MODIFICATION OF AN ENDOVASCULAR STENT GRAFT FOR ABDOMINAL AORTIC ANEURYSM

By

OLAJO-MPO BUSOLA MOLOYE

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2006
Copyright 2006

by

Olajompo Moloye
To my grandparents, parents, and sisters (ese).
ACKNOWLEDGMENTS

I would like to thank my committee members Dr. Batich, Dr. Brennan, Dr. Lee, Dr. Schultz and Dr. Tran-son-Tay for their support through out the years. I would like to especially thank Dr. Batich for all of his support and advice, and for allowing me to learn and implement research on my own. I would like to thank Dr. Lee for all of the support and encouragement, and for scheduling time to see me despite his busy schedule. I would like to thank Dr. Schultz’s for been extremely helpful (words can’t sum it up).

I would also like to thank former members of Batich group, Dr. Leamy, Dr. Santra, Dr. Willenberg, Dr. Bernd and Dr. Albina. I would like to thank all of the Batich group members for their support this includes Taili (twin), John, Anika, Chiwon, Cindy, Nakato and Glenn. I would like to thank all the members of Goldberg’s group and Brennan’s group. I would also like to thank Jennifer Wrighton for all of her help through out the years, Angel for being an angel sent to me from God, Tammy for scheduling meeting times with Dr. Lee, Nina for teaching me how to grow and harvest rabbit vascular smooth muscle cells and Priscilla in Dr. Schultz’s lab. I would like to thank Dr. Bercilli and Dr. Chegini for their encouragement and support through out the years.

I would like to thank the college of engineering, biomedical engineering department, materials science and Engineering department and major analytical instrumentation center (MAIC.). I would be remised if I did not thank Dr. Earle and Mrs. Margie for their encouragement and words of wisdom. I would like to thank my students in the STEPUP program for helping me with my research (Nnenna and Suzana). I will miss all of you.
I would like to thank Fola, my parents, sisters, friends and family in Nigeria for all of their support and prayers. Finally, I would like to thank God for giving me the strength to finish, there were times when I wanted to quit but he kept pushing me.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>10</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>11</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>16</td>
</tr>
<tr>
<td>2 BACKGROUND</td>
<td>22</td>
</tr>
<tr>
<td>The Aorta</td>
<td>22</td>
</tr>
<tr>
<td>Abdominal Aortic Aneurysm (AAA)</td>
<td>23</td>
</tr>
<tr>
<td>Pathological mechanism</td>
<td>24</td>
</tr>
<tr>
<td>Symptoms and Risk Factors</td>
<td>26</td>
</tr>
<tr>
<td>Detection Mechanism</td>
<td>28</td>
</tr>
<tr>
<td>Repair Techniques</td>
<td>29</td>
</tr>
<tr>
<td>History of Open surgery repair</td>
<td>29</td>
</tr>
<tr>
<td>Open Surgical Repair Technique</td>
<td>29</td>
</tr>
<tr>
<td>History of Endovascular Repair</td>
<td>30</td>
</tr>
<tr>
<td>Endovascular Repair Technique</td>
<td>31</td>
</tr>
<tr>
<td>Migration of Stent Grafts</td>
<td>34</td>
</tr>
<tr>
<td>Modification of Stent Grafts</td>
<td>35</td>
</tr>
<tr>
<td>3 MODIFICATION OF DACRON STENT GRAFT WITH POLY (D-L LACTIDE-CO-GLYCOLIDE)</td>
<td>44</td>
</tr>
<tr>
<td>Introduction</td>
<td>44</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>45</td>
</tr>
<tr>
<td>Materials</td>
<td>45</td>
</tr>
<tr>
<td>Modification of the Stent Graft</td>
<td>45</td>
</tr>
<tr>
<td>PLGA Modified Vascular Graft</td>
<td>45</td>
</tr>
<tr>
<td>Release Kinetics Study: Weight Loss and pH Analysis</td>
<td>46</td>
</tr>
<tr>
<td>Mechanical Analysis of the Coated Grafts: Compression Analysis</td>
<td>46</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>47</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>47</td>
</tr>
<tr>
<td>Modification of the Stent Graft</td>
<td>47</td>
</tr>
<tr>
<td>PLGA Modified Vascular Graft</td>
<td>48</td>
</tr>
<tr>
<td>Weight Loss</td>
<td>48</td>
</tr>
<tr>
<td>pH Variation</td>
<td>49</td>
</tr>
<tr>
<td>Mechanical Analysis</td>
<td>50</td>
</tr>
<tr>
<td>SEM Analysis</td>
<td>50</td>
</tr>
<tr>
<td>Conclusions</td>
<td>51</td>
</tr>
</tbody>
</table>
4 EFFECT OF VARIOUS MOLECULAR WEIGHT 50/50 POLY (D, L-LACTIC CO
GYCOLIC ACID) ON THE RELEASE OF BOVINE SERUM ALBUMIN FROM
MODIFIED VASCULAR GRAFTS

Introduction.............................................................................................................................59
Materials and Methods ...........................................................................................................60
Preparation of the vascular graft......................................................................................60
Samples for cell proliferation analysis .....................................................................61
Preparation of the coating solution...............................................................................61
Degradation study of impregnated grafts ......................................................................61
Release kinetics of BSA from impregnated grafts.........................................................62
Scanning electron microscopy (SEM) .................................................................................62
Cellular attachment and proliferation studies (qualitative)...........................................62
Statistical analysis ...........................................................................................................63
Results and Discussion ...........................................................................................................63
Degradation study of the impregnated grafts ...............................................................63
Release profiles of BSA from impregnated grafts ..........................................................64
Scanning electron microscopy.........................................................................................64
PLGA coated grafts and cellular proliferation ...............................................................65
Conclusion ..............................................................................................................................65

5 EFFECT OF SUCROSE ON PROTEIN RELEASE FROM DACRON MODIFIED
VASCULAR GRAFTS

Introduction.............................................................................................................................68
Materials and Methods ...........................................................................................................69
Preparation of vascular grafts...................................................................................69
Preparation of coating solution ................................................................................69
Dip coating ...............................................................................................................70
Release kinetics studies ............................................................................................70
Water uptake analysis ....................................................................................................70
Bicinchoninic acid (BCA analysis ...........................................................................70
Encapsulation efficiency study ................................................................................71
Surface morphology analysis .....................................................................................72
Cell culture ...............................................................................................................72
Human corneal fibroblast culture .............................................................................72
Rabbit vascular smooth muscle cell culture ...............................................................72
Statistical analysis...........................................................................................................74
Results and Discussion ...........................................................................................................74
Coated grafts....................................................................................................................74
Encapsulation Efficiency .............................................................................................74
Surface morphology .......................................................................................................75
Water uptake ................................................................................................................76
Release kinetics .............................................................................................................77
Cellular bioactivity .........................................................................................................78
Conclusion ..............................................................................................................................79
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Types of commercial available stent grafts for EVAR(77-79)</td>
<td>41</td>
</tr>
<tr>
<td>3-1</td>
<td>Types of PLGA modified vascular grafts prepared using a solvent evaporation</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>technique.</td>
<td></td>
</tr>
<tr>
<td>4-1</td>
<td>Amount coated and degraded after 7 days.</td>
<td>66</td>
</tr>
<tr>
<td>4-2</td>
<td>Molecular weight of the polymer used.</td>
<td>66</td>
</tr>
<tr>
<td>5-1</td>
<td>Composition of the aqueous phase used for water in oil emulsion.</td>
<td>80</td>
</tr>
<tr>
<td>5-2</td>
<td>Amount coated based on the different coatings.</td>
<td>80</td>
</tr>
<tr>
<td>5-3</td>
<td>Encapsulation efficiency of BSA-bFGF modified vascular grafts.</td>
<td>80</td>
</tr>
<tr>
<td>6-1</td>
<td>Contents of bFGF modified vascular grafts (n=4)</td>
<td>103</td>
</tr>
<tr>
<td>7-1</td>
<td>Reagents used for the water phase of the coating solution</td>
<td>125</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2-1</td>
<td>Different layers of the artery</td>
<td>39</td>
</tr>
<tr>
<td>2-2</td>
<td>Density of Smooth muscle cells (SMCs) in the tunica media</td>
<td>40</td>
</tr>
<tr>
<td>2-3</td>
<td>Cartoon diagram of a properly sealed stent graft after EVAR</td>
<td>42</td>
</tr>
<tr>
<td>2-4</td>
<td>Cartoon representation of the different types of endoleak</td>
<td>43</td>
</tr>
<tr>
<td>2-5</td>
<td>Gross image of Type I endoleak after EVAR</td>
<td>43</td>
</tr>
<tr>
<td>3-1</td>
<td>Mechanical analysis of modified graft</td>
<td>52</td>
</tr>
<tr>
<td>3-2</td>
<td>PLGA modified stent graft</td>
<td>52</td>
</tr>
<tr>
<td>3-3</td>
<td>Amount coating lost as function of degradation time</td>
<td>54</td>
</tr>
<tr>
<td>3-5</td>
<td>Variation of pH of PBS solution with degradation time</td>
<td>56</td>
</tr>
<tr>
<td>3-6</td>
<td>Mechanical analysis of coated vascular grafts</td>
<td>57</td>
</tr>
<tr>
<td>3-7</td>
<td>Morphological features of modified grafts</td>
<td>58</td>
</tr>
<tr>
<td>4-1</td>
<td>Release profiles of BSA from impregnated vascular grafts</td>
<td>66</td>
</tr>
<tr>
<td>4-2</td>
<td>SEM micrograph of modified vascular grafts</td>
<td>67</td>
</tr>
<tr>
<td>4-3</td>
<td>PLGA coated vascular grafts seed with human dermal fibroblast</td>
<td>67</td>
</tr>
<tr>
<td>5-1</td>
<td>Coated grafts coated</td>
<td>81</td>
</tr>
<tr>
<td>5-2</td>
<td>Water uptake kinetics of PLGA modified vascular grafts</td>
<td>82</td>
</tr>
<tr>
<td>5-3</td>
<td>Water uptake kinetics of modified vascular grafts</td>
<td>83</td>
</tr>
<tr>
<td>5-4</td>
<td>BSA standard for protein analysis</td>
<td>84</td>
</tr>
<tr>
<td>5-5</td>
<td>In vitro release profiles of BSA from modified vascular grafts</td>
<td>84</td>
</tr>
<tr>
<td>5-6</td>
<td>SEM images of B1 modified vascular graft undergoing degradation studies</td>
<td>85</td>
</tr>
<tr>
<td>5-7</td>
<td>SEM images of B2 modified vascular graft undergoing degradation studies</td>
<td>86</td>
</tr>
<tr>
<td>5-8</td>
<td>Cumulative release of BSA from B2 and B4 modified grafts</td>
<td>87</td>
</tr>
<tr>
<td>5-9</td>
<td>Cumulative release of BSA for B3 modified vascular graft</td>
<td>87</td>
</tr>
</tbody>
</table>
5-10 Cellular bioactivity of B1 and B2 modified grafts with HCF cells ...............................88
5-11 Bioactivity of B1 and B2 modified grafts with RVSMC cells. ......................................88
6-1 SEM micrographs of modified vascular graft.................................................................103
6-2 SEM micrograph of cross-sectional image .................................................................104
6-3 pH of supernatant of modified graft B1 and B2 after release kinetics study .............104
6-4 pH of supernatant obtained from PLGA and modified graft without growth factor ...105
6-5 Cumulative release of BSA from B1 and B2 modified vascular graft.........................106
6-6 Amount of bFGF released from each modified vascular graft ...................................107
6-7 Dose response of bFGF on RVSMC proliferation ..................................................108
6-8 Dose response of bFGF on HDF cell proliferation ..................................................109
6-9 Effect of bFGF released from modified vascular graft on HDF cell proliferation ....110
6-10 Effect of bFGF released from modified vascular graft on RVSMC proliferation ......111
6-11 Comparison of the effect of supernatant obtained from B1 and PLGA modified vascular grafts on RVSMC ....................................................................................112
6-12 Optical images of HDF cells seeded onto modified vascular grafts ........................113
6-13 Optical images of RVSMC on modified vascular grafts. ............................................114
7-1 Amount coated onto Dacron modified vascular graft ...........................................125
7-2 Water uptake and percent weight loss for CTGF modified vascular graft .................126
7-3 pH of supernatant obtained during release kinetics study ........................................126
7-4 SEM micrographs of coated grafts and grafts after release studies ............................127
7-5 SEM micrograph of cross section images .................................................................128
7-6 Cumulative release of BSA from C1 modified graft ..................................................128
7-7 Cumulative release of CTGF from modified vascular graft .....................................129
7-8 Effect of C1 supernatant on HDF proliferation .......................................................129
7-9 Effect of C1 supernatant on RVSMC proliferation ..................................................130
7-10 Effect of supernatant obtained from C1, C2 and C3 modified grafts on RVSMC proliferation......................................................................................................................131
7-11 Effect of various treatments on RVSMC migration. ..........................................................132
7-12 Effect of supernatant obtained from C1 on RVSMC migration. ........................................132
7-13 Optical images of HDF cells seeded onto modified vascular grafts...............................133
7-14 Optical images of RVSMC cells seeded onto modified vascular grafts..........................134
8-1 Effect of CTGF, bFGF and PLGA on vascular cell proliferation........................................141
Endovascular surgery is currently used to treat abdominal aortic aneurysms (AAA). A stent graft is deployed to exclude blood flow from the aneurysm sac. It is an effective procedure used in preventing aneurysm rupture, with reduced patient morbidity and mortality compared to open surgical repair.

Migration and leakage around the device (“endoleak”) due to poor sealing of the stent graft to the aorta have raised concerns about the long-term durability of endovascular repair.

A preliminary study of cell migration and proliferation is presented as a prelude to a more extensive in vivo testing. A method to enhance the biological seal between the stent graft and the aorta is proposed to eliminate this problem. This can be achieved by impregnating the stent graft with 50/50 poly (DL-lactide co glycolic acid) (PLGA) and growth factors such as basic fibroblast growth factor (bFGF) or connective tissue growth factor (CTGF), at the proximal and distal ends. It is hypothesized that as PLGA degrades it will release the growth factors that will promote proliferation and migration of aortic smooth muscle cells to the coated site, leading to a natural seal between the aorta and the stent graft. In addition, growth factor release should promote smooth muscle cell (SMC) contraction that will help keep the stent graft in place at the proximal and distal ends.
It is shown that a statistically significant effect of increased cell proliferation and migration is observed for CTGF release. Less of an effect is noted for bFGF or just the PLGA. The effect is estimated to be large enough to be clinically significant in a future animal study.

The long term goal of this study is to reduce migration encounter after graft deployment and to reduce secondary interventions of EVAR especially for older patients who are unfit for open surgical treatment.
CHAPTER 1
INTRODUCTION

According to the 2000 Census Bureau report, approximately 35 million people age 65 years and older live in the United States (US) (1). This represented a 12% increase since 1990. This number is projected to increase as the numbers of baby boomers reaching the age of 60 will increase in the next five years. Recently, President George Bush reported that an estimated 78 million of Americans will reach age 60 in the next few years. According to a study performed by MetLife, this aging population will represent 20% of the US population in 2030 (2). As the population rate increases, the number of patients with chronic diseases affecting the elderly will also increase. Thus, a need for medical intervention in the improvement of medical devices and drugs currently in the market will also increase. This will vastly help improve the quality of life of this aging population.

However, the medical environment is reluctant to undergo change in the medical technology field (i.e., in vivo drug delivery devices) due to the rigorous testing required by the Food and Drug administration (FDA) in approving products that are biocompatible and effective. In addition, the cost of testing the new devices for approval bars current biotechnology companies from production. A change is necessary in the medical device field because it would improve the quality and longevity of millions of people. Ample data and understanding of the mechanism of various diseases (i.e., basic science) has promoted this change. Products designed in the twentieth century in preventing diseases have been found to last 5-15 years due to the failure analysis and biocompatibility issues. As technology rapidly progresses and the number of baby boomers reaching the age of 60 years increases, the need for change or improvement in drug delivery applications and medical devices is needed.
The need for improvement is particularly acute in the cardiovascular disease arena. Cardiovascular disease is the leading cause of death in the US. In 2001, the Center for Disease Control and Prevention (CDC) reported that approximately $300 billion was spent on all cardiovascular diseases (1). This expenditure is projected to increase as the aging population steadily grows.

Abdominal aortic aneurysm (AAA) is one of the types of cardiovascular disease that affects the elderly. This disease is more common in patients over the age of 65 especially Caucasian males. According to Zarins et al., the prevalence of this disease is projected to increase in the next 5-10 years as baby boomers reach the age of 65 years and above (3). It’s the 17th leading cause of death in the US (4) AAA results in an estimated 15,000 deaths and 63,000 hospitalizations in the US each year (5). The male to female ratio with this disease is 4:1. The incidence of AAA increases between the age of range of 55-59 and 60-64, respectively. AAA is the 14 leading cause of death in males ages 55-59 and 11th in males 65-70 (1). In addition, an increase in the mortality rate is due to aortic rupture observed as the aneurysm expands. AAA is defined as a permanent localized dilation of an abdominal artery having at least a 50% increase in diameter compared with the expected normal diameter of the artery or the diameter of the segment proximal to the dilation (6). There are many speculations as to the cause of this disease; however the pathologic mechanism of this disease is not well understood. Nevertheless, general consensus on the histological examination of human AAA reveals a disorganized elastic lamina and disappearance of well-organized smooth muscle layers (6-10).

Open and endovascular surgery are two surgical procedures that are currently used to treat AAA after detection. Treating the aneurysm with an open surgical repair technique replaces the aorta with a vascular graft (i.e., Dacron vascular graft). For this procedure, the
proximal and distal ends of the aneurysm site are clamped. The aneurysm site is then excised and replaced with Dacron graft (11). Major problems encountered during and/or after this procedure include the increase blood loss during the surgical procedure, increase hospital stay, susceptibility to infection, medical cost and risk of mortality incidence in patients over the age of 70. Due to the considerable risks associated with open surgical repair, less-invasive treatment options with stent grafts are preferred.

Endovascular repair (EVAR), is a minimally invasive technique which excludes the aneurysmal site from further growth or rupture. This surgical technique is commonly used to treat at-risk patients, patients over the age of 65 years and patients with an aortic diameter greater than 5.5cm. EVAR is also preferred procedure among young healthy patients (i.e., patients below the age of 65) with this disease because of the type of technique. For this procedure, an endovascular stent graft consisting of Dacron or a poly tetrafluoroethylene (PTFE) vascular graft with a metal stent (i.e., nitinol or stainless steel) is deployed to the aneurysm site. The advantages of this less invasive procedure include minimal blood loss during the procedure and the reduced hospital stay. However, there are various drawbacks to this technique; this includes endoleaks, migration, kinks, patient-specific and cost due to the maintenance of continuing long-term function of EVAR (12). Endoleak is the leakage of blood from the stent graft. This results in an inadequate seal of the stent graft to the artery. The presence of this problem is used to determine if the repair technique is successful (13). If the repair technique fails, the aneurysm can continue to expand, leading to stent graft migration and aortic rupture. Surgical conversions with extension cuffs or open surgical procedure are ways in which the endoleak is repaired if detected early.
Modification of the stent grafts with hooks and barbs, altering the design of the stent, changing the material from which the stent is made, and the impregnation of grafts are ways in which the endovascular stent grafts have been improved. However, these modifications have not eliminated the presence of endoleak and migration (14).

In this research, the impregnation of a Dacron vascular graft with growth factors that slowly elute with the help of biodegradable polymeric matrix is proposed to stimulate the migration and proliferation of vascular cells between the stent graft and the aorta. The materials used in the modification of this graft have been previously approved for use by the FDA making it easier for approval. It is hypothesized that cellular proliferation and migration stimulated by the growth factor will promote a natural seal between the aorta and the stent graft.

In this dissertation, an in vitro analysis of the modified vascular graft that has drug delivery capability will be addressed. The following are goals that were followed in order to successful modify Dacron vascular graft:

1. To successful modify an endovascular stent graft with a biodegradable polymer. In order to achieve this goal, we prepared a solution of 50/50 Poly (DL-lactic-co glycolic acid) (PLGA) solution and dip coated the stent graft five times. Scanning electron microscope was used to observe the morphology of the modified stent graft after coating. The part of the stent graft that was modified (i.e., the stent or vascular graft) was used for further analysis.

2. To determine the mechanical properties of the modified vascular graft with a stent. This study was particularly important because we wanted to ensure that our optimal vascular graft will have similar compression forces.
with current Dacron stent grafts. The coating solution and amount of coating for the modified vascular graft with similar mechanical properties as an unmodified stent graft was used for further analysis.

3 To choose an ideal molecular weight of 50/50 PLGA that will slowly release the growth factor after modification. Various molecular weight 50/50 PLGA varying from 39kDa to 144kDa was used to prepare the coating solution for the vascular graft. Bovine serum albumin was used as a model protein to determine the effect of the various molecular weights of PLGA on protein released. The protein released was study for a period of 28 days. The polymer that slowly released the protein was used for further analysis.

4 To impregnate the vascular graft with growth factors. The growth factor used for these studies was connective tissue growth factor (CTGF) and basic fibroblast growth factor (bFGF). Various coating solution was prepared for the impregnation of the vascular grafts with growth factors. The coating solution that released the growth factor after the first day was used for further analysis.

5 To determine the bioactivity of growth factor impregnated in a cellular environment. The effect of the supernatant obtained after release studies and impregnated within the vascular graft on smooth muscle and fibroblast cell (FC) proliferation and migration was studied. This two different cell types were chosen because of their roles during vascular healing. Smooth muscle cells (SMCs) migrate to the perigraft space to promote tissue
ingrowth during the healing process. Some of the migrated cells (SMCs) differentiate into myofibroblast, so as to promote fibrosis. The supernatant obtained during the release studies for the two modified vascular graft was used to treat the cells. Its effect on cellular proliferation was measured by using a cell proliferation assay (Cell titer 96 aqueous for cell proliferation), while a modified Boyden chamber was used for migration analysis. Sterilized modified grafts were seeded with SMCs and FCs to analysis its effect on cell proliferation. The growth factor that enhanced cellular proliferation and migration was then proposed for further analysis in an in vivo environment.
CHAPTER 2
BACKGROUND

The Aorta

The aorta is the most important and largest artery of the body. It supplies oxygenated blood from the heart to other parts of the body. It also reduces cardiac workload by absorbing energy as blood is ejected from the heart. As oxygenated blood leaves the aortic arch it travels through the thoracic aorta and then to the abdominal aorta. The abdominal aorta supplies blood to the abdomen, iliac arteries and the legs. Fifty-four percent of cardiac output reaches the abdominal aorta (15), while two thirds of this cardiac output supplies the iliac arteries which supplies blood to peripheral arteries (i.e., legs). The diameter of a normal aorta is between 20-30 mm depending on gender and age.

Tunica intima, tunica media and tunica adventia are the three layers that make up the aorta (See Figure 2-1). Tunica intima is the innermost layer of the aortic wall that contacts the blood. The lining intima consists of endothelial cells with variable quantities of underlying cells and matrix elements (16). Endothelial cells in this region elongate in the direction of blood flow.

Tunica media is the thickest layer of the aortic wall. It typically measures 1mm in the segment distal to the renal arteries (16). The tunica media is separated from the intima by internal elastic lamella which is made of concentrated elastic fibers. The media is characterized by elastic fibers and vascular smooth muscle cells (SMCs) which gives the aorta circumferential resilience (16). It contains a small number of fibroblasts and extracellular matrix (ECM). SMCs cells direct the production of aortic wall elastin. Elastin, an array of interstitial collagen fibers, proteoglycans and glycosaminoglycans makes up the ECM. ECM sustains the strength and elasticity of the aortic wall. Elastin is the most abundant extracellular protein in the aortic wall. It is made of amorphous elastin and it’s deposited on a skeleton of microfibrillar proteins. SMCs
are responsible for the synthesis of elastin lamellae of the aortic media. Collagen is the second major structural protein of the aortic wall accounting for approximately 20% of the total protein in the normal aorta. The aortic wall is made of mostly Type I and Type III collagen in the ratio of 3:1. Type IV collagen is also present in the aorta. Type IV can be found within the basement membrane surrounding endothelial cells and medial SMC (17). Medial SMCs and fibroblasts in the adventitia synthesize interstitial collagen. Collagen is responsible for maintaining the aortic wall tensile strength while the elastin is responsible for elasticity. The media of the thoracic aorta contains more elastin than collagen while abdominal aorta contains more collagen than elastin (16, 18). As we age, most of the elastic fibers found in the tunica media are lost due to aging. This causes the aorta to stiffen.

Tunica adventitia is the outermost layer of the aorta that support and protect the vessels. An external elastic lamella delineates the adventitia from the media. Tunica adventitia contains perivascular connective tissue that contains fibroblast cells, vasa vasorum and nerves. Fibroblast cells that are present in the adventitia synthesize interstitial collagen type I and III. Vasa vasorum supplies the nutrients to the other layers of the aorta while the nerves regulate the function of the medial smooth muscle function. Vasa vasorum is however absent in the abdominal aorta (19). Therefore this aorta receives its nutrients from the lumen through diffusion.

**Abdominal Aortic Aneurysm (AAA)**

The first case of AAA was described over 2000 years ago by Anytullus (20). AAA can be termed the “disease of the old” because it’s prevalent in people over the age of 65. This disease is common in Caucasian males compared to males of other ethnicity. Aneurysm of the aorta can be referred to as bulging, dilating or ballooning of the aorta. There are many speculations as to the cause of this disease; the pathologic mechanism of this disease is not yet
well understood. Nevertheless, general consensus on the histological examination of human AAA reveals a disorganized elastic lamina and disappearance of well organized smooth muscle layers. Moreover, characteristic findings of AAA include degradation of medial elastin and interstitial (type I and III) collagens in the media and adventitia, chronic infiltration of inflammatory cells, local production of proinflammatory cytokines, apoptosis of SMCs resulting in the thinning of medial wall and proteolytic disruption of the tunica media (7, 21-27).

**Pathological mechanism**

Inflammatory cells such as macrophages, T-lymphocytes and plasma cells have been cited as hallmarks of AAA development. These cells are suggested to originate from the adventitial side of the artery (17). Proinflammatory cytokines produced by these cells such as Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β) and interleukin-6 (IL-6), have been shown to down regulate 1α(I) procollagen expression (28). An increase in IL-6 has been found in early aortic dilation of AAA patients. However, there is no correlation with the increase in IL-6 concentration and growth rate of aortic aneurysm. Greater levels of Interleukin 10 (IL-10) have been detected in AAA tissues (29). Moreover, inflammatory cells (i.e., macrophages) produced by aneurysm have been noted to express matrix proteinases such as matrix metalloproteinase 9 (MMP-9). MMP 9 is a 92 kDa gelatinase (gelatinase B) that degrades numerous extracellular matrix components of the vessel. MMP 9 is suggested to degrade vessel wall components such as elastin and type IV collagen. Greater amounts of MMP-9 have been found in human AAA tissue, especially moderate aortic aneurysms 5-7 cm in diameter (30). MMP 9 and MMP 2 have been reported to be necessary in aneurysm formation, especially in the stimulation of aneurysm in an animal model. MMP-2, gelatinase A, is a 72 kDa proteinase that is expressed by mesenchymal cells (i.e., vascular smooth muscle cells) and produced in small
quantities in macrophages and fibroblast (31). When vascular smooth muscle cells undergo apoptosis, MMP 2 turns off its protective effect, exacerbates inflammation and promotes aneurysm formation (32). MMP 2 has the ability to degrade both elastin and fibrillar collagen which are highly organized in the lamellar structure of aortic media. On a molecular level, MMP 2 has been found to act as a collagenase that initiates the cleavage of the triple helix into one-quarter and three-quarters length (32). The single alpha chains formed from the degradation effects of MMP 2 are then degraded by MMP 9 which releases coiled elastin, causing it to become fattened and attenuated. In MMP-9 knock-out mice, the infusion of macrophages stimulated aneurysm formation (32). This suggests that the presence of MMP 2 and MMP 9 are necessary for AAA generation. Furthermore, MMP 9 expressed by macrophages initiated the aneurysmal changes of the aortic wall. Clinical studies of human AAA have noted that MMP 2 promotes the expansion of smaller AAA’s while MMP 9 increases the risk of rupture (6).

SMCs are the most abundant cell type in the aortic media. The loss of SMCs triggered by the activation of immune cells has been linked to weakening of the aortic wall which ultimately leads to aortic rupture (9). Apoptosis of vascular SMCs has been observed in degenerate tunica media of AAAs (7, 8, 10, 27). The loss of SMCs at the media can impair the synthesis of matrix proteins needed for repair.

Compared to normal aortas, SMCs in the aneurysm dilated region reveal a disrupted and disorganize pattern in medial layer. According to numerous investigators, the density of SMC is significantly low in the aneurysmal neck compared to an occlusive aorta (7, 10, 27). Lopez-Candales et al., reported that the loss of SMC by a factor of four leads to an increase in aortic diameter greater than 5.5 cm, this causes the wall thickness to increase two-fold compared to the normal aorta (10).
Figure 2.2 shows a comparison of SMC density in normal, atherosclerotic, and aneurysm aortas.

**Symptoms and Risk Factors**

The lack of understanding the origin of this disease doesn’t help in the clear identification of the symptoms. A few of the patients diagnosed with this disease experience common symptoms such as constant abdominal pain, backache, fatigue and the feeling of fullness or pulsation in the abdomen. Since these symptoms are common in everyday people, patients with this disease do not link these symptoms with AAA development. Therefore, the majority of AAAs results in aneurysm rupture. Symptoms linked to aneurysm rupture include sudden severe pain, paleness, rapid pulse, dry mouth/skin and excessive thirst, anxiety, nausea and vomiting, lightheadness, excessive sweating and shock. Patient characteristics such as the history of smoking, history of myocardial infarction, increased weight and claudication may raise clinical suspicion of AAA (33).

The risk factor of AAA includes smoking, genetic predisposition, chronic obstructive airway disease and hypertension. Smoking is a major risk factor for the development and rupture of AAA. According to the center for disease control’s (CDC) report of the surgeon general in 2004, smoking remains the leading cause of preventable death and negatively impacts people at all stages of life (34). The duration of smoking affects the risk of aneurysm formation. According to numerous reports the risk of death from AAA increases four to five fold in current smokers compared with lifetime smokers and two-fold in former smokers (35, 36). Smoking has been found to increase the growth rate of small aneurysms by 0.07 cm per year in smokers (16). The depth of inhalation of smokers is another aspect that increases the risk of developing a large AAA. The risk of a large aortic aneurysm increased two-to-three fold for those who inhale
moderately into the lungs and four-fold for those who inhale deeply into the lungs (37). These findings suggest that smoking initiates aneurysm development by affecting elastin degradation.

A small group of patients with AAA have been positively identified with having a first-degree relative with this disease. Identification of these patients has suggested that a genetic component of this disease might exist. Efforts in identifying the gene have resulted in the detection of genetic mutations. A single base mutation which resulted in the substitution of arginine for glycine at position 619 in collagen type III molecule was identified in a single family with a history of aneurysms (16). Heritable diseases of connective tissue, such as Marfan syndrome and Ehlers-Danlos syndrome (EDS) have been linked with AAA. Marfan syndrome results from a mutation in the gene which codes for fibrillin. This genetic mutation causes the aortic media to weaken and dilate resulting in dissecting aneurysms. While EDS is a rare disorder it is associated the spontaneous rupture of large arteries (16). Diagnosis of AAA due to family history is uncommon.

Chronic obstructive pulmonary disease (COPD) has been related to the development of aortic aneurysm. In a study performed by Reilly and Tilson, the presence of COPD was found to have predictive effect on aneurysm rupture (38).

Hypertension has also been identified as a risk factor for AAA. This risk factor does not have as strong association with this disease as smoking. Patients over the age of 65 with long term history of hypertension undergoing treatment have a high frequency of developing AAA. Vardulaki et al., showed that hypertensive treated patients had an 80 percent risk of AAA compared with subjects who were never on medication. Also, an increase in the absolute growth rate of aortic aneurysm was also observed (39).
Detection Mechanism

AAA is usually diagnosed during a physical examination that is unrelated to aneurysm formation. Imaging modalities such as ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI) and angiography are ways in which AAA can be detected. Ultrasonography is the standard tool for screening patients with AAA and commonly used to detect AAA during routine physical examinations (40-42). The advantage of using this method includes sensitivity close to 100%, specificity of 96%, preferred detection technique among patients, provides physiological data and structural details of the vessel wall, gives accurate information of the aneurysm size in the longitudinal and cross-sectional directions, and can be used as a detection tool to follow the progression of AAA (41, 43, 44). However, it cannot be used to determine the relationship between renal arteries and AAA (43). It is also less expensive than CT or MRI.

Valuable and detailed information of the aneurysm prior to AAA repair is obtained with CT scan (33). This method employs the use of a contrast agent and ionization radiation as a means to obtain cross sectional images of the aorta and other structural aspects of the body. It provides accurate measurements of the aortic diameter and length necessary for aneurysm repair and exclusion. It’s also used to determine the relationship between celiac, superior, mensteric, renal and iliac arteries to the aneurysm as well as adjacent organs. Nevertheless, the exposure to radiation, cost, nephrotoxcity and suboptimal visualization of the origins of the aortic branch vessels are some of the drawbacks of this method (44). MRI, in addition to magnetic resonance angiographic (MRA), is a new method that is been currently used for AAA diagnosis. Due to its unavailability, cost and inaccuracy, MRA is not used for AAA detection or repair planning. Angiography with the help of a calibrated pigtail catheter can be used to characterize the aneurysm and iliac vessels (45). It can also be used for diagnosis purposes.
Repair Techniques

Open surgical and endovascular repair techniques are currently used for the treatment of aortic aneurysm. The selection of a suitable repair technique depends on the anatomy of the aneurysm (i.e., diameter, angulation), age and the wellbeing of the patient.

History of Open surgery repair

Open surgical technique is an invasive procedure used for AAA repair. AAA was described 2000 years ago and the first operative repair was reported 300 years after. Between the 18\textsuperscript{th}-19\textsuperscript{th} century, materials such as coil wires and cellophane wrapping were used to repair or prevent rupture of AAA (20). Even though all of these materials were excellent choices for AAA repair during the early centuries, they were however unsuccessful in the prevention of aneurysm rupture. A successful repair technique for AAA was not discovered until 1951 when Dubost and colleagues replaced an aneurysm aortic segment with frozen cadaver homograft (46). An attempt to use homografts to replace a ruptured aneurysm failed in 1953 (47). In 1952, Voorhees and colleagues reported the durability of a polyester material known as Vinyon-N for the replacement of the dilated segment (48). Dacron fabric (polyethylene terephthalate, PET) was introduced later on by Debakey in 1954 for the replacement of AAA (49). This fabric is commonly used for the replacement of small and large blood vessels.

Open Surgical Repair Technique

After Dubost et al., success and the introduction of Dacron, open surgical repair (OR) became the standard technique for AAA repair for patients of all ages. This repair technique is an invasive procedure in which the aneurysm is exposed through a transperitoneal or retroperitoneal approach, and replaced with a vascular graft. These two approaches require a midline incision, in which the aneurysm is exposed. The aneurysm is then cut longitudinally and a prosthetic tube or bifurcated graft is sutured in place to exclude the aneurysm. Bifurcated
grafts are used when an aneurysm is observed in the iliac arteries. Dacron or ePTFE are two prosthetic grafts used for the repair process. Knitted Dacron grafts impregnated with collagen or albumin are used for OR. After the graft is sutured in place, the aneurysm tissue is then wrapped around it. This procedure is currently performed in patients with moderate to excellent functional capability, below the age of 65, and aneurysm diameter below 5 cm (50, 51). This technique is effective in preventing aneurysm rupture, absence of graft complications, and can be performed in patients with various angulations of aneurysm. In addition, this procedure requires no follow-up for radiological studies and can be performed with mortality rates of 5% to 10% (13-15). Some of the shortcoming of this procedure includes, increased hospital stay, blood loss and morbidity rate (i.e., gastrointestinal and cardiac complications). An increase in the morbidity rate (i.e., >30%) was observed in patients over the age of 65 with medical complications (16-17). Due to the considerable risks associated with open surgical repair for this group of patients, less-invasive treatments options with stent grafts are preferred.

**History of Endovascular Repair**

In 1976, Parodi and colleagues, proposed the modification OR technique due to an increase in mortality and morbidity rate of patients that were at a significant risk of aneurysm rupture. These patients were regarded as older patients who could not tolerate standard surgical rupture. Parodi et al., interest in the amendment of this procedure stimulated the design of a minimal invasive device known as an endovascular stent graft. In 1991, the first successful endovascular repair procedure with an animal (canine) model and initial clinical trial results with five patients was reported (52). The first successful stent graft or endograft used consisted of a knitted Dacron fabric graft sutured onto a modified Palmaz stent that prevented it from kinking.
Endovascular Repair Technique

Since the introduction of this device, it’s become a preferred technique for “at risk” patients and patients who wish not to undergo open surgical repair procedure even though their qualifications for OR is excellent.

There are various commercialized endografts currently used for aneurysm repair, four of which are approved by the Food and Drug Administration (FDA) in the United States. These devices differ based on the device body, graft and metal exoseleton. There are two types of device bodies: unibody and modular (53). The one piece enodgraft or unibody device is easier to deploy during aneurysm repair. However, it requires contrateral occlusion and bypass grafting. Modular devices are composed of multiple pieces that are deployed through the groin. They are more flexible compared to unibody devices and are commonly used in the endograft market. Dacron (woven) and expanded polytetrafluoroethylene (ePTFE) are two grafts currently used as fabric for endografts. One difference between the two grafts is the degree of porosity (ePTFE > Dacron) which is suggested to affect the rate of type IV endoleaks. Most of the endografts use a metal skeleton that is made of stainless steel, nitinol or cobalt/chromium.

The five stent grafts that are approved for use in the US are AneuRx, Ancure, Excluder, Zenith and Powerlink stent grafts. Ancure and AneuRx stent grafts were the first two commercialized stent grafts that were approved for use in 1999. Excluder, Zenith and Powerlink are third generation stent grafts that were approved in 2003 and 2004. Table 2-1 contains the different stent grafts, approval date, type, support system and manufacturing materials.

Endovascular aortic repair (EVAR) is a minimal invasive technique that excludes the aneurysmal site from further growth or rupture through the deployment of a stent graft. The stent graft is inserted into the aorta via the femoral artery and deployed to the aneurysm site with the top of the stent close to the renal artery ostia (54). Successful deployment of the stent graft to
exclude the aneurysm is performed under fluoroscopic guidance. A radiopaque-marked blackboard or ruler is sometimes placed under the patient for reference measurements.

The performance of this procedure on patients is extremely selective and it’s based on various anatomical criteria. These criteria include (55):

1. The proximal neck must be undilated.
2. Angulation between the suprarenal aorta and proximal neck greater than 60°.
3. Limited tortuosity for the iliac vessels.
4. The size of the common and external iliac arteries must be sufficient to allow the introducer sheath.
5. The distal implantation site must be adequate for attachment.

Long term durability and success of EVAR is uncertain. Durability of this procedure depends on the presence of endoleaks, migration, kinking and dislodgement of the grafts. All of these problems, if left untreated by extension cuffs or conversion to open surgical repair can result in aneurysm rupture or graft occlusion (20, 56).

Successful treatment of also EVAR depends on the achievement of an effect “seal” between the endograft and normal vessels above and below the aneurysms (see figure 2-3) (57). Persistent blood flow to aneurysm sac is an indication of the failure of EVAR treatment. Persistent flow or perigraft flow of blood to aneurysm sac due to the endograft is known as endoleaks. It occurs in as many as 44% of all EVAR patients (58). Endoleaks can be classified into four different categories (see figure 2-4). They are (59-62):

a) Type 1 endoleaks: Inadequate seal to the attachment site (see figure 2-5).
   a. Type 1a: Inadequate seal to the proximal segment of the endograft.
   b. Type 1b: Inadequate seal at the distal site where the endograft is placed.
b) Type 2 endoleaks: retrograde inflow and outflow between patent branch vessels that feed the sac which includes lumbar artery-to-lumbar artery and inferior mesenteric-to-lumbar artery circuits.

c) Type 3 endoleaks: midgraft endoleaks through fabric holes or inadequate seal between the endograft components.

d) Type 4 endoleaks: transgraft flow due to graft porosity.

The management of endoleaks depends on the type observed. Type I endoleak is the most common endoleaks experienced after EVAR (57). Type I and type III endoleaks can be treated by deploying an extension cuff in addition to the stent graft previously deployed. However, if the problems persist then an open surgical repair technique is performed to prevent aneurysm rupture. Type II endoleaks are treated by the insertion of an embolization coil to the branch vessels. Sometimes when type II and IV endoleaks are not repaired they resolve on their own (53).

The frequency of endoleak depends on the type of device being used and the experience of the operative team in patient selection and implantation technique. Table 2-2 displays a list of complications associated with currently approved stent grafts are listed. In a study performed by AbuRahma et al., to analyze the clinical implications of endoleaks by computed tomographic angiography and color duplex ultrasound, 46% of endoleaks detected were type I endoleaks, 49% type II endoleaks and 2% for type IV endoleaks. Most of the endoleak documented were related to AneuRx stent graft (21%) compared to Ancure (14%) and Excluder (17%) stent grafts. A majority (13%) of the endoleak occurred early (i.e., within 1 month) while late endoleak occurred in 4 % (63).
Successful treatment of EVAR depends on the continuous monitoring of the stent graft with a variety of techniques such as arteriography, pressure monitoring, CT scan and duplex scanning. The cost to continuously monitor the location of the stent graft after deployment is one of the limitations of this technique. The progress of the stent graft healing is monitored monthly to yearly, depending on the patient’s age and the period of that some of complications that might have occurred after surgical repair. Complications such as the one previously mentioned, are normally observed during the normal checkup of an EVAR patient. Aortic neck dimensions, graft size mismatch, age of patient and smoking status (ex-smokers) have been suggested to cause endoleakage (57).

**Migration of Stent Grafts**

According to numerous reports, 15-30% of patients treated with endovascular stent graft experience migration during the first year of AAA repair (56, 64, 65). This might be due to dilation or shortness of the aneurysmal neck. Other migration problems might occur despite satisfactory neck anatomy, graft sizing, and stent graft deployment (56).

Stent graft migration is a recognized complication of AAA exclusion. The dislodgement of stent grafts ranges from minor (5-10 mm) to severe migration with complete descent of the stent graft into the aneurysm sac requires late conversion to open surgery.

Moreover, endovascular stent grafts frequently migrate away from the renal arteries due to natural elongation of the infrarenal aortic segment and an inadequate attachment of the proximal stent-grafts (66). Other factors that have been associated with migration include technical flaws, such as stent graft placement in a conical, heavily calcified or circumferentially thrombus lined neck; inadequate stent attachment to the vessel wall; and lack of columnar strength; and the most common reason was attributed to the dilation of the proximal neck (17, 64, 67).
Biological incorporation of endovascular grafts seems insufficient to always resist the pulsatile forces present inside the aorta. However, little or no vascular adherence is observed in the graft fiber during secondary conversion or after the death of an EVAR patient. The presence of collagen IV and laminin in the extracellular matrix is minimal after EVAR. Perigraft space between the graft and the native vessel wall has also been observed after 2.5 years of stent graft implantation (56). Grafts easily detached after the lightest touch and held in place by simple mechanical devices used at the original operation (hooks). Histopathological data from this study clearly indicated that the defective healing processes resulted from the lack of sufficient healing oriented tissue elements (fibroblasts and smooth muscle cells). This is essential for adequate tissue incorporation to the stent graft.

**Modification of Stent Grafts**

Different techniques to reduce the risk of migration of EVAR have been studied. The utilization of hooks and barbs at the proximal and distal graft are among the techniques used to prevent migration. Researchers have shown that hooks and barbs improve the fixation of endovascular grafts by ten fold in experiment models. However, they have been reports that a potential danger as a result of hooks and barbs, is the penetration to juxtaaortic structures such as the duodenum and the renal vein (68). Proximal stent graft migration (45%) of at least 5 mm has been reported for endograft designed with hooks and barbs after a 29-month follow up study (56).

Oversizing of the stent graft is routinely performed to ensure adequate proximal seal and to compensate for future conformational changes in the proximal and distal necks. However, an increase in aortic diameter has been observed during this routine period. A study performed to analyze the size of the aortic necks of patients after EVAR revealed a 20% increase in neck diameter (17).
Enlargement of the neck may predispose to graft migration and proximal seal zone failure (PSF). Aortic extension cuffs to maximize neck coverage as been used to eliminate migration and PSF if discovered prior to aneurysm reperfusion and rupture. The use of extender cuffs, in AAA patients has however, lead to a significantly higher rate of PSF (69).

**Modification of Vascular Grafts**

Unmodified Dacron grafts have been attributed to the inadequate seal experienced by EVAR patients (56). Dacron grafts have an inhibitory effect on endothelial cell proliferation and in the transinterstitial growth of fibroblast, SMCs and capillaries. It also reduces the contraction elements caused by fibroblast and collagen (70).

Attempts have been made in modifying vascular grafts surface with biological glue, growth factors and biodegradable polymers for the reduction of endoleak and migration encounter by stent grafts. The use of biological and synthetic glues as coating agent of vascular grafts promoted cell attachment; however, the non endothelialized surface became more thrombogenic due to the exposure of blood cells to the glue (71). In addition, liquid adhesive n-butyl cyanoacrylate (n-BCA) has been used for the prevention of proximal migration of the stent graft observed during type 1 endoleak. n-BCA was unable to treat distal type 1 endoleak of aneurysmal iliac arteries. The use of n-BCA in addition to EVAR is not without problems because of its tendency to undergo premature polymerization or delay the withdrawal of the delivery catheter which can result in the gluing of the catheter tip in place. In addition, further study on the effect of n-BCA adhesive on endotenion, biocompatibility with aneurysm sac and, graft fabric over time and the effect of sac remodeling has not been performed (72).

The incorporation of Dacron graft with bioresorbable grafts and the sole use of bioresorbable materials for large diameter vascular grafts have been studied. In 1972,
Ruderman’s group evaluated a woven graft composed of 24% Poly (L-lactic acid) and 76% Dacron they were implanted into aorta of dogs. After 100 days of implantation, all prosthesis was found to be patent with extensive tissue in-growth (73). Construction of woven prostheses containing Polyglycolic (PGA) and polyglactin 910 (PG910) plus Dacron components resulted in significant tissue ingrowth and inner capsule cellularity. Significant tissue ingrowth and inner capsule cellularity were observed in woven yarns containing 100% PG910, 100% PGA compared to % PG910 and 20% Dacron (5). The biodegradable yarns elicited transinterstitial migration and proliferation of primitive mesenchymal cells that differentiated into smooth muscle-like myofibroblasts and repopulation of confluent endothelial like cells that paralleled the time course of macrophage-mediated prosthetic dissolution. The ability of absorbable polymeric grafts to promote proliferation and migration of mesenchymal cells is promising; however, a total dissolution of the prosthesis is not desirable for AAA repair.

Failure of the absorbable graft to retain the structure of the artery after dissolution has promoted researchers to look into the area of growth factors (cytokines) absorption on Dacron graft. Takahasi modified Dacron vascular grafts through the adsorption of bFGF on to the prosthesis. Less than 2% of the initial bFGF adsorbed on to the graft, 40% of it was released in the first 24 hours and the rest for a period of 2 weeks. In addition, a slight migration of fibroblast and capillary blood vessels were observed on the outer layer of the graft (74).

In an attempt to modify vascular graft for EVAR, Van der Bas et al., used collagen impregnated vascular grafts with bFGF to stimulate the ingrowth of aortic vascular cells. A washout effect of the growth factor was observed in the first 3 days. However, this didn’t affect the release of bFGF factor from the modified vascular graft and its effect on tissue ingrowth. The modified vascular graft was able to release 5ng/24 hour of bFGF for a period of 28 days. In
vitro studies of the graft in aortic organ induced neointima ingrowth and formation after 28 days (75). In-growth of tissue and healing between the graft and the aorta was also observed in an in vivo study conducted for a period of 8 weeks. Microscopic evaluation demonstrated α-smooth muscle cell actin positive cells growing from the vascular wall through the graft material (76). The washout effect experienced during in vitro analysis didn’t affect the release of the growth factor in vivo. Similar amounts of growth factor were released for both studies. In addition, blood flow didn’t affect the release of growth factor in vivo. bFGF released from bFGF-impregnated grafts placed down stream from the control grafts, and it released from the impregnated grafts for a period of 5 weeks. The growth factor released also induced neointima formation between the aorta and stent after 4 weeks implantation (76). Dacron fabric was well incorporated into the vessel wall.
Figure 2-1   Different layers of the artery (Adapted from www.lab.anhb.uwa.edu.au/.../Images/VesWall.jpg) (not drawn to scale).
Figure 2-2  Density of Smooth muscle cells (SMCs) in the tunica media. Medial SMCs density is based on the number of cells (SMCs) counted per high power field (HPF). *p<0.01 significant differences between AAA tissue and normal abdominal aorta  Adapted from Lopez-Candales et al., AJP March 1997; 150(3):993-1007.
<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
<th>FDA approval</th>
<th>Type of Body</th>
<th>Support system</th>
<th>Types of graft</th>
<th>Stent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancure</td>
<td>Guidant</td>
<td>September 1999</td>
<td>Unibody</td>
<td>Hooks and barbs at the aortic iliac attachment sites</td>
<td>Woven Dacron</td>
<td>Nitinol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Removed 2003)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AneuRx</td>
<td>Medtronic</td>
<td>September 1999</td>
<td>Modular bifurcated</td>
<td>Radial and columnar support</td>
<td>Woven Dacron</td>
<td>Nitinol</td>
</tr>
<tr>
<td>Excluder</td>
<td>W.L. Gore and Associates</td>
<td>November 2003</td>
<td>Modular, 2-component system</td>
<td>PTFE (less porous)</td>
<td>Nitinol</td>
<td></td>
</tr>
<tr>
<td>Zenith</td>
<td>Cook</td>
<td>May 2003</td>
<td>Modular 3-component system</td>
<td>Anchoring barbs</td>
<td>Woven Dacron</td>
<td>Stainless steel</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Gianturo Z-stents)</td>
</tr>
<tr>
<td>Powerlink</td>
<td>Endologix</td>
<td>October 2004</td>
<td>Unibody</td>
<td>Columnar support</td>
<td>PTFE (low porosity)</td>
<td>Cobalt chromium</td>
</tr>
</tbody>
</table>
Table 2-2  Late complications reported with various endografts (80).

<table>
<thead>
<tr>
<th></th>
<th>AneuRx</th>
<th>Talent</th>
<th>Zenith</th>
<th>Excluder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoleak at 1 month</td>
<td>13.9%</td>
<td>14%</td>
<td>11%</td>
<td>22%</td>
</tr>
<tr>
<td>6 months</td>
<td>13.7%</td>
<td>12%</td>
<td></td>
<td>17%</td>
</tr>
<tr>
<td>1 year</td>
<td>13.9%</td>
<td>10%</td>
<td></td>
<td>7%</td>
</tr>
<tr>
<td>Aneurysm rupture</td>
<td>0.2%</td>
<td>1%</td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>Aneurysm expansion</td>
<td>11.5% (4 yrs)</td>
<td>0%</td>
<td></td>
<td>14.4% (2 yrs)</td>
</tr>
<tr>
<td>Conversion to open surgery</td>
<td>0.9%</td>
<td>2.8%</td>
<td>4%</td>
<td>2%</td>
</tr>
</tbody>
</table>

Note: Enough data for zenith stent grafts were not available before the publication of this article.

Figure 2-3  Cartoon diagram of a properly sealed stent graft after EVAR. Top of stent graft is proximal to the aneurysm sac (Not drawn to scale).
Figure 2-4  Cartoon representation of the different types of endoleak.

Figure 2-5  Gross image of Type I endoleak after EVAR. Obtained from (www.vacularlaparoscopy.net/index.php?pr=Phot)
CHAPTER 3
MODIFICATION OF DACRON STENT GRAFT WITH POLY (D-L LACTIDE-CO-
GLYCOLIDE)

Introduction

Endovascular stent graft repair of an abdominal aortic aneurysm (AAA) is a minimally-invasive alternative to conventional open surgical treatment of this lethal condition. Stent grafts are typically constructed of either Dacron or ePTFE surgical “graft” bound to an endo- or exoskeleton of nitinol or stainless steel alloy “stent”. One of the most significant long term complications related to endovascular AAA repair is the caudal migration (dislodgement) of the stent graft from its proximal fixation site and development of an acute endoleak and possible rupture (67, 81). Reports of migration and endoleak have been related to the lack of adequate tissue incorporation between the stent graft and the aortic wall (81, 82). Biomodification of the stent graft to promote tissue ingrowth at the sites of fixation may significantly reduce the risk of this devastating complication.

AneuRx (Medtronic, Sunnyvale, California), was one of the first stent grafts approved for AAA exclusion. According to a statistics provided by Medtronic Corporation., AneuRx has been implanted in over 45,000 patients since its approval in 1999 (83). Endoleak, migration of the stent graft and fabric tear are the major problems associated with this stent graft. The modification of AneuRx stent graft with a biodegradable polymer will be discussed in this chapter.

Copolymers of lactic acid and glycolic acid (PLGA) are among a few of the biodegradable polymers currently approved by the FDA for use in pharmaceutical products or medical devices (84). PLGA have been used in various drug targeting or prolonged drug applications because of its biocompatible and biodegradable properties (85). In an in vivo environment PLGA is metabolized/excreted via normal physiological pathways (86). PLGA has been used to modify
medical devices such as an intracranial aneurysm stent. The modification of intracranial stents resulted in the tissue incorporation of the stent with the blood vessel (87).

This chapter will highlight the feasibility of stent graft coating with PLGA, its release kinetics in phosphate buffered saline (PBS, pH 7.4) at 37°C and mechanical properties of the modified graft.

Materials and Methods

Materials

50/50 PLGA with inherent viscosity of 0.59 dl/g in hexafluorosuporopanol was obtained from Lactel Absorbable Polymers (Birmingham, AL); methylene chloride was obtained from Fisher Scientific (Pittsburgh, Pennsylvania) and phosphate buffer saline solution (pH 7.4) from Mediatech (Herndon, VA), AneuRx stent graft (Medtronic) was provided by Dr. Lee and Cooley Low Porosity Vascular grafts (size: 4 x 4 in) were obtained from Boston Scientific (Wayne, NJ).

Modification of the Stent Graft

The distal end of an AneuRx stent was modified by embedding the stent graft in 50/50 PLGA solution. Briefly, the stent graft (4cm length, 14 mm inner diameter) was dip-coated in 10 (w/v) % 50/50 PLGA coating solution. The coating solution was prepared as follow: 10 wt% amorphous 50/50 PLGA was weighed and dissolved in methylene chloride. The solution was continuously stirred. The stent graft was then dip-coated five times in the PLGA solution. The coated graft was left to air dry in a dust free environment for 24 hours and placed in a vacuum oven to remove any excess solvent for additional 24 hours. Morphological feature of the modified stent graft was then observed.

PLGA Modified Vascular Graft

Dacron vascular grafts were modified using a modified solvent casting technique, as previously described (88). Briefly, 50/50 PLGA solution in methylene chloride with a known
concentration was prepared. Woven vascular grafts (2 by 0.5 cm) were prepared by cutting with scissors. The gravimetric weight of the uncoated grafts was then noted. The grafts were then dip-coated in a known amount of the solution. The samples were then air-dried for 24 h and subsequently placed under high vacuum (20 um Hg) for 24 h to remove any remaining solvent. Four types of modified grafts prepared using this method is detailed in Table 3-1. The weight of the coated grafts was recorded after drying.

**Release Kinetics Study: Weight Loss and pH Analysis**

Coated grafts were placed in a 1.5 ml micro-centrifuge tube containing PBS (pH 7.4). The tubes containing the grafts were stored in a hybridized incubator undergoing constant rotation at 37°C. PBS was changed on the first day, day 7, day 14, 21 day and day 28. The pH of the supernatant collected was monitored during the course of degradation. At the end of each time point, three samples were removed from PBS, air dried overnight and vacuum dried for 24 h. The weight of the three samples was recorded. The following equation was used to determine the percent of weight lost for the three different samples.

\[
\text{Percent weight lost} = \frac{W_f - W_c}{W_c} \times 100
\]  

(3-1)

\[W_f=\text{final weight after drying}\]
\[W_c=\text{weight of graft after coating}\]

All measurements were expressed as means ± standard deviation relative to initial values.

**Mechanical Analysis of the Coated Grafts: Compression Analysis**

For compression analysis, coated vascular grafts were placed in between a nitinol stent (1 cm x 4cm, i.e., in the middle) with forceps. Shimpo Digital force gauge (FGV-5X, Itasca, IL) was used to obtain the force needed to compress the modified grafts. To obtain the compression force, the digital force gauge was placed on a stand, in an upside down position (Figure 3-1).
The flat attachment head was then screwed onto the attachment site for compression testing. The stent–graft containing, nitinol with modified graft was placed on a Styrofoam plate. The compression force to compress 50% of diameter of the nitinol stent with vascular graft (~0.5 mm) was recorded. Ten runs were performed on each graft to ensure the accuracy of this technique. Nitinol stent only and unmodified grafts were controls for this study. The compression force of the coated grafts was expressed in Newton (N). Compression analysis of the modified vascular graft was performed with the same displacement.

**Scanning Electron Microscopy (SEM)**

The morphology of the modified stent graft and vascular graft, and the degraded grafts were obtained using a JEOL 6500 scanning electron microscope. Modified stent graft and vascular graft were coated with gold-palladium by a sputter coater and examined at 15 kV. Degraded samples were air-and vacuum dried, and their surfaces were prepared for observation with the SEM.

**Statistical analysis**

All data values are reported as mean ± standard error about the mean (SEM). The statistical significance of difference was determined using one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison post test. Graph Pad software (Graph Pad, San Diego, CA) was used for this analysis. Differences were considered significant at p<0.05.

**Results and Discussion**

**Modification of the Stent Graft**

Modification of the stent graft with 50/50 PLGA solution was observed on the graft and not the stent (See Figure 3-2). The failure to observe any modification change on the stent might be likely to due the fact that the stent needed to be surface modified/pre-treated in other for the a
hydrophobic substrate to the adhere to the coating. Based on this finding, all subsequent studies were performed with the graft and not the stent.

**PLGA Modified Vascular Graft**

Using a modified solvent casting techniques previously described to modify Dacron vascular grafts, four types of PLGA impregnated grafts were manufactured. The amount coated on the grafts dip coated five times were $5.65 \pm 0.55$ for P5_5 and $22.30 \pm 2.35$ for P10_5; and the grafts dip coated ten times were $19.63 \pm 5.35$ and $31.82 \pm 6.82$. Increasing the number of times that the vascular graft was modified results in an increase in the thickness of the film coated on the graft.

The changes in thickness of the grafts after coating were 0.29 to 0.31 mm for P5_5; 1.01 to 1.03 mm for P5_10 and P10_5, and 1.5 to 1.61 mm for P10_10. The adhesion of the film formed by polymer coating onto the Dacron have been reported to be purely mechanical (89).

**Weight Loss**

Dacron vascular grafts were impregnated with different concentrations of PLGA solution. Grafts impregnated with higher concentration PLGA, dip-coated 10 times were stiffer than grafts dip coated five times. While grafts impregnated with a lower concentration PLGA solution were a little stiffer than non-coated grafts, but weren’t as stiff as the 10 (w/v) % 50/50 PLGA coated grafts.

The amount lost for the modified grafts were similar to each other. Initially, the weight remained relatively constant for several days; then a dramatic decrease in mass was observed. The weight of P5_5 modified graft was 95% at day 1, which significantly reduced to 18% by 4 weeks due to a significant decrease in mass after 2 weeks. The weight of the grafts coated 10
times with 5(w/v) % (P5_5) was 95% at day. Twenty five percent and 16% of the day 0 mass remained on P10_5 and P10_10 respectively. A triphasic phase was observed for the degradation of P10_10 modified grafts (see figure 3-3). An increase in the amount coated was observed between day 1 and 3 was observed. After the 3rd day, a dramatic decrease in the polymer coating was observed for this modified graft. This might have resulted from an increase in the water uptake of grafts coated with a more polymeric solution. This phenomenon was previously demonstrated with PLGA films manufactured by solvent casting (88). In a study reported by Lu et al., the degradation of films with thicker film coating was more rapid compared to thin films. A greater extent of the degradation of thick films might be due to an autocatalytic effect. Thus, the reduction in the degradation rate of vascular grafts coated with P5_5 might have been due to a thin film coating on the surface of the graft (see figure 3-4).

**pH Variation**

Little change in pH of PBS was measured up to 8 days for the modified grafts (figure 3-5). This was followed by a rapid drop in the pH due to the release of acidic polymer degradation in the solution. The rapid pH drop is due to the hydrolysis of the polymer coating which corresponds to the weight displayed in figure 3-1.

A 10% reduction in the weight of P5_5 grafts caused the pH of the release media to fall below pH 7 (~ pH 6.6). The drop in pH below 7 was also evident in P5_10 modified vascular graft. Change of pH in the various modified grafts is due to the acidic environment used to catalyze the degradation of PLGA. Li et al., suggested that once the degradation passed some characteristic time (indicated by the mass loss) the pH begins to increase back to neutral range (90).
**Mechanical Analysis**

In this study, one of the most important analyses was the compression of the modified vascular grafts. This was important because the mechanical property determines if the modified grafts can be deployed easily from the catheter to the aneurysm site while EVAR repair is being performed (68).

Compression force of grafts P5_5 modified graft was similar (within 5%) of an unmodified vascular graft. However, grafts coated 10 times and modified with higher concentration PLGA were significantly different from unmodified vascular grafts (figure 3-6). The grafts coated with higher concentration and coated ten times were stiffer than unmodified vascular grafts. This might hinder the deployment of the modified stent graft during EVAR repair.

**SEM Analysis**

Coating of the vascular graft five times with 5% PLGA solution resulted in a thin coating. (See figure 3-7). Increasing the number of times that the modified grafts were dip-coated, and the concentration of the polymeric solution used for coating, resulted in a thicker coating on the surface of the modified vascular graft. Observation of the coated weaved fibers when lower concentration polymer solution was used disappeared when the concentration and the amount of coating increased. Increase in concentration of the coating solution and amount coated changed the permeability of the fibers. Rapid deterioration of the physical properties of the coated vascular grafts with fewer coating was observed in figure 3-7. Degradation of the coating of the vascular grafts underwent heterogeneous bulk degradation.

According to Schulz et al., surface modification of poly ethylene fibers does not have any effect on its molecular or supermolecular strength, and it doesn’t affect the tensile properties of the material (91). Surface modification envelopes the fibers with a polymer layer. This was observed in the modified vascular grafts in this study.
Conclusions

Poly (DL-lactic-co glycolic acid) (50:50) modified vascular grafts were prepared by dip coating the vascular grafts in various concentrations of coating solution and with different amounts of coating. The effect of these two constituents on the *in vitro* degradation and mechanical studies of the vascular graft was determined. The concentration of the polymer and number of times the grafts were dip coated in the polymer solution affected the weight loss, pH and compression force of the modified vascular graft. The compression force of grafts modified with 10 (w/v) % PLGA solution and 5 (w/v) % dip coated 10 times were significantly different from the unmodified vascular graft. Over the course of four weeks study, most of the coating on the vascular graft was lost. The rest of the coating might be found within the interstices of the graft, which might degrade during a vigorous agitation. Further studies in the modification of vascular graft with drug delivery capabilities would be performed with 5% PLGA solution, dip coated five times.
Figure 3-1  Mechanical analysis of modified graft.

Figure 3-2  PLGA modified stent graft.
Table 3-1  Types of PLGA modified vascular grafts prepared using a solvent evaporation technique.

<table>
<thead>
<tr>
<th>Modified graft Code</th>
<th>PLGA concentration (w/v)%</th>
<th>Times dip coated</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5_5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>P5_10</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>P10_5</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>P10_10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 3-3  Amount coating lost as function of degradation time.
Figure 3-4  Morphological changes of grafts undergoing degradation. a) P5_5 modified graft at day 14; b) P5_5 modified graft at day 28; c) P5_10 modified graft at day 14; d) P5_10 modified at day 28.
Figure 3.5  Variation of pH of PBS solution with degradation time. The different types of PLGA films are presented in Table 3.1. Error bars represent means ± SEM for n=4.
Figure 3-6  Mechanical analysis of coated vascular grafts (* p<0.01, ** p<0.01) Significant difference between the stent only graft compared to grafts coated with 5% PLGA dip-coated 10 times and 10% coated grafts dip coated 10 times and 5x. In addition, significant differences occurred when stent with uncoated graft was compared to the 5% coated graft dip coated 10 times and 10% coated grafts dip coated 5 and 10 times.
Figure 3-7  Morphological features of modified grafts (2.0 kV) a) Plain graft, 80x, b) P5_5 (5(w/v) % coated vascular graft dip-coated 5 times), 80x, c) P5_10, (10(w/v) % coated vascular graft dip coated 5 times), 80x, and d) P10_10 (10 w/v)% coated vascular graft dip coated 10 times), 23x.
CHAPTER 4
EFFECT OF VARIOUS MOLECULAR WEIGHT 50/50 POLY (D, L- LACTIC CO-
GYCOLIC ACID) ON THE RELEASE OF BOVINE SERUM ALBUMIN FROM MODIFIED
VASCULAR GRAFTS

Introduction

Since its introduction in 1954 by Debakey, polyethylene-terephthalate (PET), Dacron, has
become the preferred device for replacing medium to large caliber arteries. Knitted and woven
are two types of Dacron prosthesis used in blood vessel repair.

Knitted vascular grafts are constructed from PET yarns that are interloped around each
other. These grafts are highly porous and facilitate tissue ingrowth within the intersices. The
leakage of blood from the vascular graft remains a primary concern due its porosity. Grafts are
generally preclotted to minimize blood loss (63). However, the impregnation or coating of
knitted Dacron grafts with absorbable biological materials has resulted in a leak proof prosthesis
with excellent handling characteristics for replacement of large caliber vascular prosthesis.

Low porosity vascular grafts, woven Dacron grafts, are produced by the wrap and weft of
two sets of yarns that are interlaced at right angles of each other. Woven Dacron vascular grafts
are preferred by surgeons because of its high bursting strength, low permeability to liquids,
minimal tendency to deform under stress and less proneness to kinking. Yet, woven Dacron
vascular grafts often elicit a poor healing response. It’s only used for the repair of thoracic aorta,
abdominal aortic aneurysm repair and patients with coagulation defects.

Biodegradable polymers such as poly lactide, poly glycolic acid and poly lactide-co-
glycolic acid (PLGA), have been incorporated into woven Dacron vascular graft to improve
blood vessel healing. The combination of Dacron vascular graft with vicyrl grafts has resulted in
an increased collagen formation in the descending aorta of pigs (82). However, the blend of
these two fibers has resulted in inhibitory arterial regeneration. The objective of this study was
to develop a vascular graft with drug delivery capabilities that will potentially stimulate the regeneration of vascular cells. A biodegradable system in which a drug can be encapsulated and continuously released as the degradation proceeds will be used to impregnate woven Dacron vascular grafts. Bovine serum albumin, (BSA), a model protein will be incorporated in this modified vascular graft. The effect of the different molecular weight of a 50:50 PLGA on the continuous release of the impregnated protein and the effect of polymer coating on cellular proliferation and adhesion will be evaluated.

**Materials and Methods**

Poly (D, L-lactide-co-glycolide) 50/50 with inherent viscosities of 0.39 dl/g, 0.59 dl/g and 0.82 dl/g in hexafluoroisopropanol was obtained from Lactel Absorbable Polymers (Birmingham, AL). BSA, fraction V, was purchased from Sigma (St. Louis, MO). Magnesium hydroxide (Mg (OH)₂) was purchased from Sigma-Aldrich (St. Louis, MO). Methylene chloride (Dichloromethane) and Phosphate buffer saline solution (PH 7.4) were purchased from Fischer Scientific (Fair Lawn, MJ), Bradford reagent assay and Cooley Low Porosity Vascular grafts 4 x 4 in were obtained from Boston Scientific (Wayne, NJ).

**Preparation of the vascular graft**

2 by 0.5 cm samples of vascular grafts were obtained from the 4” by 4” Cooley low porosity woven vascular graft produced by Boston Scientific.

Four samples were prepared for each experiment. The differences in samples are based on the different coating used to modify the vascular graft. This in described in the preparation of the coating solution section. The uncoated weight for each graft was recorded. Out of the 4 samples, 1 was used to study the morphological studies and the rest were used for a release kinetics study.
Samples for cell proliferation analysis

A stencil was used to trace \( \frac{3}{4} \)" diameter circle on the sheet of woven Dacron vascular graft. The circles were then cut using a scissor. Samples were rinsed in 70% ethanol and air dried before dip coating in the PLGA solution.

Preparation of the coating solution

The coating solution was prepared based on an established primary emulsion, water-in-oil emulsion, used in the preparation of protein encapsulated microspheres (83, 84). The oil phase consisted of 5(w/v) % of 50/50 PLGA dissolved in methylene chloride. The aqueous phase containing 0.5 mg/ml BSA solution and 3 (w/v) % Mg(OH)\(_2\) was prepared in phosphate buffer saline (PBS). 1 ml of the BSA solution with Mg(OH)\(_2\) was emulsified in 10 ml of PLGA solution under an ice bath. This resulted in a milky solution. Each of the uncoated grafts was then dip coated in the emulsified solution 5X at 15 seconds intervals. The coated graft was then left to air dry in a dust free environment for 24 hours and vacuum-dried for 24 hours to remove excess solvent.

Degradation study of impregnated grafts

The initial weight \( (W_i) \) of the coated grafts was recorded before the degradation study. The grafts were then rinsed in PBS for 15 minutes. Impregnated grafts were then placed in a 1.5 ml test tube containing PBS in a hybridized incubator undergoing constant rotation at 37°C. After 7 days, grafts were removed from the test tube and vacuum dried for 24 hours. The final weights of the grafts \( (W_f) \) were then reported after drying. The percent of coating lost after 7 days was determined by the following equation:

\[
\text{Percent loss} = \frac{W_i - W_f}{W_i} \times 100
\]  
\( (4-1) \)
Release kinetics of BSA from impregnated grafts

After coating, grafts were rinsed in PBS for 15 minutes. They were then placed in a 1.5 ml microcentrifuge tube containing PBS. Samples were then placed in a hybridized incubator undergoing constant rotation at 37°C. Supernatant (500 μl) from each test tube was removed and replaced with fresh PBS at 12 hours, 24 hours, 3 days, 7 days, 14 days, 21 days and 28 days. The concentration of BSA released over time was analyzed using a Bradford protein reagent assay. Absorbance of the sample with the reagent was read within 1 hour at 595 nm using UV-2410 PC spectrophotometer (Shimadzu). All samples and standards were assayed four times.

Scanning electron microscopy (SEM)

The morphology of the impregnated grafts and uncoated grafts were studied before and after incubation in PBS using a JEOL 6400 scanning electron microscope. Samples were fixed onto stubs using a carbon coated double adhesive conductive tape, and coated with gold-palladium. The voltage was set to 15.0 KV for observation.

Cellular attachment and proliferation studies (qualitative)

Human dermal fibroblast cells (K5) were cultured in RPMI medium containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. PLGA coated vascular grafts were sterilized under ultraviolet light for 1 hour. Sterilized grafts were then rinsed in serum containing medium and placed in 6-well plate. 1.5 ml of the cultured medium was added to each well before cell seeding.

Cultured cells (i.e., 70-80% confluent) were then trypsinized and seeded onto the modified grafts, ~ 2 x 10^4 cells.

Modified vascular grafts seeded with HDF were placed in then placed in a humidified environment, 5% CO₂ at 37°C, for 48 hours.
After 48 hours of incubation, grafts were removed from each well plate and rinsed with PBS without calcium and magnesium. Seeded grafts were then placed in a Petri dish containing 3.7% formaldehyde for fixation. The grafts were fixed in the formaldehyde solution for 30 minutes. They were then rinsed with deionized water. Fixed grafts were then stained with hematoxylin for 2 minutes, washed with tap water and stained with eosin of 5 minutes. Concentrated ethanol solution (95%) was the final wash for the fixed and stained grafts. Proliferated grafts on the modified grafts were observed using a Zeiss Axioplan Imaging microscope. Cells on the modified graft were imaged with 5 and 10x objectives.

**Statistical analysis**

All data values are reported as mean ± standard error about the mean (SEM). The statistical significance of difference was determined using one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison post test. Graph Pad software (Graph Pad, San Diego, CA) was used for this analysis. Differences were considered significant at p<0.05.

**Results and Discussion**

**Degradation study of the impregnated grafts**

The first water in oil emulsion used in the manufacturing of protein loaded PLGA microspheres was used to coat the vascular grafts. The water emulsion consisted of a model protein, BSA.

The amount of coating on the vascular grafts and weight loss after 7 days of incubation can be found in Table 4-1. PLGA with the same composition but varying molecular weight is used in the preparation of coating solution used in the impregnation of the vascular grafts. The amount of coating on vascular grafts increased with the molecular weight of the polymer. This phenomenon was observed in PLGA microspheres produced with the same composition but
varying molecular weights. Microspheres prepared with lower molecular weight PLGA degraded faster and encountered a rapid release of the drug (85).

**Release profiles of BSA from impregnated grafts**

Figure 4.1 shows the release profile of BSA from the impregnated grafts as a function of incubation time. The concentration of BSA released from the modified vascular graft was calculated based on standard concentration of the protein.

BSA released from the different impregnated vascular grafts depended on the molecular weight of the polymer. The molecular weight of polymer used in the preparation of the emulsion used for vascular graft modification can be found in table 4-2. Grafts impregnated with lower molecular weight 50/50 PLGA (i.e., 0.39 dl/g) exhibited a higher initial burst of protein release during the first 12 hours compared to the other modified grafts. This indicated that some of the protein was absorbed on the surface of the modified graft.

The trend of BSA release from impregnated vascular grafts was the same for coated grafts prepared with 0.59 dl/g and 0.82 dl/g 50/50 PLGA. An average of 44% of the BSA impregnated in mid-high molecular weight polymer (i.e. 0.59 dl/g and 0.82 dl/g) was released within 12 hours after placing the graft in scintillation vial containing PBS at 37°C and constant rotation. In addition, a linear increase of BSA release was observed between 12 hours and 3 days. Most of the BSA impregnated in the graft was released after the seventh day. The rest of BSA in the impregnated graft was slowly released until the complete resorption of the polymer.

**Scanning electron microscopy**

Coating of the vascular graft can be observed in figure 4-2. PLGA coating was observed within the interstices and the surface of the woven Dacron vascular graft.

The morphology of the graft coated with mid-high molecular weight polymers (i.e., 0.59 dl/g and 0.82 dl/g) is smoother than the graft coated with the lower molecular weight polymer.
Coating of the graft with higher molecular weight polymer resulted in a thicker coating compared to the other grafts.

A porous and uniform coating was observed when the graft was coated with water in oil emulsion containing BSA and 50/50 PLGA (0.82 dl/g). A mesh-like coating of the BSA impregnated vascular graft was observed at higher magnification. An increase in porosity of the impregnated vascular grafts is examined during release kinetics in PBS.

**PLGA coated grafts and cellular proliferation**

Impregnating woven Dacron vascular grafts with different molecular weights of 50/50 PLGA does not have an effect on HDF proliferation after 48 hours of incubation (See Figure 4-3). The cells were well spread out on the grafts and proliferated.

**Conclusion**

An initial burst in BSA release was observed in vascular grafts impregnated with low molecular weight 50/50 PLGA and this might be due to surface coating of the protein on the vascular graft. Vascular grafts impregnated with mid to high molecular weight PLGA (i.e., 0.59dl/g and 0.82 dl/g) exhibited a similar trend for the release of protein. Most of the protein embedded in the vascular grafts was released by day 7. The coating of woven Dacron vascular graft with 0.82 dl/g was smooth and uniform. Cells incubated on PLGA modified vascular grafts adhered and proliferated within 48 hours.
Table 4-1  Amount coated and degraded after 7 days.

<table>
<thead>
<tr>
<th>Sample (inherent viscosity)</th>
<th>Amount coated (Avg.± SEM) Mg</th>
<th>Amount loss after 7 days (Avg ± SEM) mg</th>
<th>Percent loss after 7 days (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.39 dl/g</td>
<td>2.84 ± 0.05</td>
<td>0.93 ± 0.20</td>
<td>33</td>
</tr>
<tr>
<td>0.59 dl/g</td>
<td>5.30 ± 0.39</td>
<td>0.80 ± 0.15</td>
<td>16</td>
</tr>
<tr>
<td>0.82 dl/g</td>
<td>5.60 ± 0.21</td>
<td>1.17 ± 0.07</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 4-2  Molecular weight of the polymer used (from DURECT Corporation (www.duret.com)).

<table>
<thead>
<tr>
<th>Inherent viscosity (dl/g)</th>
<th>Molecular weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.39</td>
<td>38,900</td>
</tr>
<tr>
<td>0.59</td>
<td>75,000</td>
</tr>
<tr>
<td>0.82</td>
<td>144,100</td>
</tr>
</tbody>
</table>

Figure 4-1  Release profiles of BSA from impregnated vascular grafts, (n=4)
Figure 4-2  SEM micrograph of modified vascular grafts. a) uncoated vascular graft, b) 0.39 dl/g coated vascular graft, c) 0.59 dl/g coated vascular graft d) 0.82 dl/g coated vascular graft, e) BSA impregnated vascular graft and f) BSA impregnated vascular graft after 1 day of release kinetics study.

Figure 4-3  PLGA coated vascular grafts seed with human dermal fibroblast. a) 0.59 dl/g and b) 0.82 dl/g 50/50PLGA coated vascular grafts.
CHAPTER 5
EFFECT OF SUCROSE ON PROTEIN RELEASE FROM DACRON MODIFIED VASCULAR GRAFTS

Introduction

The success of protein release and bioactivity depends on its stability when encapsulated in a biodegradable polymer matrix. Various studies have reported that PLGA microspheres can cause physical or chemical degradation of protein during polymer degradation (84, 90, 95-97). An acidic microenvironment is generated when the polymer undergoes degradation. The degradation of protein in a polymeric matrix (i.e., PLGA microspheres or millicylinders) affects its rate of release and bioactivity to stimulate cell proliferation. Various formulation strategies have been reported to prevent the protein denaturing. These include the addition of stabilizing agents such as proteins, sugars, chelating agents and inorganic salts (92, 98).

Sucrose is a well known protein stabilization excipient (70, 94, 99-103). It has been used for the stability and release of bovine serum albumin (BSA) from PLGA microspheres, and to retain the structure of basic fibroblast growth factor (bFGF) in its solid state in PLGA millicylinders (92). Sucrose has also been shown to affect the water uptake of polymeric matrix during protein release. Increasing the concentration of sucrose has been suggested to increase the viscosity of the aqueous pores of PLGA millicylinder during BSA release (92). This resulted in the slow release of the protein.

In this chapter the effect of sucrose on the water uptake of the polymeric matrix, the release of BSA and the bioactivity of bFGF encapsulated within the modified vascular graft will be discussed. In addition, the effect of acetone on the modification of vascular graft will be addressed.
Materials and Methods

Bovine serum albumin, magnesium hydroxide, sucrose, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Company. PLGA copolymer (50:50 DL, lactide:glycolide, inherent viscosity 0.82 dl/g) was obtained from DURECT Corporation. Methylene chloride, acetone, Dulbecco modified media eagle minimum, non-essential amino acids, sodium pyruvate, penicillin and streptomycin, phosphate buffer saline (pH 7.4), and fetal bovine serum were purchased from Fisher.

Preparation of vascular grafts

Vascular grafts were prepared as previously stated in Chapter 4.

Preparation of coating solution

The preparation of the coating solution was similar to the solvent evaporation method previously described in chapter 4; however, various additives including growth factor (bFGF) was added. In addition, acetone was used to dissolve the polymer used in the preparation of one of the coating solution. Five different coating solutions were prepared for modification of the vascular graft. Four of the five coating solution (B1-B4) contained: 5 (w/v) % PLGA 50/50 (inherent viscosity 0.82 dl/g) was dissolved in methylene chloride (organic phase). All of the water soluble constituents (water additives) were prepared in phosphate buffer saline (PBS) solution (pH 7.4). 400 μl of additives solution was then added to 100 μl of BSA solution. The final solution was then added to the PLGA solution. The two solutions were emulsified by ultrasonication (60% power output) for 30 seconds under an ice bath. The emulsified solution was then continuously stirred until further use.

For B5, 5 (w/v) % PLGA (50/50) solution was prepared by dissolving the polymer in acetone. The water-in-oil emulsion was then prepared as previously stated with a difference in BSA and sucrose concentration (see Table 4-1).
**Dip coating**

The samples were dip coated five times after fifteen second intervals of air drying while the coating solution was stirred continuously with forceps. After coating the samples, they were held with alligator clips and placed on a Styrofoam paper to air dry for about 5 minutes.

The coated samples were then placed in a glass Petri-dish to air dry in a dust free environment for 24 hours and subsequently vacuum dried for 24 hours to remove excess solvent. Coated grafts were stored in a desiccator under vacuum before use.

**Release kinetics studies**

The release kinetics study is similar to the one previously mentioned in chapter 4. The differences between this study and the one performed in chapter 4 are as follow: the amount of medium added, period of study and the change of release medium. For this release kinetics study, 1000 μl of PBS without calcium and magnesium was added to the microcentrifuge. After the indicated time period, 500 μl of the release medium was collected and replaced with 500 μl of fresh PBS. The release medium was stored at -20°C for further analysis.

**Water uptake analysis**

Three samples were removed at the end of the time interval for water uptake analysis. Each sample was blotted with kim-wipes and weighed to obtain the wet weight, W_{d,w}. The samples were then vacuum-dried overnight. The dry weight of the samples was obtained after drying W_d. The water uptake of the coated samples was obtained by:

\[ \frac{W_{d,w} - W_d}{W_{d,w}} \times 100 \]  

(5-1)

**Bicinchoninic acid (BCA analysis)**

The amount of BSA released at the indicated periods previously mentioned in the release kinetics study was analyzed using QuantiPro™ BCA Assay Kit (Pierce, IL).
Release medium that was frozen after the release kinetics study was thawed. Samples were vortexed to mix the supernatant. 25 μl of the release medium was placed in wells of a 96 well plate and 200 μl of BCA solution was added to each well plate. The plate was incubated for 30 minutes at 37°C for colorimetric development. The absorbance of the colorimetric solution was obtained at 590 nm using a Wallac microplate reader (Perkin-Elmer, MA). The concentration of BSA released from the modified grafts was based on the standard curve. Each sample was read in triplicate.

**Encapsulation efficiency study**

The coated sample was placed in a 1.5 ml test tube containing 500 μl of acetone. Sample was vortex for 1 minute to dissolve the coating on the vascular graft. After vortexing, the graft was removed from the test tube. The solution was then centrifuged for 30 minutes at 3000 rpm, to obtain the precipitate. The supernatant was then removed. To evaporate the solvent, the microcentrifuge tube was partially left open. The pellet, containing the protein, was then reconstituted with PBS (100 μl). The concentration of the BSA coated onto each graft was determined by BCA analysis. The encapsulation efficiency was determined by the following equation:

Theoretical protein loaded =

\[
\frac{(\text{Total protein added})}{(\text{polymer} + \text{excipients} + \text{protein})}
\]  \hspace{1cm} (5-2)

Actual protein loaded = \(\frac{(\text{protein content})}{(\text{amount of coating})}\)  \hspace{1cm} (5-3)

Efficiency = \(\frac{(\text{actual})}{(\text{theoretical})}\) * 100  \hspace{1cm} (5-4)

Amount of coating =

\[
\text{protein +polymer +excipient (i.e., the amount coated on the vascular grafts)}
\]  \hspace{1cm} (5-5)
Surface morphology analysis

The morphology of the grafts after coating and release kinetics was observed using a scanning electron microscope (JEOL 6500). Grafts were place on aluminum stubs and gold-palladium coated. Micrographs of the samples were observed at 500X, 1000X and 5000X magnification and 15 kV.

Cell culture

Human corneal fibroblast culture

Human corneal fibroblast cells (HCFC) were obtained from Dr. Schultz’s laboratory. The cells were cultured in a tissue culture flask (T-75) containing cultured medium. The Cultured medium consisted of Dulbecco modified media eagle minimum (DMEM) supplemented with L-glucose and L-glutamine without sodium pyruvate containing, 10% fetal bovine serum (FBS); penicillin (100 U/ml) and streptomycin (100 U/ml). The cells were grown at 37 °C in a humidified atmosphere of 5% CO2 in air. For experiments, HCFC at passage 4- 8 were used. The culture medium was changed every 2 days.

Rabbit vascular smooth muscle cell culture

Frozen vials of rabbit vascular smooth muscle cells (RVSMC) were obtained from Dr. Bercilli’s laboratory. The cells were explanted from rabbit aortas. The cells were then thawed and cultured in a T-25 culture flask containing RVSMC culture medium. RVSMC culture medium consisted of DMEM with L-glucose and L-glutamine without sodium pyruvate containing, 10% FBS; 1% non-essential amino acids; 1 % sodium pyruvate; penicillin (100 U/ml) and streptomycin (100 U/ml). For further analysis of cellular proliferation, RVSMC between passage 3- 8 were used. The culture medium was changed every 2 days.

Cellular bioactivity

Release media solutions up to 28 days were used for cell proliferation analysis.
These samples were placed under a UV lamp for approximately 30 minutes for sterilization of the release medium. After sterilization, the release medium was then used for cell viability studies.

For cell viability analysis, cells were subcultured (i.e., trypsinized). Approximately 2 X 10^3 cells were placed in wells of a 96 well plate for bioactivity analysis. RVSMC and HCFC were studied, respectively. To study the effect of the release medium on cell proliferation, cells in each plate were grown in cell culture medium for 24 hours, serum free medium (SF, containing 0.25 % bovine serum albumin (BSA)) for another 24 hours and then the treatments (10 μl) containing sterile release medium obtained during the release kinetics study was added to each well containing SF medium. The effect of the treatments on cell proliferation was analyzed for 48 hours for HCF and 72 hours for RVSMC. After the indicated time of study, the medium with treatments was changed to SF medium (no BSA or FBS was added). 100 μl of this medium was added to each well before cellular viability analysis. This was done to avoid any colorimetric development that might result due to the treatment. 20 μl of the Cell titer 96 aqueous one solution reagents for cell proliferation analysis (Promega, WI) was then added to each well. The wells were then covered for colorimetric development. The plate was incubated for 1-4 hours in a 37°C, 5% CO2 environment. After incubation, the absorbance of each plate was read at 490 nm using a Wallac microplate reader. Results from the cell viability analysis were normalized based on the cells cultured in a serum free environment with no treatment.

Note: All of the cell seeding, addition of the cell titer aqueous 96 reagent and media was performed with a multipipettor.
**Statistical analysis**

All data values are reported as mean ± standard error about the mean (SEM). The statistical significance of difference was determined using one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison post test. Graph Pad software (Graph Pad, San Diego, CA) was used for this analysis. Differences were considered significant at p<0.05.

**Results and Discussion**

**Coated grafts**

A water-in-oil emulsion method was used to prepare the coating solution for the modified vascular grafts. The average amount coated on the grafts ranged from 3.51-3.65 mg (see Table 5-2). The amount of protein used or solvent used for the preparation of the coating solution didn’t significantly affect the amount coating on the grafts. The amount coated on the control graft (B3) was higher than the other modified grafts, B1 and B2. The increase in mass might be due to the increase in the amount of BSA used since its molecular weight is 66 kDa, greater than the molecular weight of the other additives.

Sensory testing of the grafts revealed that B5 modified grafts were softer than B1-B4 modified grafts. The thickness of the coated grafts (i.e., B1- B5) was approximately 3 um.

**Encapsulation Efficiency**

Table 4-2 shows the encapsulation efficiency of BSA of the modified vascular grafts. Increasing the sucrose concentration from 0.6% to 1.2 % increased the encapsulation efficiency of BSA. The encapsulation efficiencies of BSA within the modified vascular grafts were 31.9% ± 0.49 and 40.2% ± 1.7 for B1 and B2 modified vascular grafts, respectively. The encapsulation efficiency of the control modified vascular graft, B3, was 79.5% ± 8.6. A reduction in the
encapsulation efficiency of B1 and B2 might be due to the interaction of the other additives with BSA.

The encapsulation efficiency for B4 modified grafts with higher concentration sucrose and lower concentration BSA was 46.5 ± 4.9%. The encapsulation efficiency reported for the modified vascular grafts represent the amount of protein that was encapsulated during the preparation of the coating solution. However, the rest of the protein not reported for the encapsulation efficiency might be absorbed on the polymer surface or loss during acetone degradation of the polymer coating.

Surface morphology

The surface morphology of the grafts was observed after modification (Figure 5-1). Porous surfaces were observed for grafts prepared with B1-B4 samples. In addition, uniformity in the coating on the grafts was also observed. B1 modified graft was more porous compared to B2 modified grafts. The increase in porosity observed for B1 modified grafts might be due to the low concentration of sucrose used in the preparation of the coating solution. Therefore, an increase in sucrose concentration results in reduction in the porosity of the coated grafts after the drying cycle. This finding was in contrast to the report by Zhu et al., who reported that an increase in sucrose increases the aqueous pores of PLGA millicylinders (92).

More pores were visible for B3 modified grafts in the absence of sucrose. An increase in porosity observed for B3 modified grafts is due to the increase in amount of protein used in the preparation of the coating solution. Reduction in the BSA and an increase in sucrose concentration produced a smoother surface observed for B4 modified graft with less pores compared to B1 and B2 modified grafts.
The coating solution of B5 modified grafts with acetone, produced a rough coating of the grafts. Particles of various sizes were observed (See Figure 5-1e). This might be due to phase separation observed after 10 seconds of ultrasonification. Dissolving the polymer in acetone might have induced phase separation of the drug. According to Jian, a phase separation technique forms very soft coacervate droplets which entrap the drugs (104). The texture of the particle form and the phase separation of the coating might have resulted in the softness of the vascular graft after coating. The flaky residue observed when the graft was bent is due to weak adhesion of the coating on the vascular graft.

**Water uptake**

The release of protein from PLGA depends on polymer erosion, osmotic events and its diffusion through aqueous pores (105). According to Kang et al., the more water the polymer absorbs, the faster the protein release (70). Hence, water uptake affects the protein stability in the polymeric environment.

In this study, the effect of BSA and sucrose on the water uptake of the polymeric coating on Dacron vascular graft was studied. As shown in figure 5-2, increasing the sucrose concentration slightly affected the water uptake of the modified grafts. An increase in water uptake of B2 modified grafts was observed between days 3 and 7 and every week after day 14. While B1 modified grafts water content didn’t change until after day 14. A slight difference in the water uptake of B2 and B1 modified grafts was observed between days 7 and 14. Based on previous research of PLGA millicylinders, the increase in sucrose concentration should induce a large osmotic pressure in polymer matrix, resulting in an increase in the water uptake (70). Therefore, the slight difference in the water uptake observed for both grafts might be due to the other additives such as Mg(OH)₂ used in the preparation of the coating solution. Addition of Mg(OH)₂ in BSA-loaded millicylinders resulted in an increase in water uptake because of the
increase in osmotic pressure generated by magnesium salts and ionization of polymer end groups (106). Hence, the presence of Mg (OH)₂ might have overshadowed the true effect of sucrose on the water uptake kinetics of the modified grafts.

In figure 5-3, the effect of BSA on water uptake kinetics of Dacron modified vascular grafts is displayed. The increase in BSA concentration didn’t affect the water uptake of the modified grafts in the presence of the Mg (OH)₂. This result is consistent with previous research in which the concentration of BSA was varied. The slight difference of water uptake was attributed to the molecular weight of BSA which causes a relative low osmotic pressure change in the polymer pores (70).

**Release kinetics**

In order to compare the effect of sucrose on protein release and stability from modified vascular grafts, formulations with constant BSA concentration were prepared.

To obtain the amount of BSA released from each graft, a standard containing 0-2000 μg/ml of BSA was prepared. In addition, to the normal standards used in the determination of protein concentration, standards containing sucrose and EDTA were also prepared. Sucrose was found to have a significant effect on the standard curve readings (See figure 5-4). Thus, the protein release kinetics is based on the standard curve generated with BSA and sucrose.

The effect of sucrose on the release of BSA from the modified vascular graft can be found in figure 5-5. The release of BSA from B1 and B2 modified exhibits a triphasic drug release pattern. A triphasic drug release pattern is characterized by an initial diffusion of the drug near the surface, a lag phase and bulk erosion of the polymer (92). An initial burst of BSA was observed until day 3 for B2 modified graft, and then a slow release of protein occurred until day 15 when a second initial burst occurred. The effect of sucrose on protein release from B2
modified graft correlated with the kinetics of its water uptake. An increase in water uptake was observed for B2 modified grafts between days 14 and 28 (see figure 5-6 and 5-7); this resulted in an increase in protein release.

Reducing the sucrose concentration, resulted in a slow release of protein from B1 modified graft. The initial burst of this modified graft was observed until day 1 and then a second initial burst occurred between days 3 and 7. The slow release of the protein followed after day 7.

Figure 5-8 displays protein release when BSA concentration is reduced and sucrose concentration is held constant. A similar trend of BSA release is observed when sucrose concentration is unchanged.

The release of BSA from the B3 modified graft was near a zero order release. Addition of the additives such as sucrose to coating solution prolonged the release of BSA from the modified grafts (see figure 5-9). The increase of the release of BSA from B3 in the first few hours might be due to its adsorption of the protein on the surface of the vascular graft or an increase in porosity of the coating.

**Cellular bioactivity**

A critical factor in controlled delivery systems is the effect of the polymer formulation on the incorporated protein. The bioactivity of bFGF protein encapsulated to induce cellular proliferation after release from the impregnated grafts was tested. Following release into PBS and quantification of the protein, the supernatant of the test samples added to appropriate cell culture system. The effect of the supernatant obtained from B1 and B2 modified vascular grafts on HCF fibroblast can be found in figure 5-10. Cellular proliferation of the supernatant was normalized by dividing the absorbance obtained by the cells treated with serum-free medium. Based on the bioactivity of the protein to stimulate cellular proliferation, the encapsulation
procedure for the modified vascular grafts didn’t affect the bioactivity of bFGF. This is
subsequently noted for RVSMC cells (see figure 5-11). bFGF released from B1 modified grafts
was more potent than that released from B2 modified grafts. The release of bFGF at the 12 hour
significantly stimulated HCF proliferation compared to B1 treated cells. Assuming that the
release profile of BSA is similar to bFGF, the increase in cellular proliferation observed between
6 hr and 1 day might be due to initial burst of protein release from the modified vascular graft.

**Conclusion**

Doubling the sucrose concentration in the preparation of the coating solution used had little
effect on the encapsulation efficiency of BSA on the modified vascular grafts. A slight
difference in water uptake was observed for B1 and B2 modified grafts. A triphasic release of
BSA was observed for the modified vascular grafts. An increase in the sucrose concentration
resulted in an increase the amount of protein released. However, increasing the sucrose
concentration resulted in a reduction in the bioactivity of bFGF on HCF cells. The bioactivity of
the supernatant obtained between 6 hours and 1 day increased the proliferation of HCF cells,
with a significant effect at the 12 hour. A similar effect was observed for RVSMC, but its effect
wasn’t significant.
Table 5-1  Composition of the aqueous phase used for water in oil emulsion.

<table>
<thead>
<tr>
<th>Label</th>
<th>bFGF</th>
<th>Heparin</th>
<th>Magnesium hydroxide</th>
<th>Sucrose</th>
<th>EDTA</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0.00025%</td>
<td>0.00025%</td>
<td>3%</td>
<td>0.6%</td>
<td>0.001%</td>
<td>15%</td>
</tr>
<tr>
<td>B2</td>
<td>0.00025%</td>
<td>0.00025%</td>
<td>3%</td>
<td>1.2%</td>
<td>0.001%</td>
<td>15%</td>
</tr>
<tr>
<td>B3</td>
<td>3%</td>
<td>3%</td>
<td>3%</td>
<td>1.2%</td>
<td>0.001%</td>
<td>15%</td>
</tr>
<tr>
<td>B4</td>
<td>0.00025%</td>
<td>0.00025%</td>
<td>3%</td>
<td>1.2%</td>
<td>0.001%</td>
<td>7.5%</td>
</tr>
<tr>
<td>B5</td>
<td>0.00025%</td>
<td>0.00025%</td>
<td>3%</td>
<td>1.2%</td>
<td>0.001%</td>
<td>7.5%</td>
</tr>
</tbody>
</table>

All of the excipient for the aqueous phase is percent weight per volume.

Table 5-2  Amount coated based on the different coatings.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount coated (mg±SEM)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1 grafts</td>
<td>3.51 ± 0.08</td>
<td>3</td>
</tr>
<tr>
<td>B2 grafts</td>
<td>3.61 ± 0.12</td>
<td>3</td>
</tr>
<tr>
<td>B3 grafts</td>
<td>3.65 ± 0.17</td>
<td>3</td>
</tr>
<tr>
<td>B4 grafts</td>
<td>3.55 ± 0.15</td>
<td>3</td>
</tr>
<tr>
<td>B5 grafts</td>
<td>3.62 ± 0.12</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 5-3  Encapsulation efficiency of BSA-bFGF modified vascular grafts.

<table>
<thead>
<tr>
<th>Sample (modified grafts)</th>
<th>Encapsulation Efficiency (%) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>36.6 ± 6.6</td>
</tr>
<tr>
<td>B2</td>
<td>40.2 ± 1.7</td>
</tr>
<tr>
<td>B3 (control)</td>
<td>79.5 ± 8.5</td>
</tr>
<tr>
<td>B4</td>
<td>46.4 ± 4.9</td>
</tr>
<tr>
<td>B5</td>
<td>85.6 ± 0.4</td>
</tr>
</tbody>
</table>

n=2
Figure 5-1  Coated grafts coated with a) B1; b) B2; c) B3; d) B4; e) B5, (B1-B4 at 500X) and f) B5 (2500X).
Figure 5-2 Water uptake kinetics of PLGA modified vascular grafts encapsulating various sucrose concentration. B1) 0.6% sucrose, and B2) 1.2% sucrose.
Figure 5-3. Water uptake kinetics of modified vascular grafts, effect of BSA. B2 - 15% BSA; B4 - 7.5% BSA
Figure 5-4  BSA standard for protein analysis

Figure 5-5  In vitro release profiles of BSA from modified vascular grafts with different concentration of sucrose (n=3).
Figure 5-6  SEM images of B1 modified vascular graft undergoing degradation studies, 250x.

a)  12 hours; b) day 1  c) day 7  d) day 14
Figure 5-7  SEM images of B2 modified vascular graft undergoing degradation studies, 250x, 10kVa)12 hours; b) day 1 c) day 7 d) day 14
Figure 5-8  Cumulative release of BSA from B2 and B4 modified grafts

Figure 5-9  Cumulative release of BSA for B3 modified vascular graft (control)
Figure 5-10  Cellular bioactivity of B1 and B2 modified grafts with HCF cells. Percent proliferation is relative to cells grown in serum free medium (control). (n=4, * p<0.05)

Figure 5-11  Bioactivity of B1 and B2 modified grafts with RVSMC cells. Percent proliferation is relative to cells grown in serum free medium (control). (n=4)
CHAPTER 6
IMPREGNATION OF WOVEN DACRON VASCULAR GRAFT WITH BASIC FIBROBLAST GROWTH FACTOR AND POLY (DL- LACTIC-CO-GLYCOLIC) ACID

Introduction

Basic fibroblast growth factor (bFGF) or fibroblast growth factor two (FGF-2), is an 18 kDa protein that is an isoform of the fibroblast growth family. It is a potent angiogenic agent associated with extracellular matrix and basement membrane (107). It’s also a known heparin binding growth factor that has peptide multifunctional properties. It’s produced by various types of cells and tissues such as macrophages and endothelial cells (108, 109). bFGF has been found to induce the proliferation and migration of endothelial, fibroblast and smooth muscle cells in vitro and in vivo. In a rat AAA model, the reduction of aneurysm size was reported to be due to the local delivery of bFGF. Gene transfer of this growth factor in vivo by electroporation significantly enhanced medial smooth muscle cell proliferation without inflammatory infiltration. However, bFGF cannot be delivered locally by electroporation in humans because of the risk of electrical damage to surrounding tissues (110). Exogenous and systemic administration of small to large amounts of this growth factor might lead to unwanted adverse effects. In addition, growth factors have a short half life in vivo (2.5 minutes) when administered systematically (111).

Poor healing of the vascular graft to the aorta has been suggested to cause endoleakage or migration of the endovascular stent after repair (112). According to Tomizawa et.al., bFGF is important for the endothelialization of the prosthesis shortly after implantation. This stimulates the ingrowth of vascular graft with the surrounding tissue (113). In an attempt to deliver bFGF from vascular grafts, Takhaski et al., soaked plain vascular grafts in 5 ng/ml bFGF solution for...
30 minutes, dried them and implanted in dogs subcutaneous layer for 5 day. bFGF accelerated the migration and capillary ingrowth of host cells into the Dacron vascular prosthesis (114).

Various vehicles such as microspheres and polymeric scaffolds made of natural and synthetic polymers have been shown to be an effective means in which to administer low doses of the growth factor for a desired period of time. For the controlled release of bFGF from vascular grafts, van der Bas et al., used collagen as a carrier for delivery. When analyzed *in vitro*, the impregnated vascular grafts induced neointima formation in porcine and human aortic organ cultures (115). Ingrowth of the neointima into the grafts was observed up to 28 days. The impregnated vascular graft also induced graft healing in an *in vivo* pig model (76). However, the use of collagen for the release the protein is costly and there are variances in the purity of collagen used. Collagen can cause an immunogenic response for patients who are allergic to animal-based products (116).

Therefore, in this study we propose to accelerate vascular graft healing by modifying woven Dacron vascular grafts with a synthetic bioabsorbable polymer (poly DL lactide-co glycolide) (PLGA) and growth factors.

In this chapter, the modification of woven Dacron grafts with bFGF will be discussed. The effect of various doses of bFGF on vascular cell proliferation (i.e., RVSMC and human dermal fibroblast (HDF)), polymeric water content of the modified graft, surface morphology of the graft before and after release kinetics, the release profile of bFGF and its bioactivity on RVSMC and HDF will be addressed.

**Materials and Methods**

**Preparation of the vascular graft**

Vascular grafts used in this study were prepared as previously described in chapter 4.
Preparation of the vascular graft

The vascular grafts were prepared as previously stated in chapter 4.

Preparation of the coating solution

The coating solution was prepared as previously described in chapter 4. Briefly, three different types of coating solution were prepared with variations in the additives. Details of the different coating solution can be found in Table 6-1. Briefly, 10(v/v) % of the water phase (i.e., additives) was added to the oil phase which consisted of 5(w/v) % PLGA in dichloromethane. The two different phases were emulsified under an ice bath by ultrasonification for 30 seconds. After emulsification, the solution was continuously stirred for the impregnation of Dacron vascular graft.

Impregnated vascular graft

Impregnation of the vascular graft with bFGF was based on techniques previously stated in chapter 4.

The amount coated on the vascular graft was measured according to the weight increase of the modified graft. The following equation was used:

\[
\text{Percent weight} = \frac{m_f - m_i}{m_f} \times 100
\]

(6-1)

\(m_i\) and \(m_f\) were the weight of the samples before and after coating, respectively.

Characterization of the coated graft

Morphological analysis of the grafts was performed as previously stated in chapter but 4 with a field emission SEM (SEM 6335F).
Release Kinetics Study

For release kinetics studies, coated grafts were placed in 1.5 ml micro-centrifuge containing 500 μl PBS (pH 7.4). The samples were then placed in a hybridized incubator, under constant rotation at 37°C for 28 days. The supernatant was removed and replaced at various time intervals (12 hours, 1 day, 3 days, 7 days, and 14 days etc for 28 days). The pH of the supernatant was measured using a pH meter (Corning, NY)

The supernatant was collected and stored at -20°C for further analysis to determine the amount of protein release.

On the final day of the release study, the vascular grafts were removed from the microcentrifuge to determine the amount of water uptake by the modified graft. The water uptake was obtained by blotting the wet modified grafts with dry kim-wipes and weighed immediately. The grafts were then placed in a vacuum oven to dry overnight. The dry weight of the graft was measured.

Water uptake of the vascular graft after 28 days was determined by the following equation:

\[ \text{Water uptake} = \frac{m_w - m_d}{m_d} \times 100 \]  

\( m_w \) and \( m_d \) is the weight of the wet and dry grafts, respectively.

In addition, the amount loss during the release kinetics study was determined as follow:

\[ \text{Percent loss} = \frac{m_d - m_i}{m_d} \times 100 \]  

\( m_i \) is the weight of the vascular graft after coating

BCA analysis

Protein (BSA) released was quantified as previously described in chapter 4 in the BCA analysis section.
Evaluation of BSA aggregation

The evaluation of BSA aggregates for the modified vascular grafts was based on Zhu et al method for evaluating BSA aggregates of microspheres (92). Briefly, the modified graft was dissolved in 500 μl acetone in a microcentrifuge tube. The microcentrifuge tube was then vortexed for 30 seconds, and the graft was removed. The acetone solution containing the polymer and protein was then centrifuged for 30 minutes at 13,400 rpm. The polymer solution was then removed, the protein pellet was air dried, and reconstituted in PBS. The amount of water-soluble BSA was then determined by BCA analysis. The amount of aggregated BSA (insoluble protein) was determined by centrifuging the reconstituted solution, removing the supernatant, and incubating the pellet in a denaturing solution (PBST, 6 M Urea, 1mM EDTA) for 30 minutes at 37°C. BCA analysis of the pellet reconstituted in the denatured solution gave the amount of noncovalently bonded BSA aggregates.

Basic FGF assay

The concentration of bFGF in the supernatant (released medium) samples was determined using a commercialized enzyme linked immunoassay kit (Quantkine bFGF, ELISA, R&D Systems, MN). The antibodies of the kit were specific for human bFGF. The assay was performed according to manufacturer’s specification. The wells were coated with mouse monoclonal antibody specific for bFGF. A small volume (100 μl) of the standard and sample were added to the appropriate wells. A polyclonal antibody, linked to peroxidase specific for bFGF was used as the conjugate. The optical density was read at 450 nm using a microplate reader (Wallac microplate reader). The optical density of the ‘blank’ was subtracted from standards and samples.
Encapsulation efficiency study

The encapsulation of BSA and bFGF were obtained as previously described in chapter 5.

Cell Culture

Human dermal fibroblast (HDF)

Human dermal fibroblast cells (CRL-2522, ATCC, and Manassas, VA) were cultured in a tissue culture flask (T-75) containing DMEM media supplemented (Gibco Invitrogen, Carlsbad, CA, ) with 10% Calf bovine serum (Mediatech Cellgro, Herndon, VA) and 1% antibiotic-antimycotic (Meditech Cellgro, Herndon, VA,).

Rabbit vascular smooth muscle cells (RVSMC)

Rabbit vascular smooth muscles cells (RVMSC) were isolated from New Zealand rabbit aortas. The tunica media obtained from the aorta was minced in 1mm² fragments. The fragments were cultured in 20% CBS supplemented with 1% sodium pyruvate, 1% non essential amino acids and 1% Antibiotic-Antimyotocin at 37 °C and 5% CO₂ and 95% air, after harvesting. After SMC migration from the tissue onto the flask, the cells were then subsequently cultured in reduced serum medium containing CBS (10%) supplemented with the other nutrients previously mentioned. The assessment of the smooth muscle cell nature of the isolated cells was performed by immunocytochemical reaction for alpha smooth muscle actin (IMMH2, Sigma, USA, See Appendix A).

Cell proliferation assay for bFGF (Quantitative)

The biological activity of bFGF released from modified vascular graft was determined by its ability to stimulate the growth of cultured HDF and RVSMC. HDF (passage 7 - 11) and RVSMC (up to passage 5) were used for this experimental study.

For proliferation analysis, cells (HDF and RVSMC) at a density of 2 x 10⁴ cells / well in culture medium were seeded in 96 well plates (Corning, Cambridge MA). Cells were grown in
serum containing medium for 48 hours and replaced with 100 μl of serum-free media (SF, DMEM media supplemented with 1% Antibiotics-Antimycotic) for 24 hours. In addition to the reagents mentioned for the preparation of SF medium, RVSMC also contained 1% sodium pyruvate and 1% non essential amino acids.

To determine the effect of supernatant on cell proliferation, 10 μl of the sterile filter release medium was then added after 96 hours. The cells were incubated with the treatments for 48 hours.

After 48 hours, the wells were replaced with 100 μl of DMEM media. Then, a small amount (20 μl) of Cell Titer 96 Aqueous One solution (MTS, promega, USA) was added to each well. Plates were covered (with a foil) incubated for 4 hours at 37 °C, followed by a spectrophotometer reading obtained at 490 nm. Cellular proliferation of the cells was normalized with the absorbance reading of the cells cultured in SF medium.

The dose response of bFGF with known concentration for cell proliferation analysis was also performed. Cells were supplemented with (10μl) of 50, 30, 20, 10 and 5 ng/ml of soluble bFGF in PBS. The effect of the different doses on cellular proliferation was determined as previously stated.

**Contact between vascular cells and modified vascular graft (Qualitative)**

Modified vascular graft shaped to cover the whole surface (0.5 inches in diameter) of a 24 well plate was prepared. The grafts were then placed in a sterile 24 well plate containing SF medium with 5% antibiotic-antimycotic for 30 minutes for sterilization. The medium was then replaced with 1.5 ml of serum containing medium.

HDF (passage 9) and RVSMC (passage 5) with cell density of 4 x 10^4 were seeded onto each graft. A negative control, vascular cells (HDF and RVSMC) cultured on unmodified
vascular graft (woven Dacron grafts only) with the same diameter was used. The samples were incubated at 37°C in 5% CO₂ atmosphere for 3 and 8 days. After incubation, the grafts were removed from the wells, fixed with 10% formalin buffered solution and stained with hematoxylin and eosin. The biocompatibility of the modified grafts including the proliferation and adhesion of cells were observed using a Zeiss Optical microscope.

**Statistical analysis**

All data values are reported as mean ± standard error about the mean (SEM). The statistical significance of difference was determined using one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison post test. Graph Pad software (Graph Pad, San Diego, CA) was used for this analysis. Differences were considered significant at p<0.05.

**Results and Discussion**

**bFGF impregnated vascular graft**

Two different coating solutions with variation in the concentration of sucrose and bFGF were used to impregnate the vascular graft. The modified vascular grafts were completely impregnated with coating solution. Figure 6-1 shows SEM micrographs of the modified grafts. The SEM micrograph in figure 6-2 shows that the modified vascular graft (B1 modified vascular graft was used as an example). Coating of the vascular graft generated a porous surface of PLGA/bFGF matrix on the surface of the graft. The amount coated on the vascular graft after modification were 4.15 ± 0.43 mg and 3.97 ± 0.92 mg for B1 and B2 modified grafts, respectively.

**Encapsulation efficiency**

The encapsulation efficiency of the water-soluble protein (BSA) within the modified vascular grafts was 56% and 42% for B1 and B2 modified vascular grafts, respectively. The amount of protein obtained for B1 modified vascular graft after the reconstitution in PBS was
similar to the amount obtained for millicylinders prepared with 15% BSA/3% Mg(OH)_2/heparin (92). The amount of protein lost in the polymer due to aggregation was obtained by treating the insoluble protein with a reducing agent (6 M Urea). The insoluble fractions obtained after urea treatment were 20% for B1 modified vascular graft and 11% for B2 modified grafts. The insoluble protein represents the amount of noncovalent aggregates formed due to the hydrophobic interaction of the protein with the polymer.

**Release Kinetics**

The pH of the supernatant after release kinetics was obtained for the modified grafts.

Figure 6-3 shows the effect of the different additives on the pH of the supernatant. A decrease in the pH of the supernatant was observed between days 3-7 for B1 modified vascular graft. After 7 days, a slight change in the pH of was measured until day 14, a drop of the pH followed after day 21. The drop of the pH might be due to an increase in acid.

A similar pattern in the pH of release medium was observed for B2 modified vascular graft. A drop in the pH (below 7) of the supernatant was observed at 7th day. The dramatic change in the pH might be due to an increase in the water uptake stimulated by the higher concentration sucrose used in the preparation of the coating solution. This might also be due to a loss in the polymer mass. According Ding et al., the acidic content of PLGA films increases near the induction time to polymer mass loss which is represented by the lag time before medium to high molecular PLGA is released into the incubation medium. The decline in pH might also be due to release of glycolic acid and hypothesized lactic acid tetramer which are major components in the supernatant.

In comparison to PLGA coated grafts, the impregnation of basic additives had a major effect on the neutralization of the pH of the release medium as the polymer degrades. (See figure
Magnesium hydroxide, an antacid, used in the preparation of the coating helps neutralize the acidic environment during polymer degradation. As the polymer degrades, the solid antacid dissolves in response to microclimate pH (92). This helps neutralize the release medium during polymer degradation.

After 28 days of incubation in PBS, the water uptake of B1 modified graft was 48.00 ± 1.77% and 36.91 ± 3.07% for B2. Increasing the sucrose concentration for B2 modified vascular graft decreased the water uptake after day 28 incubation. This was consistent with our findings in chapter 5. However, this amount cannot be used to conclusively describe the water uptake profile of the two modified vascular graft during degradation.

After the degradation studies, the amount of coating lost for the modified grafts were 48.99 ± 1.23% and 44.07 ± 5.76% for B1 and B2, respectively. Based on the percentage of coating lost, we suggest that the different concentration of additives used in the preparation of the coating solution had no significant effect on the amount of coating lost over time.

**Protein release**

Figure 6-5 displays the release of BSA from the modified vascular grafts. As previously shown in chapter 5, increasing the sucrose concentration increased the amount of BSA released from the modified vascular graft. An initial burst in the release of BSA encapsulated was observed in the first 24 hours for B1 and B2 modified grafts. This was followed by the slow release of the protein for B1 modified vascular graft. However, for B2 modified graft, a second initial burst of BSA was observed between days 1 and 3, followed by a slow release of the rest of the soluble protein. The initial burst of growth factor observed for the modified grafts is similar to the washout effect observed for bFGF-collagen impregnated grafts (115). A washout effect of
the growth factor was observed in the first 3 days for bFGF collagen impregnated grafts attached
to porcine aortic tissue samples. This was followed by a stable release of bFGF for 28 days.

As for the growth factor, bFGF, increasing the sucrose concentration reduced the amount
of growth factor released (See figure 6-6). This was consistent with the findings made by Zhu et al., which showed that an increase in sucrose concentration slowed down the release of bFGF
from PLGA millicylinders (92).

An initial release of 70% of the encapsulated bFGF was observed within the first 12 hours
for B1 modified vascular graft. This was followed by the gradual release of the bFGF (0.11 -
1.59 ng per mg of coating (1 mg of coating is equivalent to 0.23 mm² of Dacron vascular graft))
for 7 days. For B2 modified grafts, 51% of the soluble bFGF encapsulated was recovered in the
first 12 hours with the rest releasing before the 7th day.

**Dose response**

For the determination of the optimal concentration of bFGF to stimulate RVSMC and HDF
proliferation various concentration of bFGF was analyzed. The optimal concentration of bFGF
for inducing RVSMC proliferation was 5 ng/ml, while 20 ng/ml enhanced HDF proliferation. A
concentration of 10 ng/ml, 30 ng/ml and 50 ng/ml of bFGF induced less RVSMC proliferation
when compared with 5ng/ml. However, for HDF, it was very difficult to differentiate the effect
of the various doses on HDF proliferation, because there were no significant differences among
the means of the percent cell proliferation obtained for each treatment (see figure 6.7-6.8).

Increase in cell proliferation for RVSMC with 5 ng/ml is similar to findings by Yu et al.,
in which a reduction in bovine vascular smooth muscle cell growth was observed for higher
concentrations bFGF while RVSMCs with an increase in proliferation were treated with lower
concentration bFGF.
Bioactivity of bFGF

An ELISA specific for human bFGF was used to determine the amount of bFGF recovered during the release of growth factor from the modified vascular graft. The amount of bFGF released after 28 days of incubation in PBS were 50% for B1 and 40% for B2 modified vascular grafts. The amount released is based on the soluble bFGF that was obtained by during encapsulation efficiency studies. Although this amount was recovered after the release kinetics study, it doesn’t necessary indicate that the protein release was bioactive. To test this, we examined the bioactivity of the released growth factor to induce cell proliferation for both RVSMC and HDF (see figures 6.9-6.10).

The bioactivity of bFGF to stimulate HDF cell proliferation was below 100% for release medium collected at the 12th hour, day 1 and day 3. HDF cell response to the release medium for B1 and B2 was similar to those reported by Zhu et al, which showed a small inactivation of bFGF from release PLGA millicylinders between 1 and 28 days (92). However, after the 3rd day the bioactivity of B1 released medium was greater than 100%. The same trend occurred for the release medium collected for B2 modified grafts. A significant amount of HDF proliferation was observed for the supernatant collected on days 1 and 14 for B1 modified vascular graft when compared to B2 modified vascular graft. While for B2 modified vascular an increase in cell proliferation was observed on days 3 and 7. An increase in cellular proliferation observed after the release of bFGF from the modified vascular graft (3 days), might be due to by products of the degradable polymer (lactic and glycolic acid).

The bioactivity of bFGF to stimulate RVSMC proliferation was greater than 100% for cells treated with the release medium. Release medium collected (i.e., both vascular grafts) after day 3 induced a significant amount of cell proliferation compared to the supernatant collected during the initial release. A significant increase in the amount of RVSMC proliferation was
observed on days 3 and 7, for the release medium obtained on day 3 and day 7. This was similar to the response observed for supernatant collected from B2 modified vascular graft in the presence of HDF. The encapsulation of bFGF in PLGA stimulated the growth of RVSMC (>30%) more than any of the tested bolus (<10%) based on the control (serum-free treated cells). This also parallels the findings of Yu et al., which showed that the release of bFGF from microspheres stimulated the growth of smooth muscle cells more than any of the concentrations tested for dose-response studies (92).

An increase in cellular proliferation was observed for cells treated with supernatant obtained from B1 modified vascular graft compared to PLGA modified vascular graft. (See figure 6-10). This indicates that the addition of growth factor (bFGF) enhances cell proliferation in the presence of PLGA.

**Biocompatibility analysis of bFGF modified vascular grafts**

The biocompatibility of the modified vascular grafts in a cellular environment was examined by seeding sterilized modified grafts with cells for up to 8 days. Figures 6-12—6-13 show optical micrographs of the grafts seeded with cells. An increase in cellular proliferation on the surface of the modified vascular grafts was observed from day 3 to day 8. In addition, the cells that were seeded on the vascular graft migrated through the pores of the modified vascular graft during polymer degradation. An increase in cellular proliferation was observed for B1 compared to B2 modified vascular grafts. This was analogous to our findings for the cell proliferation analysis of the modified vascular grafts.

The infiltration and proliferation of cells onto the grafts is similar to findings reported by van de bas et al in which VSMC through the bFGF-collagen-coated prosthesis material. The cells also grew between the fibers of the prosthetic material (115).
Conclusion

Modification of Dacron vascular graft with drug eluting properties was successful. Dacron vascular graft was modified with a primary emulsion used in the production of the microspheres. The modified grafts with bFGF were able to release a small fraction of the growth factor embedded within the polymer matrix for 3 days. The growth factor released was bioactive and able to stimulate RVSMC and HDF proliferation, especially for cells treated with the supernatant collected from B2 modified vascular graft. The increase in sucrose concentration protected bFGF from denaturing when coating solution was prepared and underwent coating of the vascular grafts was lost after 28 days of release studies. A small amount of polymer was observed on and within the interstices after 28 day. Biocompatibility of the material with cells was observed. Cells seeded on the modified grafts for up to 8 days, showed an increase in growth compared to the 3 days. The cells started migrating into the pores of the grafts during the degradation of the polymer. In addition, treating the cells with supernatant collected from the release studies resulted in an increase in RVSMC proliferation compared to cells treated with serum free medium. In regards to HDF cell proliferation, the supernatant collected (B1 and B2 modified vascular graft) on the first 1 day inhibited cell proliferation. However, the supernatant collected on days 3 and 7 stimulated significant amount (>50%) of cell proliferation compared to cells treated with supernatant obtained at the 12 hour.

Based on our findings, we hypothesize that the modified vascular graft will promote tissue healing when implanted in vivo. Further studies to determine the effect of the supernatant collected from releases studies on VSMC migration needs to be performed. This will help in determining the effect of the coating on neointima formation, which requires the migration of the smooth muscle cell from the tunica media to the intima. Neointima formation between the stent graft and the aorta will then promote healing between the graft and aorta.
Table 6-1  Contents of bFGF modified vascular grafts (n=4)

<table>
<thead>
<tr>
<th>Sample</th>
<th>bFGF (w/v)%</th>
<th>BSA (w/v)%</th>
<th>Mg(OH)&lt;sub&gt;2&lt;/sub&gt; (w/v)%</th>
<th>Heparin (w/v)%</th>
<th>EDTA (w/v)%</th>
<th>Sucrose (w/v)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>1 E-4</td>
<td>15</td>
<td>3</td>
<td>1 E-4</td>
<td>1 E-2</td>
<td>6 E-1</td>
</tr>
<tr>
<td>B2</td>
<td>3.75 E-5</td>
<td>15</td>
<td>3</td>
<td>4 E-5</td>
<td>1 E-2</td>
<td>1.2</td>
</tr>
<tr>
<td>EW</td>
<td>15</td>
<td>3</td>
<td>4 E-5</td>
<td>1 E-2</td>
<td>6 E-1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6-1  SEM micrographs of modified vascular graft, 10 kV.
A) B1 coated vascular graft 100x; B) B1 coated vascular graft 1500x; C) B1 modified vascular graft after 28 day release kinetics 100x; D) B2 coated vascular graft 100x; E) B2 coated vascular graft, 1500x; F) B2 modified vascular graft 28 day release kinetics 100x.
Figure 6-2  SEM micrograph of cross-sectional image (10 kV) of A) Plain Dacron graft, B) B1 modified vascular graft and C) B1 modified vascular graft after 28 day release study, 200x.

Figure 6-3  pH of supernatant of modified graft B1 and B2 after release kinetics study.
Figure 6-4  pH of supernatant obtained from PLGA and modified graft without growth factor (EW).
Figure 6-5  Cumulative release of BSA from B1 and B2 modified vascular graft n=4.
Figure 6-6  Amount of bFGF released from each modified vascular graft, n = 4. (1 mg of coating is equivalent to 0.23 mm² of the vascular graft)
Figure 6-7  Dose response of bFGF on RVSMTC proliferation (n=4, * p<0.05). Cells treated with 5ng/ml are significantly different from cells treated with serum containing medium (FCS), 10 ng/ml, 30 ng/ml and 50 ng/ml. The control represents cells treated with serum-free only which is at normalized to a 100% after 48 hours incubation. Data represents mean ± standard error of mean of percent cell proliferation based on control.
Figure 6-8  Dose response of bFGF on HDF cell proliferation, n=4. (no significant differences)
The control represents cells treated with serum-free only which is at normalized to a
100% after 48 hours incubation. Data represents mean ± standard error of mean of
percent cell proliferation based on control.
Figure 6-9  Effect of bFGF released from modified vascular graft on HDF cell proliferation, n=4 (*p<0.05). The control represents cells treated with serum-free only which is normalized to a 100% after 48 hours incubation. Data represents mean ± standard error of mean of percent cell proliferation based on control.
Figure 6-10  Effect of bFGF released from modified vascular graft on RVSMC proliferation, n=4. *p<0.05, significant difference for the relative cell proliferation between B1 and B2 at day 21; ** p<0.05 and ***p <0.05:- significant difference for the cellular proliferation when comparing the effect of the supernatant individually on RVSMC proliferation (B1 and B2 only). The control represents cells treated with serum-free only which is at normalized to a 100% after 48 hours incubation. Data represents mean ± standard error of mean of percent cell proliferation based on control.
Figure 6-11  Comparison of the effect of supernatant obtained from B1 and PLGA modified vascular grafts on RVSMC, n=4. The control represents cells treated with serum-free only which is at normalized to a 100% after 48 hours incubation. Data represents mean ± standard error of mean of percent cell proliferation based on control.
Figure 6-12  Optical images of HDF cells seeded onto modified vascular grafts, H & E stained, 1000x. (A-C, 3 day incubation, D-F, 8 day incubation). A & D) Dacron only; B & E) B1 modified vascular graft C & F) B2 modified vascular graft.
Figure 6-13  Optical images of RVSMC on modified vascular grafts, H &E stained, 1000x. 
CHAPTER 7
CONNECTIVE TISSUE GROWTH FACTOR MODIFIED VASCULAR GRAFT: AN IN VITRO STUDY WITH VASCULAR CELLS

Introduction

The modification of woven Dacron vascular graft with connective tissue growth factor will be discussed in this chapter. Connective tissue growth factor is a cysteine-rich secreted heparin binding protein (38 kDa; 349 amino acids) that is part of the extracellular protein family known as the CCN (Cyr 61, CTGF and Nov) (117). CTGF and Cyr 61 are associated with the promotion of cellular growth while Nov is associated with growth inhibition. CTGF is the only known cytokine in the CCN family that was identified via biological activity. CTGF is activated by the transforming growth factor β (118). It plays an important role in stimulating the proliferation of connective tissue cells e.g. fibroblasts and extracellular matrix (ECM). CTGF is overexpressed in atherosclerotic lesions and infracted myocardium. The overexpression of CTGF stimulates collagen synthesis of fibroblast and increases the production of ECM and fibronectin (119).

The impregnation of Dacron vascular graft with CTGF was investigated and its bioactivity on vascular cell proliferation and migration will be presented in this chapter. We have demonstrated that most of the protein impregnated within the vascular graft releases in 7 days and it’s active in stimulating fibroblast and smooth muscle cell proliferation

Materials and Methods

Materials

Human recombinant connective tissue growth factor (hrCTGF) (400 μg/ml) was provided by Dr. Schultz, Institute of Wound Healing at University of Florida. Magnesium hydroxide, Ethylenediamine (EDTA), sucrose, bovine serum albumin and phosphate buffer saline were obtained from Sigma-Aldrich. Poly (DL-lactic co-glycolic acid) inherent viscosity of 0.82dl/g
(PLGA) was provided by DURECT: Absorbable Polymers International (formerly Birmingham Polymers, Fairfield, AL),

**Preparation of the vascular graft**

The vascular grafts were prepared as previously stated in chapter 4.

**Preparation of the coating solution**

The coating solution was prepared as previously described in chapter 4. Briefly, three different types of coating solution were prepared with variations in the additives. Details of the different coating solution can be found in Table 7-1. Briefly, 10(v/v) % of the water phase (i.e., additives) was added to the oil phase which consisted of 5(w/v) % PLGA in dichloromethane. The two different phases were emulsified under an ice bath by ultrasonification for 30 seconds. After emulsification, the solution was continuously stirred for the impregnation of Dacron vascular graft.

**Impregnated vascular graft**

Impregnation of the vascular graft with CTGF was based on techniques previously started in chapter 4.

**Characterization**

All of the characterization techniques were performed as previously stated in chapter 6 this excludes the CTGF release measurements which is described below.

**CTGF release measurements**

The amount of CTGF released from the modified vascular graft was determined by a non-commercially available quantitative sandwich enzyme linked immunosorbent assay (ELISA) technique previously described by Setten et al (120). Briefly, a 96-well plate was coated with 50 μl of an affinity purified polyclonal antibody specific for human CTGF in each well overnight at 4°C. Primary antibody was then removed after pre-coating. Samples or standards of CTGF in
release medium (100 μl) were added to each well and incubated for 2 hours at room temperature, followed by 1 h with biotin-linked polyclonal antibody specific for CTGF, and 1 h of incubation with alkaline-phosphate-conjugated strepavidin. The substrate alkaline phosphate substrate (p-nitrophenyl phosphate) (5mg, Tablets) was then dissolved in carbonate-bicarbonate buffer 15 minutes prior to use. 100 μl of the substrate was then added and incubated for 30 minutes. The concentration of CTGF was detected (405nm) on a plate reader. The detection limit of this assay is within 0.1 ng/ml for hrCTGF.

**Cellular migration assay**

Migration assay was performed in a “HTS Transwell for 96” samples as described (121-123). Briefly, RVSMCs were trypsinized (0.05% trypsin/0.11 mmol EDTA) and resuspended in DMEM/0.1% BSA (i.e., Serum free medium) at a density of ≈10^6 cells/mL. The chamber was then presoaked in culture media for 30 minutes. The medium (cell culture media) in the bottom chamber was then changed to DMEM/0.1% BSA (235 μl was added to each well). SMCs (2.5 x10^5 cells in 50 μl) were added to the upper wells of the chamber. 10 μl of the sterile filter release medium was then added to the bottom chamber of each experimental well, while 10 μl of serum-free media and cell media were placed in the control wells. The chambers were incubated for 5 hours at 37°C in an atmosphere of 95% air and 5% CO₂. At the end of the incubation period, migrated cells were fixed in methanol and stained with hematoxylin. Non-migrated cells on the upper chamber were wiped off with cotton swipes. Membranes were mounted and migrated cells were quantified by cell counts of 5 random (x 1000) high power fields in each membrane. Each assay was performed in quadruplicate. Cell counts in experimental wells were compared to cell counts in control wells after the 5 hour interval using a one way analysis of variance to reveal significant differences in RVSMC migration.
Statistical analysis

All data values are reported as mean ± standard error about the mean (SEM). The statistical significance of difference was determined using one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison post test. Graph Pad software (Graph Pad, San Diego, CA) was used for this analysis. Differences were considered significant at p<0.05.

Results and Discussion

Impregnated vascular graft

Three different coating solutions were prepared to impregnate Dacron vascular graft with CTGF. The first coating solution contained all the additives used previously for the impregnation of bFGF, while the other two coating solutions excluded BSA; and sucrose, EDTA, and Mg (OH)₂. The amount coated on each of the modified vascular grafts (see figure 7-1) are 4.3 ± 0.5, 2.8 ± 0.6 for C2 and 4.3 ± 0.9 for C1, C2 and C3 modified vascular grafts, respectively. The amount coated on C2 vascular graft was 1.50 times less than the amount coated on C1 and C3 vascular grafts. The reduction in the amount coated might be due to the fact that BSA was not added to the coating solution. However, the drastic change in the amount coated is not the same for C3 coated graft when BSA is eliminated. The increase in the amount coated for C3 modified graft might be due to the increase in the volume of Mg (OH)₂ used in preparation of the coating solution; approximately 135 μl of Mg OH₂ was used.

The elimination of BSA for the preparation of the coating solution resulted in a reduction of the water uptake and percent of coating lost (see figure 7-2). The percentage of coating lost for C1 modified grafts was >50%. This caused the water uptake of the polymeric matrix used in the coating of the vascular graft to increase. The pH of the supernatants was greater than 6.5 for all modified vascular grafts. A drop in the pH of C1 modified vascular graft was observed on
day 14, this is due to the degradation of polymer from the modified vascular graft (see figure 7-3). The interaction of the acidic environment within the polymer matrix with BSA might have caused the drop in pH for this modified vascular graft.

**SEM**

Surface morphology of the modified vascular grafts can be found in figure 7-4. The coating on the surface of the grafts was porous. After 28 days in PBS, the surface coating of C1 and C2 modified vascular grafts were lost. But the coating of vascular graft modified with C3 coating solution still remained. This is quite strange, because increasing the amount of Mg (OH)$_2$ used in the preparation of the coating solution should have increased the amount of water uptake. This would then lead to the degradation of the polymeric coating. Hence, based on our findings, the combination of Mg (OH)$_2$ and growth factor doesn’t increase the water uptake of the polymeric coating.

Cross section of the modified vascular grafts before and after coating results in the impregnation of the grafts (see figure 7-5)

**Protein release kinetics and encapsulation efficiency**

An initial burst of BSA used in the modification of C1 modified vascular grafts was observed within the first day. A slow release of the protein followed for 3 days, then release of the protein seized until day 14 when a small quantity of protein released (see figure 7-6).

The release profile of CTGF from C1 modified vascular graft was in contrast to the BSA release (see figure 7-7). Rapid release of the protein was observed for the first 12 hours, followed by the slow release of CTGF for 14 days. A very small fraction of the growth factor (0.8%) released on day 14.

A small amount, 15% of the coating solution prepared for C1 modified vascular graft was impregnated. The amount of growth factor impregnated within C2 was less than 1%. While for
C3 modified vascular grafts CTGF could not be detected. This might be due to absence of BSA and sucrose which have roles in protecting and carrying the protein during microsphere degradation studies. It might also be due to the size of the protein encapsulated. In a study performed by Zhu et al, an increase in BSA released was observed when the amount of Mg(OH)$_2$ encapsulated was increased in PLGA millicylinders (92). BSA, a 66 kDa protein, might be diffusing out of the polymeric matrix because of its size.

The cumulative release of protein from C2 and C3 modified vascular grafts could not be detected. The amount of CTGF released for C2 could not be detected because of the amount of PBS used. The concentration of the PBS might be diluting the amount of protein released.

**Bioactivity of protein released**

The bioactivity of CTGF from C1 modified vascular graft was determined by treating HDF and RVSMC cells with the supernatant obtained during release studies (see figures 7-8-7-9). The growth factor released was active in stimulating the proliferation of HDF and RVSMC cells. The amount of protein released on day 3 stimulated a significant amount of cellular proliferation compared to cells treated with cell culture medium. The stimulatory effect of CTGF released from modified vascular graft on HDF proliferation is supported by findings by various researchers in which CTGF was reported to be overexpressed in dermal fibrotic lesions such as hypertrophic scars, scleroderma and mesenchyme of internal organs (124-127).

The CTGF released from C1 modified vascular graft also stimulated a significant increase in RVSMC proliferation on 3$^{rd}$ and 14$^{th}$ day. Increased n RVSMC proliferation on day 3 was due to the growth factor released. CTGF has been reported to promote growth, migration and ECM expression of cultured VSMC. The result suggested that CTGF had an active role in atherogenesis (119). While, the increase RVSMC proliferation on day 14 might be due to the
combination of PLGA degradation by product and protein release. Degrading products of 50/50 PLGA films increased the proliferation and adhesion of VSMC proliferation (128).

Figure 7-10 compares the effect of CTGF released from C1, C2 and C3 modified vascular grafts. It seems that there was an initial burst of CTGF released from C2 modified vascular graft, because it stimulated more RVSMC proliferation compared to C1 modified vascular graft. In addition, the protein released from C1 modified vascular graft did not stimulate any significant cellular proliferation on day 1, but it increased after day 3. The inability of growth factor released on the 12th hour to inhibit cell proliferation might be due to the denaturing of the protein during the preparation of the coating solution (i.e., sonicated). Sonication of emulsion (i.e., water and oil phase) was suggested by van de Weert et al., to provoke cavitation stress that may destroy proteins between local temperature extremes and resulting free radical formation (98).

**Migration studies**

The effect of various doses of CTGF, and CTGF released from C1 modified vascular graft to stimulate RVSMC migration in a modified Boyden chamber was studied. For this study, migrated cells were obtained by counting cells that migrated through the 8 μm pore. The dark nucleus of the cells stained with hematoxylin was used an indicator for the cells that migrated. The cells that fell into the bottom chamber were ignored in this study because of the properties of the type of the cell that was used for migration analysis. Smooth muscle cells (SMCs) are anchoring depending cells that secrete extracellular matrix. In other for these cells to pass through the membrane and fall into the bottom chamber, they must undergoing apoptosis. In addition, the size of these cells ~ 120 μm will prevent the entire cells from migrating through the pores during the time of incubation.
Therefore, after 5 hours of incubation, a significant amount of SMCs migrated through the membrane (i.e., 8 μm) for the wells that 20 ng/ml CTGF was added as a chemoattractant. Compared to serum containing medium (FCS) and platelet derived growth factor, a known stimulant for SMCs migration, the various doses of CTGF used in this study induced SMCs migration (see figure 7-11).

The supernatant collected from the release kinetics study for C1 modified was also used for migration analysis. A significant amount of SMCs migrated through the membrane when the supernatant obtained from days 1 to 14 was used as chemoattractant. The amount of cells that migrated was significantly different from the amount of cells that migrated with serum containing medium and the supernatant obtained at the 12 hour. The stimulatory effect of the growth factor released to induce migration might be due to its bioactivity. The supernatant obtained from the days previously mentioned was able to stimulate HDF and RVSMC migration. The migratory effect of CTGF on vascular SMCs is supported by findings made by Fan et al. In a wound migratory assay, a significant amount of cells migrated to the gap (i.e., that was made to induce wound) after 5 hour of incubation(119). This was also supported by Shimo et al., findings which reported CTGF role’s in stimulating endothelial cell migration (129). The migratory effect of CTGF on VSMC cells was suggested to be depended on matrix metalloproteinase 2 (MMP 2, gelatinase A), which is involved in the promotion of VSMC during neointima formation (130).

**Biocompatibility analysis of CTGF modified vascular grafts**

C1 modified vascular grafts were seeded with HDF and RVSMC to visually observe its effect on cell adhesion and proliferation (see figures 7-12-7-13). An increase in the number of cells was observed on the modified grafts from day 3 to day 8 was observed. Cells also migrated
through the pores of the coating as the polymer degraded. The amount of VSMC proliferation quantified above with cell viability kit and the one observed after seeding onto the modified vascular graft is proposed to stimulate neointima formation during future in vivo studies.

**Conclusion**

In conclusion, we able to successfully modify woven Dacron vascular graft to control CTGF release. Three different coatings were prepared for the modification of the vascular grafts. Grafts prepared with C1 coating solution were able to successfully release CTGF. The growth factor released within the first 7 days.

Surface morphology of the grafts prepared with C3 coating solution revealed the presence of the polymer coating after 28 days of incubation in PBS solution. One of the reasons why the coating still remained on the graft is due to the increase in the amount magnesium hydroxide solution used in the preparation of the coating solution. This prevented water uptake of the polymeric matrix during the degradation studies. In addition, a small amount of the coating of C2 vascular graft degraded over 28 days. This resulted in a reduction in water uptake. The absence of BSA in the preparation of the coating solution resulted in the absorption and aggregation of the growth factor after the formation of the coating onto the vascular graft. Based on these findings we suggest that BSA, sucrose and EDTA is necessary in the preparation of PLGA/growth factor coating for Dacron vascular grafts. The supernatant obtained from C1 modified vascular graft was able to stimulate HDF and RVSMC growth. It also induced the migration of RVSMC in both a modified Boyden chamber and into the Dacron graft material (once the polymer coating became porous). These results support the likely success of using sustained delivery CTGF to more rapidly immobilize vascular grafts in the treatment of AAA.
Based on these findings we suggest that there is enough evidence that supports how hypothesis that CTGF modified graft will induce neointima formation which will optimally promote healing between the graft and the aorta.
Table 7-1  Reagents used for the water phase of the coating solution

<table>
<thead>
<tr>
<th>Sample</th>
<th>CTGF (w/v)%</th>
<th>BSA (w/v)%</th>
<th>Mg(OH)₂ (w/v)%</th>
<th>Heparin (w/v)%</th>
<th>EDTA (w/v)%</th>
<th>Sucrose (w/v)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>1 E-4</td>
<td>15</td>
<td>3</td>
<td>1 E-4</td>
<td>1 E-2</td>
<td>6 E-1</td>
</tr>
<tr>
<td>C2</td>
<td>7.5 E-5</td>
<td>3</td>
<td>7.5 E-5</td>
<td>1 E-2</td>
<td>6 E-1</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>3.75 E-6</td>
<td>3</td>
<td>3.75 E-6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 7-1  Amount coated onto Dacron modified vascular graft, n=4.
Figure 7-2  Water uptake and percent weight loss for CTGF modified vascular graft, n=4.

Figure 7-3  pH of supernatant obtained during release kinetics study, n=4.
Figure 7-4  SEM micrographs of coated grafts and grafts after release studies, 10 kV.  
A) C1 coated vascular graft 100x; B) C1 coated vascular graft 1500x; C) C1 modified vascular graft after 28 day release kinetics 100x; D) C2 coated vascular graft 100x;  
E) C2 coated vascular graft, 1500x; F) C2 modified vascular graft 28 day release kinetics 100x; G) C3 coated vascular graft 120x; H) C3 coated vascular graft 1500x;  
I) C3 modified vascular graft after 28 day release kinetics study.
Figure 7-5  SEM micrograph of cross section images (10kV) of A) Plain Dacron graft, B) C1 modified graft and C) C1 modified graft after 28 days of release study, 200x.

Figure 7-6  Cumulative release of BSA from C1 modified graft, n=4.
Figure 7-7  Cumulative release of CTGF from modified vascular graft per amount coated, n=4 (1 mg of coating is equivalent to 0.23 mm² of modified vascular graft).

Figure 7-8  Effect of C1 supernatant on HDF proliferation, n=4, *p<0.05. The control represents cells treated with serum-free only which is at normalized to a 100% after 48 hours incubation. Data represents mean ± standard error of mean of percent cell proliferation based on control.
Figure 7-9  Effect of C1 supernatant on RVSMC proliferation, n=4, *p<0.05. The control represents cells treated with serum-free only which is at normalized to a 100% after 48 hours incubation. Data represents mean ± standard error of mean of percent cell proliferation based on control.
Figure 7-10 Effect of supernatant obtained from C1, C2 and C3 modified grafts on RVSMC proliferation, n=4. The control represents cells treated with serum-free only which is normalized to a 100% after 48 hours incubation. Data represents mean ± standard error of mean of percent cell proliferation based on control.
Figure 7-11  Effect of various treatments on RVSMC migration.

SF- serum-free medium, PDGF_20 – 20 ng/ml platelet derived growth factor, CTGF 20, 20 ng/ml CTGF, and CTGF_50, 50 ng/ml CTGF.

Figure 7-12  Effect of supernatant obtained from C1 on RVSMC migration.
Figure 7-13  Optical images of HDF cells seeded onto modified vascular grafts, H&E stained 1000x.  A) Dacron only seeded RVSMC day 3; B) C1 modified vascular graft day 3; C) Dacron only day 8; and D) C1 modified vascular graft day 8.
Figure 7-14  Optical images of RVSMC cells seeded onto modified vascular grafts, H&E stained 1000x.  
A) Dacron only seeded RVSMC day 3; B) C1 modified vascular graft day 3;  
C) Dacron only day 8; and D) C1 modified vascular graft day 8.
CHAPTER 8
SUMMARY AND FUTURE WORKS

Summary of works

Modification of endovascular stent grafts is an emerging field that is being revisited in the field of tissue engineering. This is due to the fact that various questions such as the long term durability of endovascular stent grafts for AAA repair are being asked. Major problems such as endoleaks and migration of the stent grafts have been observed after implantation. If left untreated, these problems can lead to aneurysm rupture and the death of the patient. Poor healing between the stent graft and the aorta has been associated with problems observed after AAA repair. Various ways to improve the healing of endovascular stent graft was studied for this work.

To modify the vascular graft, a synthetic biodegradable polymer shown to induce neointima formation between an intracranial stent and its surrounding tissue was employed. 50/50 PLGA was used to modify the endovascular graft. The endovascular graft (stent and graft) was dipped coated in a 5% PLGA solution. The morphology of the coated stent graft revealed coating of the graft and not the stent. Based on these findings, this study’s focus shifted towards the modification of the Dacron vascular graft.

Woven Dacron vascular grafts were modified with various concentrations of 50/50 PLGA (i.e., 5 and 10 % w/v) and coated with multiple layers. In order to choose an ideal concentration of PLGA and layers to modify the vascular graft, compression testing of the coated vascular graft within nitinol stents was performed. Compression testing of the modified vascular grafts revealed no statistical differences for the compression force of grafts modified with 5% PLGA dip coated five times and plain grafts (unmodified vascular grafts). Hence, all studies performed to modify the vascular graft continued with this concentration and layers of coating.
The impregnation of growth factors within the polymeric matrix was hypothesized to improve the healing between the graft and the surrounding tissue. Thus, the modification of the vascular grafts with a model protein, BSA (Bovine Serum Albumin), was studied. BSA excluding the growth factor was used initially to modify the vascular graft because of cost and its ability to mimic the release of growth factor from biodegradable matrices. The effects of various molecular weights of 50/50 PLGA were used to control the release of protein. A slow release of protein was observed with higher molecular weight PLGA and a rapid release of the protein for lower molecular weight polymer (Mw = 39 kDa). The protein released from grafts modified with the various molecular weights within 3 days. Based on this finding, higher molecular weight molecular (Mw =144 kDa) 50/50PLGA was used for the long term elution of growth factors from vascular grafts.

Dacron grafts were successfully prepared to have drug eluting capabilities. The vascular grafts were impregnated with growth factors: basic fibroblast growth factor (bFGF) or connective tissue growth factor (CTGF). The release of this growth factor from the impregnated vascular graft and its bioactivity on vascular cells were analyzed. The coating solution used in the preparation of bFGF impregnated vascular graft varied based on sucrose concentration. Increasing sucrose concentration for the modification of B2 coated vascular graft reduced the release rate of soluble growth factor in the release medium (PBS). Release medium obtained on day 3 and 7 for B1 and B2 modified vascular graft stimulated a significant amount of RVSMC proliferation compared to those obtained at the 12 hour and 1 day. In addition, percent RVSMC proliferation for cells treated with the supernatant exceeded the control (serum-free treated cells).

However for HDF cells, some inactivity of the growth factor was observed. Cellular proliferation within the first few days were less than 80% based on the control. An increase in
HDF cells was observed for B1 modified vascular graft on day 1 and day 14, and the cellular proliferation on day 14 exceeded the control. The increase in cellular proliferation for B1 modified vascular graft was assumed to result from the release of growth factor and the by products of polymer used for the modification of the vascular graft. For B2 modified graft an increase in cellular proliferation was observed on days 3 and 7. The effect of growth factor released from B2 modified vascular graft on HDF proliferation was similar to RVSMCs. Based on this finding, we conclude that increasing the sucrose concentration maintains the activity of bFGF which stimulates HDF and RVSMC proliferation. bFGF coated vascular graft allowed HDF and RVSMC also allowed cells to adhere, proliferate and migrate for 8 days.

Connective tissue growth factor was also impregnated in Dacron vascular grafts. CTGF was used because of its ability to stimulate fibrosis. For CTGF impregnated graft, the effect of BSA on the release of the growth factor from the modified vascular graft was observed. In addition, the bioactivity of this modified graft to stimulate HDF and SMCs proliferation was analyzed. The absence of BSA in the coating solution prevented the release of CTGF from the modified graft. Some of the reasons why CTGF was not released from two modified vascular grafts prepared without BSA include: the adsorption of the growth factor to the polymer and denaturing of the growth factor during the preparation of the coating solution.

CTGF released for up to 14 days for the grafts prepared with BSA with in the coating solution. The release medium obtained from this graft stimulated HDF and RVSMC proliferation, and RVSMC migration. A similar pattern in the cellular proliferation was observed for both cell types. In regards to the migration analysis, a significant amount of cells migrated with the released medium obtained between days 1 and 7, compared to cells treated
with serum free medium and cell culture medium. The supernatant obtained from this vascular
graft is active enough to stimulate both the proliferation and migration of vascular cells.

A significant increase (40%) in cellular proliferation was observed for supernatant
collected from the C1 vascular graft compared to the PLGA modified graft. CTGF modified
vascular graft enhanced the proliferation of RVSMC compared to bFGF modified vascular graft
(see figure 8-1). Therefore, further studies to modify Dacron vascular grafts with growth factors
should be performed with CTGF.

**Future work**

**Impregnation of woven Dacron vascular graft with growth factors**

The impregnation of the grafts with growth factors (chapters 6 and 7) lacked detailed water
uptake profiles. For future studies, water uptake at the different time intervals should be
obtained so as to better understand the release of protein from the polymer matrix. This can then
be correlated with the pH of the supernatant.

Coencapsulation of the protein and polymer with nonionic block copolymer is suggested
to help increase the amount of protein released from the modified graft. In a study performed by
Blanco et al., an increase in protein release was observed when poloxamer 188 was
coenencapsulated with PLGA microspheres (131). This nonionic block copolymer should reduce
the interaction of protein with PLGA while increasing the amount released.

The use of other biodegradable polymers such as poly caprolactone or 75/25 PLGA
instead of 50/50 PLGA should be considered, as to help prolong the release of the protein from
the modified vascular graft.

A combination of both growth factors used in this study should be tried for the
modification of the vascular graft. The preparation of the coating solution for a combination of
this growth factors should be based on C1 or B2 modified vascular grafts. The amount of growth
factors released from this modify vascular graft should be analyzed using an ELISA kit. The effect of the supernatant obtained on vascular cell proliferation and migration should also be evaluated.

**Migration studies**

Cells used for the migration studies were harvested from rabbit’s aorta. During the culturing of RVSMC, we realized that the cells change phenotypes after passage 5. Therefore, for migration studies, RVSMC up to passage number 5 should be used.

Migration studies with the supernatant obtained from bFGF vascular grafts should also be studied for future work. This should be compared with the average number of migratory cells with CTGF supernatant (i.e., from modified grafts).

In addition, the effect of this supernatant on fibroblast migration through the modified Boyden chamber should be evaluated.

In regards to the technique, in this study the membranes we fixed, stained and imaged with an optical microscope. We then counted the number of migratory cells from 4 optical views using a 10x objectives. One of the problems that were observed while using this method is variance in the number of cells counted, from one person to another.

To prevent this unfairness, a person blinded to the experiment is recommended to count the migratory cells.

Another way to improving migration analysis is by modifying the way in which the cells are counted. This can be done by trypsinizing the cells that migrated through each membrane, and transferring them to a 96 well plate. A Cell Titer 96 Aqueous reagent used in the measurement of the number of viable cells can then be employed to quantitative amount of the viable cells that have migrated.
In vivo analysis

In vivo studies analysis of the modified vascular graft should based on methods described by van der Bas et al (76). This method is suggested, because it evaluates the effect of growth factor impregnated vascular graft in an *in vivo* environment.

Briefly, at least four female pigs with an average age of 10-11.5 months can be used in vivo analysis of this study. The diameter of the aortas should be measured at various levels before the construction of the impregnated graft. All of the stent grafts implanted should be oversized by 10% with respect to the site of deployment in the thoracic or abdominal aorta. Since the grafts would be hand sewn, they should be oversized by 10% to 15%. Animals should be sacrificed after 4 weeks and 8 weeks of implantation. The healing of the stent graft should be evaluated macroscopically, microscopically, and with scanning electron microscopy.

To implant the modified stent graft, a midline incision should be created to dissect the abdominal aorta. A small arteriotomy should be made for the introduction of the delivery system. A 20F endovascular sheath can be used for the introduction of the stent grafts. The stent grafts should be deployed in the aorta in a sequence: from the thoracic level to just proximal to the aortic bifurcation. To prevent the effect of flow, the control stent grafts should be placed in the thoracic aorta and the modified stent graft in the aorta below the diaphragm.
Figure 8-1  Effect of CTGF, bFGF and PLGA on vascular cell proliferation, n=4. *p<0.01
APPENDIX A

IMMUNOHISTOCHEMICAL DEMONSTRATION OF ALPHA-SMOOTH MUSCLE CELL ACTIN

Note: This protocol was used to validate the cell type harvested from the rabbit aorta.

1. Cells harvested from rabbit aorta were cultured in tissue culture flask
2. Cells were then subcultured in a 4 well chamber. The cells were seeded at low confluency.
3. At 70% confluency, cells were washed with PBS and fixed with 4% paraformaldehyde. The cells were then incubated at room temperature for at least two minutes. (Store slides in 70% ethanol if the staining process is not ran immediately).
4. Wash 3X PBS and wipe slides.
5. Permeabilize the cells with 0.5% Triton X-100 in PBS for five minutes at room temperature
6. Wash three times with PBS and wipe slides.
7. Block non specific sites by incubating with 1% BSA in PBS for at least 30 minutes in Room temperature (Add 2 drops of primary antibody if using Sigma’s - kit). Wash off excess reagent but do not wash slides.
8. Quench endogenous peroxide with 2 drops of hydrogen peroxide for 5 minutes.
9. Cover the slides with primary antibody diluted in 1% BSA for at least 1 hour (2 drops of primary antibody).
10. Wash three times with PBS and wipe slides.
11. Cover slide with biotinylated secondary antibody. Incubate for 20 minutes (if using Sigma’s kit – add 2 drops)
12. Prepare substrate reagent while waiting for the secondary antibody to bind. The substrate agent contains the following: 4 ml of deionized water; 2 drops of acetate buffer; 1 drop of AEC chromogen and 1 drop of hydrogen peroxide.
13. Wash 3X with PBS and wipe slides
15. When sufficient staining has been achieved rinse slides in deionized water for 5 minutes. Wipe off excess.
16. Counter stain with Harris hematoxylin for 2 minutes
17. Rinse in gentle running tap water to “blue” the hematoxylin.
18. If desired: apply glycerol gelatin or aqueous mounting media carefully. Cover with coverslip.
LIST OF REFERENCES


94. Alexis F. Factors affecting the degradation and drug-release of poly(lactic acid) and poly[(lactic)-co-(glycolic acid)]. Pharm Int 2005;54:36-46.


118. Brigstock D. The connective tissue growth factor/cysteine rich 61/ nephroblastoma overexpressed (CCN) family.


BIOGRAPHICAL SKETCH

Olajombo was born on January 18, 1978 to Olugbemi and Yinka Moloye in Madison, WI. She lived in Madison for two years before moving to Amerst, MA. She lived in Amerst for two years while her dad worked on his Ph.D. When her dad completed his Ph.D, she moved back to her parent’s hometown in Nigeria. She attended primary school at Bodija International School in Ibadan, Oyo State. While in primary school she enjoyed attending her math and science classes. After the completion of primary school, she attended Methodist Grammar school to specialize in science. Her time at Methodist Grammar school was cut short, because her father moved back to United States for a one-year sabbatical. Olajombo finished 9th grade in a high school at Ames, Iowa. She also ran track for her school and attended the state championship meet. In 1992, she moved to Tallahassee, Florida, where her father had an appointment as a professor at Florida Agricultural and Mechanical University (FAMU). Olajombo completed high school at Rickards High school. In high school, she was involved in Mu Alpha Theta (Math society) and the National Society for Black Engineers. She also attended various summer programs at FAMU which include a biological science program funded by the National Science Foundation. At this program her passion for science grew, especially in chemistry with the help of Mr. Richard Ford. Mr. Ford made chemistry fun and easy to learn. During this summer program (summer 1993), she decided to major in chemical engineering because of her love of math and chemistry.

She attended Florida State University in 1995, to study chemical engineering (ChE). During her second year in the program, she realized that ChE was not the field she was particularly interested in. Olajombo’s passion was to improve the quality of life of people who were sick or were born with disabilities. She started emailing companies which focused on biotechnology to understand what the field was all about. She completed her bachelors of science degree in 1999, and moved to Tampa to work on her masters in biomedical engineering.
at the University of South Florida (USF). While at USF, she sought a direction for her career path. She applied for numerous jobs and fellowships. She received the Graduate Degrees for Minorities in Engineering and Science consortium (GEM) fellowship her final year at USF. She then moved to Gainesville to pursue a doctorate degree in biomedical engineering at the University of Florida.