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Research Article

Maxillary Sinus Augmentation with Decellularized Bovine Compact Particles: A Radiological, Clinical, and Histologic Report of 4 Cases

Antonio Scarano

Department of Medical, Oral and Biotechnological Sciences and CeSi-MeT, University of Chieti-Pescara, Via dei Vestini 31, 66100 Chieti, Italy

Correspondence should be addressed to Antonio Scarano; ascarano@unich.it

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Background. One of the most problematic regions for endosseous implants is the posterior maxilla, not only having poor bone density, but also lacking adequate vertical height as a result of sinus pneumatization. The purpose of the present study was a radiologic, histological, and histomorphometrical evaluation, in humans, of specimens retrieved from sinuses augmented with decellularized bovine compact particles, after a healing period of 6 months. *Methods*. Four patients, with atrophic resorbed maxillas, underwent a sinus lift augmentation with decellularized bovine compact bone from bovine femur. The size of the particles used was 0.25–1 mm. A total of four grafts and 5 biopsies were retrieved and processed to obtain thin ground sections with the Precise 1 Automated System. *Results*. The mean volume after graft elevation calculated for each of the 4 patients was 2106 mm^3 in the immediate postoperative period (5–7 days), ranging from 1408.8 to 2946.4 mm^3. In the late postoperative period (6 months) it was 2053 mm^3, ranging from 1339.9 to 2808.9 mm^3. Histomorphometry showed that newly formed bone was 36 ± 1.6% and marrow spaces were 34 ± 1.6%, while the residual graft material was 35 ± 1.4%. *Conclusion*. In conclusion, based on the outcome of the present study, Re-Bone® can be used with success in sinus augmentation procedures and 6 months are considered an adequate time for maturation before implant placement.

1. Introduction

The rehabilitation of the edentulous posterior maxilla with dental implants often represents a clinical challenge due to the insufficient bone volume resulting from pneumatization of the maxillary sinus and crestal bone resorption. The resultant atrophic residual ridge is one of low-density trabecular bone with a minimal cortical component [1]. The maxillary sinus lifting technique is a common surgical technique to augment bone volume in atrophic posterior maxilla [2] and healing was allowed for about 6 to 8 months before implant insertion [3]. One of the most problematic regions is the posterior maxilla, not only having poor bone density, but also lacking adequate vertical height for endosseous implants as a result of sinus pneumatization. Sinus floor augmentation can provide the necessary bone mass to place and stabilize implants essential for the initial steps towards osseointegration [4].

Different materials are used in sinus lifting, such as autogenous bone grafts [5–7], allografts [8, 9], alloplast [8–11], and xenografts [8, 12, 13].

Bovine bone particles were used with success in sinus lifting [14]. No pathological inflammatory cell infiltrate or foreign body reactions were reported with the use of anorganic bone [15, 16]. Bovine bone has been shown to be highly biocompatible with hard oral tissues in animals and man [17, 18].

The aim of the present study was a radiologic, histological, and histomorphometrical evaluation, in humans, of specimens retrieved from sinuses augmented with decellularized bovine compact particles, after a healing period of 6 months.

2. Materials and Methods

Four patients, with atrophic resorbed maxillas, underwent sinus lift augmentation with decellularized bovine compact

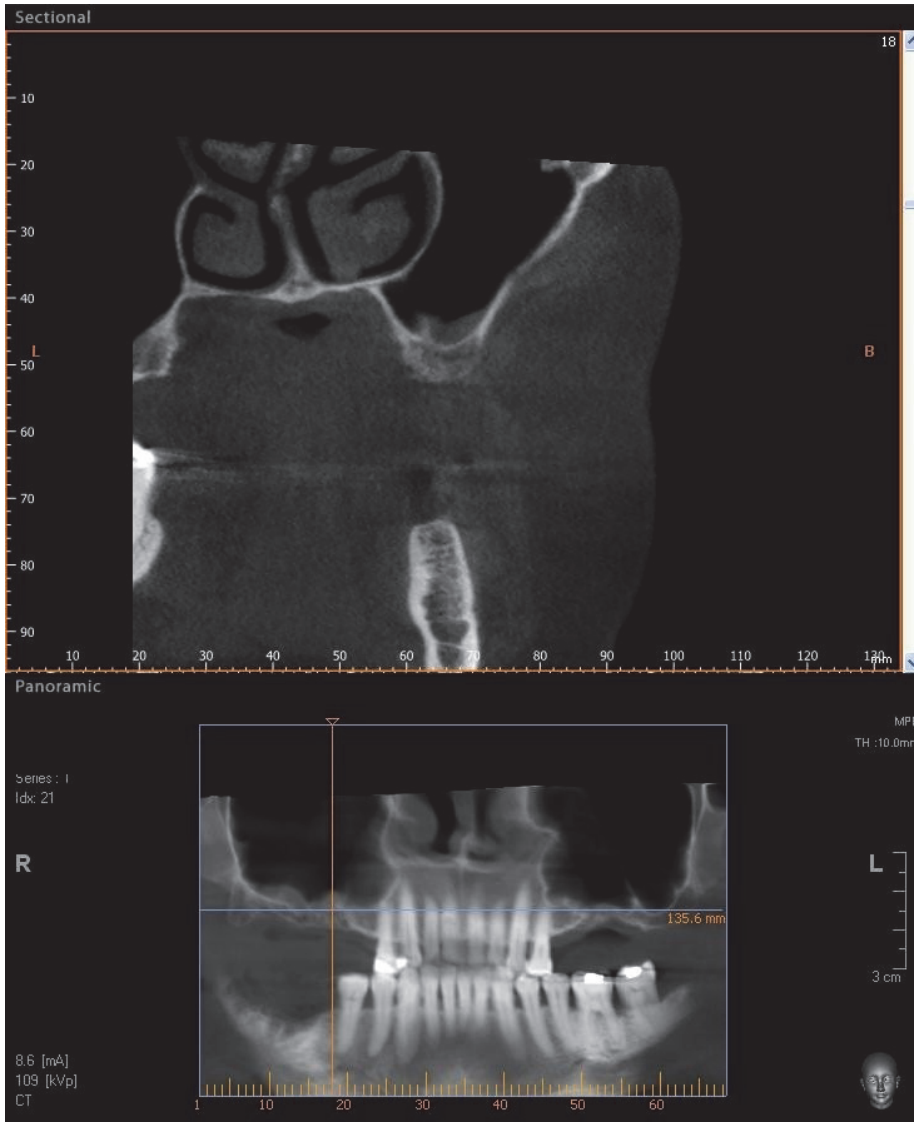


Figure 1: CBCT of an edentulous patient with bilateral severely atrophic maxilla.

bone from bovine femur (Re-Bone, UBGEN Padova, Italy) (Figures 1–4). The graft was condensed at each stage and a collagen membrane (SHELTER®, UBGEN Padova, Italy) The sizes of particles used were 0.25–1 mm. The sinus lift procedures were carried out as described by Boyne and James in 1980 (Figures 2–4). In all cases the sinus lifting procedure was considered to be successful and the insertion of implants of at least 12 mm was performed in all cases after 6 months. Biopsy specimens were retrieved at 6 months. A biopsy of the regenerated tissues was carried out with a small trephine under generous saline irrigation (Figures 5–7). A total of four grafts and 5 biopsies were retrieved. The cores were obtained at a mean depth of 12 mm. The specimens were retrieved, washed in saline solution, and immediately fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.15 M cacodylate buffer al 4°C and pH 7.4, to be processed for histology. The specimens were processed to obtain thin ground sections with the Precise 1 Automated System (Assing, Rome, Italy) [19]. The specimens were dehydrated in an ascending series of alcohol rinses and embedded in a glycolmethacrylate resin (Technovit 7200 VLC, Kulzer, Germany). After polymerization the specimens were sectioned with a high precision diamond disc at about 150 μm and ground down to about 30 μm. The slides were stained with basic fuchsin, toluidine blue, and von Kossa. The histochemical analysis of acid and alkaline phosphatases was carried out according to a previously described protocol. For general morphologic observations, sections were stained with toluidine blue and observed under light microscopy. To determine the relative distribution of the new matrix bone and osteoblast activity, morphological analyses were performed. A polarized light was used to distinguish lamellar bone and woven bone.

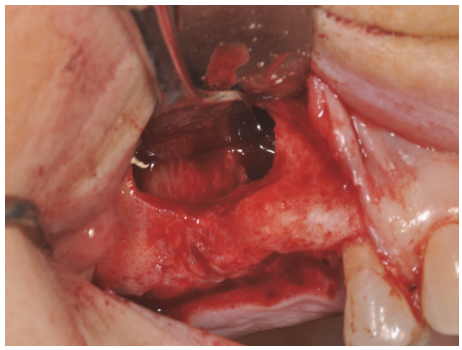


Figure 2: Sinus lifting procedure. The maxillary sinus lateral wall is exposed and a bone window is removed.

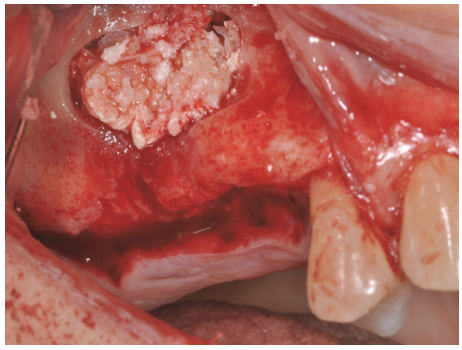


Figure 3: Sinus filled with cortical bovine bone.

Table 1: Volume after graft elevation mm^3.

N° Sinus	Immediate postoperative	After 6 months
1	1408	1339
2	2265	2265
3	1808	1800
4	2946	2808
Mean	2106,75	2053,25
SD	660	629

3. Results

The mean volume after graft elevation calculated for each of the 4 patients was 2106 mm^3 in the immediate postoperative period (5–7 days), ranging from 1408.8 to 2946.4 mm^3. In the late postoperative period (6 months) it was 2053 mm^3, ranging from 1339.9 to 2808.9 mm^3 (Figures 5 and 6). Table 1.

No perforation of the sinus membrane was evident in any of the cases. No acute infection, with pain or fever, was observed. In all cases, bone augmentation showed hyperdensity for comparison between the immediate postoperative period and the late postoperative period, with more density than native bone at both times. The statistical analysis demonstrated a significant difference in volume alterations ($P = 0.0119$).

In general, bone morphology was well present with well differentiated cellular constituents mineralized bone, osteoid, osteoblasts, osteocytes, and blood vessels. At low magnification, trabecular mature bone was observed (Figures 7 and 8). The initial formation of immature bone extending from the periphery of the bone cavities was evident. The rest of the bone cavity contained mature tissue and biomaterial with a mild inflammatory reaction.

Re-Bone particles were easily distinguished from the newly formed bone: they tended to be less stained due to the low content of collagen. The particles were surrounded by newly formed bone (Figures 8 and 9). In a few marrow space areas, in which it was possible to find small capillaries, some particles were present at the interface. In some areas osteoblasts were observed in the process of posing bone directly onto the particle surface. Some positive osteoclast for acid phosphatase and a few positive osteoblast for alkaline phosphatases were observed. Histomorphometry showed that newly formed bone was 36 ± 1.6% and marrow spaces were 34±1.6%, while the residual graft material was 35±1.4%.

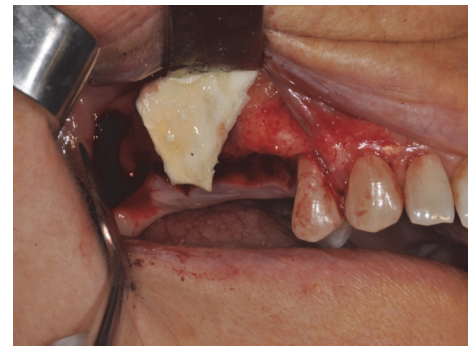


Figure 4: A membrane is placed over the antrostomy.

4. Discussion

Oral rehabilitation with osseointegrated implants is very successful and predictable in patients with normal bone volume and density, which provide adequate stabilization of implants of standard diameter and length [20]. Rehabilitation of the edentulous posterior maxilla with dental implants is often difficult because bone height is insufficient and cancellous [2].

Although there is a high risk of implant displacement/migration into the maxillary, this has been only rarely reported [10, 21]. Different biomaterials can be successfully used for sinus lifting. Many research data show that bovine bone grafting in this areas is not contraindicated and represent a procedure with low morbidity [2, 4]. This xenograft is the one most commonly used material for sinus floor augmentation and has the most powerful scientific evidence for sinus grafting [2, 4, 14, 19, 22–24] because its structure is similar to that of human [22].

In fact the outcomes of the present study showed that the Re-Bone particles appeared to be surrounded by an abundant quantity of newly formed bone. This biomaterials appeared to undergo a slow resorption process; in fact in the present study, after 6 months of observation, most of the grafting material

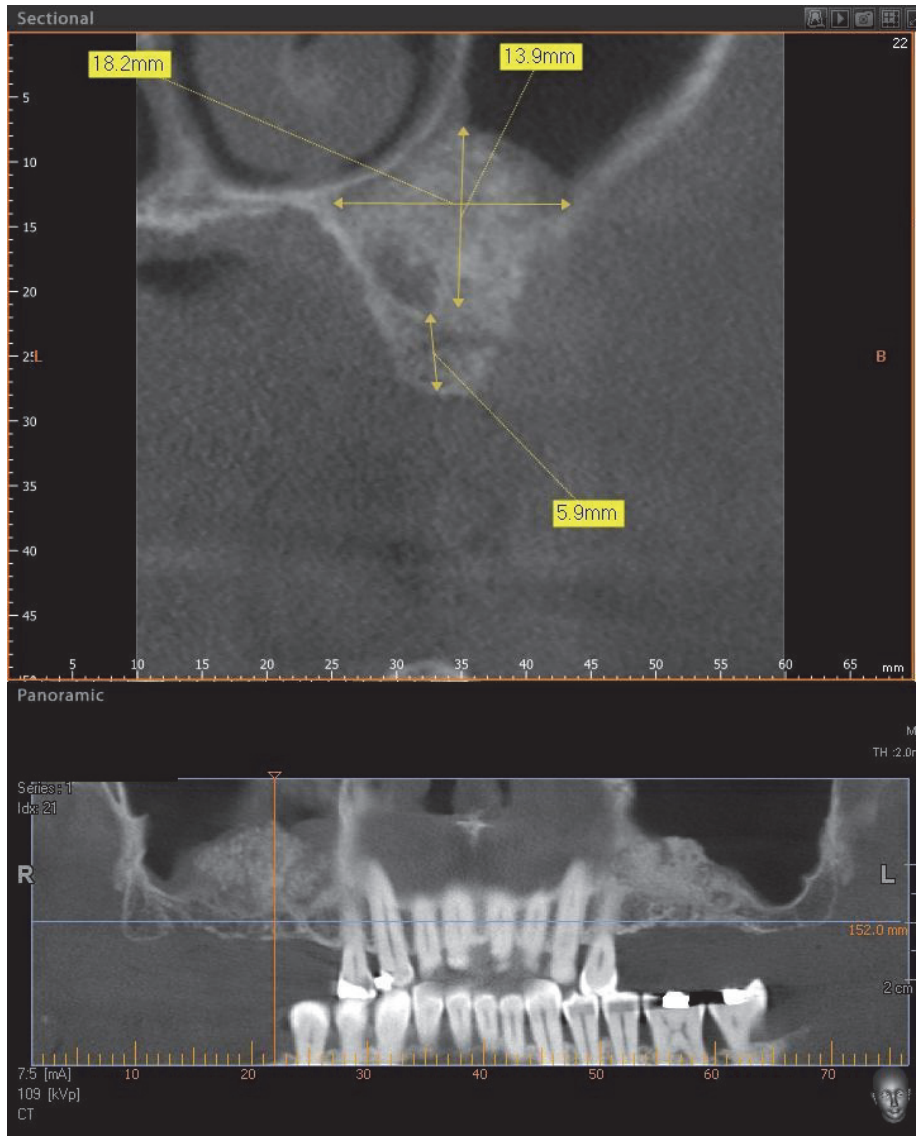


Figure 5: Postoperative CBCT scan panoramic view at 6 months after maxillary sinus lifting.

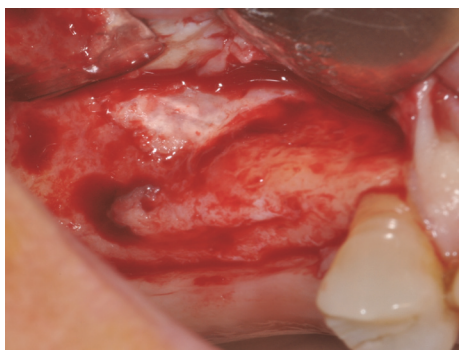
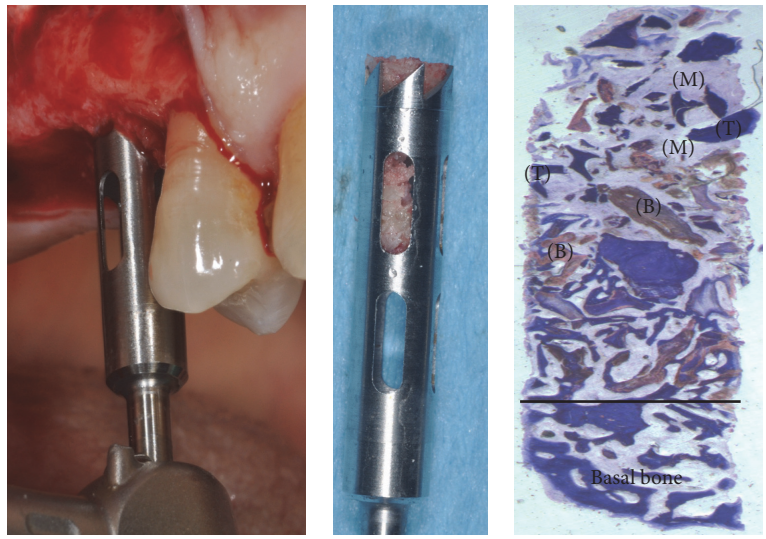


Figure 6: The lateral wall is completely closed by new hard tissues.

was still in place. This study is consistent with other studies reported that the use the bovine bone as a grafting material yielded a bone formation and no presence of inflammatory cell infiltrate [25, 26]. Close contact between most of the materials and the newly formed osseous tissue was present, near but not in contact with the implant surface [14]. Several authors have discussed the use of different graft materials and have documented results both similar and varied when compared to those in the present study [14, 23]. A biomaterial similar to Re-Bone is the Bio-Oss®; this has a similar size, structure, and biological response with conducive to vessel ingrowth [15, 21]. According to our experience and previous literature, we did not observe histological differences between Bio-Oss and Re-Bone [14, 23]. The outcomes of this study revealed new bone formation around the graft particles (36 ± 1.6%) within the maxillary sinus after six months of healing. The particles showed absence of gaps at the bone-particles interface, and the bone was always in close contact with the particles. This xenograft has excellent osteoconductive



(a) (b) (c)

Figure 7: (a-b) Bone core biopsy carried out with a small trephine. (c) Newly formed trabecular bone (T) is present, with wide marrow (M) spaces and biomaterials (B). Toluidine blue 10x.

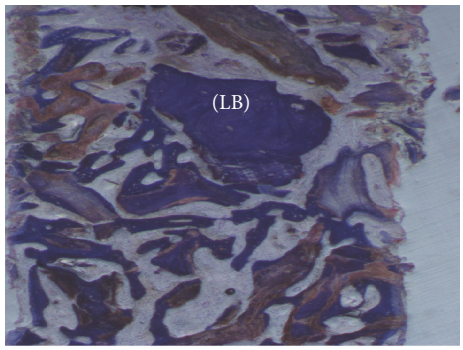


Figure 8: At higher magnification previous image: a few lamellar bones are visible (LB). Toluidine blue 50x.

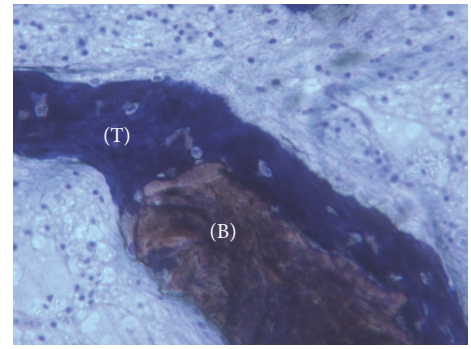


Figure 9: No gaps are present at the bone-particles interface, and newly formed bone is always in close contact with the particles. The biomaterial (B) seems to be totally incorporated in the trabecular bone (T). Toluidine blue 100x.

properties; in fact the outcomes of the present study showed that the Re-Bone particles appeared to be surrounded by an abundant quantity of newly formed bone. Probably, also Re-Bone can be resorbed by osteoclasts [21, 24]. The grafted biomaterial was clearly distinguishable from the remaining original bone due to its density and structure. This is the first case reported in the literature to use Re-Bone granules as bone grafts in sinus lifts. The granular nature of the material facilitated its application between the sinus filling and newly formed bone. *Through* surgery, the scaffold can be easily adapted to the dimension and of the sinus. During graft placement it can quickly adsorb the blood molecules and cells promoting bone formation. Its architecture favors cell attachment and proliferation. In addition, the properties exhibited make Re-Bone a valid alternative to autogenous grafting, preventing the added morbidity of a donor surgical site. Our results were similar with a recent randomized clinical trial published in 2016 to compare histological bone quality and radiographic volume stability in maxillary sinuses grafted with porcine bone and bovine bone that confirms the validity of the bovine bone when used for sinus lifting [26]. The outcomes of the present bone core histomorphometric study showed a $35 \pm 1.6\%$ presence of Re-Bone and $36 \pm 1.6\%$ newly formed bone during the 6-month healing period. This means bone formation with low standard variation between 5 biopsies was not statistically significant. Therefore, 6 months are considered adequate time for Re-Bone maturation before implant placement or the uncovering of implants placed at the same time as grafting.

Obviously, with only 4 grafts and 5 biopsies, the data presented in this study cannot be considered conclusive. However, these results help to set practice parameters that will assure a study with a large number of patients in the future. In conclusion, the findings from the present four case reports support the use of Re-Bone as a bone substitute in maxillary sinus augmentation procedures.

Competing Interests

The author declares that there are no competing interests.

Acknowledgments

The author also acknowledges the helpful technical assistance of Dr. Mauro di Berardino (X-ray Technician) in the elaboration of CBCT.

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Review

Decellularization of tissues and organs

Thomas W. Gilbert[a,b], Tiffany L. Sellaro[a,b], Stephen F. Badylak[a,c,*]

[a]McGowan Institute for Regenerative Medicine, University of Pittsburgh, 100 Technology Drive, Suite 200, Pittsburgh, PA 15219, USA
[b]Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA 15219, USA
[c]Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15219, USA

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Abstract

Decellularized tissues and organs have been successfully used in a variety of tissue engineering/regenerative medicine applications, and the decellularization methods used vary as widely as the tissues and organs of interest. The efficiency of cell removal from a tissue is dependent on the origin of the tissue and the specific physical, chemical, and enzymatic methods that are used. Each of these treatments affect the biochemical composition, tissue ultrastructure, and mechanical behavior of the remaining extracellular matrix (ECM) scaffold, which in turn, affect the host response to the material. Herein, the most commonly used decellularization methods are described, and consideration give to the effects of these methods upon the biologic scaffold material.

Keywords: Extracellular matrix; Decellularization; Tissue engineering; Scaffolds

Contents

*Corresponding author. McGowan Institute for Regenerative Medicine, University of Pittsburgh, 100 Technology Drive, Suite 200, Pittsburgh, PA 15219, USA. Tel.: +1 412 235 5145; fax: +1 412 235 5110.
E-mail address: badylaks@upmc.edu (S.F. Badylak).

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1. Introduction

Biologic scaffolds derived from decellularized tissues and organs have been successfully used in both pre-clinical animal studies and in human clinical applications [1–8]. Removal of cells from a tissue or an organ leaves the complex mixture of structural and functional proteins that constitute the extracellular matrix (ECM). The tissues from which the ECM is harvested, the species of origin, the decellularization methods and the methods of terminal sterilization for these biologic scaffolds vary widely. Each of these variables affects the composition and ultrastructure of the ECM and accordingly, affects the host tissue response to the ECM scaffold following implantation. The objective of this manuscript is to provide an overview of the various methods that have been used to decellularize tissues, and the potential effects of the various decellularization protocols on the biochemical composition, ultrastructure, and mechanical behavior of the ECM scaffold materials.

2. Rationale for decellularization of ECM

Xenogeneic and allogeneic cellular antigens are, by definition, recognized as foreign by the host and therefore induce an inflammatory response or an immune-mediated rejection of the tissue. However, components of the ECM are generally conserved among species and are tolerated well even by xenogeneic recipients [9–12]. ECM from a variety of tissues, including heart valves [13–19], blood vessels [20–23], skin [24], nerves [25,26], skeletal muscle [27], tendons [28], ligaments [29], small intestinal submucosa (SIS) [30–32], urinary bladder [2,33,34], and liver [35] have been studied for tissue engineering and regenerative medicine applications. The goal of a decellularization protocol is to efficiently remove all cellular and nuclear material while minimizing any adverse effect on the composition, biological activity, and mechanical integrity of the remaining ECM.

Any processing step intended to remove cells will alter the native three-dimensional architecture of the ECM. The most commonly utilized methods for decellularization of tissues involve a combination of physical and chemical treatments. The physical treatments can include agitation or sonication, mechanical massage or pressure, or freezing and thawing. These methods disrupt the cell membrane, release cell contents, and facilitate subsequent rinsing and removal of the cell contents from the ECM. These physical treatments are generally insufficient to achieve complete decellularization and must be combined with a chemical treatment. Enzymatic treatments, such as trypsin, and chemical treatment, such as ionic solutions and detergents, disrupt cell membranes and the bonds responsible for intercellular and extracellular connections. Tissues are composed of both cellular material and ECM arranged in variable degrees of compactness depending on the source of the tissue. The ECM must be adequately disrupted during the decellularization process to allow for adequate exposure of all cells to the chaotropic agents and to provide a path for cellular material to be removed from the tissue. The intent of most decellularization processes is to minimize the disruption and thus retain native mechanical properties and biologic properties.

3. Description of decellularization protocols

The most robust and effective decellularization protocols include a combination of physical, chemical, and enzymatic approaches. A decellularization protocol generally begins with lysis of the cell membrane using physical treatments or ionic solutions, followed by separation of cellular components from the ECM using enzymatic treatments, solubilization of cytoplasmic and nuclear cellular components using detergents, and finally removal of cellular debris from the tissue. These steps can be coupled with mechanical agitation to increase their effectiveness. Following decellularization, all residual chemicals must be removed to avoid an adverse host tissue response to the chemical. The efficiency of decellularization and preservation of the ECM can be assessed by several methods. The mechanisms of physical, enzymatic, and chemical decellularization for a variety of tissues are reviewed in the following sections and in Table 1.

3.1. Physical methods

Physical methods that can be used to facilitate decellularization of tissues include freezing, direct pressure, sonication, and agitation. Snap freezing has been used frequently for decellularization of tendinous and ligamentous tissue [36–41] and nerve tissue [42]. By rapidly freezing a tissue, intracellular ice crystals form that disrupt cellular membranes and cause cell lysis. The rate of temperature change must be carefully controlled to prevent the ice formation from disrupting the ECM as well. While freezing can be an effective method of cell lysis, it must be followed by processes to remove the cellular material from the tissue.

Cells can be lysed by applying direct pressure to tissue, but this method is only effective for tissues or organs that are not characterized by densely organized ECM (e.g., liver, lung). Mechanical force has also been used to delaminate layers of tissue from organs that are characterized by natural planes of dissection such as the small intestine and the urinary bladder. These methods are effective, and cause minimal disruption to the three-dimensional architecture of the ECM within these tissues.

Mechanical agitation and sonication have been utilized simultaneously with chemical treatment to assist in cell lysis and removal of cellular debris. Mechanical agitation can be applied by using a magnetic stir plate, an orbital shaker, or a low profile roller. There have been no studies performed to determine the optimal magnitude or frequency of sonication for disruption of cells, but a standard ultrasonic cleaner appears to be as effective at

Table 1
Commonly used decellularization methods and chaotropic agents

Method	Mode of action	Effects on ECM	References
Physical			
Snap freezing	Intracellular ice crystals disrupt cell membrane	ECM can be disrupted or fractured during rapid freezing	[36–42]
Mechanical force	Pressure can burst cells and tissue removal eliminates cells	Mechanical force can cause damage to ECM	[33,35]
Mechanical agitation	Can cause cell lysis, but more commonly used to facilitate chemical exposure and cellular material removal	Aggressive agitation or sonication can disrupt ECM as the cellular material is removed	[19,21,33,35]
Chemical			
Alkaline; acid	Solubilizes cytoplasmic components of cells; disrupts nucleic acids	Removes GAGs	[33,43–46]
Non-ionic detergents			
Triton X-100	Disrupts lipid–lipid and lipid–protein interactions, while leaving protein–protein interactions intact	Mixed results; efficiency dependent on tissue, removes GAGs	[2,21,28,29,35,43,58]
Ionic detergents			
Sodium dodecyl sulfate (SDS)	Solubilize cytoplasmic and nuclear cellular membranes; tend to denature proteins	Removes nuclear remnants and cytoplasmic proteins; tends to disrupt native tissue structure, remove GAGs and damage collagen	[18,24,25,29,35,59,60]
Sodium deoxycholate		More disruptive to tissue structure than SDS	[18,24,25,29,35,59,60]
Triton X-200		Yielded efficient cell removal when used with zwitterionic detergents	[18,24,25,29,35,59,60]
Zwitterionic detergents			
CHAPS	Exhibit properties of non-ionic and ionic detergents	Efficient cell removal with ECM disruption similar to that of Triton X-100	[21]
Sulfobetaine-10 and -16 (SB-10, SB-16)		Yielded cell removal and mild ECM disruption with Triton X-200	[18,24,25,29,35,59,60]
Tri(*n*-butyl)phosphate	Organic solvent that disrupts protein–protein interactions	Variable cell removal; loss of collagen content, although effect on mechanical properties was minimal	[28,29]
Hypotonic and hypertonic solutions	Cell lysis by osmotic shock	Efficient for cell lysis, but does not effectively remove the cellular remnants	[21,29,61–63]
EDTA, EGTA	Chelating agents that bind divalent metallic ions, thereby disrupting cell adhesion to ECM	No isolated exposure, typically used with enzymatic methods (e.g., trypsin)	[13,68–70]
Enzymatic			
Trypsin	Cleaves peptide bonds on the C-side of Arg and Lys	Prolonged exposure can disrupt ECM structure, removes laminin, fibronectin, elastin, and GAGs	[13,68–70]
Endonucleases	Catalyze the hydrolysis of the interior bonds of ribonucleotide and deoxyribonucleotide chains	Difficult to remove from the tissue and could invoke an immune response	[18,21,29,82]
Exonucleases	Catalyze the hydrolysis of the terminal bonds of ribonucleotide and deoxyribonucleotide chains		

removing cellular material as placing the tissue on an orbital shaker. In all of these procedures, the optimal speed, volume of reagent, and length of mechanical agitation is dependent on the composition, volume, and density of the tissue.

3.2. Chemical methods

3.2.1. Alkaline and acid treatments

Alkaline and acid treatments are used in decellularization protocols to solubilize the cytoplasmic component of the cells as well as remove nucleic acids such as RNA and DNA. For example, acetic acid, peracetic acid (PAA), hydrochloric acid, sulfuric acid, and ammonium hydroxide (NH_4OH) can effectively disrupt cell membranes and intracellular organelles [33,43–46]. However, these chemicals also dissociate important molecules such as GAGs from collagenous tissues.

A variety of porcine tissues including SIS and layers of the urinary bladder [e.g., submucosal layer (UBS) and the basement membrane plus tunica propria (UBM)] have been decellularized using PAA at concentrations of

approximately 0.10–0.15% (w/v). This treatment is highly efficient at removing cellular material from these thin ECM structures, and simultaneously disinfects the material by entering microorganisms and oxidizing microbial enzymes [47,48]. The effects of PAA treatment on the ECM components have been studied extensively. Several types of collagen including types I, III, IV, V, VI, and VII have been identified in SIS and/or UBM following treatment with PAA [31,49], however, the microstructure of the collagen fibers has not been closely examined following such treatment. The ECM retains many of the native GAGs including hyaluronic acid, heparin, heparin sulfate, chondroitin sulfate A, and dermatan sulfate following PAA treatment [50]. It has also been shown that laminin and fibronectin are present in the ECM scaffolds following exposure to PAA [49,51]. PAA treatment preserves the structure and function of many of the growth factors that are resident in the ECM, including transforming growth factor-β, basic fibroblast growth factor, and vascular endothelial growth factor [52,53]. PAA does not appear to have any adverse effect on the mechanical behavior of the biologic scaffold [33]. Both SIS and UBM have been shown repeatedly to serve as excellent substrates for in vitro cell culture [51,54–56], and have been successfully used for many tissue-engineering applications in vivo following decellularization and disinfection with PAA.

3.2.2. Non-ionic detergents

Non-ionic detergents have been used extensively in decellularization protocols because of their relatively mild effects upon tissue structure. Non-ionic detergents disrupt lipid–lipid and lipid–protein interactions, but leave protein–protein interactions intact so that proteins within a tissue or organ following non-ionic detergent treatment should be left in a functional conformation [57].

Triton X-100 is the most widely studied non-ionic detergent for decellularization protocols. Exposure of tissue to Triton X-100 for periods ranging from several hours to 14 days [2,21,28,29,35,43,58]. Decellularization of tissues with Triton X-100 has shown mixed results. When Triton X-100 was used to decellularize a heart valve, complete removal of nuclear material was observed with maintenance of the valvular structure after 24 h. However, cellular material was found in the adjacent myocardium and aortic wall [15]. With regard to the ECM components, Triton X-100 led to a nearly a complete loss of GAGs and decreases in the laminin and fibronectin content of the valve tissue [15]. Other studies showed that Triton X-100 was not effective at completely removing cellular material from a blood vessel, tendon, and ligament after exposure for up to 4 days [21,28,29]. Nuclear material was observed by histological staining in all of the tissues and immunohistochemical staining showed the presence of the cytoskeletal protein vimentin in the anterior cruciate ligament (ACL) after treatment with Triton X-100. It was found that treatment with Triton X-100 severely altered the tendon with respect to the tensile strength of collagen fibers isolated from the tendon. Conversely, treatment with Triton showed no effect on the collagen content in the ACL. Treatment with Triton X-100 also showed mixed results with regards to GAG content. All GAGs were removed from a heart valve after treatment with Triton X-100 for 24 h, while there was no difference in the sulfated GAG content in the ACL after treatment with Triton X-100 for 4 days. Although Triton X-100 can be an effective decellularization method, its efficacy is dependent upon the tissue being decellularized and the other methods with which it is combined in a given decellularization protocol.

3.2.3. Ionic detergents

Ionic detergents are effective for solubilizing both cytoplasmic and nuclear cellular membranes, but tend to denature proteins by disrupting protein–protein interactions [57]. The most commonly used ionic detergents are sodium dodecyl sulfate (SDS) and sodium deoxycholate and Triton X-200 [18,24,25,29,35,59,60].

SDS is very effective for removal of cellular components from tissue. Compared to other detergents, SDS yields more complete removal of nuclear remnants and cytoplasmic proteins, such as vimentin [29]. SDS tends to disrupt the native tissue structure, and causes a decrease in the GAG concentration and a loss of collagen integrity. However, it does not appear that SDS removes collagen from the tissue.

Sodium deoxycholate is also very effective for removing cellular remnants, but tends to cause greater disruption to the native tissue architecture when compared to SDS. There are no reports of tissue decellularization using sodium deoxycholate alone, so it is difficult to isolate its effect on the remaining ECM of a tissue. Sodium deoxycholate was also combined with several zwitterionic detergents to decellularize nerve tissue, however, it was found that only a combination of Triton X-200 with the zwitterionic detergents yielded a completely decellularized nerve ECM [25,60].

3.2.4. Zwitterionic detergents

Zwitterionic detergents exhibit the properties of both non-ionic and ionic detergents. Zwitterionic detergents have a greater tendency to denature proteins than non-ionic detergents. Examples of zwitterionic detergents include 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), which has been studied for decellularization of blood vessels [21], and sulfobetaine-10 (SB-10) and -16 (SB-16), which have been used for decellularization of nerves [25,60]. CHAPS treated artery tissue has histologically normal collagen and elastin morphology and the collagen content appears to remain similar to that of the native artery. CHAPS treatment results in a significant decrease in the burst pressure and maximum stress of arterial tissue, but the decrease is comparable to the burst pressure of arteries subjected to treatment with Triton X-100 and hypotonic/hypertonic solutions [21]. Peripheral nerves have been decellularized

with SB-10 and SB-16 in combination with Triton X-200, an ionic detergent. The combined treatments had a less detrimental effect on the structure of the nerve ECM than combined treatments with Triton X-100 and sodium deoxycholate [25,60].

3.2.5. Tri(n-butyl)phosphate

Tri(*n*-butyl)phosphate (TBP) is an organic solvent that is used to inactivate viruses in blood without compromising the coagulation factor activity. Recently, TBP has been used as a chaotropic agent for decellularization of tendon and ligament grafts. Treatment with TBP yielded complete removal of nuclear remnants from rat tail tendon and the midsubstance of an ACL graft, although the removal of cellular material was incomplete at the insertion of the ligament to bone. TBP treatment did not have an effect on the tensile strength of collagen fibers isolated from the rat tail tendon as compared to native control, but for the ACL, treatment with TBP led to a decrease in collagen content [28,29]. TBP appears to be a promising chaotropic agent for decellularization that has minimal effect on the mechanical behavior of the ECM and is worthy of further study.

3.2.6. Hypotonic and hypertonic treatments

Osmotic shock with a hypotonic or hypertonic solution such as deionized water or low ionic strength solution is used to lyse the cells within tissues and organs [21,29,61–63]. A treatment in a hypotonic solution (10 mM Trizma HCl, 5 mM EDTA) for 11 h followed by an 11 h treatment in a hypertonic solution (50 mM Trizma HCl, 1 M NaCl, 10 mM EDTA) can cause cell lysis, but does not generally remove the resultant cellular remnants from the tissue [21]. Additional enzymatic or chemical treatments are typically necessary to facilitate removal of cellular debris. Removal of DNA remnants from the tissue can be particularly difficult due to the "sticky" nature of DNA and its tendency to adhere to ECM proteins.

3.2.7. Chelating agents

Chelating agents, such as EDTA and EGTA, form a ring-shaped molecular complex that firmly binds and isolates a central metal ion. It has been shown that divalent cations, such as Ca^{2+} and Mg^{2+}, are necessary for cell attachment to collagen and fibronectin at the Arg–Gly–Asp receptor [64–67]. By binding the divalent cations that are present at the cell adhesions to the ECM, these agents facilitate removal of the cellular material from the tissue. EDTA is typically used in combination with trypsin.

3.3. Enzymatic methods

Enzymatic techniques of decellularization include the use of protease digestion, calcium chelating agents, and nucleases [13,68–70]. Trypsin is one of the most commonly used proteolytic enzymes in decellularization protocols. Trypsin is a highly specific enzyme that cleaves the peptide bonds on the carbon side of arginine and lysine if the next residue is not proline [71]. The maximal enzymatic activity of trypsin occurs at 37 °C and at a pH of 8. Nucleases such as endonucleases catalyze the hydrolysis of the interior bonds of the ribonucleotide or deoxyribonucleotide chains whereas exonucleases catalyze the hydrolysis of the terminal bonds of deoxyribonucleotide or ribonucleotide ultimately leading to the degradation of RNA or DNA [72].

The efficacy of enzymatic treatments for removal or separation of the cellular material from the ECM has been studied for a variety of tissues. Some studies show efficient removal of cellular material from porcine pulmonary valves after treatment with 0.05% trypsin/0.02% EDTA for 24 h with agitation [19], while other studies show less efficient decellularization. For example, porcine aortic valves subjected to treatment with 0.5% trypsin, 0.05% EDTA, 0.02% Gentamicin, 0.02 mg/ml DNase and 20 μg/ml RNAse-A in Milli-Q water for up to 17 h at 37 °C with agitation rendered the cells non-viable, but did not lead to removal of cellular material from the tissue [15].

Enzymatic methods of decellularization are not without an adverse effect upon the extracellular components of tissues and organs. Prolonged treatment with trypsin/EDTA causes disruption of the normal pulmonary valve ECM structure, but does not affect the amount of collagen in the tissue [19]. Trypsin/EDTA does, however, substantially reduced the laminin and fibronectin content of the ECM. Prolonged exposure to trypsin/EDTA greatly decreases the elastin content and GAGs over time, with *o*-sulfated GAGs (chondroitin sulfates, keratin sulfates, and dermatan sulfates) showing the greatest decrease. Such treatments can contribute to a decrease in tensile strength of up to 50%. The remaining ECM after such enzymatic decellularization protocols still supports endothelial cell growth in vitro despite the removal of ECM components [15,19]. It is desirable to limit the duration of exposure to trypsin/EDTA treatments to minimize the disruptive effects upon the ultrastructure and composition of the ECM.

3.4. Protease inhibitors

During the decellularization protocols, a number of proteases can be released from the disrupted cells. For long duration chemical treatments, the presence of proteases can cause damage to the native ECM ultrastructure. For this reason, it may be desirable to include protease inhibitors such as phenylmethylsulfonylfluoride (PMSF), aprotonin, and leupeptin to the solutions in which the tissue is immersed. A buffered solution of pH 7–8 further inhibits many proteases, and the control of temperature and time of exposure to the lysis solutions can also limit the activity of the proteases.

3.5. Antibiotics

One concern for long duration chemical decellularization methods is the presence of bacteria, which can contaminate

the remaining ECM material. A number of protocols have therefore included antibiotic solutions such as penicillin, streptomycin, or amphotericin B [29,59,73–75]. However, if antibiotics or antibiotic residues remain in the scaffold material after the decellularization protocol, regulatory agencies may consider the material a drug rather than a medical device, which increases the complexity of regulatory approval.

4. Effects of tissue variability upon decellularization

The efficiency of a given decellularization method or protocol is dependent upon the tissue of interest. Despite identical times of exposure and a greater concentration of trypsin (0.5% vs. 0.05%), Grauss et al. [15] found that a trypsin-based decellularization protocol was ineffective at removing the cellular material from the rat aortic heart valve, while Schenke-Layland et al. [19] showed complete removal of cells from a porcine pulmonary valve. It is likely that subtle nuances in the application of the protocols had an effect on the efficiency of removal that are not entirely clear, such as the application of three-dimensional agitation by Schenke-Layland et al. [19]. Studies by Cartmell [28] and Dunn and Woods and Gratzer [29], which studied decellularization of rat tail tendon and porcine ACL, respectively, showed differences in the decellularization results that cannot be easily explained by differences in the protocols studied. These studies show that the effectiveness of decellularization and the alterations to the ECM vary depending on the source of the tissue, the composition of the tissue, the tissue density, and other factors. For each tissue that is studied, it will be necessary to optimize the decellularization protocol to obtain acceptable cell removal.

5. Verification of cell removal

There are a number of methods available to determine the efficiency of the removal of cellular material from tissues. Standard histological staining with Hematoxylin and Eosin can serve as a first line of inspection to determine if nuclear structures can be observed. Alternative histological stains such as Masson's Trichome, Movat's Pentachrome, or Safrin O can be used to examine tissues for the presence of various cytoplasmic and extracellular molecules. Immunohistochemical methods can also be utilized for specific intracellular proteins, such as actin and vimentin [29].

Inspection for the presence of DNA can be performed by staining the specimen with DAPI or Hoechst, which are both fluorescent molecules that bind to the AT clusters in the minor groove of DNA [76–79]. In addition, assays using propidium iodide and PicoGreen have been developed to provide quantitative data regarding the presence of DNA within a specimen [80]. DNA probe techniques have been utilized to track the fate of DNA from allografts after implantation, and could also be utilized to determine whether any DNA is present in the decellularized tissue [81]. Polymerase chain reaction (PCR) or electron microscopic methods are possible but not typically used to examine for the presence of remnant nuclear material or cytoplasmic debris due to the technical complexity and expense of these procedures for routine work.

In addition, to determining what has been removed, it is also necessary to confirm that desirable components of the ECM are retained, such as adhesion proteins like fibronectin and laminin, GAGs, growth factors, elastic fibers, and collagens which will be required for infiltration of the matrix by cells of choice in vitro or in vivo. Mechanical testing of the ECM after treatment provides insight into presence and integrity of the structural proteins within the scaffold.

Although the above methods provide important information regarding the effectiveness of the decellularization methods, the biologic consequences of small amounts of nuclear material or cytoplasmic debris within the remaining scaffold materials is unclear. There are no reports showing a direct cause–effect relationship between such cellular remnants and an adverse host response to date.

6. Removal of residual chemicals

The decellularization methods described above include a wide variety of chemicals, which are used because of their inherent abilities to damage cells. If the chemicals remain within the tissue in high concentrations after treatment, then it is likely that they will be toxic to host cells when the scaffold is implanted in vivo. There is a need for development of assays to quantify the presence of residual chemicals in the decellularized scaffold material. Similarly, some of the processes that have been described above include enzymes commonly derived from bovine sources (i.e., DNase, RNase, trypsin). These enzymes can potentially invoke an adverse immune response by the host.

7. Conclusion

Complete decellularization of most tissues will require a combination of physical, enzymatic, and chemical treatments, and the protocol will be dependent on the tissue of interest. It is generally desirable to use the mildest protocol possible that yields an acellular material without disruption of the structural and functional component of the ECM. A typical progressive approach would be to start with treatment in a hypotonic or hypertonic solution followed by a mild non-ionic or zwitterionic detergent. If necessary, an enzymatic treatment of tryspin/EDTA can be added prior to the detergent treatment to assist in breaking the bonds between the cell membranes and the ECM. Finally, if these treatments are still inadequate to remove the cellular material, an ionic detergent such as SDS, deoxycholate, or Triton X-200 can be added to the decellularization protocol.

It is clear that physical, enzymatic, and chemical treatments can have substantial effects on the composition, mechanical behavior, and host response to biologic scaffolds derived from the decellularization of native tissue and organs, and could have important implications for subsequent use for in vitro and in vivo applications. The removal of adhesive proteins and GAGs from the scaffold could slow cell migration onto the scaffold and the bioactivity of the scaffold itself. Disruption of the collagen network can change the mechanical behavior and collagen fiber kinematics of the scaffold, which can have an effect on the load bearing capacity of the scaffold and alter the mechanical environment to which the cells are exposed. Degradation is another important factor that relates to the mechanical behavior and bioactivity of the scaffold that could be affected by decellularization treatments. The chemical treatments could compromise the ECM scaffold in a way that makes it more susceptible to enzymatic degradation in vivo, which would lead to a rapid decrease in the strength of the scaffold.

It is unlikely that any combination of methods will remove 100% of all cell components from a tissue or organ. However, it seems apparent that methods which remove most or all of the visible cellular material result in biologic scaffold materials that are safe for implantation. A number of naturally occurring ECM devices and related decellularization protocols have received regulatory approval for use in human patients, including human dermis (Alloderm®, LifeCell, Corp.), porcine SIS (SurgiSIS®, Cook Biotech, Inc.; Restore®, DePuy Orthopaedics, Inc.), porcine urinary bladder (ACell, Inc.), and porcine heart valves (Synergraft®, CryoLife, Inc.). The growing list of biologic scaffolds used for tissue engineering/regenerative medicine applications makes the continued development of decellularization protocols a clinically relevant and important effort.

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Immune Response to Biologic Scaffold Materials

Stephen F. Badylak and **Thomas W. Gilbert**
McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA

Abstract

Biologic scaffold materials composed of mammalian extracellular matrix are commonly used in regenerative medicine and in surgical procedures for the reconstruction of numerous tissue and organs. These biologic materials are typically allogeneic or xenogeneic in origin and are derived from tissues such as small intestine, urinary bladder, dermis, and pericardium. The innate and acquired host immune response to these biologic materials and the effect of the immune response upon downstream remodeling events has been largely unexplored. Variables that affect the host response include manufacturing processes, the rate of scaffold degradation, and the presence of cross species antigens. This manuscript provides an overview of studies that have evaluated the immune response to biologic scaffold materials and variables that affect this response.

Introduction

Biologic scaffold materials composed of mammalian extracellular matrix (ECM) are commonly used for the surgical reconstruction of musculotendinous, dermal, cardiovascular, gastrointestinal, and lower urinary tract tissues, among others [1–14]. Examples of commercially available products include Mosaic™, Freestyle™, Prima™, Restore™, Oasis™, Surgisis™, CuffPatch™, GraftJacket™, Alloderm™, TissueMend™, and OrthAdapt™ (Table 1). These products are all composed of ECM, but differ in their tissue source (e.g., heart valve, small intestine, dermis, pericardium), species of origin, (e.g., porcine, bovine, equine, human), and methods by which they are processed.

Despite the extensive use of allogeneic and xenogeneic biologic scaffold materials, very little is understood, and even less is published, regarding the host immune response to these materials. The present manuscript provides a review of literature relevant to the host immune response to biologic scaffold materials, and the potential relationship between the host immune response and downstream remodeling events.

Biologic materials composed of extrcellular matrix are typically processed by methods that include decellularization and/or chemical crosslinking to remove or mask antigenic epitopes, DNA, and damage associated molecular pattern (DAMP) molecules [15–17]. The effect of various processing steps upon the host immune response has not been systematically examined. In a recent study that compared five ECM products, all of which were processed by different methods, the acute host response was uniformly characterized by an intense mononuclear cell infiltrate. The long term remodeling response, however, varied from chronic inflammation, fibrosis, scarring, and encapsulation to the formation of organized, site-appropriate tissue

Corresponding author: Stephen F. Badylak, McGowan Institute for Regenerative Medicine, University of Pittsburgh, 100 Technology Drive, Suite 200, Pittsburgh, PA 15219, P: (412) 235-5144, F: (412) 235-5110, badylaks@upmc.edu.

remodeling [18]. It seems clear that biologic scaffold manufacturing methods play an important role in determining the host response. It seems just as clear that traditional histologic methods of evaluating the host cellular response are not necessarily predictive of the long term remodeling outcome.

The present manuscript will first review the role of xenogeneic antigens, specifically the Gal epitope and DNA, on the host response to biologic scaffold materials. The influence of manufacturing methods, in particular the effect of chemical crosslinking upon the host response to ECM scaffold devices will also be discussed followed by a review of the phenotypic response of T lymphocytes and macrophages to various ECM scaffold materials. Finally, the importance of scaffold degradation upon the host response to biologic scaffold materials composed of ECM will be reviewed.

The Gal epitope

Hyperacute rejection following organ xenotransplantation can be caused by the presence of cell membrane antigens, such as the oligosaccharide α-Gal (Galα1,3-Galβ1–4GlcNAc-R) (i.e., “Gal epitope”). This epitope is found in high density as a cell surface molecule in most species with notable exceptions of humans and Old World monkeys. The presence of the Gal epitope on the surface of the vascular endothelium is the primary cause of rejection of xenogeneic organ transplants [19–22]. The Gal epitope has also been found on cell associated glycoproteins and glycolipids [23,24], secreted glycoproteins including thyroglobulin, fibrinogen, and immunoglobulin G (IgG) [25,26], and basement membrane proteins such as laminin [27]. Humans and Old World monkeys do not normally express the Gal epitope due to two frameshift mutations in the the α1,3-galactosyl-transferase gene [28,29], and produce large amounts of anti-Gal antibodies (Ab), including IgG, IgM, and IgA [21,30–33], as a result of the constant exposure to intestinal bacteria that carry the Gal epitope. It has been estimated that up to 1% of circulating human IgG is anti-Gal [21,31].

In an effort to eliminate the Gal epitope as a barrier to xenotransplantation, transgenic herds of pigs have been produced in which this epitope has been knocked out [34,35]. However, xenotransplants of Gal knockout hearts [36,37] and kidneys [38] were rejected over periods of 6 months and 1 month, respectively, due to an immune response that included the formation of anti-non-Gal Ab specific to porcine antigens.

The presence of the Gal epitope in biologic scaffolds composed of xenogeneic ECM has been investigated for porcine bioprosthetic heart valves [39], porcine anterior cruciate ligament (ACL) and cartilage[40–42], and porcine SIS-ECM [43]. All of these materials were found to be Gal positive. Porcine heart valves showed presence of the Gal epitope even after treatment with glutaraldehyde and patients receiving such bioprosthetic valves showed a significant increase in anti-Gal IgM. It has been speculated that this host response to the bioprosthetic heart valve may contribute to degeneration and calcification that ultimately leads to failure of the graft, especially in younger patients [39].

Non-decellularized porcine grafts for repair of cartilage and the ACL have also been shown to contain the Gal epitope. Treatment of the xenogeneic tissue with α-galactosidase has been proposed to minimize potential adverse immune responses to these graft materials [40–42]. The enzymatic treatment can effectively remove the Gal epitope from both porcine cartilage and ACL tissue, and since the cells within the tissue graft are not viable, the Gal epitope is not replaced through natural turnover [44]. Galactosidase treated cartilage grafts [41] have been shown to reduce the proportion of T lymphocytes present at the site of remodeling from 70% of the total cell population to 10%, with the balance of cells being primarily macrophages. In both Rhesus monkeys and humans [40,42], galactosidase treated porcine ACL grafts have performed comparably with an allograft.

The Gal epitope was found to be present in SIS-ECM, a biomaterial consisting of porcine small intestinal submucosa [43]. It is not known whether the Gal epitope detected within SIS represents a secreted product of the cells originally present in the native material or cellular debris retained during preparation of the SIS. *In vitro* studies using immunoprecipitation showed that the most abundant anti-SIS Ab subtype that bound to SIS following exposure to human plasma was IgG_2, a finding which is consistent with the large percentage of IgG specific for the Gal epitope is IgG_2 [45,46]. However, complement activation was not observed either due to the low density of Gal epitopes, or the fact that IgG_2 is known to be a poor activator of complement [47–49].

To examine the potential role of the Gal epitope in the host immune response to SIS-ECM, samples of SIS-ECM were implanted subcutaneously in wild type (WT) mice and mice in which the α1,3 galactosyltransferase gene was knocked out (Gal $^{-/-}$ mice). The Gal $^{-/-}$ mice spontaneously produce anti-Gal Ab in a similar manner to that observed in humans [50]. The Gal $^{-/-}$ mice produced IgM anti-Gal antibodies in addition to IgG_1 SIS-specific antibodies, which did not bind to the Gal epitope. Histologically, the remodeling of the SIS-ECM material was complete by day 25 for the WT mice. In the Gal $^{-/-}$ mice, inflammatory cells were still present in the remodeling site after 25 days, but remodeling was complete by day 35. Immunization of the Gal $^{-/-}$ mice with sheep erythrocytes to enhance the anti-Gal Ab levels led to a more robust early inflammatory response following implantation, but did not alter the ultimate fate of the graft. Therefore, it appears that the presence of anti-Gal Ab delays, but does not prevent constructive remodeling of the ECM material.

DNA

Remnant porcine DNA within biologic scaffold materials after decellularization has been implicated as the cause of “inflammatory reactions” following the implantation of porcine derived scaffolds for orthopaedic applications [51]. Considering the manner in which cells are naturally embedded within their surrounding ECM, especially in relatively dense tissues like the dermis, it is unlikely that complete removal of all cells and cell products is possible even with the most rigorous processing methods. Most commercially available biologic scaffold materials contain trace amounts of remnant DNA, including Restore™, GraftJacket™, and TissueMend™ [51–53]. The remnant DNA is typically present as small fragments, reducing the possibility that these remnants play any substantive role in an adverse tissue remodeling response. In most of the biologic scaffold materials that were investigated in a recent study, the remnant DNA consisted of fragments less than 300 bp in length [53]. DNA fragments of this length are not likely to be of concern. The only ECM device that appeared to contain full DNA strands was GraftJacket™, an ECM material manufactured from human dermis. In addition to the small amount and abbreviated length of the remnant DNA, the noncrosslinked forms of ECM are subject to rapid degradation after placement *in vivo* [18,54,55]. Any remnant DNA is logically subject to the same degradation fate via enzymatic breakdown. Toll-like receptors may play an important role in this regard as they bind soluble DNA so that they can be broken down into nucleotides for future use by the cells [56,57].

Despite the universal presence of DNA remnants in commercially available ECM devices, the clinical efficacy of these devices for their intended application has been largely positive [1–14]. It therefore appears unlikely that the remaining DNA fragments contribute to any adverse host response or are a cause for concern.

It is plausible and even likely that cytoplasmic proteins and cell membrane components are retained in ECM scaffold materials through the processing steps, just as small amounts of the Gal epitope remain in these biomaterials. Although it is known that non-self cell products are capable of eliciting a host inflammatory response and/or stimulating an immune reaction, it is

possible that a threshold amount of material is required to adversely affect the remodeling response. The existing processing/decellularization methods are effective for preventing adverse events in host tissue [17], however, more thorough methods of decellularization are desirable and quality assurance steps for assuring removal of cell remnants are indicated.

Host Response to Biologic Scaffold Materials

The host response to biologic scaffold materials composed of ECM involves both the innate and acquired immune system and the response is affected by device specific variables including the intended clinical application, the source of the raw material/tissue from which the ECM is harvested, and the processing steps involved in manufacturing an approved medical device. A recent study examined the host response to five commercially available ECM devices [18], including GraftJacket™ (human dermis, proprietary cryogenic processing), Restore™ (porcine SIS, minimally processed), CuffPatch™ (porcine SIS, chemically crosslinked with carbodiimide), TissueMend™ (fetal bovine skin, proprietary processing), and Permacol™ (porcine dermis, chemically crosslinked with isocyanate). The results of the study showed profound differences in the acute and chronic host cellular response and in the downstream tissue remodeling outcomes. The intensity of the cell response and the temporal and spatial distribution of the cell response differed among the scaffold materials. GraftJacket™ and Restore™ elicited the most intense acute cell response, but this response was not necessarily predictive of an adverse remodeling outcome. Multinucleate giant cells, a cell type typically associated with a foreign-body response, was observed at the surgical site in which GraftJacket™, CuffPatch™, and Permacol™ was implanted. The cellular response to CuffPatch™ appeared to be predominantly a neutrophilic-type response throughout the entirety of the study, whereas the other devices showed a mainly mononuclear response. Conventional knowledge suggests that mononuclear cells follow neutrophils into a site of inflammation over time, phagocytose cellular debris and foreign material, and eventually exit from the site of inflammation [58,59]. The pattern of cell response and the remodeling outcome differed markedly for each of the ECM scaffold materials evaluated in this study. The GraftJacket™ device was replaced with fibrous connective tissue and a persistent low grade chronic inflammatory response. The host tissue response to Restore™ consisted of replacement of the biologic scaffold with a mixture of muscle cells and organized connective tissue, a finding consistent with an earlier report in which the SIS material was used as a body wall repair device in rat and dog models [60]. The CuffPatch™ device showed accumulation of dense collagenous tissue, a persistent foreign body response, and relatively slower remodeling compared to the Restore device™. The host response to TissueMend™ and Permacol™ was consistent with the expected response to a nonresorbable foreign material; that is, low grade chronic inflammation, minimal scaffold degradation, and fibrous encapsulation. This study, although limited in scope to the histomorphologic response, showed that biologic scaffolds composed of ECM differ markedly in the elicited host tissue remodeling response. There are both similarities and differences among ECM scaffold materials, but it is apparent that a more detailed investigation of the host immune response, the ECM constituents that affect the response, and the effect of these factors upon tissue/scaffold remodeling and outcomes is warranted for such materials.

Th1 vs. Th2 Lymphocyte Response

The role of T lymphcytes, especially the Th1 and Th2 lymphocyte phenotypes, in cell mediated immune responses to xenografts has been widely studied [61,62]. Th1 lymphocytes produce cytokines such as interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-β leading to macrophage activation, stimulation of complement fixing Ab isotypes (IgG_{2a} and IgG_{2b} in mice) and differentiation of $CD8^+$ cells to a cytotoxic phenotype [63,64]. Activation of this pathway is associated with both allogeneic and xenogeneic transplant rejection [61, 62,65]. Th2 lymphocytes produce IL-4, IL-5, IL-6, and IL-10, cytokines that do not activate

macrophages and that lead to production of non-complement fixing Ab isotypes (IgG_1 in mice). Activation of the Th2 pathway is associated with transplant acceptance [66–68].

The only ECM scaffold material for which the Th1/Th2 response has been characterized is SIS-ECM [69,70]. In one study, SIS-ECM was implanted subcutaneously into mice and the host response was compared to the response elicited by either xenogeneic or syngeneic muscle tissue. Histologically, the xenogeneic muscle tissue showed the presence of polymorphonuclear leukocytes (PMNs) within 1 day, with a transition to a mixed population of neutrophils, T lymphocytes, and multinucleate giant cells by day 10. After 28 days, the xenogeneic muscle showed evidence of necrosis, granuloma formation, and encapsulation; all of which are indicative of graft rejection. The syngeneic muscle tissue showed an acute inflammatory response, most of which disappeared by day 10. After 28 days, the residual mild chronic inflammatory response had resolved and the graft site showed organized tissue morphology, consistent with graft acceptance. The host response to the SIS-ECM was similar to that for the syngeneic muscle tissue. At Day 1, PMNs were present at the interface of the host tissue with the SIS-ECM device. After 10 days, the cellular infiltration was reduced and consisted primarily of mononuclear cells. By 28 days, the mononuclear cell infiltrate had diminished and the remodeling response was nearly complete. The mice did not develop an acquired adverse immune response to the SIS-ECM, and analysis of tissue cytokines showed that SIS-ECM strongly increased the expression of IL-4 (Th2), while the expression of IFN-γ (Th1) was 100 fold less than the response elicited by the xenogeneic muscle group. The SIS-ECM implanted mice developed an Ab response that was restricted to the IgG_1 isotype, which is most consistent with the Th2 pathway. Thus, although there was a vigorous immune response to the SIS-ECM after implantation, the response was dominated by the Th2 pathway mediators.

To confirm that the immune response to SIS-ECM was due to Th2 restriction rather than lack of sufficient antigen stimulation, Ab responses were measured in mice that received two sequential implants of SIS-ECM 28 days apart [69]. These animals showed a significant secondary antibody response, but the response was still exclusively of the IgG_1 isotype. There was no evidence of Th1 cytokines at the secondary graft site. No deposits of IgG_1 or IgG_{2a} Ab were found in the SIS-ECM graft. This double implantation study was also conducted with ECM derived from a different tissue source, specifically the porcine urinary bladder submucosa (UBS). The results were very similar confirming that the source of ECM did not alter the restricted Th2 immune response.

The SIS-ECM has been implanted in T cell KO mice and B cell KO mice [69]. In the T cell KO mice, no IL-4 expression was found, showing that T cells are the source of the IL-4 mRNA observed in SIS implanted in WT mice. Anti-SIS Ab were absent in both T cell KO mice and B cell KO mice; however, in both cases, the SIS-ECM scaffold was completely remodeled within 28 days. These results confirm that T and B cells do indeed respond to SIS-ECM, but are not required for SIS-ECM acceptance and a constructive remodeling response.

Two mouse models were used to examine the effects of SIS-ECM implantation upon systemic immunity [70]. Mice implanted with SIS-ECM expressed levels of influenza specific Ab of the IgG_1 and IgG_{2a} subtypes after vaccination with a T-dependent subunit vaccine. The vaccine response was comparable to that of mice not implanted with SIS-ECM. Furthermore, challenge of immunized and SIS-implanted mice showed the same survival rate as mice that did not receive the SIS implants. A second model of immune function examined the response to a deliberate bacterial infection following SIS implantation. The mice were immunized with a T-independent polysaccharide vaccine, produced Ab to *S. pneumoniae*, and survived a lethal dose of the bacteria with or without SIS-ECM implantation. In several other studies, ECM scaffolds with deliberate bacterial exposure have been shown to resist infection even without previous immunization [14,71–74]. The Th2 response elicited by SIS-ECM does not adversely affect

the host's ability to mount a protective systemic immune response to T-dependent or T-independent vaccines, and to overcome viral or bacterial infections.

Cell mediated immune responses were analyzed using delayed type hypersensitivity and cytotoxic T cell reactions [70]. In a mouse model of contact dermatitis, topical application of dinitrofluorobenzene led to similar levels of cellular infiltration in both SIS-ECM implanted mice and in mice not implanted with SIS-ECM. Similarly, SIS-ECM implantation did not increase or decrease the ability of mice to reject xenogeneic skin grafts. Thus, SIS-ECM implantation does not impair cell mediated immune responses to antigens.

Since similar studies have not been conducted for other forms of ECM scaffold materials, it is not possible to determine whether they would elicit the same type of host response. Considering the diversity of tissue sources and processing methods from which ECM scaffolds are produced, it seems likely that the host response to biologic scaffold prepared from different sources will vary to a large degree following implantation.

M1 vs. M2 Macrophage Response

Phenotypic and functional polarization of the mononuclear macrophage population has recently been described [75–78]. A distinct phenotypic polarization profile is described for the macrophage polarization, similar to the Th1/Th2 polarization schemes for lymphocytes described above [61,62,79,80]. The pro-inflammatory, cytotoxic macrophage phenotype, signified as M1, is characterized by cells that promote pathogen killing and cells that are associated with classic signs of inflammation, especially chronic inflammation. The anti-inflammatory macrophage phenotype, signified as M2, promotes immunoregulation, tissue repair, and constructive tissue remodeling. Although morphologically indistinguishable by routine methods of histologic examination, mononuclear macrophages from these two pathways can be identified and distinguished by their cell surface markers and by their cytokine and gene expression profiles [78,81,82]. M1 macrophages are characterized by $CD68^+$ and $CD80^+$ cell surface markers in rats (species differences exist), the production of large amounts of nitric oxide and other reactive oxygen intermediates, and copious amounts of pro-inflammatory cytokines such as IL-12 and TNFα. Conversely, M2 macrophages produce high levels of IL-10 and TGF-β expression, produce large amounts of arginase, inhibit release of proinflammatory cytokines, scavenge debris, promote angiogenesis, and recruit cells involved in constructive tissue remodeling. M2 macrophages express CD163 surface markers in rats, but again, species differences do exist.

A recent study was conducted to evaluate the macrophage polarization profile in response to native SIS-ECM (Restore™), SIS-ECM crosslinked with carbodiimide (CuffPatch™), and autologous abdominal wall muscle in a rat model of abdominal wall muscle repair [83]. The native SIS-ECM showed an intense mononuclear cell response at 1, 2, and 4 weeks that was predominantly of an M2 phenotype (i.e., $CD163^+$) at all time points. Only remnants of the device were distinguishable by histomorphologic examination after 4 weeks. After 16 weeks of remodeling, the implant site was characterized by organized collagenous connective tissue, islands of skeletal muscle tissue, and occasional $CD163^+$ positive mononuclear cells.

The cellular response to SIS-ECM device that had been chemically crosslinked included an abundant mononuclear cell presence with PMN leukocytes surrounding the device at 1 and 2 weeks. The mononuclear macrophages were characterized by an equal number of $CD163^+$ and $CD80^+$ cells at weeks 1 and 2, but by 4 weeks the polarization profile shifted to a shifted to a predominantly $CD80^+$ cell presence, consistent with an M1 phenotype. After 16 weeks, mononuclear cells and multinucleate giant cells were present within and surrounding the graft site and showed the classic histologic picture of chronic inflammation and fibrosis.

The acute cellular response to the autologous abdominal wall muscle tissue graft was characterized by a dense infiltration of both neutrophils and $CD68^+$ mononuclear cells at 1 and 2 weeks after implantation. Morphologically, necrosis of muscle fiber bundles was observed. The mononuclear cell population showed a predominantly M2 phenotype at 1 week. By 2 weeks, approximately equal numbers of $CD163^+$ and $CD80^+$ cells were present. By 4 weeks and all time points thereafter, the muscle tissue graft was largely replaced by moderately well organized collagenous connective tissue and the few macrophages still present showed the M2 phenotype. After 16 weeks, the fibrous connective tissue was poorly organized and consisted of a mixture of scar tissue and adipose tissue.

This study showed that macrophages respond differently to ECM scaffold materials depending upon the ECM source and processing methods. Chemical crosslinking of the SIS-ECM with carbodiimide resulted in a switch from an M2 dominant profile to an M1 dominant profile. An M2 phenotype profile was associated with constructive remodeling, while an M1 phenotype profile was associated with chronic inflammation. Interestingly, the autologous tissue graft showed an M2 response early followed by a duality of the M1 and M2 response, which may have been a consequence of pro-inflammatory cytokines produced as a product of cell death, or DAMP molecules released by dying cells within the autologous tissue graft. Additional work is needed to determine if macrophage phenotype can be predictive of downstream remodeling outcomes.

Degradation of the ECM Scaffold Materials

The length of time that a host is exposed to foreign antigens certainly affects the type of immune response that will be elicited, but the effect of bioscaffold degradation rate upon the immune response has not been investigated. Naturally-occurring biologic scaffold material, when not chemically crosslinked, is rapidly degraded after implantation. Approximately 60% of the mass is degraded and resorbed within 4 weeks of implantation and complete degradation typically occurs by 3 months [54,55]. The resorbed degradation products are eliminated completely from the body primarily via urinary excretion [54,55].

The effect of ECM scaffold persistence upon the host immune response is not known. Chemical crosslinking of ECM scaffolds provides increased strength and inhibition of degradation [8, 84–87], but recent studies suggest that degradation of the ECM scaffold is an essential component of a rapid constructive remodeling response. Low molecular weight peptides formed during the degradation of ECM scaffolds have been shown to have chemoattractant potential for several cell types *in vitro*, including multipotential progenitor cells [88]. *In vivo* studies have shown that bone marrow-derived cells are recruited to the site of healing, and that they participate in the long-term remodeling of the ECM [89,90]. Stated differently, degradation of an ECM scaffold may be a requisite process with bioactive consequences that contribute to the overall remodeling events. It is possible that chemoattraction by degradation products contributes to the recruitment of host cells, and ultimately to site specific tissue remodeling. The role of the immune response in these important biologic processes is almost totally unexplored.

Summary

In summary, allogeneic and xenogeneic biologic scaffolds composed of extracellular matrix are commonly used in numerous tissue engineering and regenerative medicine applications, and in many reconstructive surgical procedures. The effect of such scaffolds upon the host immune response has been largely unexplored. In addition, the association between the host immune response and tissue remodeling events is a factor that logically plays an important, if not determinative, role in the successful clinical application of these devices. There are many

variables in the manufacturing of matrix derived scaffolds and all of these variables can affect the host immune response. An improved understanding of the immune response to biologic scaffold materials can only lead to greater safety and efficiency of devices and applications that utilize such materials.

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Table 1

Source tissue and processing methods for commercially available devices produced from extracellular matrix scaffold material

Test Article	Species/Tissue of Origin	Crosslinking
Oasis™, Surgisis™ (Cook Biotech, Inc.)	Porcine small intestinal submucosa	n/a
Restore™ (DePuy Orthopaedics)	Porcine small intestinal submucosa	n/a
CuffPatch™ (Organogenesis, Inc)	Porcine small intestinal submucosa	Carbodiimide
Acell Vet (Acell, Inc.)	Porcine urinary bladder basement membrane and mucosa	n/a
Alloderm™ (Lifecell, Corp.)	Human dermis	n/a
GraftJacket™ (Wright Medical Techology)	Human dermis	n/a
Zimmer Collagen Repair Patch™ (Zimmer, Inc.)	Porcine dermis	Isocyanate
TissueMend® (Stryker)	Bovine dermis	Proprietary
Mosaic®, Freestyle® (Medtronic, Inc.)	Porcine heart valve	Glutaraldehyde
Prima™ (Edwards Lifesciences)	Porcine heart valve	Glutaraldehyde
OrthAdapt™ (Pegasus, Inc.)	Equine Pericardium	Proprietary

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The extracellular matrix as a scaffold for tissue reconstruction

Stephen F. Badylak

The extracellular matrix (ECM) consists of a complex mixture of structural and functional proteins and serves an important role in tissue and organ morphogenesis, maintenance of cell and tissue structure and function, and in the host response to injury. Xenogeneic and allogeneic ECM has been used as a bioscaffold for the reconstruction of many different tissue types in both pre-clinical and human clinical studies. Common features of ECM-associated tissue remodeling include extensive angiogenesis, recruitment of circulating progenitor cells, rapid scaffold degradation and constructive remodeling of damaged or missing tissues. The ECM-induced remodeling response is a distinctly different phenomenon from that of scar tissue formation.

Key words: extracellular matrix / small intestinal submucosa (SIS) / bioscaffolds / tissue engineering / urinary bladder submucosa (UBS)

Introduction

The extracellular matrix (ECM) is a complex mixture of structural and functional proteins, glycoproteins, and proteoglycans arranged in a unique, tissue specific three-dimensional ultrastructure. These proteins serve many functions including the provision of structural support and tensile strength, attachment sites for cell surface receptors, and as a reservoir for signaling factors that modulate such diverse host processes as angiogenesis and vasculogenesis, cell migration, cell proliferation and orientation, inflammation, immune responsiveness and wound healing. Stated differently, the ECM is a vital, dynamic and indispensable component of all tissues and organs and is nature's natural scaffold for tissue and organ morphogenesis, maintenance, and reconstruction following injury.

Until the mid 1960s the cell and its intracellular contents, rather than the ECM, was the focus of attention for most cell biologists, molecular biologists, developmental biologists and other life scientists. However, with the discovery that the ECM plays a role in the conversion of myoblasts to myotubes[1] and that structural proteins such as collagen and glyocosaminoglycans are important in salivary gland morphogenesis[2] it became obvious that the ECM is much more than a passive bystander in the events of tissue and organ development and in the host response to injury. The discovery of cytokines, growth factors and potent functional proteins that reside within the ECM characterized it as a virtual information highway between cells. The concept of 'dynamic reciprocity' between the ECM and intracellular cytoskeletal and nuclear elements has become widely accepted.[3–5] The translation of this phenomenon to therapeutic use of the ECM as a scaffold for tissue engineering applications has recently been attempted.

The ECM is not static. The composition and structure of the ECM are a function of location within tissues and organs, age of the host, and the physiologic requirements of the particular tissue.[6–8] Organs rich in parenchymal cells, such as the kidney, have relatively little ECM. In contrast, tissues such as tendons and ligaments with primarily structural functions have large amounts of ECM relative to their cellular component. Submucosal and dermal forms of ECM reside subjacent to structures that are rich in epithelial cells such as the mucosa of the small intestine and epidermis of the skin, respectively. These forms of ECM tend to be well vascularized, contain primarily type I collagen and site specific glycosaminoglycans, and a wide variety of growth factors including basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor (VEGF), and epidermal growth factor (EGF).

From the Department of Biomedical Engineering, Purdue University, Room 204, 1296 Potter Building, West Lafayette, IN 47907-1296, USA. E-mail: badylak@ecn.purdue.edu

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In contrast, the ECM of the basement membrane that resides immediately beneath epithelial cells such as the urothelial cells of the urinary bladder, the endothelial cells of blood vessels and the hepatocytes of the liver is comprised of distinctly different collections of proteins including laminin, collagen type IV and entactin. All ECMs share the common features of providing structural support and serving as a reservoir of growth factors and cytokines. The ECMs present these factors efficiently to resident cell surface receptors, protect the growth factors from degradation, and modulate their synthesis.[9–12] In this manner, the ECM affects local concentrations and biologic activity of growth factors and cytokines and makes the ECM an ideal scaffold for tissue repair and reconstruction.

Components of the extracellular matrix that support tissue reconstruction

Scaffolds for tissue reconstruction and replacement must have both appropriate structural and functional properties. However, the distinction between structural and functional proteins is becoming increasingly blurred. Domain peptides of proteins originally thought to have purely structural properties have been identified and found to have significant and potent modulating effects upon cell behavior. For example, the RGD peptide that promotes adhesion of numerous cell types was first identified in the fibronectin molecule;[13,14] a molecule originally described for its structural properties. Several other peptides have since been identified in 'dual function' proteins including laminin, entactin, fibrinogen, types I and VI collagen, and vitronectin, among others.[15] If one considers the ECM to be a degradable bioscaffold for implantation, both the structural and the functional components are transient due to the rapid rate of degradation of ECM scaffolds *in vivo*.[16,17] It is reasonable therefore, to consider ECM scaffolds as temporary controlled release vehicles for naturally occurring growth factors.

Collagen is the most abundant protein within the ECM. More that 20 distinct types of collagen have been identified. The primary structural collagen in mammalian tissues is type I collagen. This protein has been well characterized and is ubiquitous across the animal and plant kingdom.[18] Collagen has maintained a highly conserved amino acid sequence through the course of evolution. For this reason allogeneic and xenogeneic sources of type I collagen have been long recognized as a useful scaffold for tissue repair with low antigenic potential. Bovine type I collagen is perhaps the most widely used biologic scaffold for therapeutic applications due to its abundant source and its history of successful use.

Collagen types other than type I exist in naturally occurring ECM, albeit in much lower quantities. These alternative collagen types each provide distinct mechanical and physical properties to the ECM and contribute to the utility of the intact ECM (as opposed to isolated components of the ECM) as a scaffold for tissue repair. By way of example, type IV collagen is present within the basement membrane of all vascular structures and is an important ligand for endothelial cells. Type VII collagen is an important component of the anchoring fibrils of keratinocytes to the underlying basement membrane of the epidermis. Type VI collagen functions as a 'connector' of functional proteins and glycosaminoglycans to larger structural proteins such as type I collagen, helping to provide a gel like consistency to the ECM. Type III collagen exists within selected submucosal ECMs, such as the submucosal ECM of the urinary bladder, where less rigid structure is demanded for appropriate function. This diversity of collagens within a single scaffold material is partially responsible for the distinctive biologic activity of ECM scaffolds and is exemplary of the difficulty in recreating such a composite *in vitro*. In summary, the ECM is a rich source of numerous types of collagen and the relative concentrations and orientation of these collagens to each other provide an ideal environment for cell growth both *in vitro* and *in vivo*.

Fibronectin, one of the 'dual function' proteins mentioned earlier, represents an important component of ECM and is second only to collagen in quantity within the ECM. Fibronectin exists both in soluble and tissue isoforms and possesses many desirable properties of a tissue repair scaffold including ligands for adhesion of many cell types.[19,20] Fibronectin exists within the ECM of both submucosal structures and basement membrane structures.[21,22] The fibronectin component of the ECM scaffold derived from the porcine small intestinal submucosa (SIS) and urinary bladder submucosa (UBS) has been shown to be partially responsible for the adhesion of endothelial cells during *in vivo* constructive remodeling of this xenogeneic bioscaffold.[23] The cell friendly characteristics of this protein have made it an attractive ligand for use as a coating protein upon various synthetic scaffold materials to promote host biocompatibility.

Laminin is a complex adhesion protein found in the ECM; especially within basement membrane ECMs.[21] This trimeric cross-linked polypeptide exists

in numerous forms dependent upon the particular mixture of peptide chains (e.g. $\alpha 1$, $\beta 1$, $\gamma 1$).[24,25] The prominent role of laminin in the formation and maintenance of vascular structures is particularly noteworthy when considering the ECM as a scaffold for tissue repair.[26,27] Vascularization of scaffolds for tissue repair is one of the rate limiting steps in the field of tissue engineering and proteins such as laminin are receiving close attention as an important component of endothelial cell friendly scaffold materials.

Glycosaminoglycans (GAGs) are important components of ECM and play important roles in binding of growth factors and cytokines, water retention, and the gel properties of the ECM. The heparin binding properties of numerous cell surface receptors and of many growth factors (e.g. FGF family, VEGF) make the heparin-rich GAGs extremely desirable components of scaffolds for tissue repair. The GAG components of the SIS-ECM scaffold consist of the naturally occurring mixture of chondroitin sulfates A and B, heparin, heparan sulfate, and hyaluronic acid.[28] Hyaluronic acid has been extensively investigated as a scaffold for dermal reconstruction.

The characteristic of the intact ECM that distinguishes it from other scaffold materials is its diversity of structural proteins and associated bioactive molecules and their unique spatial distribution. Although cytokines and growth factors are present within ECM in vanishingly small quantities, they act as potent modulators of cell behavior. The list of growth factors is extensive and includes VEGF, bFGF, EGF, transforming growth factor beta (TGF-beta), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF) and platelet derived growth factor (PDGF), among others. These factors tend to exist in multiple isoforms, each with its specific biologic activity. Purified forms of growth factors and biologic peptides have been investigated in recent years as therapeutic means of encouraging blood vessel formation (VEGF), inhibiting blood vessel formation (angiostatin), stimulating deposition of granulation tissue (PDGF), and encouraging epithelialization of wounds (KGF). However, this therapeutic approach has struggled with determination of optimal dose, sustained and localized release at the desired site, and the inability to turn the factor 'on' and 'off' as needed during the course of tissue repair. An advantage of utilizing the ECM in its native state as a scaffold for tissue repair is the presence of all of the attendant growth factors (and their inhibitors) in the relative amounts that exist in nature and perhaps most importantly, in their native three-dimensional ultrastructure.

Sources of extracellular matrix and host response

ECM exists in all tissues and organs but can be harvested for use as a therapeutic scaffold from relatively few sources. The dermis of the skin, submucosa of the small intestine and urinary bladder, pericardium, basement membrane and stroma of the decellularized liver, and the decellularized Achilles tendon are all potential sources of ECM (Figure 1). The host response

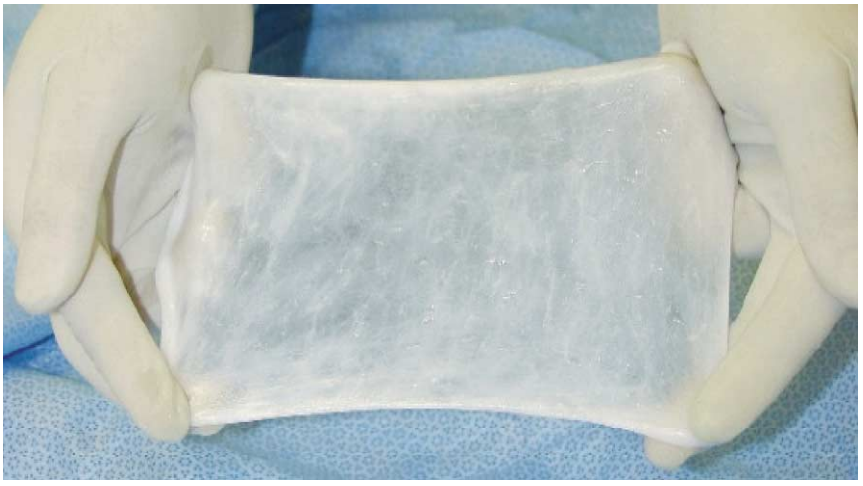

Figure 1. ECM harvested from porcine urinary bladder. This thin (60 uM) sheet of ECM is entirely free of any cellular component, has a multidirectional tensile strength of approximately 40 N, and has not been chemically cross linked or modified from its native structure.

to ECM scaffolds is largely dependent upon the methods used to process the material.

Chemical and non-chemical means of cross linking ECM proteins have been utilized extensively in an effort to modify the physical, mechanical, or immunogenic properties of naturally derived scaffolds.[29] Chemical cross-linking methods generally involve aldehyde or carbodiimide. Photochemical means of protein cross-linking have also been investigated.[30] Although these cross-linking methods can result in certain desirable mechanical or physical properties, the end result is the modification of a biologically interactive scaffold material into a relatively inert bioscaffold material. The functional tissue engineering result of this scaffold modification is typically a fibrous connective tissue response by the host to be scaffold material, complete inhibition of scaffold degradation, and inhibition of cellular infiltration into the scaffold. Although there may be clinical uses for such modified biomaterials, these properties are counter intuitive to many current approaches in the field of tissue engineering: especially those approaches in which cells are seeded upon scaffolds prior to or at the time of implantation.

In contrast, ECM scaffolds that remain essentially unchanged from native ECM elicit a host response that promotes cell infiltration and rapid scaffold degradation, deposition of host derived neomatrix, and eventually constructive tissue remodeling with a minimum of scar tissue. Therefore, the native ECM represents a fundamentally different scaffold material than ECM that has been chemically or otherwise modified.

Extracellular matrix scaffolds for tissue repair

There is abundant literature on the use of modified ECM scaffolds, especially chemically cross-linked biologic scaffolds, for tissue repair and replacement. Porcine heart valves, decellularized and cross-linked human dermis (Alloderm^{TM}), and chemically cross-linked purified bovine type I collagen (Contigen^{TM}) are examples of such products currently available for use in humans. Similarly modified ECM scaffolds have been used for the reconstitution of the cornea, skin, cartilage and bones, and nerve regeneration, among others.[30–33]

Porcine derived ECM scaffolds that have not been modified, except for the decellurization process and terminal sterilization, have been successfully used for the repair of numerous body tissues including musculotendinous structures,[34–36] lower urinary tract reconstruction,[37–39] dura mater replacement,[40,41] vascular reconstruction,[42–44] and the repair of full and partial thickness skin wounds.[45] The remodeling process in all of these applications has been remarkably similar. Immediately following implantation *in vivo*, there is an intense cellular infiltrate consisting of equal numbers of polymorphonuclear leukocytes and mononuclear cells. By 72 h post implantation, the infiltrate is almost entirely mononuclear cell in appearance with early evidence for neovascularization. Between day 3 and 14, the number of mononuclear cells increases, vascularization becomes intense, and there is a progressive degradation of the xenogeneic scaffold with associated deposition of host derived neomatrix. Following day 14, the mononuclear cell infiltrate diminishes and there is the appearance of site specific parenchymal cells that orient along lines of stress. These parenchymal cells consist of fibroblasts, smooth muscle cells, skeletal muscle cells, and epithelial cells depending upon the site in which the scaffold has been placed. It has been shown that circulating, marrow derived progenitor cells participate in this remodeling process when ECM scaffolds are used.[46] The role of environmental stressors, such as mechanical loading, have also been shown to be important in the remodeling of ECM scaffolds.[47] Of note, there is an absence of tissue necrosis and scar tissue formation during the remodeling of these xenogeneic ECM scaffolds.

Porcine derived ECM scaffolds derived from the small intestinal submucosa and the urinary bladder submucosa have been used to replace segmental defects in the esophagus of a dog model.[48] The esophagus is noteworthy for its default mechanism of scar tissue formation following injury. Remodeling of the xenogeneic ECM scaffolds showed site specific deposition and organization of skeletal muscle, intact squamous epithelial lining, and normal laminate structure of mucosa, submucosa, and muscular layers (Figures 2 and 3). Although the remodeling of this ECM scaffold did not result in perfectly normal esophageal tissue, the result was a functional structure with multiple organized tissue types. In addition, the absence of scar tissue formation suggested that the default mechanism of esophageal healing had been altered by the use of this ECM scaffold.

ECM scaffolds derived from the urinary bladder submucosa (UBS) have been used for reconstruction of the lower urinary tract with similar constructive remodeling results.[49–61] The UBS scaffolds have been either allogeneic or xenogeneic in origin and have been used both alone or with cultured autologous cells. Sections of urethra, ureter, and urinary bladder

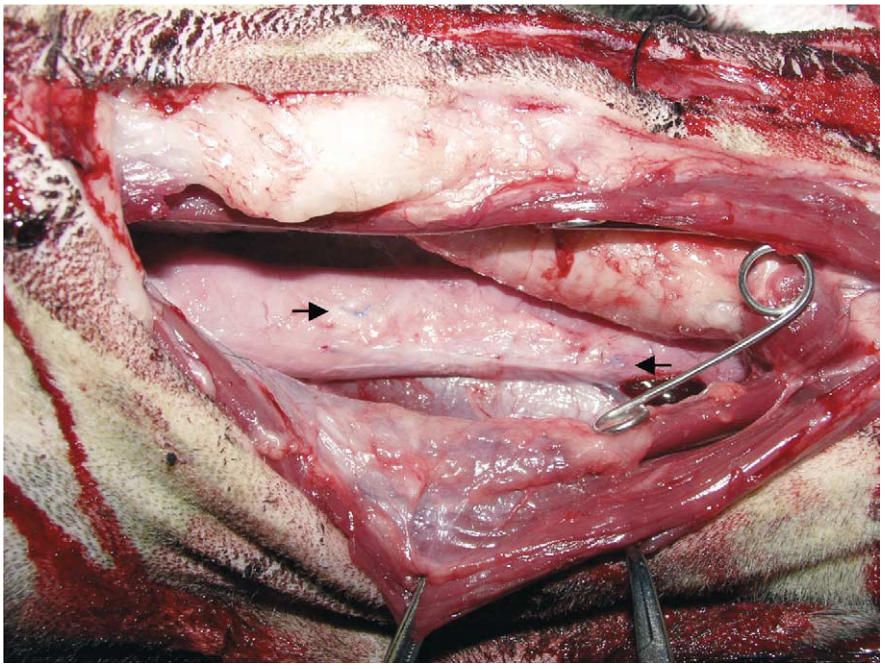

Figure 2. Five centimeter long section of cervical esophagus in a dog that represents the site of placement of a xenogeneic ECM scaffold that has now been remodeled *in vivo*. The scaffold was derived from the porcine urinary bladder. The scaffold has been replaced in 2 months by relatively normal appearing esophageal tissue without stricture, scarring or adhesions to surrounding tissues. The arrows identify sutures that represent the original anastomosis of ECM scaffold to native esophagus.

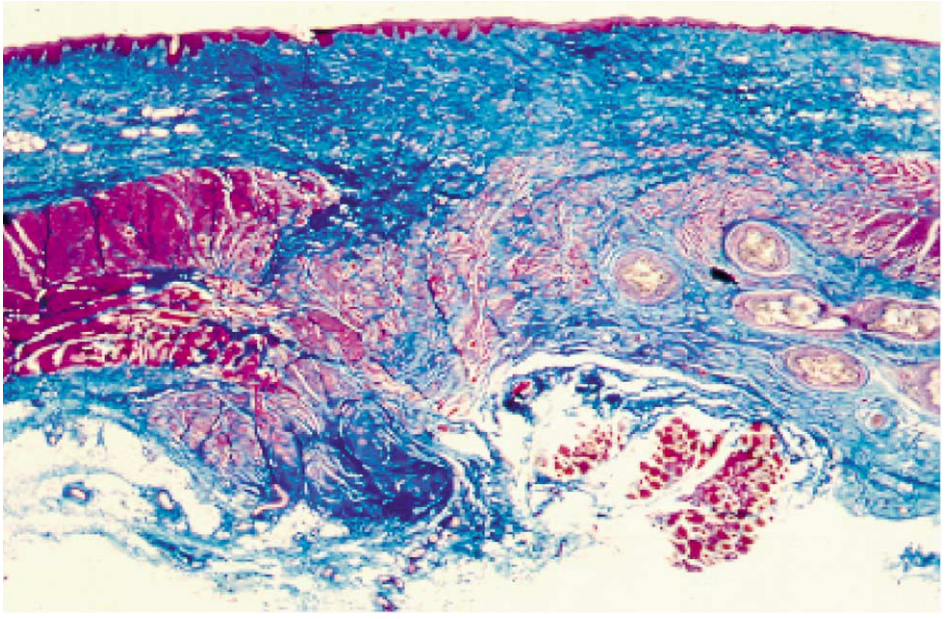

Figure 3. Photomicrograph of tissue shown in Figure 2. There is an intact but not entirely normal appearing squamous epithelium, a lack of normal complement of submucosal glands, partially organized bundles of skeletal muscle and tissue organization that resembles the normal laminar arrangement of tissue types found in the esophagus. Of note, there is a lack of inflammatory cells or scar tissue, and there is no histologic evidence of the originally implanted scaffold.

have shown excellent reconstitution with formation or organized and innervated smooth muscle. There is a substantial body of literature developing that supports the use of intact ECM as a scaffold for tissue repair. More than 100,000 human patients have now been implanted with xenogeneic ECM scaffold derived from the porcine small intestinal submucosa for a variety of applications; scaffolds are necessary components for tissue repair and reconstitution.

Conclusions

The ECM represents nature's scaffold for tissue development and tissue repair. The optimal methods for using this scaffold for clinically relevant tissue engineering applications have yet to be determined. Many questions remain to be answered including the optimal source of ECM scaffolds for clinical use, the immunologic response to allogeneic and xenogeneic scaffolds, and the optimal methods for engineering ECM scaffolds with the appropriate mechanical and physical properties. It appears that there is a fundamentally different host response to naturally occurring ECM scaffolds vs. conventional scaffold materials and that ECM has the potential to change the default scar tissue response to injury in adult mammals.

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Whole Organ Decellularization - A Tool for Bioscaffold Fabrication and Organ Bioengineering

Pedro M. Baptista, Giuseppe Orlando, Sayed-Hadi Mirmalek-Sani, Mohummad Siddiqui, Anthony Atala and Shay Soker*

Abstract— **The use of synthetic and naturally-derived scaffolds for bioengineering of solid organs has been limited due to a lack of an integrated vascular network. Here, we describe fabrication of a bioscaffold system with intact vascular tree. Animal-donor organs and tissues, ranging in size up-to thirty centimeters, were perfused with decellularization solution to selectively remove the cellular component of the tissue and leave an intact extracellular matrix and vascular network. The vascular tree demonstrated sequential fluid flow through a central inlet vessel that branched into an extensive capillary bed and coalesced back into a single outlet vessel. In one example, the liver, we used central inlet vessels to perfuse human and animal liver cells through the bioscaffold to create a functional liver tissue construct *in vitro*. These results demonstrate a novel yet simple and scalable method to obtain whole organ vascularized bioscaffolds with potential for liver, kidney, pancreas, intestine and other organs' bioengineering. These bioscaffolds can further provide a tool to study cells in their natural three-dimensional environment, which is superior for drug discovery platform compared with cells cultured in two-dimensional petri dishes.**

I. Introduction

Solid organ transplantation is a victim of its own success. As results have dramatically improved, the demand for transplantable grafts has increased but the offer has not kept pace [1]. Therefore, the gap between the number of patients who have received a transplant and those who are in the waiting list has become wider than ever; also, the mortality while on the waiting list is increasing. Over the years, alternative sources of organs were investigated, including: xenotransplantation and tissue engineering [TE]. Xenotransplantation is the transplantation of living cells, tissues or organs from one species to another; thus far, organ transplant from animals to humans has been impossible because of the overwhelming rejection and the risk of transmitting animal viral diseases to humans.

TE uses a combination of cells, biomaterials and suitable biochemical and physio-chemical factors. The goals for TE are to replace damaged and non-functioning tissues or organs with constructs obtained through the seeding of functional cells within a structure capable of: 1) supporting the three-dimensional [3D] tissue formation; and 2) mimicking the function of the natural extracellular matrix [ECM]. Such structure is referred to as scaffold. However, the main roadblock towards the production of viable constructs is the inadequacy of the current technology to reproduce: i) those signals through which cells interact with one another and with the ECM, and ii) the vasculature within the scaffold, which is essential for oxygen and nutrient supply to cells.

The ECM provides structural support to cells, segregates tissues from one another, and regulates intercellular communication and cell's dynamic behavior. Importantly, the ECM contains several bioactive molecules which, in their unique spatial distribution, provide a reservoir of biologic signals that are difficult to artificially replicate. Notably, although these biomolecules are present within ECM in very small quantities, they act as potent modulators of cell behavior.

We employed tissue decellularization to obtain collagen-rich bio-scaffolds for tissue engineering of urological tissues such as the bladder and urethra [2, 3]. However, these were relatively "thin" tissues. In order to decellularize bulky tissues such as pig liver the tissue was sectioned into thin slices which could be completely decellularized to obtain a 2D ECM preparation [4] The biomatrix was seeded with hepatocytes that preserved their function for several weeks. This data provided the evidence that liver biomatrix may be a superior alternative to existing scaffolds for tissue engineering for the following reasons: the liver ECM is bioresorbable, it can be easily prepared, and it supports long-term hepatocellular functions in vitro. These initial studies were recently followed by researchers from the University of Minnesota that have successfully produced a beating heart through seeding neonatal cardiac cells within the scaffold of a rat heart [5].

In the current study, we applied the principles of whole organ decellularization, while preserving the ECM and the vascular network, as an approach to potentially bioengineer organs such as liver, pancreas, kidney and intestine for transplantation and drug discovery.

II. METHODS AND RESULTS

A. Whole Organ Decellularization

I In order to decellularize large volume tissues without destructing the tissue by sectioning it to thin slices, we used the native vascular system to perfuse a decellularization solution (made of 1% of detergent Triton X-100 and 0.1% ammonium hydroxide in deionized water) throughout the

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PMB, GO, HSMS, MS, AA and SS are with Wake Forest Institute for Regenerative Medicine, Wake Forest University Health Sciences, Winston-Salem, NC, USA. (*corresponding author phone: 336-713-7295; fax: 336-713-7290; e-mail: ssoker@wfubmc.edu).

organs by cannulating their largest vessels for vascular access. Perfusion of the decellularization solution is preceded and followed with perfusion of deionized water, a process that takes approximately 2 days. Using this method, we were able to successfully decellularize livers, kidneys, pancreas and intestines of various sizes and animal species. These include an eight centimeter ferret liver (Fig. 1B), a twelve centimeter pig kidney (Fig. 1F), a twenty-three centimeters pig pancreas (Fig. 1J) and a twenty-five centimeters pig small intestine (Fig. 1N). This method produced completely decellularized tissues that demonstrated a preserved vascular network, which we term as an acellular vascularized bioscaffold (AVB). In the particular case of the liver, the vascular tree could be directly visualized due to the transparent parenchymal space (Fig. 1B). The use of a mild detergent Triton X-100 and ammonium hydroxide enables the quick and consistent removal of all the cellular components of the tissue, leaving behind mostly intact the ECM elements, whilst maintaining increased protein complexes in comparison to alternative detergents including SDS [sodium dodecyl (lauryl) sulfate]. Complete decellularization of AVB and the preservation of the vascular network were confirmed with several methods. H&E staining of paraffin sections of the numerous organs decellularized (Fig. 1C, G, K, O) showed the expected pink eosinophilic staining for collagen with no basophilic staining of cellular material. Further analysis of these decellularized bioscaffolds using antibodies for several types of collagen, laminin and fibronectin showed preservation of the distinctive matrix chemistry of each organ and maintenance of their spatial locations (data not shown). To confirm the integrity of the vascular network we tested if fluid injected into the vasculature can flow through the vasculature and not extravasate throughout the organ. An x-ray fluoroscopic study with radio-opaque dye demonstrated that the injected dye was flowing as expected from an intact vascular network and slowly moved from larger vessels to smaller capillaries (Fig. 1D, 1H, 1L and 1P). Approximately 5 minutes after perfusion started, the whole organs became radio-opaque, suggesting some leakage of the dye from the vascular channels into the matrix. Nonetheless, this series of experiments demonstrated that the AVB prepared from different organs maintained patency of their original vascular network. Such intact network can be used to deliver cells into the bioscaffold and subsequently to perfuse nutrients to the bioengineered organ.

B. Bioscaffold Recellularization

Besides providing vascular channels, the bioscaffolds can also provide adequate environment for cell growth. The liver is composed of two major cell types; hepatocytes and endothelial cells. To investigate if endothelial cell seeding was possible, GFP-labeled mouse endothelial cells were infused through the portal vein and the liver AVB was perfused with endothelial cell media for 3 days. The seeded AVB was visualized under fluorescent microscope, showing a single line of fluorescent endothelial cells lining the vascular channels (Fig 2A). No labeled endothelial cells were observed outside the channels. To test if we can also recellularize the portal vein vascular tract, we seeded the AVB with the same fluorescently-labeled endothelial cells (green) through the portal vein and inspected the AVB under fluorescent microscope. The peri-portal space was filled with endothelial cells (Fig 2B), showing a characteristic hexagonal arrangement of the vascular structures of the peri-portal spaces of the lobules. A picture of one of the lobes showed homogenous distribution of endothelial cells throughout the whole liver AVB (Fig. 2C).

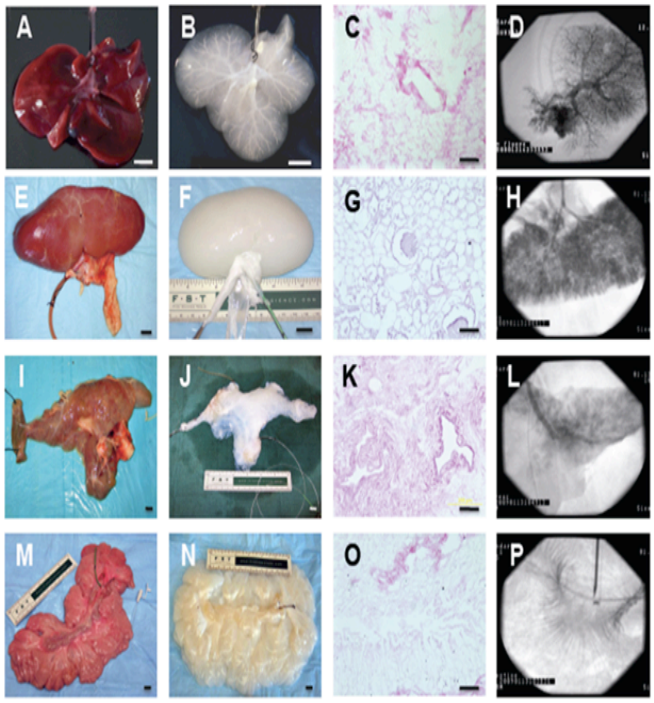

Fig. 1. Organ Decellularization. Organs were obtained en-bloc including arterial and venous structures. Vessels were cannulated and attached to a peristaltic pump, followed by overnight perfusion of ddH2O. Organs were then decellularized with 1% Triton-X / 0.1% Ammonium hydroxide in ddH2O solution at 10–60 ml per hour for 24 hrs or until translucent, and perfused with ddH2O prior to sterilization (gamma irradiation) and cell-seeding studies or processed for histological analysis. (A, B; E,F; I,J; M,N) Macroscopic view of fresh ferret liver, pig kidney, pancreas and intestine, before and after decellularization, respectively. The removal of the cellular components is observable with the transparency/ white color of the decellularized bioscaffolds. (C, G, K, O) H&E staining of histological sections of the decellularized liver, kidney, pancreas and intestine, respectively. No nuclear cellular material is observable and only pink eosinophilic staining expected from proteinous extracellular matrix is apparent. (D, H, L, P) Fluoroscopic analysis of the vascular network of the decellularized liver, kidney, pancreas and intestine, respectively. Contrast agent flows through the decellularized organ scaffolds demonstrating progressive flow from large vessels branching into medium-sized arterioles and continues to ultimately fill the fine vasculature of each organ. Scale bars are 1cm in Fig. 1A-B, E-F, I-J and M-N and 100um in Fig. 1C, G, K and O.

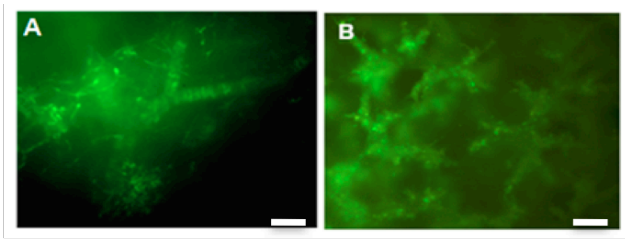

Fig. 2. Re-endothelialization of the ferret liver bioscaffold. (A) GFP-labeled EC were seeded via *vena cava* in the liver bioscaffold, demonstrating endothelial cells that are lining the bioscaffold's vascular channels. (B) GFP-labeled EC perfused via the portal vein distribute predominantly in the hexagonal shape peri-portal areas. Scale bars 100um.

Taken together, these results suggest that the perfusion method used to deliver the decellularization solution can be used to deliver cells for seeding the lumen of the vascular channels and the parenchyma of the liver lobules, by using the portal vein and the vena cava, respectively. It also confirms that the vascular channels are intact and that mostly no endothelial cells can be observed outside the vascular channels.

We further performed a series of co-seeding experiments of endothelial cells and human hepatocellular carcinoma, HepG2, cells. 30 million HepG2 and 30 million endothelial MS1 cells were seeded through portal vein of the bioscaffold by perfusion with culture medium. Culture medium (DMEM w/ 10% FBS) was then continuously perfused for 1 week at 6 ml/min. One week after seeding, high density of cells can be observed throughout the AVB with visible tissue formation (Fig. 3A). Immunohistochemical analysis showed extensive and intense albumin expression (Fig. 3B) and a large number of proliferating cells in the core of the bioscaffold, as evident by Ki67 immunostaining (Fig 3D). Von Willebrand Factor staining showed a pattern typical of a cross section through capillaries (Fig. 3C). Together, these experiments showed the potential of bioscaffold recellularization using cell perfusion as an efficient approach for the bioengineering of whole organs.

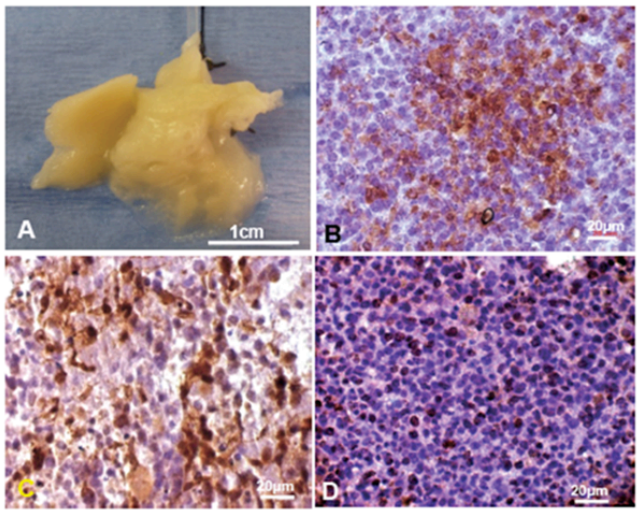

Fig. 3. Re-cellularization of the ferret liver bioscaffold. (A) Macroscopic appearance of a right lobe of a ferret liver bioscaffold seeded with human hepatocyte progenitors (HepG2) and endothelial cells. (B) Immunostaining for albumin expression of HepG2 cells engrafted in the bioscaffold. (C) Von Willebrand Factor expression by seeded endothelial cells with a pattern similar to capillary networks. (D) Anti-Ki67 immunostaining shows a large number of proliferating cells within the bioscaffold.

III. Discussion

The current study describes an effective method to fabricate organ bioscaffolds with a complete vascular system. Our data showed that the microarchitecture and the vascular network of different organs processed in this method were maintained intact and supports cell growth in vitro. This approach, which produces intact whole organ bioscaffolds with the preservation of the native microarchitecture, can provide a material with unique properties for whole organ bioengineering. The three dimensional vascularized structure can support the growth and viability of different cell types, facilitating a new level of complexity for cellular interaction, organization and perfusion; an unmet need in regenerative medicine.

Decellularization of tissues has traditionally been performed by agitating the tissue of interest in a container and allowing the cells to decellularize in bulk from an outside in approach [6]. Such an approach has been effective in completely decellularizing tissues only up to five millimeters in thickness [7]. Thicker tissues tend to decellularize well at the surface, but the core remains cellular. The reason is because the decellularized matrix at the surface forms a resistant layer preventing efficient access of the detergent to the deeper parenchyma. The method described here is less traumatic to the tissue than the classic agitation approach and thus results in an acellular matrix that demonstrates a patent vascular tree that can be used to deliver cells and nutrients into the bioscaffold. The choice of detergent for the generation of AVB by perfusion may influence the preservation of important biological activities. Harald C Ott et al recently reported the use of this technique in heart decellularization with similar results, confirming the potential of this novel method to generate scaffolds for bioartificial organ engineering [5]. Although the use of strong ionic detergents such as SDS facilitates complete removal of cells and can yield a functional bioscaffold, it is possible that it may damage some ECM components [8]. Therefore, we opted to use a mild non-ionic detergent, Triton X-100. We found that this detergent could successfully decellularize the whole liver, kidney, lungs and small intestine by the removal of approximately 98% of cellular DNA. In fact, the ability of perfused endothelial cells and hepatocytes to localize specifically to appropriate sites within the liver bioscaffolds suggests that normal physiological cues for homing and function were successfully preserved.

ECM derived from organs such as the small intestine [9], urinary bladder [2] or skin [10] are now widely used for the reconstruction of many different tissues. Lower urinary tract reconstruction, arterial graft, or skin reconstitution are amongst the numerous clinical applications. In the specific case of the liver, decellularized liver matrix sections have been used for liver tissue engineering [4]. However, these acellular tissue ECMs do not possess a natural vascular tree that is essential to support the bioengineering of a three-dimensional and bulky bioartificial organs. These results suggest that the AVB has an important advantage over other ECM preparation methods that do not preserve an intact vascular network. It makes possible to overcome the oxygen

and diffusion limitations imposed by tissue thickness. This is also true for organs like the pancreas and kidney, where so far no record has been found in the literature of a successful method to prepare a whole organ scaffold that could successfully enable kidney or pancreas bioengineering.

Finally, the major implication of this work is the simple method of generating a biodegradable, biocompatible, vascularized organ bioscaffold with the equal amount of complexity as that seen in nature. These bioscaffolds may be used for the bioengineering of other solid organs that require a vascular tree to support a large number of cells, generating new drug discovery platforms and most needed organs for transplantation.

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