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Strategies for Tissue and Organ Decellularization

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ABSTRACT

Decellularized tissues have been successfully used in a variety of tissue engineering/regenerative medicine applications, and more recently decellularized organs have been utilized in the first stages of organ engineering. The protocols used to decellularize simple tissues versus intact organs differ greatly. Herein, the most commonly used decellularization methods for both surgical mesh materials and whole organs are described, with consideration given to how these different processes affect the extracellular matrix and the host response to the scaffold. J. Cell. Biochem. 113: 2217–2222, 2012. © 2012 Wiley Periodicals, Inc.

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One of the major objectives of regenerative medicine has been the engineering of a solid organ. A variety of approaches have been investigated over the years, but recently the approach of decellularizing the organ of interest to provide a framework for building a new organ has piqued the imagination [Badylak et al., 2011; Fukumitsu et al., 2011; Song and Ott, 2011; Vacanti, 2012]. By starting with the organ of interest, the researcher has the benefit of a biologic construct that is deposited by the resident cells of that organ and largely retains the organ's complex geometry, including a relatively intact vascular network. Recent work has described the generation of acellular organs including the heart, lung, liver, and kidney, with a few early attempts at orthotopic transplantation of recellularized constructs [Macchiarini et al., 2008; Ott et al., 2008, 2010; Petersen et al., 2010; Nakayama et al., 2011; Soto-Gutierrez et al., 2011]. The approach of decellularizing whole organs builds on the extensive body of work that was performed to develop current clinical products like demineralized bone matrix, skin grafts, bioprosthetic heart valves, and more recently, acellular biologic surgical meshes [Valencia et al., 2000; Zilla et al., 2004; Pacaccio and Stern, 2005; Badylak et al., 2009]. These products have been used in millions of patients, and much is known about the intricacies of processing the tissues, the biologic properties of the scaffolds after processing, and the host response to scaffolds in a variety of body systems. Despite this wealth of knowledge, there are unique complexities to decellularization of solid organs that make direct translation of that knowledge challenging. The current review attempts to briefly summarize strategies for decellularization of complex tissues and organs with a particular emphasis on the differences that need to be considered.

WHY EXTRACELLULAR MATRIX?

Extracellular matrix consists of the proteins and other biomolecules synthesized by cells that, with the cells, make up each tissue. The cells and ECM in a tissue exist in a state of ''dynamic reciprocity,'' with cells responding to signals in the ECM to alter their behavior, and the cells in turn modifying the organization and composition of the ECM, and so on [Nelson and Bissell, 2006]. The architecture and composition of the ECM in each tissue is unique, thereby defining the functionality of that tissue. However, the structure and composition of each specific ECM protein is highly conserved among species [Bernard et al., 1983ab; Exposito et al., 1992], making the ECM recognizable within and between species largely without immune rejection. When properly processed to remove cellular antigens that would induce immune rejection without damaging the ECM, ECM scaffolds can serve as potent source of cues to promote constructive remodeling of tissue after injury. ''Constructive remodeling'' means that ECM scaffolds promote the formation of site-appropriate tissue at the site of implantation as opposed to scar tissue [Badylak, 2007].

The term constructive remodeling was coined in response to ECM scaffolds derived from porcine small intestinal submucosa (SIS) and urinary bladder matrix (UBM). These scaffolds are rather unique

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amongst ECM scaffold materials for a number of reasons. First, they are relatively thin membranes that can be easily decellularized with a single, mild acid wash (without the use of detergents) that only takes a few hours [Gilbert et al., 2006]. It is known that the process largely leaves the collagen, elastin, glycosaminoglycans, and many growth factors present and functional. Once fabricated and implanted, the scaffolds degrade rapidly, and the degradation products are comprised of matricryptic peptides that have robust biologic activity, including bacteriostatic, chemotactic, and mitogenic properties [Badylak et al., 2009]. For decellularization of more complex tissues and organs, much more rigorous protocols are be required to remove cells from the tissue [Crapo et al., 2011], and the biologic activity of and host response to these scaffolds needs to be investigated to determine the effects of these new protocols.

HOW TO DECELLULARIZE TISSUE

The objective on any decellularization process is to isolate all of the extracellular matrix components within a tissue without any loss, damage, or disruption, while removing the cellular component in its entirety [Gilbert et al., 2006; Crapo et al., 2011]. This objective is implicitly impossible as any process that disrupts cells and transports the contents from a tissue will necessarily alter the ECM. Practically, the goal is maximizing the removal of cellular material, while minimizing ECM loss and damage. Only recently have objective criteria for assessing the efficacy of decellularization been proposed, including (1) the absence of nuclei based upon histologic staining with hematoxylin and eosin and DAPI, (2) quantitative measurement of DNA at less than 50 ng/mg dry tissue weight, and (3) DNA fragment size below 200 bp [Crapo et al., 2011]. These criteria are phenomenological in that they were defined based upon years of experience that showed that when SIS and UBM scaffolds met these criteria, they promoted site-appropriate tissue remodeling. More recent work has brought these criteria into question as ineffectively decellularized tissue showed similar host remodeling as compared to effectively decellularized tissue [Keane et al., 2012].

While these criteria serve as a helpful guide, it could be argued that they define denuclearization of the tissue rather than decellularization. These criteria use DNA as a proxy for other intracellular or membranous molecules, assuming that they are removed with the same efficacy. The presence of DNA has been described as a concern since many decellularized tissues are derived from xenogeneic sources, and there is a fear (unsubstantiated to date) that the DNA could be incorporated into the recipient cells [Zheng et al., 2005]. However, intracellular and membrane components include the cell surface antigens that have been shown to invoke immune rejection in transplantation [Cooper et al., 1993; Galili, 2001]. With the exception of Gal epitope, the persistence of cellular antigens has been largely unexplored. A recent report on lung decellularization showed that peptides from multiple cell-associated proteins do persist within the ECM, including actin, tubulin, and myosin [Daly et al., 2012]. Future studies should expand upon this work to show how effectively cell-associated proteins are removed and what effects those proteins have on the host response to expand our understanding of when a tissue is adequately decellularized.

METHODS OF DECELLULARIZATION

There are a number of techniques to decellularize a tissue that generally fall into the categories of physical, chemical, and enzymatic, and these techniques are necessarily used in combination. Physical methods include agitation in solution, vascular perfusion, thermal shock, ultrasonics, and manual disruption [Gilbert et al., 2006].

Thermal shock, which involves one or more freeze–thaw cycles, ultrasonics, and mechanical disruption all cause rupture of cell membranes to facilitate transport of the cellular material from the tissue during agitation or perfusion. Mechanical disruption can also simply involve removing cell-rich layers of tissue that are not desired in the final product to increase the ease of decellularization. If used, these steps that lyse cells are generally used at the beginning of the decellularization protocol to enhance the efficacy of future efforts to transport the cellular materials from the tissue.

Agitation and perfusion both act to facilitate transport of decellularization solution to the cells and to clear cellular debris from the tissue. Hydrostatic pressure and convective flow are similar, if more sophisticated, methods of accomplishing the same goal that are used for tissue with simpler geometries [Montoya and McFetridge, 2009; Funamoto et al., 2010]. The choice of which approach to use depends on the characteristics of the tissue. For example, decellularization of SIS or trachea is commonly performed using agitation since adequate vascular network is difficult to access. For thin ECM membranes like SIS, agitation is a very effective means of decellularization. However, efficiency is greatly increased in more complex tissues when a vascular network can be preserved [Ott et al., 2008]. Logically, the vascular network is designed to facilitate diffusional transport to the cells, so use of this network to deliver decellularization agents is extremely effective. Vascular perfusion has the added advantage of leaving the structure of the tissue largely intact, although various changes do occur, such as distention of the tissue.

In most complex tissues, the vascular network is the most efficient means of delivering the decellularization agents to the cells, however any system that facilitates decreasing the diffusion distance can be employed. One of the most accessible systems is the airway of the lung, which can be accessed through intratracheal perfusion [Ott et al., 2010; Petersen et al., 2010; Price et al., 2010]. Similarly, the bile ducts in the liver and ureter in the kidney provide additional access. An aspect of the perfusion decellularization strategies that is sometimes overlooked is the mechanical pressures that are generated during the process, which can further decrease the diffusion distance by thinning the tissue walls and could act to drive decellularization agents through the tissue. An associated caution is that excessive pressures can burst of the vessels leaving portions of the tissue incompletely decellularized.

Various chemicals have been investigated for their ability to decellularize tissues. These chemicals are generally selected in order

to solubilize the cell membranes and to transport cellular components from the tissue by agitation or perfusion. The chemicals fall into multiple general categories, namely detergents, solvents, acidic and alkaline solutions, and ionic solutions. For the purposes of this paper, the focus will be on those that are most commonly used. More complete reviews of these agents can be found elsewhere [Gilbert et al., 2006; Crapo et al., 2011]. It is extremely rare that decellularization protocols rely on only one chemical to decellularize a tissue, and those protocols generally are used for thin membranous ECM like SIS and UBM. For more complex tissues, it is generally more advantageous to combine numerous chemicals through a series of short washes in cycles to increase the efficiency of each chemical and to reduce the overall time that the tissue is exposed to any one chemical.

High molarity and low molarity ionic solutions are generally used in succession to induce osmotic shock to rupture the cells. When used, these steps are typically among the first steps in a decellularization protocol to release the cellular contents from the cells so that they are easier to transport from the tissue in subsequent washes. Because of the ionic strength of these solutions, there is potential for them to disrupt the matrix proteins, but there is not much work published investigating these effects. Generally, this step can greatly increase the efficiency of later steps, but it is desired to keep the duration of exposure as short as possible.

Detergents are by far the most commonly used chemicals used for tissue decellularization since they are known to solubilize the cell membrane [Gilbert et al., 2006; Crapo et al., 2011]. Detergents can be classified into three categories: nonionic, zwitterionic, and ionic. Nonionic detergents, such as Triton X-100, are theoretically the most desirable detergents to use since they should have the least impact on the protein structure due to the lack of ionic charge. Conversely, ionic detergents, such as sodium dodecyl sulfate (SDS) and sodium deoxycholate, are generally thought to be harsher to the tissue with greater disruption of protein structure and loss of matrix components, particularly glycosaminoglycans. Zwitterionic detergents, for example, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), have features of both ionic and nonionic detergents, which suggests that the effects should be less harsh than ionic detergents, but greater than nonionic detergents.

In practice, these classifications are not particularly helpful. The literature is full of conflicting results on the effects that each of these detergents has on the tissue of interest in the study [Gilbert et al., 2006; Crapo et al., 2011]. Results can be difficult to interpret, due in part to variations in the concentration of detergent used, the combination of other physical and chemical methods employed, inconsistencies in the means of analysis, and the tissue studied. Controlled studies are needed to determine the effects of each detergent on each tissue of interest. Ultimately, there is unlikely to be one detergent that is the best for decellularization of every tissue.

Acidic and alkaline solutions are also commonly used for tissue decellularization. The most commonly used is peracetic acid, which has been used at low concentrations of approximately 0.1% for decellularization of SIS and UBM [Hodde and Hiles, 2002; Freytes et al., 2008ab]. Since it is an oxidizer, PAA is used in combination with low concentration of ethanol (~4%) primarily to reduce the generation of bubbles due to gaseous evolution. PAA is not particularly effective for decellularization of more complex tissues, but is still frequently used as a disinfection step [Hodde and Hiles, 2002]. Some evidence is also becoming available that suggests that the PAA may modify the ECM in such a way that in promotes a more desirable immune response. It was described above that inefficient decellularization showed similar in vivo host results to efficient decellularization when PAA was used for different lengths of time. Interestingly, the control for the study showed that phosphate-buffered saline (PBS) was more effective for decellularization than the shorter exposure to PAA, yet the host response to the PBS treated SIS led to fibrotic tissue deposition as opposed to constructive remodeling [Keane et al., 2012].

Enzymes are frequently used in tissue decellularization either to disrupt the interactions between the cells and the ECM, or to specifically target proteins for which removal is desirable. Trypsin, a serine protease, is commonly used to disrupt cell–matrix interactions in tissues. Exposure to trypsin should be avoided if possible and limited if not, since it is known that trypsin can specifically target collagen, leading to decreased mechanical strength [Yang et al., 2009]. However, there are some tissues, such as porcine heart, for which exposure to trypsin is a critical step in the decellularization process [Wainwright et al., 2010]. Collagenases have also been used, but usually at very low concentrations unless maintenance of the structure is not required. Collagenase and trypsin are most effectively used as an early step when needed to help disrupt the structure to allow for transport of cells from the tissue.

Nucleases, particularly DNase, are used to break down nucleic acid sequences within the tissue to facilitate their removal or to eliminate their function. DNase has become ubiquitous in decellularization of complex organs, allowing for the total process time to be dramatically reduced. Recent work has shown that after rinsing, there is no functional DNase retained within the decellularized tissue [Daly et al., 2012], obviating previous concerns about persistence of the DNase within the tissue that could affect cellular response to the tissue.

Other enzymes that have been used for removal of components from tissue are lipase and α-galactosidase. Lipase specifically targets lipids can be challenging to remove from fatty tissues. Recently, lipase has been used along with alcohols and other solvents to derive adipose derived ECM [Flynn, 2010; Brown et al., 2011]. The use of α-galactosidase has been used to remove the galactose-α-(1,3)-galactose, also known as Gal, epitope [Stone et al., 1998; Xu et al., 2009]. The Gal epitope is a cellular antigen that is known to cause xenorejection in humans [Cooper et al., 1993; Galili, 2001], and is found in small amounts in decellularized tissues [McPherson et al., 2000; Daly et al., 2009]. Given the mild immune response, it is not clear that treatment with α-galactosidase is necessary in simple tissues, but further work is required to determine whether it would be beneficial in complex organs. A more complete review of the immune response to the Gal epitope is available elsewhere [Badylak and Gilbert, 2008].

An important consideration with the use of all of these chemicals for tissue decellularization is the potential for residues to remain within the tissue. SDS in particular has a high affinity for proteins, even at low concentrations [Grefrath and Reynolds, 1974; Krejci,

2007]. Therefore, it is questionable whether complete removal is possible, although it has been suggested that secondary rinsing with Triton X-100 decreases the SDS content in decellularized heart [Ott et al., 2008]. If residues persist through the rinsing steps, the chemical remaining in the tissue rather than the ECM will dictate the response of cells seeded onto the scaffold or that migrate into the scaffold after implantation. As such, there is a need for assays that can accurately detect the presence of chemicals used in any protocol, preferably in a quantitative fashion. Alternatively, development of new approaches for decellularization of tissue that do not require chemicals that leave residues would be extremely attractive.

Ultimately, the methods for decellularization of any tissue of interest are going to have to be developed through systematic, controlled experiments with a broad range of assessments to determine the efficacy of cellular removal, the effects of processing on the matrix proteins, and the cellular response to the scaffold after preparation. To date, most decellularization protocols have been proposed with little insight into how the process was developed. It is still quite rare that manuscripts compare multiple decellularization protocols, and when this occurs, the processes include so many different reagents that it is often impossible to determine the effects of any one decellularization step on the scaffold. Controlled studies for various tissue types will dramatically improve our understanding of tissue and organ decellularization.

HOST RESPONSE TO DECELLULARIZED TISSUES AND ORGANS

Processing and tissue source are among the most important variables that impact the host response to and remodeling outcome for an ECM scaffold [Badylak et al., 2009]. Unfortunately, broad generalities are made about ECM scaffolds based upon the existing literature about a limited number of tissue sources and processed by a narrow set of conditions, specifically SIS and UBM processed with peracetic acid. These scaffolds retain a variety of collagens, growth factors (TGF-beta, b-FGF, VEGF), glycosaminoglycans, and other matrix proteins, and the arrangement of the proteins is minimally disturbed through the process [Hodde et al., 1996, 2001, 2002; Voytik-Harbin et al., 1997; McDevitt et al., 2003]. Upon implantation, mononuclear cells migrate into the scaffold and degrade it within approximately 90 days while concomitantly depositing new site-appropriate tissue [Badylak et al., 2002; Gilbert et al., 2007]. The process of scaffold degradation provides positive feedback to the process of site-appropriate tissue deposition by releasing matricryptic peptides that are chemotactic for progenitor cells and bacteriostatic [Brennan et al., 2006; Reing et al., 2009; Agrawal et al., 2010]. However, the limitation to the claim of site-appropriate tissue remodeling is that it tends to form dense, simple tissues. Stated differently, the scaffolds can promote formation of esophageal tissue or a musculotendinous junction, both of which have fairly simple geometries, but it has not been observed that the scaffolds can guide tissue formation into complex geometries. This point emphasizes the need for scaffolds with more complex geometries.

In contrast, ECM scaffolds derived from denser tissues, such as dermis, tend to require much longer and harsher decellularization processes, including various ionic solutions, detergents and enzymes [Reing et al., 2010]. Consequently, the structure of these scaffolds may be similar, but the scaffolds are stripped of many components, particularly growth factors and glycosaminoglycans [Reing et al., 2010]. The degradation time for dermis after implantation has not been explicitly tested, but various studies have shown that the scaffolds tend to persist for a long time, and become integrated into the surrounding tissue rather than being rapidly degraded and replaced by host tissue [Xu et al., 2009]. At this point it is still unclear whether these differences are due to inherent differences in the ECM from each tissue, or whether the differences are due to changes in the matrix that occur in response to exposure to harsher decellularization protocols and persistence of the chemicals within the scaffold.

Much of the recent published work on decellularized whole organs has synthesized concepts about the bioactivity and site-appropriate remodeling that are derived from our knowledge about SIS and UBM, but the techniques used to generate ECM from these organs is more comparable to processing for dermis. Since it is not known whether it is the tissue source or processing that is the primary factor determining the host response to a scaffold, it is difficult to predict what the remodeling response to these scaffolds will be.

The objective of whole organ engineering is to develop a transplantable organ that will remodel slowly over time and maintain the structure and function that is developed in vitro. The decellularized scaffold serves as a substrate for attachment, proliferation, and differentiation of the particular cell populations of interest, and the remodeling should occur on the timescale of normal tissue homeostasis. In fact, a return to homeostasis is the ultimate goal so that the morphology and function of the engineered tissue remains largely unchanged over time. If the ECM scaffolds behave more similarly to scaffolds like dermis, then this objective may be achieved. However, if the ECM scaffold behaves like SIS or UBM, and promotes ''constructive remodeling,'' then the likely outcome will be degradation of the scaffold and repopulation by a dense connective tissue that may not retain the characteristics of the complex organ [Wagner and Griffith, 2010]. None of the existing current approaches to organ engineering have advanced to the stage where the data can provide answers to this question. However, given the inherent role that ECM scaffolds play in tissue architecture and function, it is reasonable to expect that decellularization and recellularization strategies exist that will yield an ECM scaffold that is capable of resuming its original role.

The realization of this goal is going to take years of close collaboration among multidisciplinary teams. Efforts are already well under way within the cell biology and tissue engineering communities. Recent technological advances in matrix biology, structural biology, and imaging are also going to be critical to the success. Imaging techniques like electron microscopy, atomic force microscopy, and multiphoton microscopy will provide important insight into the effects of decellularization on the ECM at the molecular level, while advances in MRI and CT will enable a better understanding of the macrostructure of the scaffold through the decellularization and recellularization process. Advances in proteomics and structural biology are beginning to create opportunities

to understand the complex composition the decellularized organs, the effects that the decellularization process has on individual ECM components, and the protein-protein interactions, all of which will help with our understanding of the cell–matrix interactions during seeding and in vivo implantation.

CONCLUSION

Clearly, there is much work to be done to realize the goal of transplantable organs based upon whole organ engineering, but there is also significant promise. The success of decellularized tissues for surgical mesh and the recent reports of decellularized tracheas being used in clinical tracheal reconstruction are important examples that should motivate the field. With multidisciplinary teams performing focused, systematic studies to address critical questions about how to improve decellularization of whole organs, there is great hope that success will be within our grasp.

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