Cardiovascular Tissue Engineered Constructs for Patients With Diabetes

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Clinical translation of cardiovascular tissue engineering (CVTE) is rapidly shifting from concept to application, granting a myriad of opportunities for the treatment of cardiovascular disorder (CVD). There remains, however, a critical hurdle to overcome: the application of tissue engineering to a comprised patient – more specifically, a patient with diabetes mellitus (DM). The alarming prevalence of DM is of great concern due to its dual threat as both a risk factor for CVD and a predictor of biomedical device failure. Elevated levels of inflammation and impaired wound healing are hallmarks of DM contributing to cardiomyopathy, atherosclerosis, and valve disease. The primary focus of my research was two-fold: 1) to evaluate diabetes-related complications to scaffolds and stem cells used for cardiovascular tissue engineering; and 2) to attenuate these complications by addition of a non-toxic matrix-binding polyphenolic antioxidant, pentagalloyl glucose (PGG).

Two types of extracellular matrix (ECM) scaffolds were investigated in this study: collagen-based and elastin-based. *In vivo* biocompatibility studies revealed that the diabetic environment invoked detrimental alterations to the matrix scaffolds including crosslinking, advanced glycation end product (AGE) accumulation, and elevated inflammation. However, these complications could be mitigated by scaffold pretreatment with PGG. By virtue of its antioxidant properties, PGG halted diabetes-related stiffening, AGE accumulation, inflammation, and calcification.

The effects of seeded autologous adipose stem cells (ASCs) were also investigated *in vivo*. We observed immunomodulatory capabilities of ASCs to the
implanted constructs by reducing the pro-inflammatory response, shifting the polarization of macrophages towards constructive remodeling, and preventing inflammation-driven calcification. The combination of ASCs with PGG formed a truly diabetic-resistant construct capable of combating glycoxidation, crosslinking, destructive inflammation, and calcification.

The overall goal of this research was to establish the framework of clinical translation of tissue engineering. Tissue engineering is often heralded as a patient-tailored approach for disease treatment; however, our translational efforts are useless if we cannot address the comorbidities associated with the patient. This research takes a step towards the development of a deliverable and robust tissue engineered construct for use in treatment of cardiovascular disease.
DEDICATION

This dissertation is dedicated to my fiancée and future wife, Rachael – thank you for your relentless encouragement, support, and awesome quirkiness. You have been my greatest source of inspiration and strength, and I would not be where I am without you.

Also to my parents and brothers, thank you for your love and support throughout my entire life. You have instilled in me an unparalleled strive for tenacity and success. But more importantly, you’ve taught me how to do good in the world. I will carry these life lessons with me wherever I go in all of life’s avenues.
ACKNOWLEDGEMENTS

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I would also like to thank my committee members Dr. John Bruch, Dr. Christopher Wright, and Dr. Martine LaBerge. Under your advice, my research has been grounded in real-world applications and clinical practicality. You all have consistently reminded me that the purpose of our work is to better the world and improve patient lives. You have provided not only your support for this research project, but also invaluable contributions to my personal graduate education and professional development.

Laboratory research and functionality is only as great as the people it operates under. I have so many people to be thankful for in this respect. Thank you to my fellow graduate students, past and present, in the Biocompatibility and Tissue Regeneration Lab – Jeremy Mercuri, Lee Sierad, Jason Schulte, Mike Jaeggli, Katy Jaeggli, George Fercana, Chris DeBorde, Laura McCallum, Allison Kennamer, Tasha Topoluk, Richard Pascal, Grace Dion, Satyam Patel, Betsy Tedder, and Tom Chuang. Thank you for your friendship and your priceless input on my project. I would also like to express a special thanks to my past and current undergraduate students who contributed directly to this
research project – Harleigh Warner, Anna Lu Carter, and Irina Geiculescu. I hope I have not emotionally or scientifically scarred you too much during our research endeavors together. Your companionship and dedication had been the crux of my doctoral work. Thank you to Maria Torres, who made every single gear turn smoothly in the logistics of my graduate studies.

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CHAPTER 1: REVIEW OF LITERATURE

1.1. The Rise and Impact of Diabetes

Diabetes Mellitus (DM) is quickly gaining prominence as an epidemic disease and a major risk factor for the occurrence of cardiovascular diseases (CVD). Patients with DM tend to have a significantly greater frequency of CVD along with surgical interventions for CVD repair. In 2011, the Center for Disease Control (CDC) estimated 25.6 million people (8.3% of the US population) with diabetes. Of these, 18.8 million were diagnosed and 7.0 million were undiagnosed. An estimated 79 million people were considered to have prediabetes, a precursor to type 2 DM\(^1\). More alarming, the prevalence of DM is not just limited to the US; it is spreading worldwide to high population countries such as China, India, and parts of the Middle East. The worldwide prevalence of diabetes was estimated to be 2.8% (171 million) in 2000 and is expected to rise to 4.4% (366 million) by the year 2030. In the US, it is expected to more than double from 5.6% in 2005 to 12% by the year 2050\(^2\).

The socioeconomic impact of diabetes will have a major effect on healthcare costs and insurance policies, especially if numbers continue to rise. In 2007, the total cost of diabetes was $174 billion in both direct and indirect costs\(^3\). That same year, it was pronounced the 7\(^{th}\) leading cause of death in the US. It was noted that the risk of death among people with diabetes was twice that of people of similar age, but without diabetes. This ranking is based on the 71,382 death certificates in 2007 in which diabetes was the underlying cause of death. These numbers do no include the additional 160,022 death certificates where diabetes was listed as a contributing cause of a death\(^3\).
Diabetes is a major factor in promoting elevated frequencies of CVD. This situation is a vicious cycle that requires diabetic patients to have more surgeries for repair or replacement of major cardiovascular features such as heart valves and blood vessels. Such surgery has been documented to be more problematic in diabetic patients\textsuperscript{4}.

1.2. Clinical Focus and Characterization of Diabetes Mellitus

DM is categorized into three main classes: type 1, type 2, and gestational. Type 1 DM is an autoimmune disorder wherein pancreatic beta cells are destroyed thus resulting in lack of insulin production. Type 2 DM, formerly known as adult-onset diabetes, is the most prevalent form of DM, accounting for 90 – 95% of all diagnosed cases of DM in adults\textsuperscript{1}. Type 2 DM is characterized by insulin resistance and eventual inability for pancreatic insulin production. Gestational diabetes is characterized by glucose intolerance during pregnancy. The exact cause of gestational DM remains unclear; however, it is believed to be influenced by a combination of risk factors such as obesity and hormone imbalances\textsuperscript{5}. In each class, DM is characterized by high levels of glucose in the bloodstream caused by either lack of insulin or insulin resistance (Fig 1.1).

\textbf{Figure 1.1.} Elevated glucose molecules circulating in the bloodstream (hyperglycemia)
1.2.1. Management and Treatment

Currently there is no comprehensive cure for diabetes. The best strategy lies with a diligent and aggressive management of blood glucose level. For patients with type 1 DM, management includes an exogenous insulin regimen, a diabetic diet, and exercise. For patients with type 2 DM, management includes immediate weight reduction combined with a diabetic diet and exercise.\(^6\)

Adjuvant therapy in the form of pharmaceutical medications may also be administered if glucose management, alone, is insufficient. These drugs may be used individually or in oral combination therapy (Table 1.1)\(^7\). Oral combination therapy, however, also increases the occurrence and severity of respective side effects. Sulfonylureas and meglitinides act by stimulating beta cells to secrete more insulin. Biguanides and thiazolidinediones act by stimulating other cells (fat and muscle cells) to become more responsive to insulin. Alpha-glucosidase inhibitors act directly on the glucose itself to prevent its formation from the breakdown of starches.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Effect</th>
<th>Market Name</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonylureas</td>
<td>Stimulates beta cells to secrete more insulin</td>
<td>Diabinese, Glucotrol, Micronase, Glynase, Diabeta, Amaryl</td>
<td>Differs depending on person, dosage, and co-interaction with other drugs</td>
</tr>
<tr>
<td>Meglitinides</td>
<td>Stimulates beta cells to secrete more insulin</td>
<td>Prandin, Starlix</td>
<td>Vomiting, flushing, or sickness</td>
</tr>
<tr>
<td>Biguanides</td>
<td>Decreases glucose production in liver; sensitizes muscle cells to insulin</td>
<td>Glucophage</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>Thiazolidinediones</td>
<td>Decreases glucose production in liver; sensitizes muscle and fat to insulin</td>
<td>Avandia, ACTOS</td>
<td>Increased risk of heart failure</td>
</tr>
<tr>
<td>Alpha-glucosidase inhibitors</td>
<td>Blocks the breakdown of starches to glucose</td>
<td>Precose, Glyset</td>
<td>Diarrhea, gas</td>
</tr>
<tr>
<td>DPP-4 Inhibitors</td>
<td>Prevents breakdown of GLP-1</td>
<td>Januvia, Onglyza</td>
<td>Some negative effects on cholesterol levels</td>
</tr>
</tbody>
</table>

**Table 1.1.** Approved drugs for the pharmacological treatment of diabetes mellitus
DPP-4 inhibitors prevent the breakdown of GLP-1, an incretin that reduces blood glucose levels in the body. DPP-4 inhibitors, however, are unstable and breakdown rapidly and is thus frequently used in combination with other drugs. Thiazolidinedione medications are a class of antihyperglycemic drug currently being investigated for the resolve of cardiovascular disease. Thiazolidinedione medications such as Troglitazone, rosiglitazone (Avandia), and pioglitazone (Actos) have been introduced into clinical practice. Troglitazone has been withdrawn due to its hepatotoxicity effects, while the others have yet to demonstrate adverse effects\(^8\). However until additional safety and efficacy data are investigated, the use of these medications for the treatment of hyperglycemia in diabetic patients is still not recommended.

**1.2.2. Other Current and Investigational Treatment Options**

An alternative option for treatment is organ transplantation. Diabetes heavily damages the kidneys and attributes to end-stage renal disease (ESRD)\(^9\). The success rate of a renal transplant is variable; according to the American Diabetes Association, 75% of patients live past 5 years of receiving transplantation. Though it seems like a daunting percentage, it is overwhelmingly higher than the 33% survival rate of the patients with ESRD who choose dialysis treatment methods. Though renal transplantation can be risky, it still offers significant chances of survival for ESRD patients. There are, however, limitations including organ availability and organ matching\(^10\).

Another transplant option is pancreas transplantation. Pancreas transplantation has demonstrated to improve the quality of life for diabetic patients and minimized secondary
complications due to diabetes; however, there are still disadvantages including surgical risks and the dependence on life-long immunosuppressive therapy\textsuperscript{11,12}. In the same light, islet transplantation is another option in the realm of transplants. Though islet transplantation has had less success, these successes have yielded both regulated insulin secretion and the establishment of insulin independence\textsuperscript{11}. As with pancreas transplants, islet transplantation also possesses surgical risks and immunosuppressive therapy. In order for such transplantation to achieve clinical satisfactoriness, a clear advantage over exogenous insulin therapy remains to be demonstrated.

Biomolecular manipulation, such as gene therapy, holds the potential key in treating diabetes. Riedel \textit{et al.} has suggested that glucagon-like peptide (GLP)-1 is a natural peptide that can be used to effectively combat diabetes\textsuperscript{13}. Delivery methods for this peptide using gene therapy are currently being investigated so that targeted production of GLP-1 using tissue-specific promoters can improve therapeutic efficacy\textsuperscript{13}. Furuhashi \textit{et al.}\textsuperscript{14} has investigated the treatment of diabetes and atherosclerosis by the inhibition of fatty-acid-binding protein aP2 (FABP4). This protein is expressed in adipocytes and macrophages, linking inflammatory and metabolic responses. They demonstrated \textit{in vivo} that a small-molecule inhibitor aP2 can be an effective therapeutic agent against type II diabetes and atherosclerosis.

1.3. Diabetes Biochemistry – AGEs, ROS, and RAGE

The pathogenesis of diabetes-related cardiovascular diseases is a complex and multifaceted process\textsuperscript{14-20}. Dysfunction at the cellular level induced by altered metabolites
in the body reflects the importance to understand the biochemical interactions in the hyperglycemic environment. It is believed that a combination of advanced glycation end products and oxidative stress elicit adverse cellular responses, which dictate tissue and organ disease\textsuperscript{21-25}.

1.3.1. Advanced Glycation End Products (AGEs)

Advanced glycation end products (AGEs) accumulate gradually during the aging process of a human; this accumulation is greatly accelerated in diabetes. The pathologic formation of AGEs by diabetes is believed to be responsible for inducing the pathogenesis of many cardiovascular disorders including coronary artery disease (CAD), peripheral artery disease (PAD), cardiomyopathy, and valve disease\textsuperscript{9,26-28}. Glucose binds nonenzymatically to proteins, lipids, and nucleic acids via the Maillard reaction. AGEs are characterized for their ability to form crosslinks to and between amino groups capable of being reduced\textsuperscript{18}. In AGE formation, the reaction of proteins and glucose form a Schiff base adduct. This Schiff base will then rearrange to form an

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1_2.png}
\caption{Schematic of AGE formation to result in the crosslink of two proteins\textsuperscript{18}}
\end{figure}
Amadori product, which at this point, is still reversible. Once the Amadori product completes the Maillard reaction, the AGE is irreversibly formed (Fig 1.2). Oxidation accompanies glycation and assists in the formation of permanent glycoxidation products such as (3,4)\(\text{Ne-}(\text{carboxymethyl})\text{lysine (CML)}\) and pentosidine\(^{29,30}\).

1.3.2. Reactive Oxygen Species (ROS)

Capable of undergoing dismutation to hydrogen peroxide, reactive oxygen species (ROS) are believed to be formed by two main pathways\(^{31}\). In the first pathway, ROS may be generated along the mitochondrial respiratory chain in cells rendered dysfunctional by AGE formation\(^{32}\). A recent study demonstrated that hyperglycemia-induced generation of reactive oxygen at the mitochondrial level is the initial trigger of the vicious cycle of oxidative stress in diabetes\(^{33}\). In another study by Nishikawa et al, the generation of excess pyruvate during accelerated glycolysis under hyperglycemic conditions floods the mitochondria and causes oxygen radicals in the respiratory chain\(^{34}\). The second pathway for ROS generation lies with the autoxidation of glucose molecules to generate \(\text{OH}\)-radicals. In hyperglycemia, there exists an enhanced metabolism of glucose through the polyol pathway, which also results in enhanced production of reactive oxygen\(^{35}\). Superoxide dismutase (SOD) and glutathione are natural antioxidants, which help combat oxidation in the body. SOD converts \(\text{O}_2^-\) to \(\text{H}_2\text{O}_2\), which is then detoxified by water to catalase or glutathione peroxidase in the mitochondria. Glutathione reductase generates glutathione that is used as a hydrogen donor by glutathione peroxidase during elimination.
of H$_2$O$_2$. In diabetes, however, SOD and glutathione peroxidase expression and activity are significantly decreased$^{36, 37}$. An aggressive assault of oxidative stress and AGE formation combined with an impaired endogenous antioxidant defense system can lead to devastating CVDs (Table 1.2).

1.3.3. Receptors for Advanced Glycation End Products (RAGE)

Receptors of AGEs (RAGEs) are linked with the development of atherogenesis in diabetes. RAGE is a multiligand cell surface molecule, and its activation has been inferred to contribute to diabetic complications including atherosclerosis$^{38}$. Soro-Paavonen et al. has demonstrated that the deletion of RAGE, causing RAGE deficiency, attenuates the development of atherosclerosis in diabetic apoE$^{-/-}$ mice, a model of accelerated atherosclerosis$^{39}$. RAGE activates both pro-inflammatory responses as well as down-regulating cellular defense mechanisms. The ligation of RAGE with AGE contributes to increased intracellular oxidant stress$^{18, 38, 40}$. RAGE has the ability to sustain cellular activation; thus, RAGE is able to turn an acute pro-inflammatory response into sustained cellular dysfunction. The sustained stress to the cell introduces
**Table 1.2.** Effects of AGEs that are relevant in vascular disease and diabetes mellitus

<table>
<thead>
<tr>
<th>Effects of advanced glycation endproducts relevant in vascular disease and diabetes mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stabilisation of collagen by conferring high resistance to collagenase [48,49]</td>
</tr>
<tr>
<td>Increased vascular matrix formation and narrowing of the vessel lumen [50]</td>
</tr>
<tr>
<td>Increased basement membrane deposition [4]</td>
</tr>
<tr>
<td>Glomerular hypertrophy and glomerulosclerosis [51]</td>
</tr>
<tr>
<td>Increased mesangial matrix secretion [52,53]</td>
</tr>
<tr>
<td>Impairment of matrix-bound heparansulfateproteoglycans [54]</td>
</tr>
<tr>
<td>Increased endothelial permeability [55]</td>
</tr>
<tr>
<td>Induction of cytokines and growth factors (e.g., IL-1α, TNF, IGF-1A, PDGF) by monocytes/macrophages followed by vascular cell proliferation [56–58]</td>
</tr>
<tr>
<td>Induction of smooth muscle cell proliferation [59]</td>
</tr>
<tr>
<td>Induction of fibroblast proliferation [2]</td>
</tr>
<tr>
<td>T-cell stimulation and induction of interferon-γ synthesis [60]</td>
</tr>
<tr>
<td>Induction of RAGE, one of the known receptors for AGE [61]</td>
</tr>
<tr>
<td>Induction of binding of AGE-loaded erythrocytes to endothelial cells [62]</td>
</tr>
<tr>
<td>Loss of endothelial cell mitogenic activity due to changes in bFGF [47]</td>
</tr>
<tr>
<td>Induction of autocrine vascular VEGF synthesis [49]</td>
</tr>
<tr>
<td>Increased procoagulant activity (e.g., Tissue Factor expression) [55,61]</td>
</tr>
<tr>
<td>Impairment of anticoagulant activity (e.g., Thrombomodulin expression) [55]</td>
</tr>
<tr>
<td>Induction of adhesion molecules (e.g., VCAM-1) [63,64]</td>
</tr>
<tr>
<td>Induction of the chemotactic polypeptide JE/MCP-1 in smooth muscle cells</td>
</tr>
<tr>
<td>Induction of mononuclear cell chemotaxis, activation and transendothelial migration [65,66]</td>
</tr>
<tr>
<td>Impairment of vasoconstrictor effects by quenching NO [67]</td>
</tr>
<tr>
<td>Increased vasoconstriction by inducing endothelin-1 [68]</td>
</tr>
<tr>
<td>Induction of intracellular oxidative stress and activation of the transcription factor NF-κB [69]</td>
</tr>
<tr>
<td>Lipid peroxidation [36,41]</td>
</tr>
<tr>
<td>Reduction of intracellular antioxidant defense mechanisms (e.g., GSH, vitamin C) [70]</td>
</tr>
<tr>
<td>Increased macrophage uptake of AGE-LDL and possible atheroma formation [71,72]</td>
</tr>
<tr>
<td>Complement activation by trapping non-AGE molecules [2]</td>
</tr>
<tr>
<td>Enhancement of the DNA mutation rate [73]</td>
</tr>
</tbody>
</table>

the formation of ROS to induce further inflammation\(^{38,41}\). For this reason, a pro-inflammatory environment such as that found in DM can devastatingly trigger a cascade of chronic inflammation and wound healing. Oxidative stress results in the alteration of the pro-oxidant and anti-oxidant balance. This perturbation of balance has been implicated atherosclerosis in both human and animal studies\(^{42,43}\). The acceleration of atherosclerosis can also be attributed to plasma proteins such as lipoproteins being trapped by the crosslinking of collagen molecules in blood vessels. The AGE-mediated crosslinking can trap these unwanted plasma proteins\(^{44}\).
1.4. Effect of AGEs and Oxidative Stress on CVD

AGE formation was originally thought to be specific to proteins, thus modulating a specific signal pathway for macromolecules. It was also believed to tag senescent proteins for degradation and removal. However, more recent studies suggest that the interaction of AGE modified proteins with various AGE receptors activate signal transduction pathways that promote the expression of cytokines and growth factors responsible for tissue repair, cell chemotaxis, and cell proliferation. This biological response contributes to the development of vascular disease and atherosclerosis\(^\text{18}\). Occurring both intracellularly and extracellularly, the formation of AGEs promotes endothelial dysfunction, accelerated atherosclerosis, inflammation, and calcification\(^\text{19, 45}\). Diabetes is also considered a major risk factor for retinopathy, neuropathy, and nephropathy. Macrovascular disease, such as atherosclerosis, is not specific to diabetes; however, the presence of diabetes tremendously accelerates atherosclerotic lesions and inflammation\(^\text{46}\). Effects of AGEs relevant to the aforementioned diseases are summarized in Table 1.3.

Hyperglycemia impairs endothelial function by increasing ROS, attenuating NO signaling, and inducing glycoxidative stress. Though molecular and cellular mechanisms for diabetic vascular disease are not well understood, it is believed that endothelial dysfunction and inflammation are the two crucial factors governing the pathophysiology of atherothrombosis. These two factors are a link connecting mitigated
Table 1.3. Consequence of AGE formation linked with its respective biological effect in atherosclerosis, nephropathy, neuropathy, and retinopathy

<table>
<thead>
<tr>
<th>Atherosclerosis</th>
<th>Biological Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accumulation in vascular matrix</td>
<td>Narrowing and occlusion</td>
</tr>
<tr>
<td>Endothelial dysfunction</td>
<td>Vasconstriction, hypertension</td>
</tr>
<tr>
<td>LDL Glyoxidation</td>
<td>Lipid peroxidation, oxidative stress</td>
</tr>
<tr>
<td>Monocyte activation</td>
<td>Cell proliferation, cytokines, oxidative stress</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diabetic Nephropathy</th>
<th>Biological Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix expansion and basement membrane thickening</td>
<td>Glomerular hypertrophy and sclerosis</td>
</tr>
<tr>
<td>Glomerular sclerosis</td>
<td>Albuminuria</td>
</tr>
<tr>
<td>Delayed clearance of AGE peptides</td>
<td>Uremic complications</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diabetic Neuropathy</th>
<th>Biological Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accumulation in vasa nervorum</td>
<td>Wall thickening and occlusion</td>
</tr>
<tr>
<td>Endothelial dysfunction</td>
<td>Occlusion and ischemia</td>
</tr>
<tr>
<td>Myelin Glycation</td>
<td>Myelin damage</td>
</tr>
<tr>
<td>Growth Factor Glycation (NGF, FGF)</td>
<td>Loss of function</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diabetic Retinopathy</th>
<th>Biological Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased endothelial cell permeability</td>
<td>Vascular leakage and retinal damage</td>
</tr>
<tr>
<td>Vessel wall thickening</td>
<td>Occlusion, ischemia</td>
</tr>
<tr>
<td>Coagulation</td>
<td>Occlusion, ischemia</td>
</tr>
</tbody>
</table>

Development of heart failure and early and late stage death are higher following a myocardial infarction in diabetics than in non-diabetics\textsuperscript{27}. Dyslipidemia associated with diabetes plays a key role in the development of CAD. High levels of low-dense low density lipoprotein (LDL) and low levels of high density lipoprotein (HDL) are well-established factors for the pathogenesis of atherosclerosis. High levels of free fatty acids can cause oxidative damage of HDL and impair its function\textsuperscript{49}. In addition, altered levels of metabolic substrates and products in diabetes can perturb the function of cells that are central to atherosclerotic lesion formation\textsuperscript{50}. Endothelial dysfunction promoted by oxidative stress causes further atherogenesis and inflammation. Abnormal metabolites in diabetes may also promote lesion progression due to negative effects on monocyte or...
foam cell function as well as altered signaling pathways for vascular calcification\textsuperscript{24, 51}.

While vascular complications and diabetic cardiomyopathy are the hallmarks of diabetes, this is also considered a predictor of aortic valve calcification as well as valve stiffening and stenosis\textsuperscript{52, 53}.

Furthermore, AGE interactions with specific receptors on cell surfaces (RAGE) trigger continuous ROS formation, inflammation, and progressive vascular complications\textsuperscript{38, 22}. It has also been shown that this activation of AGE-RAGE pathway induces osteoblastic differentiation of pericytes and alter the alignment of endothelial cells, contributing to vascular calcification\textsuperscript{54} and atherosclerosis\textsuperscript{55}, respectively. AGE-RAGE interaction contributes to impaired angiogenesis and wound healing due to modified basement membrane and endothelial progenitor cell interaction\textsuperscript{56}. AGE-RAGE interaction also increases the adhesion of neutrophils, contributing to the weakening of host-defense capacity in patients with diabetes\textsuperscript{57}.

\textbf{1.4.1. Valve Calcification}

A contribution to decreased elasticity and stenosis is calcification. In cardiac valves, valvular interstitial cells (VICs) are the most prevalent cells and found in all three layers – fibrosa, spongiosa, and ventricularis – of the heart valve. These cells are known to maintain and regulate valve integrity and functionality, but there is still much to be explored in terms of cellular characterization. It has been suggested that there are five identifiable phenotypes of VICs including embryonic progenitor endothelial/mesenchymal cells, quiescent VICs (qVICs), actived VICs (aVICs),
progenitor VICs (pVICs), and osteoblastic VICs (obVICs). These VICs have the ability to convert from one form to another (Fig. 1.3)\(^{58}\). Heart valve calcification occurs when VICs express osteoblastic phenotypes leading to chondrogenesis and osteogensis. Alkaline phosphatase, osteocalcin, osteopontin, and bone sialoprotein are secreted\(^{58,59}\).

The role of diabetes in shaping valve calcification has not been extremely well analyzed until it was recently suggested that aortic valve calcification is not a passive and degenerative process, but an active and highly regulated process\(^{52}\). It has been shown that aortic valve calcification is more frequent with aging and atherosclerotic risk factors. It is also a marker of subclinical coronary artery disease\(^{52}\). Because diabetes has been known a notorious risk factor for atherosclerosis\(^{60}\), the link between diabetes and accelerated valve calcification can be established.

Fig 1.3. Calcification of aortic valve leaflets causing valvular stenosis
1.4.2. Vascular Calcification

In terms of vascular calcification, the same principles of osteoblastic phenotype guiding vascular pathology can be applied as with valve pathology. Recent evidence also suggests that arterial medial calcification in diabetes is a highly regulated and active process, very similar to that observed in patients in end-stage renal disease (ESRD). Vascular smooth muscle cells (VSMCs) express bone matrix proteins to facilitate the calcification process such as osteopontin, bone sialoprotein, alkaline phosphatase, collagen type I, and osteocalcin. It has been shown that these proteins are correlated with the degree of both vascular calcification and diabetes. In vitro studies have shown that high glucose levels induce cell proliferation and the expression of osteopontin in cultured VSMCs. Hunt et al. demonstrated that the bone formation in carotid plaques of patients correlates with diabetes. High glucose, alone, cannot be blamed for diabetic vascular complications, but rather a combination of high glucose, altered metabolic activity, increased AGE formation, hyperinsulinemia, and elevated levels of fibronectin
in the arterial media\textsuperscript{64}. It has been shown that insulin and fibronectin accompanied with high levels of glucose yields increased osteopontin secretion in rat aortic smooth muscle cells\textsuperscript{65}. Hypoxia is believed to be another factor in promoting atherosclerotic lesions. Arterial wall hypoxia has been shown to be induced by diabetes preceding the formation of atherosclerotic lesion in animal models\textsuperscript{66}. Sodhi \textit{et al.} has demonstrated that not only does hyperglycemia induce vascular smooth muscle cell calcification, but also hypoxia in diabetes stimulates osteopontin synthesis\textsuperscript{62}. Elevated levels of oxidized LDL has been suggested to play a role in vascular calcification \textit{in vitro}\textsuperscript{67}; this result may correlate to patients with diabetes who also exhibit elevated levels of oxidized LDL due to glycoxidation. It is unclear the underlying mechanism of pathogenesis for vascular complication, but it is known that such vascular calcification in patients with diabetes can be attributed to a multitude of factors including hyperglycemia, adverse cellular stimulation, hypoxia, and glycoxidation.

\textbf{1.5. Diabetes vs. Modern Intervention}

The adverse effects of diabetes are not limited to the cardiovascular system. Its effects also extend to the biomaterials and surgical procedures used to repair cardiovascular diseases. It has been reported that diabetic populations have worse outcomes of percutaneous coronary interventions (PCI) and coronary artery bypass grafting (CABG). Specifically, there are more frequent occlusions in artery bypass grafts and angioplasty\textsuperscript{16}. Though CABG is worse among diabetic populations, it remains preferable over angioplasty, particularly those with multivessel disease. Luminal
renarrowing and occlusion is a major problem after PCI\textsuperscript{113}. Diabetes has also been reported as a predictor for increased risk of long term mortality and limb loss after revascularization surgery in patients with critical limb ischemia\textsuperscript{114}. These adverse events are associated with impaired wound healing along with increased rate of infection in diabetic patients. There are also higher rates of restenosis with both bare-metal and drug-eluting stents in diabetic populations\textsuperscript{115,116}. Diabetes mellitus has also been associated with faster structural valve degeneration of implanted bioprosthetic valves leading to a high risk of early and long-term mortality\textsuperscript{117}.

The mechanism behind exactly how diabetes causes these complex biomaterial and surgical-related complications is not fully understood. Diabetes is believed to inhibit biomaterial construct integration by leading a relentless assault of inflammation, metabolite imbalance, dysfunctional cell remodeling, and oxidative stress. Furthermore the ability of diabetes to impair wound healing ensures lack of integration of the biomaterial as well as introduces other risks such as infection and mortality.

\textbf{1.6. Tissue Engineering – Paradigm and Principles}

Tissue engineering holds vast potential for the treatment of CVD; much progress is being made in the engineering of blood vessels, heart valves, and cardiac muscle\textsuperscript{68}. The ultimate goal of tissue engineering is to replace diseased tissue with a biocompatible tissue that has the ability regenerate over time, integrate with the patient, and restore physiological function. The basic tenets of tissue engineering include the creation of a suitable scaffold, repopulation of the scaffold with appropriate cells, and physiologic
preconditioning in a bioreactor. Tissue engineering and regenerative medicine therapies are considered patient-tailored because the regenerative capacity and host-tissue integration is specific to the patient. Therefore in such cases, no immunomatching or immunosuppressive medication is necessary due to the guided natural restoration of both structure and function of diseased tissue. The selection of biological scaffolds for tissue engineering applications must include considerations in biocompatibility, bioresorbability (with safe by-products), and high porosity (to allow cell growth, waste removal, and nutrient supply). It is important also to optimize scaffold porosity with scaffold mechanical strength so that the tissue may be stable enough to suit function.

By no means is it an “easy” feat to create a suitable, alternative cardiovascular component (such as a cardiac valve), much less make it conform to native biological function. For instance, the aortic valve can be considered the most stressed tissue in the body – it undergoes a lifetime of continuous cyclic loading and unloading. Synthetically engineered replacements tend to fail when subjected to demanding conditions, whereas tissue engineering holds the potential to provide a cardiac valve capable of growing, repairing, and remodeling while maintaining proper mechanical function over a lifetime. The preservation of this mechanical function post implantation is crucial to the longevity of both the construct and the patient; failure of the construct could yield life-threatening situations for the patient.

1.6.1. Tissue Engineering vs. Current Engineering

Much progress has been made in the engineering of cardiovascular components
such as blood vessels, heart valves, and myocardium. Currently, adults needing replacement of diseased tissue, such as valves, will receive either a bioprosthetic valve (xenografts, allografts, or autografts) or a mechanical valve (Fig 1.5). Both types of replacements have heralded varying levels of success and enhanced quality of life. Unfortunately, both valve types have their own respective limitations. For instance, mechanical valves require life-long anticoagulation to prevent the chances of thrombosis and distal embolism. Bioprosthetic valves are prone to calcification and structural compromise.

As previously mentioned, most bioprosthetic valves commercially available are: (1) porcine xenograft based, (2) bovine pericardial based, and (3) allograft or autograft valves. The porcine xenograft is comprised of a whole pig aortic valve treated in low-concentration gluteraldehyde. Often, this valve is configured with integrated suture rings to make anchoring and fixation more stable. The bovine pericardial valves are also based on gluteraldehyde treated tissues. Glutaraldehyde serves to reduce antigenicity, stabilize the tissue to prevent from degradation, and give it mechanical strength. The
homograft valves are preserved human valves, usually cryopreserved, which are cut and trimmed to size prior to transplantation\textsuperscript{74, 77}. It is important to note, however, that regardless of treatments and implant procedure, the bioprosthetic valves will never be 100 percent immune from disease and calcification. The same rationale can be applied to the mechanical valves. There have been great strides in polymer and materials fabrication. However, all materials will undergo some biocompatibility response and elicit adverse reactions such as thrombosis, hematoma, embolism, and wound healing\textsuperscript{71, 74, 76}. Tissue engineering options seek to remedy these adverse issues seen in conventional replacement options (Table 1.4)

Perhaps the most relevant and dire is the need for a suitable replacement of vascular tissues, particularly small-diameter\textsuperscript{78}. A myriad of synthetic (ePTFE, PET, polyurethanes, PGA and PLA) and bioprosthetic grafts (autograft, allograft, xenograft) have been developed (Fig. 1.6), but there remains an unmet clinical need of small-diameter vascular grafts\textsuperscript{79}. Such grafts must exhibit excellent mechanical properties including adequate burst-pressure strength, suture retention strength, and compliance. In addition to possessing adequate mechanical properties, these grafts must also be non-cytotoxic, yield safe degradation products, possess long-term patency, and exhibit resistance to thrombosis and calcification\textsuperscript{80-82}. While great advances have been made in large and medium caliber vascular grafts, small-diameter grafts have seen little
Table 1.4. Features of conventional valve replacement vs. tissue engineered replacement\textsuperscript{71}

<table>
<thead>
<tr>
<th>Feature to optimize</th>
<th>Conventional (Mechanical, bioprosthetic)</th>
<th>Tissue engineered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Closure of leaflets</td>
<td>Rapid and complete</td>
<td>Rapid and complete</td>
</tr>
<tr>
<td>Size of orifice area</td>
<td>Less than that of natural valves</td>
<td>Better</td>
</tr>
<tr>
<td>Mechanical properties</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>Surgical insertion</td>
<td>Easy and permanent</td>
<td>Easy and permanent</td>
</tr>
<tr>
<td>Risk of thrombosis</td>
<td>Yes, especially mechanical valves, which require anticoagulation, causing vulnerability to hemorrhage</td>
<td>No; endothelial surface to inhibit thrombogenesis</td>
</tr>
<tr>
<td>Risk of structural dysfunction</td>
<td>Degradation of synthetic materials rare with mechanical valves</td>
<td>Resistant to degradation and calcification</td>
</tr>
<tr>
<td>Risk of Infection</td>
<td>Ever present</td>
<td>Resistant to infection</td>
</tr>
<tr>
<td>Viability</td>
<td>No</td>
<td>Yes, able to repair injury, remodel, and potentially grow with patient</td>
</tr>
</tbody>
</table>

Fig 1.6. Example of synthetic vascular graft made from ePTFE (by W.L. Gore)
success. A tissue engineered small-diameter vascular graft is a viable option for the replacement of peripheral and coronary vasculature, especially when demanded by an aggressive atheroprone environment elicited by diabetes\textsuperscript{14,83}.

1.6.2. Biologic Scaffolds for Tissue Engineering

Biologic scaffolds have been proposed as a unique scaffold source due to characteristics such as biocompatibility, biodegradability with safe by-products, mechanical stability, and natural ECM architecture\textsuperscript{69}. Biologic scaffolds derived from allogeneic or xenogeneic sources have piqued much interest since they can be eliminated of cells and immunogenic material while simultaneously retaining matrix integrity, mechanical properties, and key cell signaling molecules.

ECM-based scaffolds have been popularly used in tissue engineering applications due to its natural architecture as being a biological scaffold in tissues and organs. ECM is the natural framework during tissue maintenance and reconstruction following injury. It is also part of the constructive tissue remodeling during scar tissue formation. The three-dimensional architecture of ECM is what sets it apart from traditional synthetic scaffolds. The constructive remodeling capabilities of ECM scaffolds lies with its ability to be quickly and completely degraded\textsuperscript{84}. To process the ECM scaffold, the xenogeneic tissue must be rendered acellular via decellularization process to remove all cellular epitopes and cellular debris – anything that would elicit an immunogenic response. Various detergents and enzymatic agents have been widely used in decellularization procedures. Commonly used decellularization techniques for heart valves include a non-
ionic detergent, Triton X-100, an anionic detergent, SDS (sodium dodecyl sulfate), and an enzymatic agent, trypsin\textsuperscript{85-87}. Other enzymatic agents such as deoxyribonuclease and ribonuclease have been used to fully digest away nucleic acids\textsuperscript{88}. The decellularization of vascular components such as arteries has also utilized detergents such as Triton X-100 and SDS. Another decellularization agent that has shown to eliminate both cells and collagen is sodium hydroxide. This decellularization method leaves the vascular elastin intact to achieve a mostly elastin ECM scaffold\textsuperscript{89}. It is therefore important to note that there are various decellularization methods being used – each method may be utilized to achieve a unique extracellular matrix scaffold. Decellularization techniques must be carefully performed so as to not jeopardize structural integrity of the ECM while simultaneously eliminating immunogenic rejection\textsuperscript{85}.

\textbf{1.6.3. Stem cells for Tissue Engineering}

After an appropriate scaffold has been developed, the next step is the revitalization of these scaffolds with appropriate stem cells and then the exposure of this construct to mechanical and biochemical cues to spur differentiation into specific target cells. These cells may come from several sources – embryonic, adult mesenchymal, or adult progenitor – all of which are capable of self-renewal and differentiation into a variety of cell types. The cells used for seeding the developing tissue must be non-immunogenic, easy to proliferate, easy to harvest, and able to be differentiated into tissue-specific cells to carry out specialized actions\textsuperscript{69}. Patient autologous cells provide the most reliable source of cells due to the eliminated risk of immunorejection. The drawbacks to
autologous cells, particularly in elderly patients, include limited availability, time-consuming cell expansion, high variability, and weakened proliferative capacity. These cells can come from one of five sources: (1) differentiated cells from primary tissues, (2) adult stem cells, (3) bone marrow stromal cells, (4) bone marrow derived, circulating stem cells, and (5) embryonic stem cells.\textsuperscript{69, 90}

Typically in tissue engineering, the appropriate, undifferentiated cells are seeded onto the scaffold whereupon stimulation of cell growth and differentiation takes place \textit{in vitro}. Thus once implantation \textit{in vivo} is performed, the entire construct will be able to remodel and establish itself as native, functional tissue.\textsuperscript{91} In the cardiovascular realm, one of the most important cell types is the endothelial cell. The endothelium forms a monolayer to line the entire vascular system to provide a non-thrombogenic, blood-contacting surface as well as provides a medium for the regulation of immune reactions, nutrients, and inflammation. The endothelium also regulates the growth of other cells, namely smooth muscle cells in the tunica media of vasculature.\textsuperscript{19} Vascular smooth muscle cells (VSMCs) are also a vital component of the vascular system. They are responsible for the vasoconstriction and vasodilation of vessels, synthesis of collagen, elastin, and proteoglycans. Under normal conditions VSMCs proliferate at a very slow rate with low synthetic activity,\textsuperscript{92} but are also capable of exhibiting high plasticity in phenotype as a result of environmental changes and stimuli.\textsuperscript{93} There is strong evidence that this phenotypic switching of VSMCs and alteration of characteristic function is what leads to vascular disease, such as atherosclerosis.\textsuperscript{92, 93} In heart valves, the major cell population is comprised of valvular interstitial cells (VICs). VICs are responsible for the
secretion of collagen types I and III, glycosaminoglycans (GAGs), and other matrix components. Not only do they secrete matrix products, but they also contribute matrix degrading enzymes such as matrix metalloproteinases (MMPs) and GAG-degrading enzymes for remodeling capabilities\textsuperscript{94-97}. As mentioned before, VICs display a wide range of phenotypes, all of which exhibit varying function; one particular phenotype expressed by the obVIC is believed to be responsible for valvular disease (calcification)\textsuperscript{58}. Duplication of a structure where cells and matrix work together in a symbiotic relationship is the goal of tissue engineers.

Adipose-derived stem cells (ADSCs), has until recently, been an underappreciated source of stem cells\textsuperscript{98}. The principal advantage of adipose tissue as a cell source is the ease at which it can be obtained with minimal morbidity. ADSCs have shown the potential for multilineage differentiation as well as the expression of multiple growth factors\textsuperscript{98, 99}. Adipose tissue may easily be obtained via minimally invasive liposuction procedures, and the cells obtained come in large quantities (50 times more than that of bone-marrow biopsies)\textsuperscript{99}. ADSCs have also shown the potential for differentiation into endothelial cells, cardiac muscle cells, smooth muscle cells, and VIC-like cells\textsuperscript{100}. ADSCs also show amazing potential for use in vascular tissue engineering\textsuperscript{101, 102}. Adipose tissue is easily obtained via minimally invasive techniques such as liposuction or lipectomy with negligible morbidity. Clinically relevant cell numbers obtained from lipectomy is significantly higher than that from bone marrow biopsies (approximately 50X more), suggesting the possibility for the elimination of in vitro cell expansion. ADSCs have demonstrated the potential for differentiation into smooth muscle cells,
endothelial cells, and fibroblasts especially once isolated from the stromal vascular fraction\textsuperscript{99, 101}. The phenotypic characteristics of ADSCs are similar to that of bone marrow stromal cells\textsuperscript{99}.

The use of human embryonic stem cells still remains a topic for ethical debate; however, these cells are still actively being investigated. Their multipotency is considered much more expansive and would eliminate the hassle of cell phenotype pre-selection\textsuperscript{69}. Embryonic stem cells have shown particular potential for differentiation into vascular endothelial cells\textsuperscript{103}. Much more investigation in the characterization of these cells and the extent of differentiation abilities contributing to function is still needed before their use becomes clinically significant in tissue engineering applications.

\textbf{1.6.4. Bioreactors and Conditioning}

Prior to functional implantation into either preclinical or clinical models, the stem cell-revitalized scaffold must be adequately preconditioned to ensure construct maturation and stem cell differentiation into target vascular cells. Much effort has been devoted to the development of a suitable bioreactor to induce both physiologically relevant mechanical forces such as shear stress, pulsatile flow, pressure\textsuperscript{104-106}. In addition to providing mechanical stimuli to the construct, biomechanical stimuli such as growth factors may also be added to the medium to encourage stem cell differentiation toward a specific cell lineage. The goal of the bioreactor is to replicate pertinent environmental factors as seen in the human biologic system and ease the transition of the tissue engineered construct from \textit{in vitro} to \textit{in vivo}. The combination of mechanotransduction
and growth factor signaling in the bioreactor is expected to allow stem cells to adjust and adapt to their new environmental niche and begin guided differentiation and construct remodeling.

Mass transport of selected biochemicals such as oxygen and growth factors may be provided in the bioreactor along with mechanical stimulation such as pulsatile stress, shear stress, tension, and compression to elicit an appropriate physiological cell phenotype and morphology\(^69, 107, 108\). The bioreactor is responsible for initial cell viability, proliferation, and ECM structure remodeling in revitalized tissue engineered constructs. Recent studies have shown that pulsatile stress during incubation upreglates ECM deposition\(^109\).

Bioreactors have been developed for dynamic preconditioning of various cardiovascular components such as vascular grafts\(^105\), heart valves (Fig. 1.7)\(^110\), and myocardial patches\(^111\). Early bioreactors were designed to pump culture medium to nourish the developing tissue. With the development of bioreactor technology came the dynamic conditions to simulate physiological relevant environments. Pulsatile flow is popular design input as it has been shown to promote the increase of mechanical strength\(^109\) and modulation of cellular function\(^112\). While progress is being made to guide stem cells towards appropriate differentiation, there are still some obstacles to overcome such as lack of control, sterility, and the inability to tailor to device-specific variability\(^109\). However, because mechanical forces and control of culture medium with various stimulants and growth factors are so central to cellular proliferation and phenotypic expression, the use of ever-improving bioreactors vital to tissue survival.
1.7. Cardiovascular Tissue Engineering Treatments for Diabetic Patients

While tissue engineering has the potential to cure diabetes (i.e. tissue engineered pancreas), the end goal of cardiovascular tissue engineering is to make such tissue engineering methods a viable option for diabetic patients with CVD. The promise tissue engineering holds for the treatment of cardiovascular disorders would be rendered useless if it did not take into consideration the complications associated with the comorbidities of the patient (e.g. diabetes). As it stands, very little information exists on the fate of tissue
engineered constructs in patients with diabetes. Tissue engineering has been met with some dramatic failures in the past such as the Synergraft™ decellularized porcine heart valve in pediatric patients. In diabetic patients, surgeries and organ transplantation have demonstrated to be more problematic. Thus the challenge of a viable tissue engineered device combined with the challenge of diabetic conditions poses a serious obstacle. Taking both factors into consideration, tissue engineered scaffolds and components are expected to fail comprehensively in diabetic conditions. It is, therefore, necessary to tailor such cardiovascular tissue engineered constructs to become resistant to diabetes and its associated glycoxidation, oxidative stress, and adverse cellular reactions. Several possibilities exist for making CVTE a valid consideration for diabetic patients.

1.7.1. AGE Breakers

The restriction of the Maillard reaction with the use of “AGE breakers” have been proposed as a potential means of deglycating proteins, lipids, and nucleic acids. These “AGE breakers” would be Amadoriase enzymes and other agents capable of reversing the Amadori products formed by the Maillard reaction. Examples of such AGE breakers include ALT-711 (dimethyl-3-phenacyl-thiazolium chloride) and pyridoxamine; these agents are capable of breaking dicarbonyl bonds responsible for crosslinking during glycation. There are, however, several shortcomings indicating that the concept of AGE breakers is a flawed concept. ALT-711, though able to break dicarbonyl bonds, has only been able to target 1,4-dideoxy-2,3-dioxoglucosone crosslink precursor; this target has not been found to form during the Maillard reaction. ALT-711 has also been found
unable to cleave AGE-crosslinks \textit{in vitro} and \textit{in vivo}\textsuperscript{121}. More investigation on cleaving agents similar to ALT-711 is needed, specifically with the purpose of breaking the crosslinkages associated with AGE-modified proteins caused by diabetes.

\textbf{1.7.2. Antioxidants}

The use of antioxidant treatment in diabetes also has potential in giving tissue engineered constructs a fighting chance for viability. Decreased anti-oxidant mechanisms combined with the increased generation of free radicals is believed to cause the oxidative stress associated with diabetes by increasing levels of reactive oxygen species\textsuperscript{122}. Studies have proven that stobadine (STB), a synthetic pyridoindole, is an efficient antioxidant\textsuperscript{123}. STB was shown to have a protective effect against glycoxidative damage \textit{in vitro}\textsuperscript{124} and matrix collagen crosslinking\textsuperscript{125}. The use of STB, however, would not be localized to the site of cardiovascular repair, but rather function as a non-specific antioxidant. Furthermore, its effects are not permanent and only aid in preventing or delaying long term cardiovascular diabetic complications\textsuperscript{122}. Another use of antioxidants pertains to the treatment of CVTE scaffolds to protect ECM scaffolds from protein glycoxidation. One such treatment includes the use of phenolic tannins to not only stabilize the scaffold, but also to render the scaffold resistant to harmful hyperglycemic effects. Polyphenols are strong antioxidants, inhibit proteases, and reduce inflammation and antigenicity\textsuperscript{126}. Furthermore polyphenols, particularly penta-galloyl glucose (PGG), has been shown to have a high affinity for proline-rich proteins of elastin and collagen\textsuperscript{88, 89, 127, 128} and are not cytotoxic\textsuperscript{129}. Both collagen scaffolds\textsuperscript{88} and elastin scaffolds\textsuperscript{89} have
used PGG as a stabilization agent. Much more investigation is needed in determining the efficacy of phenolic coatings of CVTE constructs as a means to achieve diabetic resistance of scaffolds.

Protective effects of antioxidant treatments for endothelial and vascular aspects have been elucidated by several literature reviews. Physiological antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) are responsible for regulating the amount of reactive oxygen species. In diabetes, however, these biological enzymes are not only overloaded with free radicals, but also their production is compromised due to cellular dysfunction and damage. Exogenous antioxidant treatment such as vitamin E, rutin, and green tea catechins have also seen limited success in the prevention of diabetes-related complications. These successes have been observed more so in the laboratory setting rather than the clinical setting. Treating the antioxidant vitamins as a single class of compounds with expected similar effects inappropriately disregards their wide range of chemical properties and pharmacodynamics. Most clinical trials focus on the use of vitamin E.

1.7.3. Polyphenols – PGG

There has been comprehensive examination of the detrimental effects of diabetes on cardiovascular collagen and elastin scaffolds, but also elucidation of a promising solution to the diabetes-related complications – the use of a matrix-binding polyphenol antioxidant, PGG. PGG is a gallotannin with five ester bonds formed between carboxylic groups of gallic acids and aliphatic hydroxyl groups of a glucose core.
Polyphenols exhibit high affinity for proline-rich proteins such as collagen and elastin\textsuperscript{127, 128}, which have open structures and form strong peptide-phenolic hydrogen bonds. These interactions make PGG strong candidates for stabilizing biologic cardiovascular scaffolds. In addition to being strong antioxidants, PGG also exhibits properties in anti-inflammation, anti-mutagenicity, anti-carcinogenicity, insulin-mimicry, and anti-infection\textsuperscript{135-139}.

\textbf{Fig. 1.8.} a Sterical configuration of \(\alpha\)-PGG and \(\beta\)-PGG. b Structure and 3D model of \(\beta\)-PGG.\textsuperscript{135}

Studies both \textit{in vivo} and \textit{in vitro} have shown that PGG exhibits multiple biological activities, which implicate a great potential for PGG in the therapy and
prevention of several major human diseases including cancer and diabetes. PGG has been shown to strongly inhibit the binding of VEGF to its receptor tyrosine kinase (DKR/Flk-1) and block VEGF-induced HUVEC proliferation and the growth of cells thus implicating anti-cancer potential via anti-angiogenesis abilities\textsuperscript{140}. Li et al reported that both that both α-PGG and β-PGG possessed insulin-mimicking activity in the absence of insulin, and that α-PGG was more potent than β-PGG. In these studies, α-PGG itself stimulated glucose uptake in 3T3-L1 adipocytes but weakened the activity of insulin if treated together\textsuperscript{136}. Tannins have also been shown to be good direct antioxidants; the protein-tannin complex can act as radical scavengers and radical sinks. In one study, free radical scavenging capability has shown efficacy more potent than that of vitamin E\textsuperscript{141}. In the same study, it was also seen to inhibit lipid peroxidation induced by hydrogen peroxide. The anti-inflammatory properties of PGG has been shown to attenuate the stimulating effects of pro-inflammatory cytokines such as TNF-α and IL-6\textsuperscript{142}. Similarly, PGG is able to inhibit iNOS activity and NO production as well as suppress the vascular inflammatory process by acting as an anti-arteriosclerosis agent\textsuperscript{143,144}. While the mechanisms underlying the anti-inflammatory effects of PGG are unclear, it is believed that the inhibition of nuclear factor-κB (NF-κB) plays an important\textsuperscript{139}.

1.8. Conclusions

Progress is being made towards finding the potential cure for diabetes. Whether
this cure lies with gene therapy, pharmaceutic therapy, or regenerative medicine still remains unknown. It is clear, however, that patients with diabetes currently have no pathology-specific treatment for cardiovascular disease. Tissue engineering in the cardiovascular realm holds immense potential for treating pathologies and disorders; however these treatment options would be rendered ineffective if they cannot address the complications associated with a compromised patient (e.g. a patient with DM). It is clear that DM is a major risk factor for cardiovascular disorders such as atherosclerosis, end stage renal disease, and calcification. The body chemistry of a diabetic patient is characterized by extreme levels glycoxidation and oxidative stress which can take a devastating toll on proteins, lipids, and nucleic acids – virtually all tissues and cells comprising the human body. Therefore, there is no reason to believe that a tissue engineered construct would not encounter the same complications and be subjected to the same diabetic scrutiny once implanted into the patient. While great strides are being made in CVTE, more investigation is needed to devise a method to combat glycoxidative conditions in order to give patients with diabetes the same opportunistic chance as patients without diabetes.

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CHAPTER 2: RESEARCH MOTIVATION, SPECIFIC AIDS, AND PROJECT SIGNIFICANCE

2.1. Introduction

Clinical translation of cardiovascular tissue engineering is rapidly shifting from concept to application, granting a myriad of opportunities for the treatment of cardiovascular disease (CVD). There remains, however, a critical hurdle to overcome: the application of tissue engineering to a compromised patient – more specifically, a patient with diabetes mellitus (DM). Diabetes poses a major risk factor for cardiovascular diseases, particularly vascular disease. High levels of glucose non-enzymatically interact with extracellular matrix (ECM) proteins, such as collagen and elastin, to form irreversible crosslinks resulting in the formation of two detriments: advanced glycation end products (AGEs) and reactive oxygen species (ROS). AGEs and ROS are believed to be responsible for the adverse fate of vascular disease and vascular stiffening. The severe cell and extracellular matrix (ECM) alterations induced by glycooxidation lead to endothelial dysfunction, inflammation, atherosclerosis, impaired wound healing, fibrosis, and calcification – all of which are counter-conducive to the integration of tissue engineered constructs.

Our long-term goal is to develop tissue engineered vascular and valvular ECM-based scaffolds, revitalized with adult stem cells, which are capable of withstanding the effects of diabetes-related glycooxidation. To achieve this goal, we will investigate the
effects of diabetes on both collagen-elastin scaffolds and stem cells used for scaffold revitalization as well as the protective effects of scaffold pre-treatment with polyphenols.

**Preliminary studies** performed in our lab have highlighted the protective effects of pentagalloyl glucose (PGG), an antioxidant polyphenol capable of preventing the formation of AGEs as well as lessen stiffening and calcification when implanted subdermally into diabetic rats\(^8\). These results have encouraged us to transition to clinically relevant scenarios, which include the introduction of autologous stem cells to the cardiovascular constructs. We choose to focus on vascular constructs for the *in vitro* 2D and bioreactor (3D) testing due to the large demand for vascular grafts and because vascular disease in diabetes is the most devastating. We believe that by seeding autologous stem cells onto these acellular vascular scaffolds, we will promote differentiation and support construct remodeling into a native tissue replacement. We hypothesize that diabetes will alter the ECM-derived scaffolds and their in vivo remodeling, but scaffold pre-treatment with PGG and seeding with autologous stem cells will mitigate diabetes-related complications.

### 2.2. Specific Aims

**Aim 1:** To identify biochemical and mechanical alterations of cardiovascular scaffolds in diabetes (Presented in Chapter 3)

**Hypothesis:** Diabetes induces chemical crosslinking and stiffening of the collagen and elastin based biological scaffolds
**Approach:** Normal and diabetic (STZ-induced) adult male Sprague Dawley rats will be used for these studies. Diabetic Collagen scaffolds and elastin scaffolds will be prepared by complete decellularization of adult porcine aortic valve leaflets and adult porcine carotid arteries, respectively, from local abattoirs. Scaffolds will be implanted subcutaneously on the backs of the rats and incubated for four weeks. At the end of this time, scaffolds will be retrieved and evaluated for stiffness and crosslinking, AGE products, host cell infiltration, and matrix remodeling.

**Innovative Features:** We will report for the first time on diabetes-related complications induced to ECM-based scaffolds used for cardiovascular tissue engineering.

**Aim 2: To mitigate diabetes-induced alterations of cardiovascular scaffolds by treatment with PGG (Presented in Chapter 3)**

**Hypothesis:** PGG protects ECM proteins from irreversible cross-link formation, by virtue of its anti-oxidative and matrix-binding properties.

**Approach:** Similar to Aim 1, normal and diabetic (STZ-induced) adult male Sprague Dawley rats will be used for these studies. Both collagen and elastin scaffolds will be obtained from the same decellularization procedures as described in Aim 1. After decellularization, scaffolds will be mildly treated with PGG. Scaffolds will be implanted subcutaneously on the backs of the rats and incubated for four weeks. At the end of this time, scaffolds will be retrieved and evaluated for stiffness and crosslinking, AGE products, host cell infiltration, and matrix remodeling.
**Innovative Features:** We will report for the first time on the use of PGG as an ECM-binding agent that renders cardiovascular scaffolds resistant from diabetes-related complications.

**Aim 3: To Evaluate the Effect of Autologous Stem Cell Seeding on Scaffold Remodeling in Diabetic Environment (Presented in Chapter 4)**

**Hypothesis:** Autologous stem cells seeded on PGG-treated scaffolds will alleviate diabetes-related inflammation and oxidation.

**Approach:** Adipose stem cells (ASCs) will be isolated from normal and diabetic rats and seeded, via injection, into non-treated and PGG-treated scaffolds comprised of collagen and elastin (see Aim 2). Seeded constructs will be placed subdermally into each rat as scaffold-supported autologous implants. Upon four weeks, constructs will be explanted and evaluated for phenotypic modulations, ECM degradation, mechanical properties, glycoxidation products, and host response such as calcification and inflammation.

**Innovative Features:** The fate of autologous stem cell-seeded scaffolds have not been investigated in *in vivo* diabetic environments. Moreover, this will be the first reported exploration of the immunomodulatory properties of ASCs in these scaffolds.
Aim 4: To assess the effect of hyperglycemic environments on adipose stem cell differentiation into target vascular cells in 2D and 3D (Presented in Chapter 5)

**Hypothesis:** The efficacy of differentiation of stem cells into target vascular cells will be altered in a diabetic environment and will require pre-differentiation in a controlled biochemical and biomechanical millieu.

**Approach:** Human ASCs, obtained and expanded in either normal or diabetic conditions, will be seeded onto all tunics of the vascular scaffold and subjected to biomechanical stresses in a vascular bioreactor in normoglycemic and hyperglycemic conditions. The effect of mechanical conditioning on modulations in cell phenotype will be examined by various markers relevant to smooth muscle cells and endothelial cells. Likewise, human ASCs will be cultured in 2D plates and subjected to growth factor-enriched media to encourage either smooth muscle cell or endothelial cell differentiation. Differentiation will be analyzed by markers relevant to endothelial cells and smooth muscle cells.

**Innovative Features:** This will be the first reported attempt to differentiate and characterize adipose stem cells in a diabetic setting in 2D and 3D.

**2.3. Significance of Proposed Project**

This project is expected to have a significant impact by addressing the patient-tailored approach to translational regenerative medicine. Tissue engineering therapies are often heralded as being specifically tailored according to each patient; however, many of these assertions neglect to address the comorbidities associated with the patient. Diabetes
is expected to not only trigger disease, but also cause cutting edge treatments in regenerative medicine to fail. The creation of a diabetes-resistant tissue engineered construct for regenerative medicine will not only revolutionize the field of disease-modeled tissue engineering, but also pave the way for a readily available and much needed autologous valve replacements and vascular grafts for the treatment of diabetic patients with cardiovascular disease.

2.4. References

CHAPTER 3: DEVELOPMENT AND CHARACTERIZATION OF DIABETIC–RESISTANT CARDIOVASCULAR SCAFFOLDS FOR TISSUE ENGINEERING

(This work has been published in Biomaterials, 2013. 34(3): p. 685-695)

3.1 Introduction

Diabetic hyperglycemia, resulting from the deficiency in insulin secretion (Type 1 diabetes) or insulin resistance (Type 2 diabetes), combined with dyslipidemia, oxidative stress, and inflammation, significantly increases the risk of atherosclerotic vascular disease\(^1\), aortic valve disease\(^2, 3\) and cardiomyopathy\(^4\). Studies have shown that, despite great advances in diagnosis and treatment of CVD, over the last several years diabetic patients have not shared the same decline in coronary artery disease-related mortality as non-diabetic patients\(^1\).

The primary cause of cardiovascular tissue damage occurring in diabetes is the formation of advanced glycation end products (AGEs), which generate irreversible cross-links on long-lived proteins, such as collagen and elastin\(^5, 6\). Glucose and lipid molecules undergo a series of oxidant-induced fragmentation, leading to the formation of short-chain reactive compounds that react with proteins and form AGEs, such as carboxy-methyl lysine (CML) and pentosidine\(^7\). Malondialdehyde (MDA) is a marker for oxidative stress and a well known by-product of lipid peroxidation\(^8\). AGEs impair wound healing and induce excessive inflammation\(^9\), fibrosis, and tissue stiffness\(^10-12\). As a result, the outcome of reparative surgery and tissue transplantation is more problematic in diabetic patients\(^13\).

Tissue engineering holds great promise to treat cardiovascular diseases\(^14, 15\).
Significant progress has been made in the field of blood vessel\textsuperscript{16-18}, heart valve\textsuperscript{19,20}, and cardiac tissue engineering\textsuperscript{21,22}. It is critical that replacements for damaged cardiovascular structures possess appropriate biomechanical properties from the outset of implantation. Therefore, there is increased interest in collagen and elastin-based biological scaffolds derived from xenogeneic or allogeneic extracellular matrices (ECM), which have optimal physical properties. Furthermore, the 3D structure of the ECM can be preserved with an optimal decellularization technique that removes cells without damaging the matrix components\textsuperscript{23-25}. Ideally, basement membrane proteins are also retained, as their presence is essential to tissue regeneration\textsuperscript{26}.

For pre-clinical evaluation, tissue engineered constructs and their remodeling are typically tested in healthy animals\textsuperscript{27-29}. However, there are great expectations that TE and regenerative medicine research will offer solutions for patients affected by the cardiovascular complications of diabetes. The complex glycooxidative environment could affect tissue remodeling since the ECM proteins, especially collagen and elastin as well as the matrix metalloproteinases (MMPs) involved in matrix remodeling, might be modified by the formation of AGEs. Matrix alterations that result in activation of inflammation, fibrosis, and impaired healing might not be conducive to the desired integration and remodeling of tissue engineered constructs. These aspects can only be assessed in diabetic animal models with very strict glycemic control\textsuperscript{30-32}.

We hypothesized that AGEs could alter the properties of matrix-derived scaffolds, such as collagen scaffolds used for heart valve tissue engineering and elastin scaffolds for blood vessel tissue engineering. This could affect the outcome of tissue engineering
products based on biological scaffolds.

3.2. Materials and Methods

3.2.1. Materials

Streptozotocin was from Sigma (S0130). The insulin preparation used for rats in this study was Humulin N U-100 NPH, Human Insulin of rDNA origin Isophane suspension from Lilly (Indianapolis, IN). Electrophoresis apparatus, chemicals, and molecular weight standards were from Bio-Rad (Hercules, CA). Bicinchoninic acid protein assay kit was from Pierce Biotech (Rockford, IL). The Vectastain Elite kit and the ABC diaminobenzidine tetrahydrochloride peroxidase substrate kit were purchased from Vector Laboratories (Burlingame, CA). We used the following antibodies: rabbit anti-collagen IV (Abcam, #ab6586), rabbit anti-laminin (Abcam, #ab11575), monoclonal anti-N-epsilon-(carboxymethyl)lysine (CML) antibody (MAB3247, R&D Systems), monoclonal anti-vimentin (V5255, Sigma), mouse anti-CD8 (GTX76218, GeneTex Inc, Irvine, CA), mouse anti-CD68 anti-macrophage/monocyte antibody, clone ED-1 (MAB1435, Millipore, Billerica, MA). Deoxyribonuclease I was from Worthington Biochemical Corporation (Lakewood, NJ). AlphaTRAK (Gen II) test strip and the AlphaTRAK Blood Glucose Monitoring System was from Abbott Laboratories, Animal Health (Abbott Park, IL). All other chemicals were of highest purity available and were obtained from Sigma Aldrich Corporation (Lakewood, NJ).

3.2.2. Heart Valve Collagen Scaffold Preparation

Collagen scaffolds were prepared following a protocol described previously with
minor modifications. Briefly, fresh porcine aortic roots were harvested from a local slaughterhouse, cleaned over ice, and placed in double-distilled water overnight at 4°C to induce hypotonic shock and cell lysis. Next, for complete cell removal, the valves were placed on an orbital shaker at room temperature and treated with 0.05M NaOH for 2 h followed by 70% ethanol for 20 min and an overnight incubation in a mixture of detergents: 0.5% sodium dodecyl sulfate, 0.5% Triton X-100, 0.5% deoxycholate, 0.2% ethylenediaminetetra-acetic acid in 50 mM TRIS, pH7.5. After rinsing five times with double-distilled water and 70% ethanol to remove detergents, valves were treated with deoxyribonuclease/ribonuclease mixture (360mU/ml for each enzyme) for 2 days at 37°C, to complete the removal of nucleic acids. After rinsing with double-distilled water, valves were sterilized in 70% ethanol overnight at room temperature. Under sterile conditions, the aortic cusps were dissected away from the aortic wall and stored in sterile ddH2O with 1% antibiotic/antimycotic (Pen-Strep) at 4°C. Each individual cusp served as a collagen scaffold. This decellularization method effectively removed cells, while preserving valve matrix components and eliminating the porcine a-Gal epitope.

3.2.3. Arterial Elastin Scaffold Preparation

Elastin scaffolds were prepared following an alkaline extraction protocol described before, with minor modifications. Briefly, fresh porcine carotid arteries (60x80 mm long, 5x6 mm diameter) obtained from Animal Technologies, Inc. (Tyler, TX) were rendered acellular by incubation in 0.1M NaOH solution at 37°C for 24 h followed by extensive rinsing with deionized water until pH dropped to neutral. Scaffolds
were then rinsed and stored in sterile PBS. This treatment removed all cells and most of the collagen, leaving vascular elastin intact. Scaffolds were also completely devoid of the a-Gal epitope (data not shown).

3.2.4. Rat Model of STZ-Induced Diabetes

Adult male Sprague-Dawley rats (n = 20, weight 300-350 g) were rendered diabetic via a single dose of sterile filtered 55 mg/kg streptozotocin solution in 0.1M citrate buffer (pH 5) by tail vein injection. Control rats (n = 20) received an equal volume of vehicle (sterile citrate buffer). Starting on day 3, levels of blood glucose were determined 3e4 times per week, using AlphaTRAK (Gen II) test strips on the AlphaTRAK Blood Glucose Monitoring System, designed specifically for animals. Diabetes was established (>400 mg glucose/dL blood), and diabetic rats were given subcutaneous injections of long-lasting insulin (2-4 U Isophane) every other day to maintain blood glucose level in a desirable range (400-600 mg glucose/dL blood) and prevent development of ketonuria and weight loss. Glucose levels, individual weights, hydration status, and food and water consumption were monitored closely and continuously graphed to ensure adequate health parameters. Animals were provided with food and water ad libitum and were cared for by the attending university veterinarian and associated staff at the Godley-Snell Research Center animal facility. The Animal Research Committee at Clemson University approved the animal protocol, and National Institute of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication #86-23 Rev. 1996) were observed throughout the experiment.
3.2.5. **Subdermal Implantation**

Four weeks after STZ administration, rats were prepped for surgery and anesthetized using 1-2% Isoflurane. A small, transverse incision was made on the back of the rats, and two subdermal pouches were created by blunt dissection. The acellular scaffolds were implanted – one whole acellular aortic cusp (collagen scaffold) in each pocket (n = 2 implants per rat), and the incision was closed with surgical staples. Acellular arteries (elastin scaffolds) were cut open longitudinally and 1 x 3 cm samples were implanted subdermally, as described above for cusps (n = 2 per rat). Diabetic rats were given 1U of insulin pre-operatively. The rats were allowed to recover, provided with food and water ad libitum, and were cared for by the attending veterinarian and associated staff at the Godley-Snell Research Center animal facility. Post-operative levels of blood glucose were determined 3-4 times per week, and diabetic rats were given insulin as described above. After four weeks, the rats were humanely euthanized by CO₂ asphyxiation and the scaffolds explanted and collected according to their respective assay application as follows: scaffolds for histological analysis were placed in Karnovksy’s Fixative (2.5% glutaraldehyde, 2% formalin, 0.1M cacodylic acid, pH 7.4) and paraffin embedded; samples designated for mechanical analysis were collected in sterile PBS with 0.02% NaN₃; and samples for protein, calcium and AGE analysis were flash frozen in liquid nitrogen and kept on dry ice until transferred to -20°C for storage.

3.2.6. **Histological Analysis**

Rehydrated paraffin sections (5 mm) were stained with Hematoxylin and Eosin
(H&E) for a general overview of morphology and to confirm cellular removal. Movat’s Pentachrome stain was used to evaluate the extracellular matrix composition and integrity after decellularization and after implantation (n = 4 per implant group).

Immunohistochemistry (IHC) was performed for detection of laminin and type IV collagen in acellular scaffolds, and the results were compared to native cusps and arteries (n = 4 per implant group). Briefly, rehydrated paraffin sections (5 mm) were exposed to 0.1% Proteinase K in 10 mM TRIS buffer, pH 7.5, at room temperature for 30s to unmask the antigens. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in 0.3% horse normal serum. Sections were treated with 0.025% Triton X-100 for 10 min and then incubated with normal blocking serum for 20 min. Primary antibodies (rabbit anti-laminin, 4 mg/mL dilution, or rabbit anti-collagen type IV, 2 mg/mL dilution) were applied for 1 h at room temperature. Negative staining controls were obtained by the omission of the primary antibody. The Vector ABC peroxidase substrate kit was then used to visualize the antibody staining, and sections were lightly counterstained with Hematoxylin, prior to mounting. Digital images were obtained at various magnifications (25 to 200) on a Zeiss Axiovert 40CFL microscope using AxioVision Release 4.6.3 digital imaging software (Carl Zeiss MicroImaging, Inc. Thornwood, NY).

3.2.7. Mechanical Testing

For collagen scaffolds, a 12 mm x 12 mm square was cut from a central region of the cusp, with one edge aligned along the circumferential direction and another edge aligned along the radial direction (n = 5). Similarly, a 12 mm x 12 mm square specimen
was cut from the arterial scaffolds, maintaining orientation of the circumferential and longitudinal axes of the artery. The biaxial testing method has been reported previously\textsuperscript{27}. Briefly, four markers were placed in the center of the specimen to track tissue deformation. A total of 8 loops of 00 polyester suture of equal length were attached to the sample via stainless steel hooks, with two loops on each side of the square specimen. Specimens were first preconditioned for 10 contiguous cycles, then loaded up to 60:60 N/m equibiaxial tension for collagen scaffolds and 20:20 N/m tension for elastin scaffolds. Tissue extensibility was characterized by $\lambda_{\text{circ}}$ and $\lambda_{\text{rad}}$, the maximum stretch ratio along the circumferential and radial directions, respectively. The biaxial testing was implemented with the samples completely immersed in PBS (pH 7.4) at physiological temperature (37°C).

3.2.8. *Differential Scanning Calorimetry*

To determine the thermal denaturation temperature ($T_d$), also known as shrinkage temperature, a well-known indicator of collagen crosslinking\textsuperscript{33}, samples ($n = 3$) were subjected to differential scanning calorimetry (DSC, model 131 Setaram Instrumentation, Caluire, France) at a heating rate of 10°C/min from 20°C to 110°C in a N$_2$ gas environment. $T_2$ was defined as the temperature at the endothermic peak.

3.2.9. *Detection of AGEs and MDA*

N-epsilon-(carboxymethyl)lysine (CML) was detected by IHC, (ABC kit, Vector Laboratories). Briefly, rehydrated paraffin sections (5 mm, $n = 4$ per group) were exposed
to 0.1% Proteinase K in 10 mM Tris buffer, pH 7.5, at room temperature for 30s to unmask the antigens. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in 0.3% horse normal serum. Sections were treated with 0.025% Triton X-100 for 10 min and then incubated with normal blocking serum for 20 min. Primary antibody (4 mg/mL mouse anti-CML) was applied for 1h at room temperature. Rat-adsorbed biotinylated anti-mouse IgG was used as a secondary antibody. Negative staining controls were obtained by the omission of the primary antibody. The ABC peroxidase substrate kit was then used to visualize the antibody staining, and sections were lightly counterstained with hematoxylin before mounting.

To detect and measure pentosidine and MDA in explanted scaffolds, tissues were weighed and their mass recorded. Samples were then incubated with collagenase type I (100 U/sample) in 50 mM HEPES buffer with 10 mM CaCl₂, pH = 7.5 at 37°C until fully digested (2-4 days). Samples were then centrifuged for 10 min at 12000 rpm at 22°C and the supernatant collected. Fluorescence of the supernatant was measured at 335/385 for pentosidine and at 390/460 for MDA⁸ and expressed as relative fluorescence units per milligram original tissue wet weight.

3.2.10. Evaluation of Infiltrated Cell Phenotype

In order to identify the cells infiltrated in the scaffolds, we used IHC (n = 4 samples per group), following the protocol described above for CML, and specific antibodies for fibroblasts (vimentin), T-lymphocytes (CD8) and macrophages (CD68).
3.2.11. **MMP and TIMP Detection**

MMPs were detected in explanted scaffolds as described before\(^{20}\). Briefly, proteins were extracted by pulverizing liquid nitrogen-frozen tissue samples and homogenizing them in RIPA extraction buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS, with protease inhibitor cocktail). Protein concentration was determined using BCA assay. For each sample, 6 mg per lane were loaded, alongside pre-stained molecular weight standards. After staining, the MMP clear bands on a dark background were evaluated by densitometry on a FluorChem SP imager and the Alpha EaseFC Software v. 4.1.0 by Alpha Innotech Corporation (Protein Simple, Santa Clara, CA) and expressed as relative density units normalized to protein content. Tissue inhibitors of MMPs (TIMP) levels were measured in the same protein extracts (n = 6 per group, all 6 pooled into one assay sample) using a Rat Cytokine Array Panel (Proteome Profiler Antibody Array Panel A, R&D Systems, Minneapolis, MN).

3.2.12. **Calcium Analysis**

Alizarin Red histology staining for calcium deposits was performed on sections of explanted scaffolds (n = 4 per group) as described previously\(^ {34,35}\). Calcium content was analyzed in tissue protein extracts (n = 4 per group, see above method for MMP analysis) using a QuantiChrom Calcium Assay Kit (BioAssay Systems, Hayward, CA).
3.2.13. Statistical Analysis

Results are expressed as means ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA). Differences between means were determined using the least significant difference (LSD) with an alpha ($\alpha$) value of 0.05.

3.3. Results

3.3.1. Collagen and Elastin Scaffold Characterization

Initial studies focused on characterization of the two scaffolds before pursuing implantation studies. Porcine aortic cusps and carotid arteries were chemically treated in order to remove all cells, but preserve the ECM components (methods of decellularization have been published by us previously)$^{24, 25}$. As seen in Fig. 3.1.1, scaffolds showed complete elimination of cells (H&E staining); DNA analysis (agarose gel electrophoresis followed by densitometry, PicoGreen quantitative DNA assay, and Agilent Bioanalyzer lab-on-a-chip DNA kit) revealed complete DNA reduction after decellularization treatment in both tissues (results not shown). The scaffold exhibited typical “pores” (i.e. areas devoid of content where the original cells used to be), while maintaining most of the original matrix proteins, as confirmed by Movat’s Pentachrome staining. In the cusp scaffolds, collagen predominated and was well preserved. Intact elastin fibers in the ventricularis layer were also visible. In the spongiosa layer, we noticed the loss of glycosaminoglycans. In the arterial scaffolds, elastin sheets were well preserved in all arterial tunics, while collagen was less visible (Fig. 3.1). IHC staining for
Fig. 3.1.1. Macroscopic and histological images of fresh and decellularized (decell) porcine aortic valve cusps (collagen scaffolds) and carotid arteries (elastin scaffolds) used in this study. For histological analysis, tissues were stained with Hematoxylin and Eosin (H&E, dark purple = nuclei, pink = background substance) and Movat’s Pentachrome (yellow = collagen, blue = glycosaminoglycans, dark purple = elastin, bright red = nuclei). Tissues and scaffolds were also stained by immunohistochemistry for laminin and collagen type IV (brown = positive). Cusp layers: V = ventricularis, S = spongiosa, F = fibrosa. Arterial layers: I = intima, M = media, A = adventitia.

Fig. 3.1.2. Graphs depicting average blood glucose level (left) and average weight (right) of both control and diabetic rat models over course of study
basal lamina components revealed good preservation of collagen type IV in both scaffolds, while laminin showed only moderate retention in the cusp scaffolds.

### 3.3.2. Diabetes-related Scaffold Stiffening and Crosslinking

Biaxial tensile test analysis showed that collagen and elastin scaffolds implanted in diabetic rats exhibited markedly increased stiffness in both radial and circumferential directions compared to scaffolds implanted into control (non-diabetic) rats (Fig. 3.2). Remarkably, PGG-treatment appeared to halt the pathological stiffening effect observed in scaffolds implanted into diabetic rats. Thus, there were no statistical differences in mechanical properties in either the radial or circumferential directions of PGG-treated acellular cusps and arteries implanted in diabetic rats as compared to their controls. Differential scanning calorimetry (DSC) showed significantly higher thermal denaturation temperatures ($T_d$) of scaffolds explanted from diabetic environments (Fig. 3.2), strongly suggesting that diabetes induced stiffening and crosslink formation in collagen and elastin scaffolds.

### 3.3.3. Glycoxidation and Lipid Peroxidation

Having demonstrated that diabetes induced matrix protein crosslinking, we sought to further define diabetes-related changes in scaffolds implanted subdermally in diabetic rats. The presence of AGEs, specifically CML and pentosidine, and lipid peroxidation products such as MDA were demonstrated in implanted scaffolds (Fig 3.3). Baseline levels of CML were detected by IHC in all scaffolds, regardless of glycemic conditions.
Fig. 3.2. Mechanical properties and matrix cross-linking in explanted scaffolds. Biaxial stress strain analysis showing tension vs. stretch plots, and differential scanning calorimetry (DSC) showing thermal denaturation temperatures ($T_d$) of collagen scaffolds (top panel) and elastin scaffolds (bottom panel) after subdermal implantation in control rats and in diabetic rats. *Indicates statistical significance.
Remarkably, CML antigen was highly expressed in non-PGG-treated scaffolds implanted in diabetic conditions, and was associated with both infiltrated host cells and the implanted extracellular matrix. However, PGG-treatment of the scaffolds before implantation markedly decreased the accumulation of CML in diabetic environments, particularly in the extracellular matrix (Fig. 3.3). Pentosidine and MDA detection in scaffold extracts essentially confirmed IHC results, showing that PGG-treated scaffolds allowed significantly less AGE formation and accumulation in diabetic environments when compared to non-PGG-treated scaffolds (p > 0.001).

3.3.4. Cellular Infiltration

In order to learn how the diabetic host cells react to the implanted collagen and elastin scaffolds and whether the treatment with PGG affects the inflammatory cell infiltration, we analyzed the scaffolds by H&E staining and IHC four weeks after implantation. H&E staining documented host cell infiltration permeating the scaffolds, preferentially through the ventricularis layer in the cusp and the adventitia in the artery (Figs. 3.4 and 3.5). Host cells appeared to have an affinity for the pore spaces in the collagen scaffolds and spaces between elastin fibers. No differences were observed in cell infiltration patterns between non-PGG-treated scaffolds implanted in diabetic rats as compared to those implanted in control, non-diabetic rats. PGG-treatment of scaffolds before implantation slightly reduced, but did not inhibit cellular infiltration.

IHC stain for vimentin documented an influx of fibroblasts into all scaffolds regardless of glycemic environments. Fibroblasts were seen to migrate throughout the
Fig. 3.3. Advanced glycation end products and lipid peroxidation products in explanted scaffolds. (top panel) Immunohistochemical detection of carboxymethyl lysine (CML) in non-treated and PGG-treated collagen and elastin scaffolds implanted in control and diabetic rats (positive ¼ brown). (bottom panel) Pentosidine (left) and malondialdehyde (MDA, right) content in non-treated and PGG-treated collagen and elastin scaffolds after implantation in diabetic rats. *Indicates statistical significance.
scaffolds unhindered, and cell spreading was clearly seen in most areas analyzed. Treatment of scaffolds with PGG did not appear to inhibit fibroblast infiltration or spreading (Figs. 3.4 and 3.5). IHC for CD8 showed presence of few T-cells in all scaffolds, but they were constrained to the edges of the scaffolds. Diabetic environments appeared to elicit a greater T-cell response in non-treated scaffolds compared with control non-treated scaffolds. PGG-treatment of scaffolds appeared to discourage T-cell infiltration, though it did not completely inhibit it. IHC staining for CD68 (a pan-macrophage antibody) showed presence of macrophages in all scaffolds, indicating an inflammatory response. This was noticeably more aggressive in diabetic non-treated scaffolds (Figs. 3.4 and 3.5). An evident decrease in general macrophage presence was observed in PGG-treated tissues, suggesting that PGG might discourage the macrophage inflammatory response of the host to the scaffolds.

3.3.5. ECM Remodeling

Non-PGG-treated scaffold ECM integrity was slightly compromised after implantation, with signs of matrix degradation visible in both collagen and elastin scaffolds stained with Movat’s Pentachrome (Fig. 3.6). Conversely, treatment of scaffolds with PGG before implantation preserved the structural integrity of the ECM in both control and diabetic conditions. Collagen and elastin scaffolds were also analyzed for MMP activities and amounts of TIMPs. Results showed higher MMP activities in diabetic vs. non-diabetic rats and significantly reduced protease levels in PGG-treated scaffolds. The highest reduction in MMP activity was noticed in PGG-treated scaffold
Fig. 3.4. Cell infiltration in collagen scaffolds. Explants were stained by H&E and IHC (brown = positive reaction)

Fig. 3.5. Cell infiltration in elastin scaffolds. Explants were stained by H&E and IHC (brown = positive reaction)
samples implanted in diabetic rats (Fig. 3.6). TIMP levels were highest in non-PGG-treated scaffolds implanted in control, non-diabetic rats (0.27 RDU per mg wet weight) and lowest in PGG-treated scaffolds implanted in diabetic rats (0.19 RDU per mg wet weight).

Fig. 3.6. ECM remodeling in implanted scaffolds. Scaffolds treated with PGG or non-treated controls were implanted subdermally in control and diabetic rats. (upper panel) Explants were stained with Movat’s Pentachrome histology stain (yellow = collagen, blue = glycosaminoglycans, dark purple = elastin, bright red = nuclei). (bottom panel) Protein extracts from collagen scaffolds (left) and elastin scaffolds (right) were analyzed for matrix metalloproteinase activity by gelatin zymography followed by densitometry (inserts, positive = white bands); results are shown as relative density units (RDU). *Indicates statistical significance.
3.3.6. Calcification

To test for calcification, samples from all groups were stained with Alizarin Red histological stain and also analyzed for calcium content using a colorimetric kit. Results revealed no accumulation of calcium in any of the collagen scaffolds (baseline levels of calcium) irrespective of glycemic status (Fig. 3.7). Elastin scaffolds however calcified significantly after being implanted in both normal and diabetic rats (17 mg Ca/mg dry weight) but did not accumulate any calcium deposits if pre-treated with PGG before implantation (baseline levels, no red staining after Alizarin). These results point to important structural differences between the two scaffolds and the outstanding effect of PGG on prevention of elastin calcification.

**Fig. 3.7.** Calcification in implanted scaffolds. Scaffolds treated with PGG or non-treated controls were implanted subdermally in control and diabetic rats. Explants were stained with Alizarin Red histology stain for calcium (positive = red).
3.4. Discussions

Biologic scaffolds composed of ECM proteins have been used in numerous regenerative medicine applications, for both preclinical animal studies and clinical purposes\textsuperscript{23, 36}. The ECM is the natural 3D structure that assists the functions of cells and tissues, not only providing structural support for cells, but also regulating processes such as cell proliferation, survival, migration, and differentiation. Cell-matrix interactions constantly determine the remodeling of ECM superstructures during the normal processes of development and wound repair. However, when cells detect and respond to altered matrix biochemical or mechanical stimuli from their environment, the dysfunctional remodeling that might occur can contribute to the onset and progression of disease\textsuperscript{37}. One of the most daunting environments that could damage the ECM and its interaction with cells is progressively built in diabetes. In the presence of high glucose concentrations, long-lived ECM proteins such as collagen and elastin undergo irreversible crosslinking by the formation of AGEs through non-enzymatic glycation (Maillard reaction). Reactive oxygen species and free metal ions were identified as key participants in glycoxidation and lipid peroxidation processes\textsuperscript{38}. Furthermore, AGEs interact with specific receptors on cell surfaces (RAGE), triggering continuous reactive oxygen species formation, inflammation, and progressive vascular complications\textsuperscript{10}. The consequences of severe matrix alterations in diabetes are impaired healing, remodeling, and tissue regeneration, all being key processes targeted in tissue engineering and regenerative medicine.

In studies presented here, we show that matrix-based scaffolds used for heart valve and blood vessel tissue engineering accumulate AGEs, become crosslinked, change
their mechanical and biochemical properties, trigger inflammatory reactions, and change their matrix remodeling abilities when subjected to experimentally induced diabetes in rats.

In order to prepare our collagen and elastin scaffolds, we developed suitable methods that remove all cells, but do not disturb the overall scaffold architecture\textsuperscript{24, 25}. Not only did the major collagen and elastin components of the ECM maintain their structures, but some basal lamina components, predominantly collagen type IV was also retained post-decellularization. Several studies have shown that if the basal lamina is not damaged, it provides a scaffold along which regenerating cells can migrate and regenerate injured tissues such as muscle, nerves, and epithelia\textsuperscript{39-41}. Since the composition and architecture of the matrix are not compromised during decellularization, our scaffolds have similar mechanical properties compared to fresh tissues. These results are in agreement with several other studies that use decellularization methods, which do not induce significant changes in the ECM composition vs. the native tissues and, consequently, do not impair tissue strength\textsuperscript{28, 42}. However, when implanted subcutaneously for four weeks in diabetic rats, both the collagen and the elastin scaffolds showed significantly altered mechanical properties, becoming stiffer, compared to those scaffolds implanted in non-diabetic surroundings. These physical changes are similar to those already noticed in tissues obtained from diabetic patients. Without doubt, one of the most prominent complications of hyperglycemia is associated with the alteration of the vascular wall and, consequently, vascular stiffening is considered the hallmark of diabetes\textsuperscript{43}. There is much ongoing research examining the mechanical properties of large
and peripheral arteries in diseased states\textsuperscript{44, 45}, with particular emphasis on the aorta\textsuperscript{46}. However, there is currently little research examining mechanical properties of aortic valve cusps in a pathological state such as diabetes, although aortic valve cusp thickening and calcification were shown to be accelerated in diabetic patients, eventually ending in heavily calcified, stiff cusps causing severe valve stenosis\textsuperscript{47, 48}.

We noticed in our animal studies that the diabetic environment elicited a pathological stiffening of collagen scaffolds, likely due to the crosslinking nature of AGEs. DSC confirmed the crosslinked nature of the scaffolds by illustrating an increased thermal denaturation temperature ($T_d$) of 6-10$^\circ$C, which can be considered significant. In fact, in vitro studies have also reported the formation of AGEs by Fe$^{2+}$-catalyzed, non-enzymatic glycation\textsuperscript{7, 49, 50} of collagen type I incubated in vitro in solutions with high glucose concentrations.

As diabetes is increasing to epidemic proportions worldwide with severe consequences, numerous studies were performed to elucidate the nature of AGEs, their involvement in the generation of permanent crosslinks of extracellular matrix proteins, as well as their association with diabetes-induced complications. Concurrently, numerous studies are focused on finding effective means of attenuating the glucose adduct formation and the damage they are bringing to proteins\textsuperscript{38}. Several strategies and agents were considered such as aspirin, glutathione, and dibasic amino acids (lysine and arginine) to block glucose adduct formation: amino- guanidine and pyridoxamine\textsuperscript{51}, to trap the sugar fragmentation products; N-phenacylthiazolium bromide, to break AGEs; vitamin E and selenium, as agents with antioxidant supplements because the antioxidant
defense system is perturbed in diabetes. As alterations in iron and copper homeostasis are a characteristic feature of diabetes, chelators, such as triethylenetetramine or citrate, may inhibit AGE formation\textsuperscript{51}.

A number of publications report on the protection against diabetic complications by several plant extracts, such as polyphenols, rutin\textsuperscript{52}, and resveratrol. It is possible that they may work in part by limiting the uptake or promoting the excretion of metal ions through chelating activities\textsuperscript{51}. Given that high levels of CML, pentosidine and MDA were noticed in the collagen and elastin scaffolds exposed to the harmful environment in diabetic rats (Figs. 3.2 and 3.3), we treated our scaffolds with PGG, a polyphenolic, antioxidant stabilizing agent with high affinity for proline-rich proteins\textsuperscript{53}, and implanted them in diabetic rats. The reduced quantities of CML, pentosidine, and MDA in implanted PGG-treated collagen scaffolds compared to non-treated scaffolds suggest that PGG might protect cardiovascular tissue engineering scaffolds from diabetic complications.

It is known that inflammatory cells are often present in altered diabetic tissues\textsuperscript{54} Neutrophil, macrophage, and T-cell accumulation was also noticed in the collagen and elastin-based scaffolds implanted in diabetic rats. However, in the PGG-treated scaffolds, we noticed less cell infiltration. As there is a highly regulated connection between AGEs, oxidative stress, and inflammation, it is possible that the antioxidant nature of PGG is halting the glycooxidation, which in turn, ceases the further acceleration of AGE accumulation and inflammation. These results are in agreement with other papers that describe PGG as an anti-inflammatory and anti-oxidative agent\textsuperscript{55}. 


PGG was also efficient in preventing calcium accumulation in the elastin scaffolds. Many studies have demonstrated that elastin calcification is a widespread feature of vascular pathology\textsuperscript{35, 56, 57} and that the glycoxidative modification of elastin is a potential accelerating factor for diabetic macroangiopathy\textsuperscript{58, 59}. In our studies, the acellular elastin scaffolds exhibited massive calcium deposits in the media after implantation in both control and diabetic rats. Auspiciously, the PGG-treated elastin scaffolds revealed no detectable calcium accumulation in vivo. These results were in agreement with studies done by Chuang et al., who showed that there were no detectable deposits of calcium salts at four weeks after subcutaneous implantation in normal rats\textsuperscript{24}. In this study, we show that PGG is able to prevent calcification of elastin scaffolds in diabetic rats as well. Similarly, it was demonstrated earlier that tannic acid (a chemical derivative of PGG) decreased calcification of glutaraldehyde treated aorta implanted in the rat subdermal model\textsuperscript{60}. The same authors revealed that stabilization of abdominal aorta with PGG reduced the onset and progression of abdominal aorta aneurysms and that PGG-treated aortas exhibited improved preservation of elastic laminar integrity, waviness, and overall preserved tissue architecture in a well-established abdominal aortic aneurysm model in rats\textsuperscript{34}.

On the other hand, our collagen scaffolds were not readily susceptible to calcification upon four weeks implantation in diabetic rats, although studies have shown that diabetes is a strong predictor for aortic valve calcification\textsuperscript{61}. The acellular collagen scaffolds proved to be resistant to calcification, possibly as a result of the mild detergent-based method that we used to decellularize the tissue (it has been shown that degraded
collagen fibers are associated with valve calcification)\textsuperscript{62, 63}. In addition, PGG might prevent the further disorganization of matrix components, reducing the infiltration of inflammatory cells and the synthesis of matrix proteases, which play a significant role in elastin and collagen calcification\textsuperscript{35}. The activity of MMPs was slightly higher in scaffolds implanted in diabetic rats, compared to non-diabetic rats; as expected, TIMP levels were lower in diabetic conditions. These results are in agreement with studies that show that both MMP activities and TIMP protein levels are altered in different cell types isolated from diabetic patients\textsuperscript{11}. In our scaffolds, MMP levels were about 50\% lower in PGG-treated scaffolds implanted in both non-diabetic and diabetic rats. This indicates that the remodeling process would be likely decelerated, allowing prolonged scaffold retention, an essential characteristic for use in replacement of cardiovascular tissues.

3.5. Conclusions

Collagen and elastin-based scaffolds used for heart valve and blood vessel tissue engineering accumulate AGEs, become stiffer, and change their matrix remodeling abilities when subjected to experimentally induced diabetes in rats. Pre-implantation treatment of scaffolds with PGG, an antioxidant matrix-binding polyphenol, stabilizes the scaffolds and protects them from diabetes-related complications thereby supporting their future use for cardiovascular tissue engineering in diabetic patients.

3.6. References


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CHAPTER 4: EVALUATION OF AUTOLOGOUS ADIPOSE STEM CELL REVITALIZATION ON SCAFFOLDS IN DIABETES

(This work has been submitted to Biomaterials)

4.1. Introduction

Diabetes is a well-known major risk factor for the progression of cardiovascular disease\textsuperscript{1-5}. More alarming, the prevalence of diabetes is globally on the rise, expected to increase from 366 million people in 2011 to 552 million by 2030\textsuperscript{6}. The central mechanism driving vascular complications in diabetes is chronic inflammation. Hyperglycemia and dyslipidemia induce activation of the endothelium and modifications of matrix components, both changes resulting in inflammation. The formation of advanced glycation end products (AGEs) and their interaction with RAGE, their specific cell surface receptor, stimulate the formation of reactive oxygen species (ROS), which, together with dysfunctions of mitochondria, contribute to the oxidative stress in diabetes; indeed, superoxide generation is believed to accelerate the pathogenesis of cardiovascular diseases in diabetes\textsuperscript{7-11}. Furthermore, increased ROS production positively correlates with activation of innate immunity\textsuperscript{12} and, subsequently, monocytes and macrophages infiltrate in diabetic aorta\textsuperscript{13}. In addition, the pro inflammatory state of diabetes has been recognized for its pro-calcific potential\textsuperscript{14, 15}. Elevated levels and faster progression of arterial calcification are consistently reported in populations with diabetes\textsuperscript{16}. Vascular calcification can occur in either the intima, caused by atherosclerosis, or the media of the vessel wall, more associated with diabetic conditions\textsuperscript{17}. 
Tissue engineering is rapidly emerging as a potential solution for the treatment of vascular diseases\textsuperscript{18, 19}, however, there is a gap in the understanding of how tissue engineered vascular products will perform when implanted in patients with diabetes. We have previously reported on the grim fate of collagen and elastin scaffolds used for cardiovascular tissue engineering which might ultimately lead to their failure after implantation into a diabetic environment\textsuperscript{20}. Surely, the observed matrix stiffening, the elevated state of inflammation and calcification are not conducive to graft integration and vascular tissue regeneration. We suggested previously a treatment for the scaffolds with penta-galloyl glucose (PGG), a matrix-binding polyphenol with intrinsic antioxidant properties, which protected the matrix from pathological diabetes-induced adverse reactions.

Scaffolds, however, are just one key ingredient in the tissue-engineering paradigm; they must be seeded with cells in order to promote new tissue generation and remodeling, leading to construct integration\textsuperscript{21, 22}. Adipose tissue-derived stem cells (ASCs) have recently been identified as a promising stem cell source due to their immunomodulatory properties, ability to differentiate into various vascular cells and their availability\textsuperscript{23-26}. Stem cell immunomodulation, in particular, holds high potential for tissue engineered construct integration by reducing inflammatory cytokine production, suppressing cytotoxic T-cell proliferation, and stimulating the secretion of anti-inflammatory cytokines such as IL-4 and IL-10\textsuperscript{26-29}. Furthermore, ASCs have been shown to regulate macrophage polarization by reduction of classically activated pro-inflammatory M1 macrophage phenotype in favor of the alternatively activated pro
healing M2 macrophage phenotype\textsuperscript{30-32}. M1 macrophages are responsible for chronic inflammation, secreting abundant amounts of IL-6, TNF-\(\alpha\), as well as toxic reactive oxygen intermediates and nitric oxide, generated by activated iNOS\textsuperscript{33,34}.

Suppression of chronic inflammation is key for tissue engineered construct survival and integration. However, the ability of ASCs to moderate the aggressive inflammatory environment that diabetes elicits in response to tissue-engineered implants has not been explored. Hence, we hypothesize that the immunosuppressive capabilities of autologous ASCs seeded on vascular scaffolds would temperate the diabetes-intensified inflammatory reactions. Furthermore, the combined effect of immunomodulatory ASCs and antioxidant PGG would inhibit calcification and positively impact remodeling of tissue-engineered vascular constructs when implanted subcutaneously in diabetic rats.

4.2. Materials and Methods

4.2.1. Materials

High-purity penta-galloyl glucose was a generous gift from N.V. Ajinomoto OmniChem S.A., Wetteren, Belgium (www.omnichem.be). Streptozotocin was from Sigma (S0130). The insulin preparation used for rats in this study was Humulin N U-100 NPH, Human Insulin of rDNA origin isophane suspension from Lilly (Indianapolis, IN). AlphaTRAK (Gen II) test strip and the AlphaTRAK Blood Glucose Monitoring System was from Abbott Laboratories, Animal Health (Abbott Park, IL). Electrophoresis apparatus, chemicals, and molecular weight standards were from Bio-Rad (Hercules, CA). Bicinchoninic acid protein assay kit was from Pierce Biotech (Rockford, IL). The
VECTASTAIN Elite ABC reagent, R.T.U. (#PK-7100) and the diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate kit (#SK-4100) were purchased from Vector Laboratories (Burlingame, CA). We used the following antibodies: rabbit anti-laminin (ab11575, Abcam), monoclonal anti-vimentin (V5255, Sigma), mouse anti-CD8 (GTX76218, GeneTex Inc, Irvine, CA), mouse anti-CD68 (ab31630, Abcam), rabbit anti-iNOS (ab15323, Abcam), rabbit anti-CCR7 (CG1678, Cell Applications Inc, San Diego, CA), mouse anti-CD163 (ab111250, Abcam), rabbit anti-osteopontin (ab8448, Abcam), rabbit anti-alkaline phosphatase (ab108337, Abcam), rabbit anti-RAGE (ab37647, Abcam), sheep anti-CD34 (AF6518, R&D Systems, Minneapolis, MN), and rabbit anti-alpha-smooth muscle actin (ab5694, Abcam). We used the following ELISA kits: TNF-α Rat ELISA Kit (ab100785, Abcam) and Prostaglandin E2 Express EIA Kit (#500141, Cayman Chemical, San Antonio, TX). Alizarin Red 1% aqueous staining solution was obtained from Poly Scientific R&D Corp (#s2007, Bay Shore, NY). All other chemicals were of highest purity available and were obtained from Sigma Aldrich Corporation (Lakewood, NJ).

4.2.2. Arterial Elastin Scaffold Preparation

Elastin scaffolds were prepared following an alkaline extraction protocol described before, with minor modifications [25]. Briefly, fresh porcine carotid arteries (6-8 cm long, 5-6mm diameter) obtained from Animal Technologies, Inc. (Tyler, TX) were rendered acellular by incubation in 0.1M NaOH solution at 37°C for 24 h followed by extensive rinsing with deionized water until pH dropped to neutral. Scaffolds were stored
in sterile PBS. This treatment removed all cells and most of the collagen, leaving vascular elastin intact. Scaffolds were also completely devoid of the α-Gal epitope (data not shown).

4.2.3. PGG Treatment

Acellular scaffolds were treated with sterile 0.1% PGG in 50mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 5.5, containing 20% isopropanol, overnight at room temperature under agitation and protected from light. Scaffolds were then rinsed in sterile PBS and stored in sterile PBS containing 1% protease inhibitor and 1% Pen-Strep at 4°C. The efficiency of PGG binding and tissue stabilization was assessed previously by testing tissue resistance to collagenase and elastase digestion [25,26]. Untreated scaffolds were used as controls.

4.2.4. Rat Model of STZ-induced Diabetes

Adult male Sprague Dawley rats (n = 40, weight 300-350 g) were rendered diabetic via a single dose of sterile filtered 55 mg/kg streptozotocin solution in 0.1M citrate buffer (pH 5) by tail vein injection. Control rats (n = 20) received an equal volume of vehicle (sterile citrate buffer). Starting on day 3, levels of blood glucose were determined 3-4 times per week, using AlphaTRAK (Gen II) test strips on the AlphaTRAK Blood Glucose Monitoring System, designed specifically for animals. Diabetes was established (>400 mg glucose/dL blood), and diabetic rats were given subcutaneous injections of long-lasting insulin (2-4U Isophane) every other day to
maintain blood glucose level in a desirable range (400-600 mg glucose/dL blood) and prevent development of ketonuria and weight loss. Glucose levels, individual weights, hydration status, and food and water consumption were monitored closely and continuously graphed to ensure adequate health parameters. Animals were provided with food and water ad libitum and were cared for by the attending university veterinarian and associated staff at the Godley-Snell Research Center animal facility. The Animal Research Committee at Clemson University approved the animal protocol, and National Institute of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication #86-23 Rev. 1996) were observed throughout the experiment.

4.2.5. Adipose Stem Cell Isolation and Injection Into Scaffolds

A small amount of subcutaneous belly fat (~30mg) was harvested from each rat 2 weeks after STZ administration via lipectomy procedure and immediately processed according to the Zuk procedure. Briefly, adipose tissue was minced, washed with ammonium chloride to remove red blood cells, incubated in collagenase, and centrifuged. The stromal vascular fraction pellet was plated in tissue culture flasks and cultured for 2 weeks to propagate the adipose stem cells (ASCs). ASCs were then seeded into each elastin scaffold at approximately 1X10^6 cells per scaffold (~5X10^4 cells per cm^2) via injection with 30G syringe at multiple sites along the scaffold. Recellularized scaffolds were maintained in culture media overnight prior to subcutaneous implantation.
4.2.6. Subdermal Implantation of Elastin Scaffolds

Four weeks after STZ administration, rats were prepped for surgery and anesthetized using 1-2% isoflurane. Diabetic rats were given 1U of insulin preoperatively. A small, transverse incision was made on the back of the rats, and two subdermal pouches were created by blunt dissection. Elastin scaffolds were cut open longitudinally and 2-3 cm samples were implanted subdermally into each pouch (n=2 scaffolds per rat). Constructs with injected autologous ASC were also implanted into each respective rat. The rats were allowed to recover, provided with food and water \textit{ad libitum}, and were cared for by the attending veterinarian and associated staff at the Godley-Snell Research Center animal facility. Post-operative levels of blood glucose were determined 3-4 times per week, and diabetic rats were given insulin as described above. After four weeks, the rats were humanely euthanized by CO2 asphyxiation, and the scaffolds were explanted and collected according to their respective assay application as follows: scaffolds for histological analysis were placed in Karnovksy’s Fixative and paraffin embedded, while samples for protein analysis were flash frozen in liquid nitrogen and transferred to -20°C for storage.

4.2.7. Experimental Implant Groups

Scaffolds were divided into four groups as follows: a) non-treated elastin scaffolds without ASCs; b) PGG-treated elastin scaffolds without ASCs; c) non-treated elastin scaffolds with ASCs; d) PGG-treated elastin scaffolds with ASCs. Samples from each group were implanted subdermally into control and diabetic rats (n = 20 implants
per group) as detailed below.

4.2.8. Histology and Immunohistochemistry (IHC)

Paraffin-embedded scaffolds sections (5µm) were rehydrated and stained with hematoxylin and eosin (Richard-Allen Scientific, Thermo Scientific) for a general overview of scaffold integrity and to confirm presence of ASCs. For IHC, heat-mediated antigen retrieval was implemented by immersing sections in 95-100°C solution of 10mM citrate buffer (pH 6.0) for 20 min. Sections were then treated with 0.025% Triton X-100 for 10 min and blocked in 1.5% normal horse serum for 30 min. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in 0.3% horse normal serum for 30 min then primary antibody was applied for 1 hr at room temperature, followed by incubation in appropriate biotinylated secondary antibody (1:200 dilution, Vector Labs) for 30 min. VECTASTAIN Elite ABC Reagent, R.T.U. was applied, and the chromogen was visualized using the DAB Peroxidase Substrate Kit. Negative staining controls were obtained by the omission of the primary antibody. Sections were lightly counterstained with hematoxylin, prior to mounting. Digital images were obtained at various magnifications (25X to 200X) on a Zeiss Axiovert 40CFL microscope using AxioVision Release 4.6.3 digital imaging software (Carl Zeiss MicroImaging, Inc. Thornwood, NY). We used the following antibody dilutions and concentrations: CD34 (1:200), CD68 (1:100), iNOS (1:100), CD163 (4µg/mL) CD8 (4µg/mL), vimentin (1:250), α-smooth muscle actin (1:200) and laminin (1:25).
4.2.9. Calcium and Bone Protein Analysis

Alizarin Red histology staining for calcium deposits was performed on sections of explanted elastin scaffolds (n = 5 per group). Osteogenic markers, osteopontin (1:250 dilution) and alkaline phosphatase (1:300 dilution), were determined in elastin scaffolds via IHC.

4.2.10. IHC Quantification

Relative quantification for immunohistochemistry stains was performed on ImageJ (provided by NIH) using the ImmunoRatio plugin (Touminen and Isola, University of Tampere, Finland). Quantities are expressed as a percentage of DAB to nuclear area or ECM area, depending on stain type (cellular stain vs. matrix stain). Each image was adjusted individually for brown threshold and blue threshold to fine-tune each component. Proper instructions for use provided by the developer was followed.

4.2.11. Mechanical Testing

A 12mm x 12mm square specimen was cut from the elastin scaffolds, maintaining orientation of the circumferential and longitudinal axes of the artery (n=5 per group). The biaxial testing method has been reported previously [40]. Briefly, four markers were placed in the center of the specimen to track tissue deformation. A total of 8 loops of 000 polyester suture of equal length were attached to the sample via stainless steel hooks, with two loops on each side of the square specimen. Specimens were first preconditioned for 10 contiguous cycles, and then loaded up 20:20 N/m tension for elastin scaffolds.
Tissue extensibility was characterized by $\lambda_{\text{circ}}$ and $\lambda_{\text{rad}}$, the maximum stretch ratio along the circumferential and radial directions, respectively. The biaxial testing was implemented with the samples completely immersed in PBS (pH 7.4) at physiologic temperature (37°C).

4.3. Results

4.3.1. In Vitro and In Vivo Imaging of ASCs Seeded Into Scaffolds

ASCs were injected into the scaffolds (~5X10$^4$ cells per cm$^2$) and incubated overnight in cell culture media. To confirm the presence of cells in the scaffolds before implantation, three pieces were prepared for histology and analyzed by H&E and IHC for CD34. Positive IHC staining for CD34 confirms presence of ASCs in the elastin scaffold (Fig. 4.1C). Compared to unseeded scaffolds (Fig. 4.1A), the seeded samples showed cell alignment along the elastin fibers (Fig.1B) that stained positive for CD34 (Fig. 4.1C). Cells prepared for injecting into six scaffolds were tagged with CFDA-SE and then seeded. The fluorescently tagged cells could be imaged via IVIS before seeding (Fig. 4.1 D), and monitored for up to two weeks post-implantation (Fig. 4.1 E and F). After four weeks, however, the fluorescence of the tagged ASCs could not be detected.

4.3.2. ASCs Impact on Inflammatory Cell Infiltration

In order to evaluate the effect of implanted autologous ASC on the host reaction, 2 separate ASC-seeded scaffolds were implanted in each diabetic and non-diabetic rat for four weeks; the types of infiltrated inflammatory cells were analyzed and compared to
Figure 4.1. Histological images of (A) a decellularized elastin scaffold stained with H&E, (B) ASC-recellularized elastin scaffolds stained with H&E, and (B) CD34+ stain for ASCs via immunohistochemistry (brown = positive). (D) Fluorescently tagged ASCs prior to elastin scaffold recellularization. (E) IVIS image showing fluorescently labeled constructs after subcutaneous implantation. (F) IVIS image of a rat with no implant as a negative control.
acellular scaffolds implanted in non-diabetic and diabetic rats as well. The explants were prepared for histology and sections were stained for T cells and macrophages. Elevated levels of CD8$^+$ T-cells (29.3%) and CD68$^+$ pan-macrophages (14.2%) were noticed to infiltrate the scaffolds implanted in the diabetic environment compared to the non-diabetic (13.5% CD8$^+$ and 1.9% CD68$^+$). (Fig. 4.2 A, B, E, F). However, scaffolds seeded with autologous ASCs lower the level of inflammatory reaction elicited in diabetic rats, as less accumulation of both T-cells (10.2%) and pan-macrophages (5.0%) was noticed in cell-seeded versus non-seeded scaffolds implanted in diabetic conditions (Fig. 4.2 C, D, G, H).

**Figure 4.2.** IHC and relative DAB:Nuclear Area quantification for CD8 (left panel – A,B,C,D) and CD68 (right panel – E,F,G,H) as markers for T-cells and pan-macrophages, respectively (taken at 200X magnification; brown = positive).
### Table 4.1. Relative quantification of IHC images from Fig. 4.2 – (left) CD8 and (right) CD68

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**4.3.3. ASCs Effect on M1 and M2 Macrophage Polarization**

To appraise the number of M1 and M2 macrophages present in implants, sections were stained for inducible nitric oxide synthase (iNOS), a marker for M1 macrophages, and CD163, a marker for M2 macrophages. M1 macrophages were seen in abundance in scaffolds implanted in diabetic rats (20.8%) (Fig. 4.3B) compared to scaffolds implanted in non-diabetic rats (6.2%) (Fig. 4.3A). In ASC-seeded scaffolds, however, iNOS expression was significantly lowered (2.1% in control and 3.3% in diabetic) (Fig. 4.3C&D). The opposite trend was seen in expression of CD163: no CD163+ cells (0%) were detected in acellular elastin scaffolds of glycemic condition (Fig. 4.3E&F). Conversely, a significant amount of CD163+ cells were observed in elastin scaffolds with ASCs (20.2% in control and 20.0% in diabetic rats) (Fig. 4.3G&H).

**4.3.4. Non-inflammatory Cell Infiltration and ECM Remodeling**

In order to evaluate the type of cells involved in tissue remodeling, explanted samples were assessed by IHC for the presence of smooth muscle and fibroblast-like
Figure 4.3. IHC and relative DAB:Nuclear Area quantification for inducible nitric oxide synthase (iNOS) (left panel – A,B,C,D) and CD163 (right panel – E,F,G,H) as markers for M1 phenotype macrophage and M2 phenotype macrophage polarization, respectively (taken at 200X magnification; brown = positive).

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Table 4.2. Relative quantification of IHC images from Fig. 4.3 – (left) iNOS and (right) CD163
cells. Cells positive for α-smooth muscle actin (Fig. 4.4B) and vimentin (Fig. 4.4C) as well as CD34+ cells (Fig. 4.4A) were detected in the adventitia of vascular scaffold. These cell types did not appear to have a preference for a particular condition, either control or diabetic. ASC-recellularized scaffolds, however, exhibited greater expression of CD34 (28% increase in control and 41.6% increase in diabetic) and α-smooth muscle actin (14.1% increase in control and 12.7% increase in diabetic). Vimentin expression in either scaffold group remained relatively unchained regardless of glycemic condition of the rat. Laminin production was also observed throughout the ASC-recellularized elastin scaffold, implying that the injected and/or infiltrated cells are attached to the scaffold. Scaffolds without ASC-recellularization exhibited no endogenous laminin production (Fig. 4.4).

4.3.5. Calcification and Osteogenic Responses

Alizarin red staining indicates presence of calcium in elastin scaffolds without ASCs, regardless of glycemic conditions (Fig. 4.5A&D). IHC reveals presence of both OPN and ALP in control elastin scaffolds without ASCs (Fig. 4.5B&C). OPN and ALP levels were even more abundant in diabetic elastin scaffolds (Fig. 4.5E&F). No visible calcium was observed in the control ASC-recellularized elastin scaffolds (Fig. 4.5G). Similarly, little traces of OPN and ALP (Fig. 4.5H&I) were detected in these scaffolds. Contrastingly, a significant amount of calcium was observed in the diabetic ASC-recellularized elastin scaffolds (Fig. 4.5J) with high levels of both OPN and ALP (Fig. 4.5K&L). Alizarin red stain indicates no visible traces of calcium regardless of glycemic
condition or recellularization status after treatment with PGG, a known anti-inflammatory and anti-oxidative agent (Fig. 4.6A).

*Fig. 4.4.* IHC for CD34 (A), α-smooth muscle actin (B), vimentin (C), and laminin (D) in elastin scaffolds with and without ASC-recellularization implanted control and diabetic rats.

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*Table 4.3.* Relative quantification of IHC images from Fig. 4.4.
Figure 4.5. (Top panel) Implanted elastin scaffolds without ASCs in control (first row) and diabetic (second row) rats. (Bottom panel) ASC-recellularized elastin scaffolds implanted in control and diabetic rats. (First column – A,D,G,J) Alizarin Red histological stain for calcium. (Second column – B,E,H,K) IHC stain for osteopontin (brown = positive). (Third column – C,F,I,L) IHC stain for alkaline phosphatase (brown = positive).
Table 4.4. Relative quantification of IHC images from Fig. 4.5 – (left) OPN and (right) ALP.

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4.3.6. Receptor for Advanced Glycation End Products

Receptors for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily of cell surface receptors, play an essential role in the biology of diabetic pathology. We observed higher traces of RAGE in non-treated elastin scaffolds with ASCs in diabetic conditions (11.3%) compared to control conditions (2.6%). These amounts were decreased to 0.9% and 0.6% in control and diabetic conditions, respectively, in PGG-treated elastin scaffolds with ASCs (Fig. 4.6B).

4.3.7. Cytokines and Mechanical Properties

PGG pre-treatment of scaffolds also significantly inhibit both TGF-β and TNF-α (Fig. 4.7A&B). The effect of glycemic environment and ASCs on TGF-β and TNF-α levels are unclear in comparison to the overshadowing effects of PGG on these cytokines. Diabetes also induced stiffening of ASC-recellularized elastin scaffolds (Fig. 4.7C). ASCs did not appear to have an effect on the mechanical properties of the scaffold. Pre-treatment of scaffolds with PGG prior to recellularization and implantation, however, was able to prevent diabetes-related stiffening without inducing any significant changes to
native mechanical properties. These results are consistent with the non-revitalized scaffolds in Chapter 3.

**Figure 4.6.** (A) Alizarin Red histological stain of PGG-treated elastin scaffolds with and without ASC-recellularization in control and diabetic rats. (red = positive). (B) IHC for receptors for advanced glycation end products (RAGE) of non-treated and PGG-treated elastin scaffolds recellularized with ASCs in control and diabetic rats (brown = positive).

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**Table 4.5.** Relative quantification of IHC for RAGE from Fig. 4.6
Figure 4.7. (A) ELISAs for TNF-α and (B) TGF-β content in extracted protein, normalized to total protein. (C) Biaxial tensile tests showing tension (N/m) vs. stretch in both circumferential and longitudinal directions of ASC-recellularized elastin scaffolds.

4.4. Discussion

Recellularization of acellular matrix scaffolds with an appropriate cell source is actively being researched as a key component in many cardiovascular tissue engineering paradigms\textsuperscript{36, 37}. The use of adipose stem cells was chosen due to its high clinical potential\textsuperscript{23, 25}. Our ASC-recellularization did not achieve vascular cell density equal to native conditions; however, complete recellularization was not the goal of this study.
Rather, the objective was to seed all of the isolated ASCs into the scaffold to examine the effect of these stem cells on elastin scaffolds in diabetes.

We have previously highlighted the increased inflammation caused by diabetes on acellular elastin scaffolds\textsuperscript{20}. The source of increased inflammation stems from a variety of factors including elevated oxidative stress and decreased activity of endogenous antioxidants in diabetes\textsuperscript{7, 9, 38}. Specifically, glucose can undergo autoxidation and generate OH- radicals. In addition, glucose can react with proteins to form advanced glycation end products (AGEs). ROS is generated at multiple steps during AGE formation. In hyperglycemic conditions, there is enhanced metabolism of glucose through the polyol pathway, which also results in enhanced production of reactive oxygen\textsuperscript{4, 7, 39}. It is believed that the increased oxidative stress in diabetes is the primary source of chronic inflammation and cardiovascular disease\textsuperscript{12}. Previously, we have shown anti-inflammatory effects of PGG as a matrix-binding stabilizing agent in diabetes\textsuperscript{20}. Because ASCs play a pivotal role in our tissue engineering paradigm, or goal was to investigate the potential immunomodulatory properties ASCs possess to aid in rendering cardiovascular tissue engineered constructs diabetes-resistant. ASCs have been documented to downregulate Th1 cytokines and improve overall Th1/Th2 balance\textsuperscript{40} by stimulating production of anti-inflammatory cytokines such as IL-4 and IL-10\textsuperscript{28}. Moreover, both ASCs and other mesenchymal stem cells have been shown to suppress T-cell proliferation and cytokine production\textsuperscript{40}. Though initial studies have suggested that ASCs can trans-differentiate along a particular lineage pathway to replace target cell populations, many tracking studies in animal models do not consistently support this
It has now been suggested that ASCs act through paracrine release of growth factors required to accelerate and direct tissue repair by endogenous or host-derived cells\textsuperscript{41-43}. Furthermore, ASC have been shown to secrete immunomodulatory factors such as prostaglandin to suppress inflammatory responses following ischemic events, thus enhancing recovery\textsuperscript{44}. We noticed an excessive inflammatory response to the acellular elastin scaffolds implanted in diabetic rats (Table 4.1): 29.3% T-cells and 14.2% macrophages of total cells infiltrated; none of these cells were M2 macrophages (Fig. 4.3). These inflammatory cells are an increase from the 13.5% T-cells and 1.9% macrophages of total cells infiltrated from non-diabetic rats; again, none of these cells were M2 macrophages. Consequently, we presumed that tissue engineered ECM-based vascular scaffolds implanted in diabetic conditions might undergo impaired remodeling and healing, due to chronic inflammation. It has been demonstrated that the host innate immune system responds to implanted biomaterials by recruiting macrophages that amplify the inflammatory response and subsequently send signals to T-cells. However, the ECM-based scaffolds have been shown to promote a switch in the macrophage population from a predominantly pro-inflammatory M1 phenotype to predominantly reparative M2 macrophages, which secrete anti-inflammatory mediators\textsuperscript{45, 46}. We noticed these facts in scaffolds implanted in control rats, but not in diabetic rats. These observation are in agreement with studies that show that in diabetes, the combination of persistent hyperglycemia and oxidative stress associated with decreased activity of endogenous antioxidants, lead to chronic inflammation, delayed or impaired wound
healing and reduced ability to transition from a M1 to a M2 phenotype macrophages \(^7,^9,^{38}\).

As expected, higher levels of M1 phenotype macrophages are seen in scaffolds implanted in diabetic rats (Fig. 4.3B) compared to that of control rats (Fig. 4.3A). Moreover, the ratio of M1 to M2 macrophages is heavily skewed in diabetic environments towards M1 polarization with little to no expression of CD163+ M2 macrophages (Fig. 4.3F). The pro-inflammatory diabetic environment is conducive to M1 phenotype polarization of macrophages. M1 macrophages are responsible for chronic inflammation, promoting muscle damage, and generating copious amounts of toxic reactive oxygen intermediates and cytokines such as IL-6, TNF-\(\alpha\), and nitric oxide which is generated by an overabundance of iNOS \(^{33,34}\). However, we demonstrate the immunomodulatory properties of the seeded ASCs onto the elastin scaffold prior to implantation are inhibiting the M1 macrophage polarization that was so prevalent in diabetic conditions (Fig. 4.3D). Even more interesting, we see much greater polarization of macrophages towards the M2 phenotype in both control (Fig. 4.3G) and diabetic conditions (Fig. 4.3H). The ability of ASCs to shift the Th1/Th2 balance in favor of constructive tissue remodeling in not only control environments but also diabetic environments is extremely promising for future applications of tissue engineering therapies for a wide variety of patient populations.

The presence of CD34\(^+\) cells (Fig. 4.4) is indicative of potential constructive tissue remodeling and cell recruitment. In addition, these CD34\(^+\) cells also indicate possible continued immunomodulation at the implant site after implantation. It is also
important to note the ability of ASC-recellularized elastin constructs to encourage relevant vascular cell infiltration such as smooth muscle cells and fibroblasts. ASCs do not prevent or hinder infiltration of target cell types such as smooth muscle cells (SMCs) (Fig. 4.4). We have also previously reported the loss of laminin after decellularization of the porcine carotid artery to produce the elastin scaffold\textsuperscript{20}. The loss of this basal lamina protein presented a potential drawback to the future use of our scaffold due to its vital role in cell adhesion and signaling pathways\textsuperscript{19, 47, 48}. Laminin is an integral ECM component and has been shown to be involved in adhesion-mediated events such as cell proliferation, differentiation, and maturation\textsuperscript{49, 50}. We see, however, endogenous production of laminin after implantation (Fig. 4.4) suggesting a positive outlook on the use of our elastin scaffold for vascular applications.

Elastin calcification is another threat to the downstream application of our elastin scaffold. Elastin scaffolds have been shown to readily calcify \textit{in vivo} \textsuperscript{20, 51, 52}. What’s more alarming is that vascular calcification and atherosclerosis is already prevalent, especially in patients with diabetes\textsuperscript{53, 54}. It makes little sense to replace a calcified artery with a calcification-prone elastin graft. The mechanism of diabetic calcification \textit{in vivo} is still not fully understood, however it is believed to be caused by chronic inflammation and oxidative stress similar to that seen in atherosclerosis and end stage renal disease (ESRD)\textsuperscript{55-58}. Furthermore, osteogenic transformation of medial graft SMCs was shown to occur alongside up-regulated pro-inflammatory response caused by TNF-\(\alpha\)\textsuperscript{59} and TGF-\(\beta\)\textsuperscript{60, 61}. Also, osteogenic differentiation of mesenchymal stem cells was shown to be possible in inflammatory environments\textsuperscript{62}. Specifically, osteopontin is reported to be
upregulated at inflammatory sites, particularly biomaterial implant sites. Aspects of diabetes such as hyperglycemia, excessive insulin, and hypoxia can all lead to increased osteopontin expression in SMCs. We have previously reported in vivo calcification of elastin scaffolds regardless of glycemic environment with calcium staining (Fig. 4.5 A,D). The presence of osteogenic markers osteopontin (Fig. 4.5B,E) and alkaline phosphatase (Fig. 4.5C,F) are prevalent in both control and diabetic rats. From these results, it is still unclear whether or not there are higher levels of calcification in the diabetic conditions compared to control. Astonishingly, we see no visible traces of calcium in ASC-recellularized elastin scaffolds implanted in control rats along with significantly reduced levels of osteopontin and alkaline phosphatase. ASC-recellularization seems to have little effect on calcium deposition (Fig. 4.5J) and osteogenic expression (Fig. 4.5K,L) in diabetic rats. It is possible that the immunomodulation provided by the ASCs are actively inhibiting calcification in the mild-inflammatory environment in control conditions. However, the propensity of ASC immunomodulation is overwhelmed in the aggressive inflammatory environment afforded by diabetic conditions thus allowing inflammation-driven calcification to occur.

PGG has been demonstrated to have a multitude of beneficial pharmacologic and biologic properties including anti-inflammatory, anti-oxidant, and anti-cancer. Our goal was to utilize, in particular, anti-oxidant and anti-inflammatory properties of PGG to reduce diabetic complications of tissue engineered constructs. Remarkably, all histologically detectable calcification was inhibited after PGG-treatment of either non-seeded or ASC-seeded elastin scaffolds in both control and diabetic conditions (Fig.
In diabetes, besides the excessive inflammatory, there is also an increased oxidative environment, well illustrated by the activation of RAGE, which stimulates an overproduction of ROS\textsuperscript{67}. The amount of RAGE was also reduced in the presence of PGG. (Fig. 4.6B). Many publications have showed that stimulation of RAGE results in NADPH oxidase and mitochondrial dependent ROS generation\textsuperscript{68, 69}; superoxide generation by dysfunctional mitochondria in diabetes has been postulated as the initiating event in the development of diabetic complications\textsuperscript{38}. It is possible that, while ASCs reduce inflammation, the anti oxidative treatment of scaffolds with PGG prevents the expression of RAGE on injected and/or infiltrated cells, inhibiting the process of calcification.

A drastic decrease in both TNF-$\alpha$ (Fig. 4.7A) and TGF-$\beta$ (Fig. 4.7B) was seen in PGG-treated scaffolds. No outstanding difference in these cytokines was observed between non-seeded and ASC-recellularized elastin scaffolds. While it is possible that the ASCs are actively participating in cytokine release and modulation, their activity is dwarfed by the effect of PGG. PGG has a markedly noticeable effect on both TGF-$\beta$ and TNF-$\alpha$, suggesting its ability to overpower the aggressive inflammation in diabetes. ASC-recellularization does not seem protect the scaffold from diabetes-related stiffening (Fig. 4.7C); however, the protection from stiffening is still offered by PGG-treatment. The ASCs do not contribute significantly to mechanical properties of implanted elastin scaffolds (Fig. 4.7C) compared to previously reported non-seeded counterparts\textsuperscript{20} suggesting that ASCs do not significantly prevent scaffold cross-linking in diabetes. The
preservation of mechanical properties akin to that of PGG-treated scaffolds implanted in control rats is seen in PGG-treated scaffolds implanted in diabetic rats.

While the immunomodulation of ASCs ameliorate some aspects of inflammation and calcification, PGG stabilization of biologic scaffolds remains a good option to achieve a truly diabetic resistant tissue engineered construct. Tissue engineering for the treatment of cardiovascular disease holds a promising future; however, its fate rests on its ability to not only address the disease but also the comorbidities of the patient. Diabetes, a major risk factor for cardiovascular disease, must be addressed as therapies in tissue engineering approach clinical feasibility. Rampant inflammation in diabetes will lead to chronic wound healing of the implant site, scaffold calcification, and ultimately graft failure. We have demonstrated that recellularization of scaffolds with autologous stem cells, a key component of the tissue engineering paradigm, may intrinsically aid in fighting diabetes-related complications seen in implanted grafts.

4.5. Conclusion

The immunomodulatory capabilities of adipose stem cells may, indeed, contribute to overall favorable outcomes post-implantation into a diabetic environment by shifting to a constructive Th2 response. Nonetheless, the chronic oxidative, inflammatory milieu that diabetes elicits can still overwhelm ASC’s immunomodulatory properties therefore necessitating the need PGG, an antioxidant polyphenol capable of binding to extracellular matrix scaffolds. PGG not only provides an extra means for anti-inflammation, but also is essential in preventing elastin scaffold calcification, particularly in diabetes. A
foreseeable end goal is the synergistic harmony between PGG-treatment and ASC-recellularization of elastin scaffolds for a practical means to achieve clinical translation of vascular tissue engineering in patients with diabetes.

4.6. References


CHAPTER 5: CHARACTERIZATION OF ADIPOSE STEM CELL DIFFERENTIATION IN THE 2D AND 3D DIABETIC ENVIRONMENT

5.1. Introduction

In order for tissue-engineered constructs to survive long-term implantation, they must be capable of not only host integration, but also gradual remodeling and maintenance. The human body is considered a harsh environment not only because of complex homeostatic mechanisms, but also constant mechanical stress and acute injury. For example, the aortic heart valve is considered the most mechanically stressed tissue in the human body\(^1\). In normal activity, the valve experiences pressure change from 10 to 120 mmHg in less than 0.1 seconds. Furthermore, in the valve undergoes this cycle of extreme pressure and shear stress for an average of 8 billion times in a lifetime. There is currently no synthetic construct – either valvular or vascular – on the market that can withstand the demanding environment afforded by the human body.

There also exists the problem of pediatric patients. Cardiovascular prostheses on the market have no remodeling capability that will grow with these patients. As a result, multiple follow-up surgeries for size-appropriate device replacements are required for pediatric patients, which can be both painful and traumatic.

Tissue engineered structures require a resident cell population that is capable of remodeling and maintaining the local site during growth and wound healing. In a common paradigm of patient-tailored tissue engineering (Fig. 5.1), we combine autologous adult adipose stem cells with a biologic scaffold together to form a tissue
engineered construct. The characterization of scaffolds and the effect of stem cells have been reviewed in the previous chapters (chapters 3 and 4).

**Fig. 5.1.** Our overall approach to the tissue engineering paradigm – starts with the patients and ends with the patient.

Functional (circulatory) implantation of tissue engineered constructs, however, are not expected to integrate in the human body without mechanical preconditioning. Preconditioning in a bioreactor subjects the construct to relevant physiologic stresses and strains akin to that experienced in the native environment. The role of mechanical inputs has shown to be vital in determining cell metabolism and phenotype due to
mechanotransduction and tensegrity-based mechanosensing\textsuperscript{2, 3}. In vitro maturation of constructs in a bioreactor also plays an essential role in cell proliferation, cell activation, establishing early ECM elaboration\textsuperscript{4}. In addition to providing mechanical stresses, the bioreactor also allows for three-dimensional biochemical mass transport of oxygen and growth factors to further mimic the native physiological environment. The combinations of various stimuli in the bioreactor are expected to provide the relevant signals necessary for ASC differentiation into target cardiovascular cell (e.g. VICs for valves, SMCs and ECs for vessels). ASCs have demonstrated the potential for differentiation into vascular and valvular cells especially once isolated from the stromal vascular fraction\textsuperscript{5, 6}. The phenotypic characteristics of ASCs are similar to that of bone marrow stromal cells\textsuperscript{6}.

As briefly mentioned in Chapter 4, ASCs show excellent potential for use in vascular tissue engineering. Importantly, ASCs have the ability to differentiate into endothelial cells, creating a non-thrombogenic blood-tissue interface that regulates inflammatory and immune reactions\textsuperscript{7}; into smooth muscle cells, responsible for vasoconstriction and dilation in response to normal and pharmacologic stimuli; and into fibroblasts, involved in synthesis of collagen, elastin, and proteoglycans and secretion of growth factors and cytokines. Adipose stem cell immunomodulation holds high potential for tissue engineered construct integration. ASCs are able to suppress T-cell proliferation, reduce inflammatory cytokines, and stimulate production of anti-inflammatory cytokines such as IL-4 and IL-10. The immunomodulatory impact of ASC has already been demonstrated in a variety of experimental models of disease, including spinal cord injury, neurodegenerative diseases, and GvHD. Results from clinical trials have confirmed the
safety and efficacy of ASC in treating a variety of diseases, including diabetes mellitus. Diabetic patients infused with bone marrow and ASCs had a 40% decrease in insulin requirements with no adverse effects in a 3 months follow-up period. Studies evaluating the therapeutic impact of ASC in patients with DM foot showed improved rest pain score, walking time, and evidence of increased vascular collateral networks within 6 months of intramuscular ASC injection. Besides immunosuppressive molecules, ASCs also secrete soluble factors that promote tissue regeneration at the injury site via a paracrine mechanism: angiogenic factors (VEGF), anti-apoptotic factors (IGF-1), hematopoietic factors (colony stimulating factors and interleukins), transforming growth factor-β1 and hepatic growth factor. Furthermore, adipose stem cells have been shown to regulate macrophage activation by suppression of classically activated M1 macrophages in favor of alternatively activated M2 macrophages, providing a therapeutic effect in wound healing.\(^8\) In order to precondition the cell seeded scaffolds and induce tissue maturation, mechanical and biochemical stimuli are required before implantation\(^9,10\) in animals\(^11-13\).

We hypothesize that the efficacy of differentiation of adipose stem cells into target vascular cells will be altered by diabetes in a controlled biochemical and biomechanical environment.

5.2. Materials and Methods

5.2.1. 2D Endothelial and Smooth Muscle Cell Culture

Human aortic endothelial cells (hAECs) (#PCS-100-011, ATCC, passage 1) were obtained and expanded in Endothelial Cell Growth Kit (VEGF enriched) (#PCS-100-041,
ATCC) with 1% antibiotic solution (Corning – Cellgro). HAECs were maintained and subcultured at subconfluent conditions on tissue culture plastic with Trypsin-EDTA for Primary Cells (#PCS-999-003, ATCC) and Trypsin Neutralizing Solution (#PCS-999-004, ATCC). To obtain high glucose media, D-(+)-Glucose Monohydrate (#EM1.08342.1000, VWR) was added to the Endothelial Cell Growth Kit to a concentration of 5.5g/L. HAECs were cultured for up to 6 weeks in both control and high glucose concentrations.

Human smooth muscle cells (hSMCs) (#C-007-5C, Life Technologies) were obtained and expanded in media comprised of Dulbecco’s Modification of Eagle Medium (DMEM, Corning-Cellgro) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, Georgia) and 1% antibiotic solution. HSMCs were maintained and subcultured on tissue culture plastic with Trypsin-EDTA 1X (#25-053-Cl, Corning-Cellgro). To obtain high glucose media, D-(+)-Glucose Monohydrate (#EM1.08342.1000, VWR) was added to the media to a concentration of 5.5g/L. HSMCs were cultured for up to 6 weeks in both control and hyperglycemic conditions.

5.2.2. 2D ASC Culture

Human adipose tissue was collected according to the procedures within an approved IRB protocol (Institutional Review Board) with the Greenville Health System (GHS). The fat was minced into fine pieces using scalpel blades and placed into a solution of collagenase type I (1 mg/mL, #NC96336323, Fisher Scientific) in 1x PBS and incubated at 37°C for 1 hour under agitation. Following digestion, the solution was filtered through 100 µm sieves and centrifuged at 1000 rpm for 5 minutes. Once the
supernatant was decanted, the cell pellet was resuspended in a solution of 155 mM ammonium chloride (NH₄Cl, #BDH0208, Fisher Scientific) and 0.1 mM EDTA (#E6511, Fisher Scientific) in 1x PBS for 5 minutes at room temperature to lyse any residual erythrocytes. The solution was then centrifuged at 1000 rpm for 10 minutes, the supernatant decanted, and the pellet resuspended in MesenPRO RS Medium (#12746-012, Life Technologies), the cells counted, and plated in tissue culture-treated flasks at a density of 5000 cells/cm². Adherent adipose stem cells (ASCs) were maintained at sub-confluent levels and replated at 5000 cells/cm² twice to yield 4 separate flasks of ASCs. Each flask was then designated to (1) control SMC differentiation, (2) control EC differentiation, (3) diabetic SMC differentiation, or (4) diabetic EC differentiation.

5.2.3. 2D EC and SMC Differentiation

For differentiation of ASCs to endothelial-like cells, ASCs were cultured for up to 4 weeks in EC differentiation media comprised of DMEM, 2% FBS, and 1% antibiotic solution supplemented with 0.5 ng/mL vascular endothelial growth factor (VEGF, #100-20B, PreproTech Inc) and 20 ng/mL insulin-like growth factor-1 (IGF-1, #AF-100-11, PeproTech Inc). Growth factors were freshly added to the media at the time of each media change. Diabetic EC differentiation media was generated by adding D-(+)-Glucose Monohydrate to a concentration of 5.5 g/L. ASCs were cultured in either control or diabetic EC differentiation media for up to 4 weeks on tissue culture plastic.

For differentiation of ASCs to smooth muscle-like cells, ASCs were cultured for up in SMC differentiation media comprised of DMEM, 1% FBS, and 1% antibiotic
solution supplemented with 5 ng/mL transforming growth factor beta (TGF-β1, #100-21C, PeproTech Inc) and 2.5 ng/mL bone morphogenic protein 4 (BMP-4, #120-05, PeproTech Inc). Growth factors were freshly added to the media at the time of each media change. Diabetic SMC differentiation media was generated by adding D-(+)-Glucose Monohydrate to a concentration of 5.5 g/L. ASCs were cultured in either control or diabetic SMC differentiation media for up to 4 weeks on tissue culture plastic.

5.2.4. Investigation of Cell Phenotype (Immunofluorescence Visualization)

Immunofluorescence (IF) was performed on the ASCs undergoing EC and SMC differentiation at three separate time points: time zero, 2 weeks, and 4 weeks. Prior to IF, cells were passaged, plated on 6-well plates, and cultured until the end of each time point. To perform IF, media was removed and wells were washed with 1X PBS one time. After removal of PBS, cells were covered with 4% paraformaldehyde and incubated for 15 min at room temperature. After the fixative was removed, cells were rinsed 5 times with 1X PBS for 5 min each. Cells were then blocked in blocking buffer (1X PBS, 5% Normal Horse Serum, 0.3% Triton X-100) for 60 min at room temperature. After removal of blocking buffer, diluted primary antibody was added. The antibody diluent buffer (1X PBS, 1% bovine serum albumin, 0.3% Triton X-100) was used to dilute stock antibodies: rabbit anti-CCR7 (CCR7, 1:250 dilution, #CG1678, Cell Applications), Mouse/Rat Polyclonal to CD34 (CD34, 3µg/mL working concentration, #AF6518, R&D Systems), rabbit polyclonal to α-smooth muscle actin (α-SMA, 1:100 dilution, #ab5694, Abcam), mouse monoclonal to smooth muscle myosin heavy chain
(SMM-hc, 1:250 dilution, #ab683, Abcam), rabbit polyclonal to CD31 (CD31, 1:20 dilution, #ab28364, Abcam), and rabbit polyclonal to vascular endothelial cadherin (VE-Cadherin, 5µg/mL working concentration, #ab33168, Abcam). Cells were incubated in diluted primary antibody overnight at 4°C under slight agitation. After rinsing 3 times in 1X PBS, cells were incubated in fluorochrome-conjugated secondary antibody diluted in antibody diluent buffer for 1 hour at room temperature in the dark. Fluorescent secondary antibodies – Goat anti-rabbit IgG DyLight 488 (#ab96899, Abcam), donkey anti-sheep IgG AlexaFluor 647 (#A21448, Life Technologies), and donkey anti-mouse IgG AlexaFluor 488 (#A21202, Life Technologies) – were diluted to 4µg/mL prior to incubation. Cells were then rinsed in 1X PBS once and incubated in DAPI solution at a concentration of 1µg/mL (#D9542, Sigma) for 15 minutes. Cells were rinsed one last time in 1X PBS and imaged via fluorescence microscopy immediately.

5.2.5. Vascular Elastin Graft Preparation and Seeding

Decellularized elastin scaffolds (see Chapters 3 & 4) were seeded with expanded hASCs not subjected to growth factors to yield a vascular graft. Ten vascular grafts (~6-7cm in length) were seeded to both the adventitia and the lumen. A total of 2.5X10⁶ ASCs were carefully seeded into the media at multiple sites along the scaffold media with a 30G syringe needle. Another 2.5X10⁶ ASCs were injected into the lumen of the graft; subsequently, the grafts were sealed shut by capped barbed luers. Vascular grafts were maintained in a cell culture incubator in DMEM, 10% FBS, and 1% antibiotic solution and turned 90° every 4 hours to ensure adequate luminal coverage by the ASCs.
5.2.6. Vascular Bioreactor Design

To mimic physiological conditions in vitro, we developed a vascular bioreactor based on a similar design by Lee Sierad. The vascular bioreactor was constructed in SolidWorks and machined by the Clemson Machining and Technical Services. Designed to accommodate 5 vascular grafts up to 4 inches long, the vascular bioreactor features some improvements over its predecessor including stainless steel “quick” barbed fittings (fittings with O-ring seal rather than threading seal), larger size, aluminum base, acrylic chamber, polycarbonate lid, and air bubble catchers. Overall, this bioreactor was designed to be constructed quickly with less risk of contamination.

5.2.7. Bioreactor Conditioning

Vascular grafts were mounted into the bioreactor (5 per bioreactor) by attaching each graft end to the stainless barbed fittings. Grafts were fastened to each barb using flush-fit zip ties. Two bioreactors were used to mechanically condition the vascular grafts – one diabetic and one control. The diabetic media was comprised of DMEM, 10% FBS, and 1% antibiotic solution and supplemented with glucose to a concentration of 5.5 g/L. Control media was made the same way sans additional glucose. A peristaltic pump (Cole Parmer) was used to perfuse media at a rate of 1 mL/min through the lumen and throughout the chamber to fully bathe the grafts in media. The vascular grafts were dynamically cultured for up to 2 weeks in a cell culture incubator with static grafts serving as a control.
5.2.8. Histological Analysis of Vascular Constructs

Cell viability and cytotoxicity of the vascular grafts were evaluated using Live/Dead Viability Assay Kit (Life Technologies). A small section was cut from the center of the graft and stained with contents of the kit according to the procedure provided by the manufacturer. The luminal side of the section (the area exposed to dynamic flow) was imaged via fluorescence microscopy.

Both dynamically and statically conditioned vascular grafts were placed in 10% neutral buffered formalin (Fisher Scientific) for over 24 hours and processed through a tissue processor for histological evaluation. Immunohistochemistry (IHC) was performed on rehydrated paraffin sections (5µm) to examine presence of α-SMA, SMM-hc, CD31, VE-cadherin, CCR7, and CD34. Briefly, heat-mediated antigen retrieval was implemented by immersing sections in 95-100°C solution of 10mM citrate buffer (pH 6.0) for 20 min. Sections were then treated with 0.025% Triton X-100 for 10 min and blocked in 1.5% normal horse serum for 30min. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in 0.3% horse normal serum for 30 min then primary antibody was applied for 1 hour at room temperature, followed by incubation in appropriate biotinylated secondary antibody (1:200 dilution, Vector Labs) for 30 min. VECTASTAIN Elite ABC Reagent, R.T.U. was applied, and the chromogen was visualized using the DAB Peroxidase Substrate Kit. Negative staining controls were obtained by the omission of the primary antibody. Sections were lightly counterstained with hematoxylin, prior to mounting. Digital images were obtained at various
magnifications (100X to 200X) on a Zeiss Axiovert 40CFL microscope using AxioVision Release 4.6.3 digital imaging software (Carl Zeiss MicroImaging, Inc. Thornwood, NY).

5.3. Results

5.3.1. Effect of High Glucose Concentrations on ECs and SMCs in 2D

High glucose media did not appear to have much effect on hECs after 6 weeks. Expression of CD31 and VE-Cadherin remained fairly consistent after 4 weeks of culture in the diabetic culture environment (Fig. 5.2). Expression of both α-SMA and SMM-hc was also seen after 6 weeks in high glucose conditions with no apparent difference from the 6-week control in terms of fluorescence (Fig. 5.2). However, a slightly higher rate of proliferation of the SMCs was observed in the diabetic conditions with a cell count approximately 13% higher after each passage. This higher proliferation rate was not exhibited by the diabetic hECs.
5.3.2. Effect of High Glucose Concentration on ASC Differentiation in 2D

At time zero, it was established that low passage hASCs strongly express of CCR7, a known mesenchymal stem cell marker; however, expression of CD34 was not observed. No expression of SMC markers (α-SMA and SMM-hc) or EC markers (CD31 and VE-cadherin) was seen in these hASCs at time zero (Fig. 5.3A).
After 4 weeks of culture without growth factors, CCR7 expression still remained prevalent in the hASCs in both control and diabetic conditions. Slight expression of α-SMA was also observed after 4-week culture in both conditions. However, no smooth muscle myosin or endothelial cell markers were observed in either condition (Fig. 5.3B).

After 4 weeks of culture with growth factors specific to either endothelial cells or smooth muscle cells, some phenotypic modulations were seen via immunofluorescence. For hASCs cultured in EC differentiation media for 4 weeks, no CD31 was observed in either control or diabetic conditions. Conversely, VE-cadherin was seen in both conditions (Fig. 5.3B). No expression of α-SMA and SMM-hc was seen in the ASCs cultured in SMC differentiation media for 4 weeks, suggesting that high glucose impairs this particular differentiation (Fig. 5.3B).

5.3.3. Efficacy of New Vascular Bioreactor

During dynamic culturing of the vascular grafts with the vascular bioreactor, no observable leaks were detected, indicating adequate size matching of the O-rings on both the chamber lid and the quick fittings. Full set up time for this bioreactor was 30 minutes, whereas with the old vascular bioreactor, at least 1 hour was necessary for full installation (Fig. 5.4).
Fig. 5.3A. Immunofluorescence stain for CCR7, CD34, alpha smooth muscle actin, smooth muscle myosin heavy chain, CD31, and vascular endothelial cadherin in low passage hASCs (time zero) cultured in 2D. Blue = Nuclei. Green = Positive Stain.
Fig. 5.3B. Immunofluorescence of hASCs subjected to 4 weeks in growth factor-enriched media (right side) – endothelial differentiation media (stained with CD31 and vascular endothelial cadherin) and smooth muscle cell differentiation media (stained with alpha-smooth muscle actin and smooth muscle myosin heavy chain) in control and diabetic conditions. HASCs cultured for 4 weeks without differentiation media shown as a control (left side). Blue = Nuclei. Green/Red = Positive stain.
Fig. 5.4. SolidWorks drawing (top left) and macro images of bioreactor chamber, base, and lid with “quick-connect” barbed luers.
Live cells were seen exclusively in the bioreactor conditioned vascular grafts in both control and diabetic conditions, whereas only dead cells were observed in the static controls. While alignment of live cells in the direction of media flow was witnessed in both conditions, the alignment was much more significant in control conditions (Fig. 5.5A).

**Fig. 5.5A.** Live/Dead stain of calcein AM (green) to indicate live cells and ethidium homodimer (red) to indicate dead cells in control and diabetic conditions of dynamically and statically cultured vascular grafts. Green = Live. Red = Dead.
5.3.4. Morphology and Phenotype Evaluation of Conditioned Vascular Constructs in 3D

At time zero (after 16 hours post-seeding incubation), boluses of hASCs could be seen in the cross section of the grafts. A monolayer of hASCs lining the internal elastic lamina in the lumen could also be detected. After the 2-week dynamic culture, the boluses of cells were less apparent as the cells seemed to migrate from the bolus site to adjacent areas in the media. Fewer cells were detected in the lumen after 2 weeks than at time zero (Fig. 5.5B).

α-SMA was detected at all phases of seeding – from time zero to 2 weeks, with no observable difference between control and diabetic conditions. More α-SMA was detected at time zero due to presence of the cell bolus; as the cells migrated, the density of α-SMA⁺ cells became sparser. Unlike smooth muscle actin, expression of SMM-hc was not readily seen; the elastin fibers exhibited traces of pseudo-positive staining, even in areas where no cells were present. Little to no expression of VE-cadherin was seen at time zero, but did being to appear after 2-week dynamic culture. Upon 2 weeks, VE-cadherin was seen both along the lumen as well as in the cell boluses. Expression of CD31 by the cells remained largely absent throughout all phases of the culture in both the media and the lumen (Fig. 5.5B).

The effects of high glucose conditions in this experiment remain unclear; the diabetic environment did not induce any noticeable modulation of hASC phenotype towards SMCs or ECs. It is clear, however, that the mechanical stresses afforded by the vascular bioreactor induces significant changes to the vascular construct in terms of cell retention and migration.
Fig. 5.5B. IHC of vascular constructs at (left column) time zero, (middle column) 2-week post control bioreactor, and (right column) 2-week post diabetic bioreactor stained for CCR7, CD31, VE-Cadherin, $\alpha$-SMA, and SMM-hc. Brown = Positive. Purple = Nuclei.
5.4. Discussions

As it stands with the study, the term “diabetic” environment is used quite loosely. When we refer to this term, we are focusing directly on the high glucose aspect that’s prevalent in patients with diabetes. The non-enzymatic crosslinking reaction of glucose with proteins, lipids, and nucleic acids is thought to be one of the root causes for the development of cellular dysfunction which leads to cardiovascular disease\textsuperscript{14}. We are not taking into consideration the other metabolic imbalances associated with the diabetic environment involving insulin, triglycerides, and cholesterol. Indeed, the combination of metabolic abnormalities along with glycoxidation and reactive oxygen radicals intensely exacerbates the pathogenesis of diabetes-related disease\textsuperscript{15-17}. For the purposes of this study, however, we focused on the effects of the high glucose concentration environment on stem cell differentiation and viability in 3D coupled with relevant mechanical forces that would be seen in native physiologic conditions. Our objective was to see scrutinize stem cell differentiation in both 2D vs. 3D to determine optimum vascular graft development. We noticed that growth factors were necessary for differentiation of hASCs into SMCs and ECs. While CD31 was not readily expressed after exposure to EC differentiation media, VE-cadherin expression was seen. While VE-cadherin is usually only seen at cell-cell interactions, the upregulation of VE-cadherin has also been shown to be associated with significant accumulation of \( \beta \)-catenin, which can also yield a VE-cadherin positive stain\textsuperscript{18}. This result suggests that the high glucose environment either inhibits or slows endothelial cell differentiation of ASCs.
The presence of α-SMA in ASCs without SMC differentiation media is not alarming; this result has been supported by several other groups who claim that ASCs express α-SMA upon prolonged culture\textsuperscript{19,20}. Therefore, the use of α-SMA, alone, as a marker for the determination of SMC differentiation is insufficient. However, when coupled with smooth muscle myosin heavy chain, a late marker for smooth muscle cells\textsuperscript{21}, SMC differentiation looks much more promising particularly since time zero controls exhibited no SMM-hc expression.

Clearly, the effectiveness of growth factor-enriched media specific for SMC and EC differentiation of ASCs cannot be ignored. The limitation to this technique, however, is that it is constrained only to two-dimensional cultures. In native physiologic environments, cells interact within a three-dimensional matrix framework, which plays a large role in cell signaling, communication, remodeling, and phenotypic modulation\textsuperscript{3,22,23}. The role of bioreactor-induced mechanical signals has also been shown to play a vital role in stem cell differentiation to target vascular cells\textsuperscript{24-26}. In our studies, we did not observe drastic SMC or EC differentiation of stem cells on vascular constructs due to mechanical stress alone. Similar to the 2D differentiation cultures, CD31 was not seen throughout the vascular grafts while VE-cadherin was. Both SMC markers also stained positive in the dynamically conditioned vascular grafts in both control and diabetic conditions. The most drastic change to the grafts was elicited by the mechanical flow and shear stress, thus overshadowing any potential effects of diabetes. While the efficacy of differentiation of ASCs into relevant vascular cells still remains unclear, we see major changes to cell morphology, viability, and migration; static counterparts did not survive
the 2-week culture (Fig. 5A). Cell survival and proliferation are essential to vascular graft viability post-implantation in a translational scenario.

There is some controversy of the use of CD34 as a marker for ASCs\textsuperscript{27} because it has been shown to stain positive for other progenitor cells found in the same population as adipose derived tissue stromal cells (hematopoietic cells, endothelial progenitor cells, etc.). ASCs have been shown to be CCR7\textsuperscript{+}, CD34\textsuperscript{+}, CD90\textsuperscript{+}, CD140\textsuperscript{+}, CD133\textsuperscript{-}, and CD45\textsuperscript{-}\textsuperscript{19, 28}; isolation of pure ASCs from the stromal vascular fraction would require a much more thorough screening process and separation via flow cytometry. The lack of CD34 expression (Fig. 5.3A&B) does not necessarily indicate the lack of ASCs in the culture; in fact, studies have shown ASCs devoid of CD34 expression in 2D cultures\textsuperscript{29, 30} and full of CD34 expression in 3D and some 2D cultures\textsuperscript{31, 32}. We have seen firsthand the reason for this controversy with varying expressions of CD34 (See Chapter 4). The lack of CD31 expression combined with the positive expression of CCR7, however, is a positive indication that the population of cells are primarily a homogeneous culture of ASCs with no endothelial progenitor cells\textsuperscript{33}.

While the high glucose diabetic environment did not appear to have an effect on the phenotype of somatic human SMCs (Fig. 5.2), it did cause a higher rate proliferation, which was not observed by the endothelial cells. One of the effects of advanced glycation end products has relevant to vascular disease is the induction of smooth muscle cell proliferation\textsuperscript{14, 34}. It is possible that endothelial cells are able to withstand the effects of AGEs more readily than SMCs; after all, ECs are the first line of defense in the blood-contacting surface of the endothelium. It is clear, however, that endothelial cells are not
immune to dysfunction, which is one of the major mechanisms underlying accelerated atherosclerosis in patients with diabetes\textsuperscript{35}.

5.5. Conclusions

More sensitive biochemical assays will be necessary to elucidate the potential adverse effects of diabetic conditions on ASC differentiation into target vascular cells. Perhaps the best method for the creation of a viable vascular construct lies with combination of relevant growth factors necessary for differentiation and mechanical conditioning and mass transport of nutrients offered by the vascular bioreactor for construct viability and graft survival. A synergistic effect by growth factors and bioreactor holds high potential to yield the next generation of personalized medicine by paving the way for a robust and much-needed tissue engineered vascular replacement.

5.6. References


CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1. Conclusions

Cardiovascular disease remains one of the top reasons for hospitalizations in the United States and is the leading cause of death in the nation\(^1\). Tissue engineering holds vast potential for the treatment of these diseases by regenerating the diseased tissue and restoring structure and function to the system. Because tissue engineered options in our research focus on autologous stem cells, it is considered a patient-tailored approach to regenerative medicine. However, the complications presented by diabetes seem to be the elephant in the room when it comes to hurdles in treating patients. Our tissue engineering therapies cannot be considered patient-tailored if we cannot take into consideration the comorbidities associated with the patient. Not only does diabetes impair the body’s metabolic homeostasis and cause CVD, but it also complicates the reparative biomaterial and surgical measures used treat the respective CVD. The daunting reality is that diabetes mellitus targets the biochemistry and biological components of the human body. Our tissue engineering paradigm includes 2 ingredients: biologic scaffolds comprised of long-lived ECM proteins and adult autologous stem cells – both of which are just as susceptible to AGE formation and glycoxidation as native tissues in the human body. There is an alarming gap in the understanding of how tissue engineering approaches, which utilize biologic scaffolds and stem cells, perform in diabetic populations. This dilemma begs the question: how do we combat diabetes-related complications in our
tissue engineering therapies? We believe the key to answering this question lies in the protection of the scaffolds.

**Biologic scaffolds** have been proposed as an ideal source due to characteristics such as biocompatibility, biodegradability with safe by-products, mechanical stability, and natural ECM architecture\(^2\). Biologic scaffolds derived from allogeneic or xenogeneic sources have piqued much interest since they can be eliminated of cells and immunogenic material while simultaneously retaining matrix integrity, mechanical properties, and key cell signaling molecules\(^3,4\). Tissue engineered bioprostheses are promising alternatives for the creation of viable constructs in cardiovascular applications. The balance between the resiliency of the scaffold to retain functional mechanical properties and the biodegradability of the scaffold to allow remodeling remain a key consideration in tissue engineering applications. The motivation underlying the use of matrix-based vascular scaffolds lies with the attainment of the cardiovascular “niche” which will be essential in providing the optimal environment to stem cells by presenting cell adhesion motifs and providing physiologically relevant substrate rigidity. This niche will drive stem cell differentiation into target cardiovascular cells when combined with relevant biochemical and dynamic mechanical stimuli provide by a bioreactor. Though there is an increase in the popularity of stem cell-based cardiovascular therapies, it has been recognized that the lack of an appropriate niche for the stem cells was at fault for poor *in vivo* outcomes\(^5,6\). We believe that the ideal niche will be presented by acellular biologic scaffolds. We have developed several optimal decellularization procedures that wholly remove cells and antigenic proteins while preserving the natural 3D architecture,
the major matrix components (collagen and elastin), and key basal lamina proteins (collagen type IV, laminin, and fibronectin)\textsuperscript{7-10}. By focusing on the preservation of cardiovascular niches, we address the 3 main components of the tissue engineering paradigm: scaffolds, stem cells, and bioreactors.

We have shown that, if left untreated, diabetes will structurally alter the biologic scaffold thereby changing the “vascular niche” to a pathological state. Furthermore, we have shown that diabetes leads an inflammatory insult by the host to the implanted scaffold. These altered scaffold structural and inflammatory conditions are not conducive to stem cell adaptation and remodeling. By treating the scaffolds with PGG, we halted the pathological structural changes of the scaffold to preserve the vascular niche environment as well as mitigate inflammation during host implantation. The antioxidant properties of PGG combated glycoxidation and AGE formation thus rendering our tissue engineered construct “diabetic-resistant” to allow for constructive stem cell remodeling, tissue integration, and functionality.

We have also shown the potent immunomodulatory properties of adipose stem cells (ASCs) by mitigating inflammatory cells and shifting macrophage polarization from destructive to constructive. While the mechanisms and cytokines of immunomodulation remains unclear, it is believed that these stem cells act as paracrine signaling factories\textsuperscript{11, 12}. We saw that ASCs are able to prevent calcification in the control rats, but not in the diabetic rat. Asserting that inflammation and calcification are linked processes\textsuperscript{13-15}, the conclusion is that the immunomodulatory properties of ASCs inhibit the mild inflammation-driven calcification in control rats; however, the inflammation presented by
the diabetic rat is so severe, it overwhelms the stem cells’ propensity for immunomodulation and continues to cause calcification. For this reason, the PGG-treatment of the scaffolds is still necessary to provide the extra means of antioxidation and anti-inflammation. The end goal is a synergistic harmony between PGG and stem cells to produce a practical tissue engineered device for clinical translation of cardiovascular tissue engineering in patients with diabetes.

6.2. Alternative Perspectives

Chapter 5 focused on the differentiation of ASCs directly to target cardiovascular cells, namely endothelial cells and smooth muscle cells. While this avenue in the tissue engineering paradigm is still actively being pursued, recent evidence suggests that the regenerative properties of stem cells lies in their paracrine signaling. It is thought that, in cell-based therapies, the paracrine mechanism of ASCs recruit relevant progenitor cells to the local area, which then differentiate into the target cell. Regardless of the mechanism, the ideal end result would be the full repopulation of native host cells to the scaffold.

Much of the research has concentrated on a fairly narrow scope of diabetes, where the effect of hyperglycemia was mainly investigated. The small animal studies mimicked type 1 diabetes due to the destruction of pancreatic islets by STZ and subsequent insulin removal. Moreover, the blood glucose levels of the diabetic rats were fairly high (~600mg/dL) – much higher than that seen in diabetic human patients. In order to further characterize the effects of diabetes on tissue engineering, use of animal models for type 2
diabetes would be necessary for the *in vivo* studies. For *in vitro* studies, insulin and free fatty acids should be added to the media to simulate hyperinsulinemia and hyperlipidemia.

As the goal of our research is to bridge the gap from bench to bedside, realistic efforts must be taken to meet quality and regulatory standards set forth by the FDA. As it currently stands, stem cells receive significant scrutiny from the FDA by only allowing “minimal manipulation” to the cells. This implies the elimination of 2-phase stem cell culturing where stem cells are isolated and propagated *in vitro*, a common approach in the laboratory. Efforts must be taken to seek ways to achieve a point-of-care approach to tissue engineering in order to comply with the FDA as it stands today.

### 6.3. Recommendations for Future Work

#### 6.3.1. Further Assessment on the Effect of Diabetes on Adipose Stem Cell Differentiation

Additional analysis on the phenotypic modulations during diabetic hASC differentiation, diabetic hSMC culture, and diabetic hEC culture may be investigated with protein and gene analysis. Once an adequate number of cells have been cultured, they will be seeded into 6-well plates at 3x10^5 cells/well and cultured until confluency (~1x10^6 cells after 3-4 days). The plate will be divided for protein extraction for western blot and RNA extraction for RT-PCR (Figure 6.1). A total of two 6-well plates (one control and one diabetic) will be used for both hECs and hSMCs. RNA will be isolated using the
Figure 6.1. 6-well plate layout for RT-PCR and western blot analysis. (Row A) Cells for RNA isolation. (Row B) Cells for protein isolation. This will be performed for all cell groups discussed in Chapter 5.

RNeasy Mini Kit (QIAGEN Life Sciences) and quantified via NanoDrop (Thermo Scientific). All samples for RT-PCR will utilize target-specific PCR primers for GAPDH as a housekeeping gene. Primers for α-smooth muscle actin (α-SMA) and heavy chain myosin will be used for hSMCs. Primers for CD31 and VE-Cadherin will be used for hECs. Real-time PCR amplifications will be performed using a SYBR Green PCR kit in a Rotorgene 3000 thermal cycler, and the 2−ΔΔCt method will be used to analyze the relative changes in gene expression.

Total protein will be isolated from the designated wells using M-PER Mammalian Protein Extraction Reagent (Pierce Protein Biology Products, Thermo Scientific). Total protein will be quantified using a bicinchoninic acid assay (BCA assay) and separated
using SDS-PAGE in Laemmlı Buffer. Western blot will be performed using BM Chemiluminescence Western Blotting Kit (Roche Applied Science). Proteins to be examined will mirror that of RT-PCR: CD31, VE-Cadherin for ECs and α-SMA, heavy chain myosin for SMCs.

6.3.2. Further Assessment on the Effect of Diabetes and Mechanical Stress on Vascular Constructs

The same experimental setup as the bioreactor conditioned group seen in Chapter 5 will be used, however with the addition of growth factors VEGF + IGF-1 for endothelial cell development and TGF-β1 + BMP-4 for smooth muscle cell development. RNA isolation will still be performed with the RNAeasy Mini Kit using the protocol prescribed for RNA isolation from animal tissues. Protein extraction will be performed by an established protocol. Briefly, the tissue will be frozen with liquid nitrogen and pulverized with a tissue pulverizer. The sample will then be homogenized with a tissue homogenizer in tubes containing 1mL RIPA extraction buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS). The homogenized solution will then sit on ice for 30 minutes and centrifuged at 12000G for 15 minutes. The supernatant (protein extract) will be collected and stored at -20°C.

SDS-PAGE and western blotting will be performed in the same fashion as with protein extract collected using M-PER. Immunohistochemistry (IHC) will be performed on the constructs in lieu of immunofluorescence. In this case, constructs will be formalin-fixed and paraffin-embedded. Sections will be cut at 5µm and dried on treated
microscopy slides. IHC will be performed by the same protocol outlined in the previous chapters. The same markers used for detecting smooth muscle cells and endothelial cells from Chapter 5 will be used for gene analysis with RT-PCR and protein analysis with western blotting.

6.3.3. Testing of Diabetic-Resistant Vascular Constructs in a Preclinical Animal Model

Yucatan mini-swine (Sinclair Bioresources) will be used to model in situ vascular graft implants. We choose these particular minipigs due to their resemblance to human anatomy/physiology. Also, they are the largest animals in which diabetes can be reproducibly induced with strict glycemic control. To test for implant and surgical viability, we will implant an acellular scaffold based from a porcine femoral artery (developed by George Fercana, PhD) into the peripheral vasculature of a heparinized minipig for a period of 1 week. These acellular scaffolds have been well-characterized and is expected to create a vascular niche similar to that of decellularized carotid arteries. Explantation analysis will involve detection for endothelial cells, smooth muscle cells, fibroblasts, macrophages, and lymphocytes. ECM integrity will also be analyzed by Movat’s Pentachrome staining, H&E, and Verhoeff van Gieson (VVG) stain for elastin. Detection of calcification and osteogenic markers such as alkaline phosphatase, osteopontin, and osteocalcin will also be performed via IHC. To gain further insight into the hemocompatibility of the scaffold, the luminal side will be examined for platelet activation and cell distribution by scanning electron microscopy.
After implant viability is established, a longer-term implant term may be employed. We will order a total of 12 male Yucatan minipigs from Sinclair Bioresources. Six of these minipigs will be rendered diabetic by the supplier, while the other 6 will remain as control specimens. Type 1 diabetes is induced using a single alloxan injection, which partially destroys the insulin producing beta cells. Diabetes will be confirmed by daily glucose checks and insulin administration to maintain glucose levels within 300-400 mg/dL. Once these minipigs are stabilized, they will be shipped to us with unilateral jugular vein access ports for painless glycemia monitoring and insulin delivery. Upon arrival to our animal facility, Godley-Snell Animal Research Center, animals will be allowed to acclimate 7-10 days. During this time, baseline blood glucose levels will be established using AlphaTRAK (Abbott Laboratories, Abbott Park, IL). Diabetic minipigs will be monitored on a daily basis for food and water consumption, behavior, and blood glucose (checked in the a.m. before feeding time). Insulin will be administered daily at 0.1U/kg for glycemic levels between 300-400mg/dL. For levels <400mg/dL, daily insulin dosage will be increased by 0.1U/kg for every increase in 50mg/dL.

The same femoral artery scaffolds as discussed previously will be treated sterilized with 0.1% peracetic acid, treated with PGG, and seeded by a process prescribed by George Fercana. ASCs will be isolated and propagated from adipose tissue of the minipigs via lipectomy 2 weeks prior to implant surgery. Endothelialization will be performed in the same manner as the Dynamic ASC Differentiation Studies constructs, with the exception that instead of 1 hour incubation per 90° turn, we will seed ~5x10^6
cells per turn and incubate overnight per turn. For introducing cells into the media of the scaffold, we will seed at a density of $5 \times 10^6$ cells with a syringe and a repeating dispenser (Hamilton Company, Reno, Nevada). Cells will be injected into multiple sites along the graft to ensure adequate, uniform coverage. The adventitia will be seeded in a drop-wise fashion at a density of $5.5 \times 10^6$ cells. After all tunics have been seeded, the construct will be cultured in static conditions for 4 days to allow for sufficient cell attachment and adaptation. Constructs will be implanted, and each minipig will receive one autologously cell seeded vascular scaffold, leaving the contra-lateral artery as control. A summary chart of implants is shown in Table 6.1. Anesthesia will be administered with an intramuscular injection of Ketamin (33 mg/kg), Acepromazine (1.1 mg/kg), Atropine (0.02-0.05 mg/kg), and Buprenex (0.05 mg/kg). Animals will be masked with 4-5% isoflurane in oxygen until intubation. After intubation, animals will be maintained on 2-3% isoflurane and connected to a ventilator. After exposure, the femoral artery will be clamped and heparin will be administered (200 IU/kg) via IV. The grafts will be anastomosed in an end-to-side fashion with continuous 4-0 nylon sutures. After blood flow is re-established, grafts will be analyzed for leaks and abnormalities. Post-operative medications include aspirin (10 mg/kg) once a day for 2 days, Plavix (75 mg/kg) once a day for 1 month, and subermal Fragmin (75 U/kg) once a day for 7 days. Doppler ultrasound will be performed every week for 4 weeks to determine graft patency.
After 4 weeks, animals will be sedated and anesthetized. Heparin (200 IU/kg) will be administered and an overdose of sodium pentobarbitol (1mL / 10 lbs) will be given as a barbiturate. Once the cessation of breathing has been determined, the graft site will be exposed and excised together with ~20mm of native artery attached to the anastomotic sites. All animals will receive humane care according to protocols approved by the Clemson IACUC (AUP#2012-069, approved 1/9/2013) in compliance with NIH guidelines.

For explant analysis we will examine mechanical properties, host cell infiltration, AGE accumulation, calcification, matrix integrity, and inflammation. Mechanical properties will be determined by stress-strain characteristics from biaxial tensile tests and thermal denaturation temperature from DSC. AGE products such as CML and Pentosidine will be determined via IHC and fluorescence, respectively. To determine thrombogenicity, samples (with native artery attached) will be fixed luminal side up in Karnovsky’s Fixative solution and imaged via SEM for platelet activation, thrombus formation, and endothelium confluency. Immunogenicity and inflammation will be determined by performing IHC stains for macrophages and lymphocytes. Calcification will be analyzed by Alizarin Red staining, and other osteogenic markers such as ALP,
osteopontin, and osteocalcin will be analyzed by IHC. Color-based matrix stains such as H&E, Movat’s Pentachrome, and VVG will be used to examine matrix integrity of the graft. Presence of tunic specific cells (endothelial cells, smooth muscle cells, and fibroblasts) will also be examined via IHC.

6.4. References


