completely biological TEBV constituted of living human cells in the absence of any synthetic or exogenous material (L'Heureux et al., 1998). Entirely dependent upon the self-assembly method, the recon-

structed blood vessel possesses all three histological layers of arteries and veins: the external tunica named the adventitia, the middle tunica called the media, and the inner lining referred to either as the tunica intima or the endothelium.

Briefly, self-assembled cellular sheets are formed by culturing juxtaposed fibroblasts and SMCs for three to four weeks. The cellular sheets are then rolled, SMC-side first, around a mandrel (Gauvin et al., 2010). After about three weeks of maturation and fusion of the layers, endothelial cells are seeded into the lumen. The vessel is then perfused for approximately one week using a home-made bioreactor to form the endothelium. The endothelial cells then are reported to form a confluent and functional endothelium. Endothelial cells expressed von Willebrand factor, incorporate acetylated low-density lipoproteins, and also expressed anti-thrombotic factors (L'Heureux et al., 1998, 2001; Rémy-Zolghadri et al., 2004). Albeit devoid of biomaterials, this TEBV is resistant and easy to handle. Alternately, the cells can be aligned before rolling them into a tubular form, conveying properties to the TEBV that mimic native vessels (Auger, 2004; Grenier et al., 2005; Guillemette et al., 2009).

4 Replicating the *vasa vasorum*

The adventitial layer, made mostly of fibroblasts, can contribute to the nourishment of larger vessels by acting as a support for the establishment of a capillary network. This network, called the *vasa vasorum*, exists in large and metabolically active arteries and veins where the diffusion of intraluminal blood is insufficient to provide oxygen and other nutrients to the outermost layers. By plating endothelial cells on top of self-assembly fibroblast sheets, it was possible to observe the formation of capillary-like structures (Guillemette et al., 2010). The formation *in vitro* of capillaries has been reported to be associated with a favorable functional phenotypic change in endothelial cells, as the cells that could not uptake low-density lipoproteins when cultured in monolayer could do so when forming capillary-like structures (Arnaoutova et al., 2009). Furthermore, it has been shown that the capillary-like structures recruited *in vitro* pericytes, i.e., vascular cells that wrap around capillaries (Berthod et al., 2012). Guillemette et al. (2010) demonstrated the feasibility of using the technique to produce a self-assembly tissue-engineered adventitia with a vasa vasorum.

5 Source of cells

The main cell types that compose a native blood vessel, i.e., the fibroblasts, SMC, and endothelial cells, can be obtained from a single human or animal vein biopsy (Grenier et al., 2003). Using *in vitro* expansion with the appropriate cell medium, thousands of cells can proliferate to millions of cells by doubling approximately once every 24 hours. A single biopsy can be used to fabricate dozens of TEBV. However, one sometimes has to limit the replication of cells, as some of their functional characteristics can be lost in expansion. The blood vessels of the umbilical cord serve as a major source of vascular cells in tissue engineering. Indeed, umbilical cords are readily available, and their cells are known to be ideal for cell culture, as they usually proliferate easily compared to cells from aged adults. One drawback is that they might not behave like the highly differentiated cells of an adult blood vessel. Consequently, alternative cellular sources include residual human saphenous veins and other tissues that are sometimes disposed of following surgeries, and, of course, cadaveric tissue. However, the cells of aged adults have shown limitations in proliferation and collagen secretion (Petersen et al., 2010). Recently, a study emerging from Niklason's group suggested the transient delivery of a mutated form of telomerase, an enzyme comparable to a cellular fountain of youth, as a means to effectively improve the proliferation capacity and collagen deposition of cells (Petersen et al., 2010). Another avenue could be the use of differentiated adult mesenchymal stem cells, such as those derived from bone marrow (Jerareungrattan et al., 2005) or adipose tissue (Labbé et al., 2011). Indeed, all vascular cell types have been reported to be obtainable through mesenchymal stem cell differentiation. However, the differentiation process of those cells is still not fully understood, and it is difficult to know whether they are fully comparable to the cells of native tissues. It has been reported that isolated cells present functional properties similar to those of their native blood vessels (Laflamme et al., 2006b; Gauvin et al., 2011a). Consequently, when planning studies, it may be deemed advantageous to choose the cells that are the most pertinent.

6 The serum conundrum

Serum is usually needed in cell culture to provide various growth factors, cytokines, hormones, vitamins, and proteins that are essential for cell growth and cell proliferation (Gstraunthaler, 2003; Brunner et al., 2010). Despite the fact that serum is essential for cell proliferation, it also has many non-negligible disadvantages. First, serum is an ill-defined medium supplement, and its composition is inconsistent from one batch to the next, which can increase result variability (Enomoto et al., 2002). Second, serum production is costly and inefficient. About 44-144 cows must be slaughtered to obtain 1 l of fetal bovine serum (Hodgson, 1991). Third, fetal calf serum can contain different amounts of endotoxins, hemoglobin, and infectious agents (fungi, bacteria, viruses, and prions) (van der Valk et al., 2004). The objective of reducing serum in culture medium thus would be beneficial not only to reduce animal use, but also to optimize culture conditions, reproducibility of results, and production costs. Once again, the clinical goals of blood vessel tissue engineering parallel the needs of fundamental research.

A three-dimensional engineered tissue may provide the optimal conditions for its own maintenance, in some cases ren-

dering the need for exogenous serum factors superfluous. For example, evidence suggested that avoiding serum when co-culturing epidermal keratinocytes with dermal fibroblasts improved epidermal differentiation (Black et al., 2005). A study by Jean et al. (2011) confirmed that serum was not necessary during the final epidermal differentiation step of self-assembly tissue engineered skin. This kind of study is a step toward diminishing serum use when possible. Indeed, there is an acknowledged need to fully understand and define the conditions for optimal cell growth. We consider the elimination of serum to be one of the most understated challenges of cell culture and tissue engineering.

7 Advantages and pitfalls of self-assembly tissue-engineered blood vessels

Most *in vitro* models are based on single cell type 2D cultures, which lack the physiologically relevant 3D structures of investigated tissues and the interactions of the different cell types normally present. The self-assembly approach has the advantage of providing cells with their own extracellular matrix. This very own matrix could provide the natural habitat for the cells to thrive and express their rightful phenotypes. An interesting case can be made for vascular SMCs. SMCs can exist in two different phenotypes. One is a synthetic or dedifferentiated state in which the cells are in a state of proliferation; this is the phenotype of conventional two-dimensional culture; it can also occur in the formation of a neointima, leading to vascular stenosis and blocking blood flow. The other one is called the contractile or differentiated state, the type in which mature and healthy vascular SMCs are normally found. Interestingly, SMCs cultured in a flask as a living tissue sheet express markers of the differentiated state. When the tissue sheets are detached from their support, the release of the residual stress results in a mechanical unloading, and cells within the extracellular matrix are dedifferentiated as shown by a downregulation of markers. The rolling procedure onto a tubular mandrel results in the reintroduction of a mechanical load, leading to a cohesive compacted tissue comprised of redifferentiated cells (Grenier et al., 2006). It seems therefore that the three-dimensional environment brought forth by self-assembly can be a needed mechanical trigger to the formation of a physiologically relevant vascular media. The 3D advantage also extends to desmin, a protein component of the cellular intermediate filaments known to be lost in culture, which is re-expressed in the SMC of the TEBV (L'Heureux et al., 1998; Auger et al., 2000).

A major component responsible for the mechanical behavior of native arteries is the formation of elastin fibers (Patel et al., 2006). Elastin in native vessels was organized into clearly identifiable concentric sheets (Dahl et al., 2007). In contrast, elastin in engineered vessels was predominantly intracellular and did not form concentric sheets. It seems that the elastin precursor, tropoelastin, fails to form mature elastin fibers (Debelle et al., 1998). There is currently a lack of knowledge of material and methods to promote the assembly of tropoelastin into mature elastic fibers (Bashur et al., 2012). It seems that undifferentiated SMCs, such as when SMCs are cultured on two-dimensional surfaces or within scaffolds like fibrin and polyglycolic acid (PGA), are not conducive to the production of elastin (Bashur et al., 2012). In contrast, the differentiated SMCs of self-assembly TEBV may have their elastin expression inhibited by the collagenous environment. However, mature elastic fibers have been reported when fibrin was used as a tissue scaffold (Long and Tranquillo, 2003; Swartz et al., 2005). The increase of synthesis by the SMCs seemed to occur in parallel with fibrin degradation (Ahmann et al., 2010).

Low levels of staining for mature elastin in engineered vessels may be partly explained by the use of ascorbic acid, which promotes collagen synthesis but simultaneously inhibits tropoelastin gene expression (Debelle et al., 1998). One study combined the absence of ascorbic acid with the cellular overexpression of a versican isoform (splice variant 3) in self-assembly vascular media and found an increase in elastic fibers (Keire et al., 2010). This study suggests not only a palliative to the lack of elastin, but it also underlines the feasibility of creating TEBV models using genetically modified cells.

A major advantage of a self-assembly blood vessel is the possibility of layering its tunicae. Indeed, self-assembly allows the study of isolated adventitia and media, as seen in multiple studies discussed later (L'Heureux et al., 2001; Laflamme et al., 2005; Laflamme et al., 2006a; Diebolt et al., 2005, 2007; Pricci et al., 2009).

Two of the major improvements we wish to bring to self-assembly TEBV are to reduce the cost and time of production, which could lead to wider use. Along with cost and time, another barrier is the high level of technical expertise currently required by the technique

8 Applications to vascular research

The similarity of self-assembly TEBV to native vessels did not escape attention by the scientific community, and it begot a number of publications. The contractile capabilities of a self-assembly cell-sheet technology reconstructed media have been well characterized, showing contractile response to various agonists as well as a nitric oxide-mediated relaxation occurring through the normal vascular pathways (L'Heureux et al., 2001; Laflamme et al., 2005). One such study contributed to the understanding of blood microparticles that derive from T lymphocytes. Interestingly, it was found that microparticles affected the adventitia and the media differently (Pricci et al., 2009). The ability to use self-assembly TEBV to study the different tunicae of a blood vessel has been a subject of predilection of various other studies. Laflamme et al. (2006a) demonstrated that a human tissue-engineered vascular adventitia has the capacity to contract and relax. Even though other studies had suggested that the adventitia could contribute to the vascular function, it was the first time that a direct demonstra-

tion was made of this capacity in response to vasoactive agents (Auger et al., 2007). Thus, the understanding that the adventitia might play a greater role than expected in vascular tone might pave the way to novel strategies against hypertension. New insights into understanding the metastatic process, involving the regulation of the diapedesis of circulating cancer cells by E-selectin, have been gained using a laminar flow chamber and a self-assembly TEBV (Tremblay et al., 2006). Reconstructed blood vessel media have also been challenged with red wine polyphenols, bringing new knowledge about their effects and possibly pinpointing potential therapeutic targets (Diebolt et al., 2005, 2007).

9 Recreating the tissue environment

The cellular environment is mostly understood in terms of nutrients and growth factors, currently provided in great part by serum. However, the mechanical components seem to be the cornerstone of the tissue environment (Akst, 2009). Multiple studies suggest that the tissue environment is of paramount importance for promoting epithelial maturation (Auger et al., 2004; Dubé et al., 2011; Cattan et al., 2011) and extracellular matrix organization (Seliktar et al., 2003). Accumulating evidence suggests that mechanical strain regulates vascular SMC phenotype, function, and matrix remodeling (Kurpinski et al., 2006). A stretch alone is sufficient to remodel self-assembly fibroblast and SMCs sheets and make them more resistant (Grenier et al., 2005; Gauvin et al., 2011b). The stretch also induces an alignment of the SMCs cellular proteins, which is conserved when the sheets are rolled to form a vascular media (Grenier et al., 2006). Apart from a pulsed axial stretch, blood vessels have to endure other forces, such as the shear stress that is consequent to the blood flow and viscosity. Unsurprisingly, many novel complex bioreactors reproducing physiological conditions have been developed in recent years (Niklason et al., 2010; Zaucha et al., 2009; Syedain et al., 2011).

10 Other methods

It seems reasonable to conclude that, for vascular studies, cells producing their own extracellular environment are optimal. Other methods indirectly exploit the self-assembly capacity of cells. One major group of methods involves biodegradable scaffolds. The degradation of the synthetic materials can occur either *in vitro* or *in vivo*. The most interesting models for vascular research probably would be those that degrade *in vitro* to avoid interference from synthetic elements. PGA, for instance, has been used in medicine and surgery for decades as suture material (Niklason and Langer, 1997). PGA scaffolds, seeded with SMCs, are stimulated within a bioreactor to provide optimal conditions for the cellular secretion of their extracellular environment that slowly replaces the synthetic scaffold (Niklason, 1999). Indeed, PGA can be made to degrade spontaneously in aqueous environments. However, one problem that arises with the use of any synthetic material is the persistence of partially degraded remnant polymer fragments. Those remnants make engineered tissues weaker (Dahl et al., 2007). During the vessel's growth, the polymer and tissue components become inextricable. It has been reported that, in such constructs, SMCs that were near remaining PGA did not express smooth muscle α-actin or calponin, two major markers of SMCs. Hence, SMCs in proximity to residual PGA fragments displayed an undifferentiated phenotype, as evidenced by a high mitotic rate and low expression of contractile proteins (Niklason et al., 2001). Many other biodegradable biomaterials exist or are in development. Biodegradable scaffolds akin to PGA have been developed that may degrade faster (Koch et al., 2010; Gui et al., 2011). One major advantage of those biomaterials is that the TEBV can undergo dynamic stimulation almost readily, since the tubular structure is already formed and the cells are already evolving in a three-dimensional environment. While the use of bioreactors introduces intricacy to the making of the TEBV, it could be argued that the physiological environment brought by bioreactors is a prerequisite for the development of optimal vascular models.

Fibrin is a biological compound naturally produced in wounded tissues by circulatory fibrinogen, and it can be degraded by the cells themselves. Similarly, entirely biological fibrin-based vascular constructs can be made. Tranquillo's group developed a tissue-engineered artery based on human dermal fibroblasts entrapped in fibrin gels (Ahmann et al., 2010).

Promising new models may emerge from the bioprinting techniques (Norotte et al., 2009). Bioprinting is a recent innovation that can print macrovascular tissue by physically depositing multicellular and agarose cylinders using a bioprinter. Constructed tissue then can be put into a bioreactor, not unlike techniques based on biodegradable scaffold, to achieve the optimal self-assembly of extracellular matrix in the presence of ascorbic acid.

11 Concluding remarks

The human cells used in TEBV construction make them an ideal replacement for animals in research. However, TEBVs lack some of the more complex features found in native vessels, such as resident immune cells and nerve endings (Auger et al., 2007). It is therefore unclear whether they will ever fully replace the use of animals in this area of research.

Tissue engineering, in most cases, is still at the stage of preliminary studies and "the drawing board." We hope that the upcoming decades will see a vast increase in the clinical applications of tissue engineering derived products. We also believe that the recreation of tissue engineered organs and grafts helps to stimulate the acquisition of knowledge about human physiology and pharmacology. We cannot deny that the development of tissue engineering itself often leads to the use of animals for preclinical studies. However, we expect that the

development of complex biological models that will reduce and, in some cases, replace animal use in fundamental research will be an inevitable outcome in the long term.

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