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RICE UNIVERSITY

POLYMERIC DELIVERY OF INHIBITORS OF SMOOTH MUSCLE CELL PROLIFERATION

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

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ABSTRACT

Polymeric Delivery of Inhibitors of Smooth Muscle Cell Proliferation

by

Robert L. Cleek

Restenosis, currently estimated to occur in 30-50% of dilated lesions, continues to be a major limitation to the success of current interventional cardiovascular procedures. Presently, no established therapy prevents or ameliorates this complication. We propose an alternate method for reducing the rate of restenosis that utilizes localized delivery of inhibitors of smooth muscle cell (SMC) growth to injured arteries. SMCs were targeted because they are recognized as the major proliferative component of the stenotic lesion. The work in this thesis was specifically aimed at creating a biodegradable implantable microparticle delivery system, which when loaded with inhibitors of SMC growth, could be used to reduce the lesion. The effect of controlled delivery of two novel therapeutic compounds from this microparticle system on SMC growth was included in this investigation.

A method was developed to fabricate poly(DL-lactic-co-glycolic acid)/poly(ethylene glycol) (PLGA/PEG) blend microparticles that involves a double-emulsion-solvent-extraction technique to investigate the effect of polymer blend ratio on release kinetics. Two model drugs (FITC-IgG and FITC-dextran) were entrapped using this technique with high efficiency. In vitro release studies showed that the initial burst effect was dependent on the PLGA/PEG blend ratio. Moreover, the release rate increased
in direct relation to PEG content. A linear release profile was obtained for microparticles loaded with FITC-IgG for initial PEG weight fractions up to 5 wt% and a biphasic release profile was obtained for FITC-dextran loaded microparticles with rates dependent on the PEG content. These results demonstrate the feasibility of modulating the release profile of entrapped drug compounds from biodegradable microparticles by adjusting the PLGA/PEG blend ratio.

Tenascin, a large extracellular matrix glycoprotein, is thought to play an important role in SMC growth. Hence, we proceeded to investigate the inhibition of SMC proliferation and migration in vitro by an antisense (AS) oligodeoxynucleotide (ODN) targeted to the tenascin mRNA and released from PLGA/PEG blend microparticles. Release of AS-ODN was characterized by a small initial burst effect followed by a period of controlled release. SMC proliferation studies exhibited dose dependent growth inhibition with AS-ODN loaded microparticles. Microparticles loaded with scrambled (SC) ODN showed less growth inhibition than AS-ODN. Moreover, only the AS-ODN loaded microparticles inhibited migration. These results demonstrate the feasibility of entrapping an AS-ODN to rat tenascin into PLGA/PEG microparticles for controlled delivery to inhibit SMC proliferation and migration.

Basic fibroblast growth factor (bFGF) is a potent mitogen for SMCs and is believed to play a key role in neointimal formation. Consequently, we fabricated PLGA/PEG blend microparticles loaded with an antibody (Ab) against bFGF, and determined the effect of the Ab-bFGF released on smooth muscle cell proliferation in vitro. The release of Ab-bFGF from PLGA/PEG microparticles was characterized by a small initial burst effect followed by a period of controlled release. SMC proliferation studies exhibited dose dependent growth inhibition for Ab-bFGF loaded microparticles. Negative controls containing IgG showed no growth inhibition. A significant portion of the Ab-bFGF released from the PLGA/PEG microparticles was determined to be active.
for the time period investigated. This work demonstrated the feasibility of entrapping an
Ab-bFGF into PLGA/PEG microparticles for controlled delivery to inhibit bFGF
stimulated SMC proliferation.

Lastly, preliminary in vivo studies demonstrated that PLGA/PEG microparticles
can be implanted and immobilized adventitiously in the rat carotid artery. Ab-bFGF loaded
microparticles implanted in this manner did not reduce intimal hyperplasia in the
ballooned rat carotid artery model as compared to controls.
ACKNOWLEDGMENTS

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<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
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<tr>
<td>ANOVA</td>
<td>single-factor analysis</td>
</tr>
<tr>
<td>AS</td>
<td>antisense</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid protein assay</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>DCA</td>
<td>directional coronary atherectomy</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Media</td>
</tr>
<tr>
<td>EVAc</td>
<td>ethylene vinyl acetate</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>I</td>
<td>intima</td>
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<tr>
<td>IgG</td>
<td>gamma immunoglobulin</td>
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<tr>
<td>L</td>
<td>lumen</td>
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<tr>
<td>M</td>
<td>media</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>Mw</td>
<td>weight average molecular weight</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>ODN</td>
<td>oligodeoxynucleotide</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
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<tr>
<td>PTCA</td>
<td>percutaneous transluminal coronary angioplasty</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>SC</td>
<td>scrambled</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TN</td>
<td>tenascin</td>
</tr>
<tr>
<td>W/O/O</td>
<td>water-in-oil-in-water emulsion</td>
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</table>
CHAPTER 1
INTRODUCTION

1.1 Significance and Restenosis Therapies

Vascular proliferative diseases result from an excessive, inflammatory, fibroproliferative response to injury of the endothelial and smooth muscle cells (SMCs) of the arterial wall. The SMCs which are stimulated to migrate from media to intima are the principal fibroproliferative component of the lesion (Ross 1993). Various invasive treatments to alleviate the resulting stenosis have included percutaneous transluminal angioplasty, endarterectomy, and vascular grafting. However, a significant portion of the arteries reclose within the first 6 months after treatment and require repeat procedures. Percutaneous transluminal angioplasty, for example, has a restenosis rate of 30-50% (Beatt et al. 1990). Moreover, the financial implications of restenosis are astounding—estimated cost to the US is $9 billion.

Because of this unacceptable high restenosis rate and its astounding cost to society, there has been intense research into identifying clinical treatments that will reduce the rate of restenosis. Current clinical treatment methods to prevent restenosis include the administration of drugs and the applications of intracoronary interventional devices.

Many pharmacological approaches to preventing restenosis have been proposed such as antiplatelet agents and anticoagulants (Schwartz et al. 1988; Ellis et al. 1989), antiproliferative agents (O'Keefe et al. 1992; Desmet et al. 1994), and hypolipidemic agents (Austin et al. 1989; Rozenman et al. 1993). A subset of these compounds have proven to be effective in animal models of restenosis (Ferns et al. 1991; Simons et al. 1992; Abe et al. 1994), however, no clinical study has provided demonstrative proof that
any of the drugs benefit mankind. The implication of these findings seem to indicate that the dose and manner in which these compounds are administered may be suboptimal requiring the development of new delivery modalities and technologies.

Because no drug regimen has convincingly succeeded in overcoming restenosis, many clinicians have opted for intracoronary devices. In the use of mechanical intracoronary devices it is believed that the best results may be obtained from producing the largest lumen diameters. Of the various new devices currently available, stents and directional coronary atherectomy (DCA) produce the largest luminal diameters and thus make them potentially the most useful. In addition to a large lumen diameter, the stent is believed to provide a stable scaffold to prevent elastic recoil, limit the exposure of deep tissue to blood components, and improve the rheological properties by providing a smooth contoured lumen for blood flow. Observational studies, however, have reported high restenosis rates of 47% (Schatz et al. 1991; Laskey et al. 1993) for stents. DCA often results in the removal of deep arterial wall components such as medial and adventitial tissues (Garratt et al. 1990). This extensive injury is believed to stimulate intimal proliferation that results in restenosis rates of approximately 50% for DCA (Topol et al. 1993). The overwhelming fact remains that the use of mechanical intracoronary devices has not significantly reduced restenosis, leading researchers to explore other treatment methods.

1.2 New Methods to Treat Restenosis

The inadequacies of the existing pharmacological and mechanical methods have led us and others to search for alternative approaches to treat restenosis. These new methods to reduce restenosis focus on the use of synthetic materials to deliver active agents locally to the injured artery site. The work contained in this thesis focuses on the
development of a drug delivery system and the use of several novel pharmacological agents with this system. This work also has relevance to mechanical interventional treatments as it also seems probable that mechanical devices must also be capable of locally instilling agents that promote vascular healing in order to reduce restenosis.

Systemic delivery of pharmacological agents to limit lesion formation has for the most part been ineffective. This ineffectiveness may be largely attributed to the inability of systemic delivery to provide high localized drug concentrations. Localized drug delivery, however, addresses this deficiency by allowing the delivery of drugs at high local concentrations without causing severe systemic side effects. For this reason it is an attractive approach that may be more efficacious than systemic treatment in eventually reducing restenosis. Furthermore, potentially useful but toxic agents that would otherwise be unlikely candidates because of systemic concentration from injections can be used because of the benign low systemic concentration that would result from localized delivery.

Ideally, a delivery system could provide controlled release for both high and low molecular weight compounds. A number of possibly important SMC inhibitors have low molecular weights (Mw < 20,000) such as antisense oligodeoxynucleotides (Shi et al. 1993; Burgess et al. 1995). Moreover, gamma immunoglobulin antibodies to cytokines, growth factors, and receptors have high molecular weights of approximately 150,000 (Kuby 1994). Furthermore, if this drug delivery system could be made with polymers that are both biodegradable and biocompatible, the delivery system would eventually be absorbed or eliminated, and hence, removal would not be an issue.

Many different delivery motifs and device configurations could be used for the delivery of therapeutic compounds to the injured artery via a biodegradable polymer. However, microparticles are unique in that they have been used to delivery a wide range of compounds with the retention of bioactivity. Biodegradable microparticles prepared
from polyesters such as poly(DL-lactic-co-glycolic acid) (PLGA) and poly(lactic acid) (PLA) have been investigated extensively as controlled release delivery systems. The popularity of these microparticles arises out of the fact that PLGA and PLA are biocompatible, biodegradable, and among the few synthetic polymers which are approved for human clinical use (Holland et al. 1986). Furthermore, these microparticles can be administered vascularly for site specific delivery (Flandroy et al. 1993; Wilensky et al. 1995).
CHAPTER 2
BACKGROUND

2.1 Physiology of the Normal Muscular Artery

A schematic representation of the normal muscular artery is shown in Figure 2-1. The normal muscular artery is composed of three layers: the intima, the media, and the adventitia. The tunica intima forms the inner lining of the vessel. This layer is composed of a single-cell thickness of squamous endothelial cells, with a thin underlining basal lamina. The lining endothelial cells have an elongated shape, with a long axis parallel to the direction of blood flow.

Intimal endothelium cells play an important active role both in normal vascular function and in the pathogenesis of atherosclerosis and its complications. Endothelial cells maintain a nonthrombogenic surface on the luminal aspect of the vessel. Disruption of the endothelium predisposes the vessel towards thrombus formation.

Muscular arteries have a media defined by the internal and external elastic laminae, which separate the media from the intima and adventitia, respectively. The internal and external laminae are fenestrated elastic sheets, similar to the elastic lamina of elastic sheets within the media, and those present do not appear to be concentric. The major cellular element of the media is the spindle shape smooth muscle cell. Although these cells are arranged concentrically, the pitch of the continuous spirals relative to the vascular axis remains controversial. The normal media does not contain fibroblasts.

The function of the medial smooth muscle of muscular arteries is to augment or reduce flow by changing the lumen diameter. This allows the control of blood pressure in addition to flow. The SMCs, normally in a near-quiescent state, are surrounded by a
basement membrane composed of collagen type IV, laminin, entactin, and proteoglycans (Table 2-1).

The outlying tunica adventitia is composed of dense fibrous tissue that anchors and stabilizes the vessel. Collagen and elastin fibrils of the adventitia run longitudinally and aid in retaining vessel shape. The predominant cell of the adventitia is the fibroblast. The adventitia carries the afferent and efferent nerves and is nourished by minute vessels called the vasa vasorum.
Figure 2-1. Cross section showing the internal structure of the normal muscular artery. (From Rehabilitation In Ischemic Heart Disease (1983). Blocker, W.P. and Cardus, D. (eds.). New York: Spectrum Publications)
2.2 Pathophysiology of Restenosis

2.2.1 Cellular Events in Restenosis

Animal (Clowes et al. 1983) and human (Ip et al. 1991; Nobuyoshi et al. 1991) pathological studies have provided demonstrative proof that excessive smooth muscle cell proliferation and the synthesis of large volumes of extracellular matrix represents the final common pathway and the principle cause of restenosis. The early events and major triggering factors causing excessive repair response to the injured artery are not well defined and poorly understood. While much of the information obtained on this subject is derived from animal injury models rather than from human studies, the prevailing consensus for the genesis of restenosis is that of an accelerated version of atherosclerosis (Ross 1993). Both share similarities in key events which are a result of a typical response of the artery to injury. However, this response is accelerated in restenosis. The current postulated mechanism of restenosis may be categorized into an early phase consisting of platelet activation and thrombus formation, which occurs within minutes to hours after injury; an intermediate phase of cellular recruitment, which occurs within hours to days; and a late proliferative phase, which occurs from days to months.

2.2.1.1 Early Phase of Platelet Activation and Thrombus Formation

Interventional cardiovascular procedures lead to endothelium denudation, plaque fracture and medial dissection (Uchida et al. 1989). This damage exposes the highly thrombogenic subintimal tissue to serum components and leukocytes. Within minutes of injury, there is platelet adhesion that leads to aggregation and eventually thrombus formation (Steele et al. 1985). The response is proportionate to the severity of the injury and the degree of unfavorable rheological factors present at the lesion site (Kohler and
Jawein 1992). Platelet activation leads to secretion of various factors that include platelet-derived growth factor (PDGF) (Nakao et al. 1982) and transforming growth factor-β1 (TGF-β1) (Shuman 1986). TGF-β1 promotes the synthesis of collagen and proteoglycans in the extracellular matrix. The growth factor PDGF affects the stimulation of platelets enhancing further platelet aggregation. Platelets also activate leukocytes to release vasoconstrictor leukotrienes. Other cellular and secretory factors are likely to also be involved.

2.2.1.2 Intermediate Phase of Cellular Recruitment

Leukocytes from the blood are recruited by the thrombus and damaged endothelium. These cells then migrate into the degenerating thrombus and release growth factors and cytokines that affect SMC migration and proliferation (Nathan 1987). Growth factors include PDGF, FGF, endothelial growth factor, and TGF (Rappolee et al. 1988). Enzymes responsible for the dissolution of the thrombus are released from monocytes. During this phase there is neoendothelialization of the thrombus usually within a few days after balloon injury that prevents further platelet and thrombus deposition.

2.2.1.3 Late Proliferative Phase

The key events of the final phase involve SMC proliferation and extracellular matrix formation (Snow et al. 1990). When the reparative response is excessive the result is termed restenosis. Histologically, restenotic lesions consist of excessive neointimal volume of SMCs and extracellular matrix. It is postulated that medial SMCs from the site of injury, migrate through breaks in the internal elastic lamina, and excessively proliferate. Furthermore, once activated, SMCs undergo a characteristic phenotypical
transformation from a contractile to a synthetic phenotype (Pauly et al. 1992). It is these synthetic cells that are responsible for the production of excessive extracellular matrix.

Although SMC proliferation and synthesis of excessive amounts of extracellular matrix are the central cause of restenosis, the exact underlying mechanisms leading to it have not been clearly identified.

2.2.2 Extracellular Matrix Components

Extracellular matrix is a necessary component of the vascular repair process. This is evidenced by a growing body of research that suggests matrix expansion and remodeling may be as important as SMC proliferation in lesion formation. In particular, attention has been directed toward matrix changes that alter the ability of SMCs to proliferate and migrate in response to various mitogens. These events have been shown to be associated with the production of a number of extracellular matrix proteins including tenascin, thrombospondin, type I and type IV collagen, fibronectin, and proteoglycans (Table 2-1). The production of proteoglycans may be particularly important since proteoglycans of the extracellular matrix are capable of binding and sequestering heparin-binding growth factors such as FGFs (Salmivirta et al. 1992; Dinbergs et al. 1996) and VEGF (Gitay-Goren et al. 1992).

Two distinct phases are required for the reconstruction of the vasculature. SMCs must first alter focal points to permit migration into the repair site. This process depends primarily on proteases to cleave focal adhesion proteins. Secondly, new matrix must be produced once migration into the site has occurred. Initially this new matrix is counter adhesive to allow migration of SMCs and later is altered by the SMCs to become strongly adhesive. Much attention has been given to tenascin and thrombospondin because they appear to be major counteradhesive proteins.
2.2.2.1 Tenascin

Tenascin (TN) appears to be a major counter adhesive protein within the early matrix. In fact, tenascin production is induced concurrently with changes in smooth muscle phenotype both in vivo and in vitro (Hedin et al. 1991). Structurally, TN a large hexameric protein with disulfide linked multidomain subunits of 190 to 240 kd (Cliquet et al. 1991). It seems to be re-expressed in association with tissue growth and reorganization, e.g., in healing wounds and vascular tissue. In vascular tissue, TN is observed in the arterial media during vasculogenesis (Hoffman et al. 1990).

2.2.2.2 Thrombospondin

Thrombospondin is a counteradhesive protein that appears to be synthesis by SMCs after arterial injury (Raugi et al. 1990). The protein acts as a counteradhesion molecule by interfering with focal adhesions of fibroblasts, endothelial cells, and SMCs. This disruption allows the SMCs to migrate.
Table 2-1 Vascular extracellular matrix components and their function

<table>
<thead>
<tr>
<th>Component</th>
<th>Function</th>
<th>Location</th>
</tr>
</thead>
</table>
| Collagens (I, III, IV, V, VI, VII) | Tissue architecture  
                          | Tensile strength  
                          | Vascular permeability  
                          | Regulation of cell shape and phenotype | Media, adventitia, basement membrane |
| Elastin            | Tissue architecture  
                          | Vessel elasticity  
                          | Vascular permeability | Principally in the media |
| Fibronectin        | Cell adhesion  
                          | Cell spreading  
                          | Tissue architecture | Ubiquitous |
| Proteoglycans      | Collagen fibril formation  
                          | Cell-matrix contacts  
                          | Binding/storage of growth factors  
                          | Regulation of cell shape  
                          | Cell migration | Ubiquitous |
| Laminin            | Cell migration, adhesion, and differentiation | Basement membrane |
| Thrombospondin     | Cell adhesion; proliferation, and migration of SMCs | Appears in tissue remodeling |
| Tenascin           | Linked to cell rounding and migration | Appears in tissue remodeling and healing |
| SPAC               | Inhibits cell binding and spreading | Basement membrane, and appears in tissue remodeling |
| Integrins          | Cell adhesion  
                          | Structural link between cytoskeleton and cell surface | Cell surface molecules |
| Fibrillin          | Component of elastic microfibrils | Media |

2.2.3 Role of Growth Factors in Restenosis

Various growth factors play a number of important roles in the process of restenosis. Here we will discuss the significance of some of the major candidates of pathological significance.

2.2.3.1 Basic Fibroblast Growth Factor

Basic FGF is a potent SMC mitogen both in vitro and in vivo (Lindner et al. 1991; Reidy 1992). This is demonstrated by the fact that exposure of the vessel wall to the growth factor leads to abundant neointimal proliferation (Edelman et al. 1992). Furthermore, during angioplasty, ballooning results in cell death and destruction that releases bFGF and other growth factors. Heparanases and proteases from infiltrating macrophages also have the potential to degrade the extracellular matrix and release stored bFGF. Rats treated with a blocking antibody to bFGF has decreased SMC proliferation after balloon catheter injury, suggesting that this growth factor may be significant in the process of vessel repair and restenosis (Lindner and Reidy 1991). This treatment, however, did not effect eventual neointimal lesion formation suggesting that other growth factors are capable of stimulating the process in the absence of bFGF.

2.2.3.2 Platelet-Derived Growth Factors

The activation of platelets leads to the secretion of various mitogens to the damaged vessel wall. Platelets directly release PDGF-AB and -BB which serve as an initial stimulus to both SMC migration and proliferation. Once SMCs are activated they produce PDGF-AA which further induces migration and proliferation. The importance of platelet-derived PDGF is demonstrated by experiments in which neointimal lesion
formation is reduced in thrombocytopenic rats (Fingerle et al. 1989). Furthermore, a reduction in neointimal formation was shown for mice that were treated with anti-PDGF antibody (Ferns et al. 1991).

2.2.3.3 Transforming Growth Factor Beta

TGF-β is released by cells localized at the site of tissue repair, such as macrophages, lymphocytes, and possibly vascular SMCs (Sporn et al. 1987). It is important in lesion formation because it is a major regulator of extracellular matrix production (Chen et al. 1987).

2.3 Inhibitors of Restenosis

Many new pharmacological compounds have been proposed for decreasing the rate of restenosis. The most promising of these inhibitors are the oligodeoxyribonucleotides, which block gene expression, and monoclonal antibodies, which can be targeted to key proteins and growth factors.

Antisense (AS) oligodeoxyribonucleotides (ODNs) offer the potential to block specific gene expression within cells. The development of AS techniques will allow researchers to explore the function of genes and may also produce new therapies for human diseases such as restenosis. Inhibition of gene expression by AS-ODNs depends on the ability of an ODN to bind effectively to a complementary messenger ribonucleic acid (mRNA) sequence and prevent translation of the mRNA. AS-ODNs represents a potential therapeutic agent for inhibition of SMC proliferation and migration. AS-ODN effectiveness against SMC proliferation and migration has been demonstrated \textit{in vitro} using c-myc AS-ODNs (Biro et al. 1993; Shi et al. 1993; Bennett et al. 1994). Furthermore, animal studies using rat, rabbit, and pig models have shown AS-ODNs to
be somewhat efficacious in suppressing intimal thickening (Simons et al. 1992; Shi et al. 1994; Burgess et al. 1995). There are, however, many none specific biological effects of ODNs that can not be attributed to antisense mechanisms (Wagner 1994). Such effects may compromise the usefulness of AS-ODNs as therapeutic agents.

Studies in animal models have used neutralizing antibodies targeted against mitogenic growth factors to inhibit neointimal formation (Ferns et al. 1991; Lindner and Reidy 1991). An anti-PDGF polyclonal antibody administered to rats after ballooning of the carotid artery resulted in nearly 100% blockage of SMC migration in the neointima 8 days postinjury (Ferns et al. 1991), effectively inhibiting PDGF signaling. Systemic delivery of Ab-bFGF to injured arteries to reduce SMC replication has been proposed (Lindner et al. 1991; Lindner and Reidy 1991).

The principal problem with monoclonal antibody therapies has been recognition of therapeutic antibodies as foreign proteins by the immune system. If such difficulties can be resolved, however, neutralizing antibody therapies may become clinically viable. Several groups have made alternatives to classical murine monoclonal antibodies that may eliminate or reduce immune system recognition. For example, murine antibodies have been humanized by recombinant expression of modified murine antibody variable regions linked to human Fe (constant) regions (Lockwood et al. 1993). There has also been success in creating transgenic mice that express human immunoglobulin genes (Morrison 1994). These new approaches may allow for a greater variety of monoclonal antibodies to become available for clinical trials and eventual use in humans.

2.4 Local Delivery Using Polymeric Implants

The use of polymer stents or catheters has been attempted for intraluminal drug delivery (Goldman et al. 1987; Wolinsky and Thung 1990; Lincoff et al. 1994). However,
washout of the drug occurs rapidly from the arterial wall by convection and diffusion with this delivery mode. Furthermore, the use of perforated or infusion balloons for drug delivery can cause significant local tissue damage and cell necrosis particularly when high delivery pressures and large infusate volumes are utilized.

An alternative method is to surgically implant a biomaterial for adventitial delivery of drugs. Polymer-based delivery systems offer the potential for quantifiable sustained and controlled release from devices that can be implanted adjacent to target tissues. For example, Edelman and Karnovsky (Edelman et al. 1990) used ethylene vinyl acetate (EVAc) matrices to deliver heparin and provided evidence that continuous delivery decreases intimal hyperplasia. Villa et al. (Villa et al. 1994) developed a periadventitial silicone polymer collar to deliver and maintain high tissue levels of dexamethasone, and showed that prolonged drug delivery was necessary to prevent restenosis. Although studies with EVAc and silicone polymer demonstrated the feasibility and benefits of local controlled adventitial delivery of SMC inhibitors, it would be preferable to use biodegradable polymers as the drug carrier because the long-term implantation of a non-degradable material may result in an inflammatory response. Poly(α-hydroxy esters) are a class of biodegradable and biocompatible polymers that can be used and are discussed in section 2.4.1.

Microparticles are a unique delivery system in that they have been used to deliver a wide range of compounds with the retention of bioactivity. Furthermore, vascular site specific delivery to the media has been achieved with the use of microparticles (Flandroy et al. 1993; Wilensky et al. 1995), and they can be manufactured with biodegradable polymers such as any member for the poly(α-hydroxy esters) family.
2.4.1 Poly(α-hydroxy esters)

Poly(α-hydroxy esters) are a family of linear aliphatic polymers. The most common polymers in this family are poly(glycolic acid) (PGA) (Fig. 2-2a), poly(lactic acid) (PLA) (Fig. 2-2b), and copolymers of poly(lactic-co-glycolic acids) (PLGA) (Fig. 2-2c). These polymers have been proposed for numerous drug delivery systems. This is largely because PGA, PLGA, and their copolymers are biocompatible, biodegradable, and are among the few synthetic degradable polymers approved by the Food and Drug Administration (FDA) for human clinical use. Degradation occurs by random hydrolysis of the backbone, and to a lesser extent enzymatic degradation in vivo (Holland et al. 1986). Degradation products are eliminated from the body either through metabolic pathways or by direct renal excretion (Hollinger and Battistone 1986). The implant location must be well characterized and chosen with care in order to obtain predictable degradation characteristics: because acidic and basic environmental conditions can catalyze hydrolysis, and thus, greatly increase the degradation rate (Chu 1982).

The range of physical and mechanical properties achieved with poly(α-hydroxy esters) can be increased by copolymerizing the precursor ring molecules. For example, copolymers have higher degradation rates as compared to the homopolymers because crystallinity is rapidly lost in the copolymers. The degradation rate continues to increase in a nonlinear fashion (Miller et al. 1977), as the copolymers get closer to an equimolar ratio.

Poly(α-hydroxy esters) fulfill many of the material requirements necessary for the fabrication of drug delivery systems. These include biocompatibility, biodegradability, and ease of processing. Furthermore, a wide range of degradation rates can be achieved by the copolymers suggesting that it may be possible to fabricate a polymeric delivery system to degrade at a rate which would allow for controlled drug delivery.
Figure 2-2. Chemical repeat units of (a) poly(glycolic acid) and (b) poly(lactic acid).

\[
\begin{aligned}
(a) & \quad \left[ \text{CH}_2 - \text{C} - \text{O} \right]_n \\
(b) & \quad \left[ \text{CH}_3 - \text{C} - \text{O} \right]_n
\end{aligned}
\]

Poly(Lactic-co-Glycolic Acid)

\[
\begin{aligned}
+ \text{H}_2\text{O} \\
\end{aligned}
\]

Lactic Acid

Glycolic Acid

Figure 2-2c. Hydrolytic degradation of poly(lactic-co-glycolic acids) into lactic and glycolic acid.
2.4.2 Poly(ethylene glycol) (PEG)

PEG is defined as polyethylene oxides having hydroxyl end groups and a molecular weight of 20,000 or less. PEG is nontoxic and cleared by the FDA for internal use in the human body. While it is not biodegradable PEG can be passively excreted from the body for molecular weights below 20,000 with no toxic side effects (Herold et al. 1989). Furthermore, PEG is unique in that it is soluble in organic solvents such as dichloromethane and aqueous solutions.

2.4.3 Polymer Blends

Blending two polymers represents a different approach for controlled drug delivery applications. For example, poly(L-lactic acid) (PLA) has been blended with a series of nonionic Pluronic surfactants to obtain modified controlled release profiles (Park et al. 1992), and blends of PLGA with PEG have improved the delivery capacity and release profiles of microparticles (Yeh et al. 1995). Furthermore, two poly(DL-lactide) samples of a very different molecular weight have been combined (Bodmeier et al. 1989; Grandfils et al. 1996) to alter biodegradation rate in an attempt to modify release. Blends yield advantageous physical and mechanical properties that each individual polymer alone does not have, and often thermodynamic compatibilities are used to obtain different morphologies and matrix characteristics that have resulted in modifying release. Consequently, blending of PLGA and PEG may be useful in the design of a new drug delivery system to alter physicochemical properties.
CHAPTER 3

OBJECTIVES

The specific intention of the work contained within this thesis was to develop and characterize the use of a biodegradable microparticle delivery system for inhibitors of smooth muscle cell proliferation. We also intended to investigate the potential of two novel compounds at inhibiting SMC growth when delivered via our microparticle system. This was done to support our hypothesis that the localized delivery of inhibitors of smooth muscle cell proliferation may reduce the rate of restenosis occurring after interventional cardiovascular procedures.

The first step in our research was the design and characterization of biodegradable microparticles based on physical blends of PLGA and PEG fabricated from a water-in-oil-in-water (W/O/W) double-emulsion-solvent extraction technique for the controlled release of high and low molecular weight model drug compounds that may be used in inhibiting SMC growth. We aimed to deliver model compounds in a controlled manner using PLGA/PEG blend microparticles. In addition, we wished to characterize how the PLGA/PEG blend ratio modulates release kinetics, and how the release kinetics depend on the nature of the drug.

Antisense oligodeoxynucleotides represent a potential therapeutic agent for inhibition of SMC proliferation and migration. In the next phase of research, we investigated the inhibition of SMC proliferation and migration in vitro by an AS-ODN targeted to the tenasin mRNA. The aims of this study were to deliver a 24-mer ODN in a controlled manner using PLGA/PEG microparticles, characterize inhibition of SMC proliferation and migration by an AS-ODN released from PLGA/PEG microparticles, and
determine if inhibition of SMC proliferation by an ODN is sequence specific and dose dependent.

After interventional cardiovascular procedures, injured SMCs are believed to release bFGF, which stimulates adjacent viable SMCs to proliferate excessively. For this reason we chose to use an Ab to bFGF in this in vitro study. We aimed to deliver an Ab-bFGF in a controlled manner using PLGA/PEG blend microparticles, characterize the bioactive state of the Ab-bFGF released, and determine if we can inhibit SMC proliferation by an Ab-bFGF released from PLGA/PEG blend microparticles.

In order to provide a sound foundation for the continuation of this work, we performed preliminary in vivo studies. These studies sought to develop a method to immobilize PLGA/PEG microparticles adventitially, and test whether Ab-bFGF loaded microparticles inhibit neointimal formation in the balloon rat carotid artery model.
CHAPTER 4

MICROPARTICLES OF POLY(DL-LACTIC-CO-GLYCOLIC ACID) / POLY(ETHYLENE GLYCOL) BLENDS FOR CONTROLLED DRUG DELIVERY

4.1 Introduction

The success of adventitial drug delivery for the treatment of restenosis depends heavily on the ability to design a drug delivery platform that provides an ample dose of drug for a sufficient time period. Furthermore, the system should also be able to deliver high and low molecular weight compounds. In this chapter, we report on the design and characterization of biodegradable microparticles based on physical blends of PLGA and PEG fabricated by a W/O/W double-emulsion-solvent-extraction technique for the controlled release of high and low molecular weight model drug compounds. We sought to deliver model drug compounds in a controlled manner using PLGA/PEG blend microparticles, modulate the release kinetics with the PLGA/PEG blend ratio, and to characterize how the release kinetics depend on the nature of the drug.

4.2 Materials

4.2.1 Polymers and Reagents

Poly(DL-lactic-co-glycolic acid) (PLGA) of 50:50 lactic to glycolic acid copolymer ratio was supplied by Medisorb (Cincinnati, OH). The polymer weight average molecular weight (Mw) was measured by gel permeation chromatography as 42,200. Poly(ethylene glycol) (PEG) with nominal Mw of 4,600 (Milwaukee, WI) and poly(vinyl alcohol) (PVA), 88% mole hydrolyzed, with a Mw of 25,000 were purchased
from Aldrich. Two model drugs each of molecular weight comparable to that of growth factors or antibodies were utilized: Fluorescein isothiocyanate labeled dextran (FITC-dextran) of Mw = 19,600 (Sigma Chemical. St. Louis. MO) and FITC conjugated rabbit gamma immunoglobulin (FITC-IgG) of Mw = 150,000 (Sigma). Bicinchoninic acid (BCA) protein assay kit to measure the FITC-IgG concentration in an aqueous solution was purchased from Pierce Chemical (Rockford. IL).

4.3 Methods

4.3.1 Preparation and Characterization of Microparticles

Microparticles were prepared by a modification of a double-emulsion-solvent-extraction technique (Alonso et al. 1994). Briefly. 247.5 mg total mixture of PLGA and PEG was dissolved in 1 ml dichloromethane contained in a flint glass test tube. A model drug compound (2.5 mg) was dissolved in 125 μl water and injected into the test tube containing the polymer solution. The mixture was vortexed for 30 sec. The solution was transferred to a 400 ml beaker, containing 98 ml 0.3% aqueous PVA. by vortexing 2 ml of 0.3% aqueous PVA in the test tube for not more than 30 sec and pouring the mixture into the beaker. The resulting solution was vigorously mixed using a 2 inch magnetic bar at 530 rpm. The re-emulsification resulted in a double emulsion to which 100 ml of 2% aqueous isopropanol was added and continuously stirred for 1 hr. The extraction of the dichloromethane to the external alcoholic phase precipitated the dissolved polymer which in turn resulted in formation of microparticles. The formed microparticles were finally sieved to sizes smaller than 125 μm. rinsed in water, and collected by centrifugation. Four microparticle formulations were examined of different initial PEG content expressed on a dry basis for the total mass of polymer and drug: 0, 1, 2, and 5 wt% PEG.
Size distribution of the microparticles was measured with a Coulter Multisizer (Coulter Electronics, Hialeah, FL) after microparticles were redispersed in an Isoton II solution (Coulter Electronics). This analysis was carried out with a 200 μm aperture orifice tube and the results were reported as a mean microparticle diameter.

The entrapment efficiency of the model drug was determined by comparing the amount of starting FITC-labeled compound with the quantity actually entrapped by a solvent extraction technique (Cohen et al. 1991; Iwata and McGinity 1993). Approximately 10 mg of microparticles were added to 1 ml dichloromethane and allowed to dissolve over 6 hrs. The FITC-labeled compound was extracted by adding 1 ml water and shaken every 6 hrs over 24 hrs. The concentration of FITC-labeled compound in the aqueous phase was determined by absorption at 496 nm in a UV-VIS Beckman spectrophotometer (Fullerton, CA).

4.3.1.1 Nuclear magnetic Resonance (NMR)

NMR spectroscopy was utilized to determine the actual percentage of PEG remaining in the microparticles after preparation because a portion of the PEG was extracted during fabrication. Microparticle preparations weighing at least 10 mg were placed into glass NMR sample tubes with 1 ml of deuterated chloroform solution (Acros Organics, NJ) containing 1% v/v tetramethylsilane. The proton NMR spectra were acquired on a NMR spectrometer (Bruker AC250, Germany). The hydrogen of the methine group of the lactic acid unit of the PLGA copolymer resonated at 5.2 ppm while those of the methylene group of the glycolic acid unit appeared at 4.8 ppm. and the hydrogens of the methylene group of the PEG homopolymer appeared at 3.6 ppm. The areas under the peaks were integrated to determine the blend composition (Silverstein et al. 1991).
4.3.2 *In Vitro* Degradation of Microparticles

4.3.2.1 Gel Permeation Chromatography

Microparticles fabricated with different amounts of PEG of approximately 30 mg were placed into 1 ml water in 1.5 ml microvials and maintained at 37°C. Every 2 days the samples were decanted off and fresh water was added. At 9, 18, and 27 days samples of each formulation were collected, frozen and vacuum-dried for 24 hrs.

The PLGA molecular weight distribution was determined by gel permeation chromatography (Waters. Milford, MA) equipped with a differential refractor (Waters. Series 410). The samples were dissolved in chloroform and eluted in a series configuration through a Phenogel guard column (model 494386. 50x7.8 mm. 5 μm particle diameter. Phenomenex. Torrance. CA) and a Phenogel column (linear 0 - 100,000 mixed bed. 7.8x300 mm. 5 μm particle diameter) at a flow rate of 1 ml/min. The molecular weight distribution curve representing the PLGA was selected on the chromatogram and the molecular weight was determined relative to polystyrene standards (Tosoh. Tokyo. Japan). The half-life of each formulation was calculated by fitting the data for the number average molecular weight to an exponential function of time (Pitt and Gu 1987).

4.3.2.2 Scanning Electron Microscopy (SEM)

The morphology of degrading microparticles at 0, 3, and 9 days was observed by scanning electron microscopy (Model JSM-5300. JEOL. Tokyo. Japan) at 25 kV. Microparticle formulations (25 mg) of 1% FITC-dextran or FITC-IgG and 0 or 5 wt% initial PEG content were placed into 1 ml water in 1.5 ml microvials and maintained at 37°C. Every 3 days the samples were decanted off and fresh water was added. At 3 and 9 days microparticle samples were collected, frozen and vacuum-dried for 24 hrs. Before
viewing on SEM. samples were freeze-dried, mounted on glass coverslips with nickel print (GC electronics, Rockford, IL), and coated with a Au-Pd film of 300-600 Å thickness. Cross-sections of microparticles were obtained by embedding specimens in freezing media, freezing at -20°C for 10 min. and sectioning on a microtome. Then, they were washed to remove freezing media, centrifuged, freeze-dried, and mounted as described above.

4.3.3 In Vitro Release Studies

A sample of 25 (±0.09) mg of FITC-IgG loaded and 20 (±0.04) mg FITC-dextran loaded microparticles was weighed, placed into 1 ml water in 1.5 ml microvials, and maintained at 37°C on a shaker table (60 rpm). The supernatant was collected, after centrifugation, and replaced at 6, 12, and 24 hours, daily thereafter until day 8, and then every two days. The removed supernatant was filtered and stored at 4°C until analyzed. The FITC-dextran concentration was measured at 490 nm using a UV-VIS Beckman spectrophotometer. The FITC-IgG concentration was measured using the microplate BCA assay at 470 nm. These studies were conducted in water as a first approximation of physiologic conditions recognizing that degradation products could turn the supernatant acidic and accelerate the polymer degradation (as might happen in implantation sites that are not well perfused) (Suggs and Mikos 1996).

4.3.4 Statistics

All samples were run in triplicate, except for the determination of PEG weight percentage from NMR spectroscopy (n=1), and expressed as means ± standard deviation (SD). Different groups were compared at a given level using the unpaired two group t-test. Single-factor analysis of variance (ANOVA) was employed to assess statistical significance of the PLGA degradation half-life studies. A significance level of 0.05 was used in all the statistical tests performed.
4.4 Results and Discussion

4.4.1 Initial Microparticle Characterization

SEM revealed that smooth spherical microparticles were produced from all formulations whether or not PEG was included (Figure 4-1 a and b). The concentration of the polymer organic mixture was chosen so as to fix the solution viscosity in the range where microparticles slightly less than 100 μm in diameter would be produced and prevent phagocytosis (Tabata and Ikada 1988). The particle size of FITC-loaded microparticles decreased with increasing formulation weight percent PEG (Table 4-1) as determined by single factor ANOVA (p<0.05). The microparticle size was also dependent on the model drug used. FITC-IgG loaded microparticle formulations made with 2 and 5 wt% initial PEG content were smaller than their FITC-dextran counterparts (p<0.05). Differences in loading were also noted (Table 4-1). The entrapment efficiency of FITC-dextran loaded microparticles decreased with increasing PEG content (p<0.05). The entrapment efficiency of the FITC-IgG loaded microparticles did not depend on PEG content as determined by single factor ANOVA (p>0.05).

NMR studies showed that a substantial fraction of the PEG originally dissolved in the organic phase of the emulsion was extracted into the aqueous phase and not incorporated into the final microparticle (Table 4-2). However, the weight percent of PEG incorporated into the final microparticle and the corresponding percentage remaining were a function of the initial PEG weight percentage. Microparticle formulations
Table 4-1. Entrapment efficiency, entrapment, and mean diameter of FITC-dextran and FITC-IgG loaded microparticles.

<table>
<thead>
<tr>
<th>Initial PEG Weight Percent (wt%)</th>
<th>FITC-dextran</th>
<th>FITC-IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Entrapment Efficiency * (%)</td>
<td>Entrapment (wt%)</td>
</tr>
<tr>
<td>0</td>
<td>77 ± 2</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>1</td>
<td>76 ± 3</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>64 ± 1§</td>
<td>0.64 ± 0.01§</td>
</tr>
<tr>
<td>5</td>
<td>67 ± 2§</td>
<td>0.67 ± 0.02§</td>
</tr>
</tbody>
</table>

*Theoretical loading was 1 wt%.
§Statistically different from value corresponding to immediately smaller initial PEG content.

Table 4-2. Percentage of PEG incorporated in microparticles.

<table>
<thead>
<tr>
<th>Initial PEG Weight Percent (wt%)</th>
<th>Final PEG Weight Percent (wt%)</th>
<th>Percent of Initial PEG Remaining After Fabrication *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.49</td>
<td>49</td>
</tr>
<tr>
<td>2</td>
<td>0.65</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>1.49</td>
<td>30</td>
</tr>
</tbody>
</table>

*Final PEG wt% divided by initial PEG wt%.
manufactured with 1, 2, and 5 wt% initial PEG content contained 0.49, 0.65, and 1.49 wt% PEG in the final microparticles, and the corresponding percentage of original PEG incorporated was 49.33, and 30 wt%. Thus, the higher the initial PEG content, the lower the percentage incorporated in microparticles.

4.4.2 In Vitro Degradation of Microparticles

The decrease in the PLGA weight average molecular weight of microparticles made with 0, 1, 2, and 5 wt% initial PEG content during incubation over a 27 day period is shown in Figure 4-2a. A reduction in the initial weight average molecular weight of approximately 10 fold was observed after 27 days of degradation for all samples. The half-life for PLGA 50:50 in microparticle formulations of 0, 1, 2, and 5 wt% initial PEG content was calculated (Figure 4-2b) to be 10.4 ± 0.4, 10.8 ± 0.4, 12.8 ± 0.8, and 12.6 ± 0.8 days, respectively. The PEG content had no significant effect on the PLGA half-life (p>0.05).

SEM micrographs of FITC-IgG loaded PLGA 50:50 microparticle formulations of 0 and 5 wt% initial PEG content incubated for 0, 3, and 9 days are shown in Figure 4-1 (a, b, c, d, e, and f). The shape and integrity of the microparticles were maintained throughout the 9 day study, but significant morphological changes were exhibited as early as day 3. The microparticles at day 0 exhibited a smooth surface and a spherical shape while at day 3 surface pores became apparent and by day 9 the pores comprised a large percentage of the surface area revealing the internal structure of the microparticles for both formulations. Notably, microparticles made with 5 wt% initial PEG content exhibited a similar structure to 0 wt% PEG throughout the time course of this study. Moreover, SEM examination of microparticle cross-sections revealed similar structural features (data not shown). This suggests that any changes resulting from the addition of PEG are below the resolution of SEM at 500X magnification, and not macroscopic.
Figure 4-1. Scanning electron micrographs of FITC-IgG loaded PLGA/PEG blend microparticle formulations of 0 (a) or 5 wt% (b) initial PEG content after 0 days of incubation at 37°C. 500X.
Figure 4-1. Scanning electron micrographs of FITC-IgG loaded PLGA/PEG blend microparticle formulations of 0 (c) or 5 wt% (d) initial PEG content after 3 days of incubation at 37°C. 500X.
Figure 4-1. Scanning electron micrographs of FITC-IgG loaded PLGA/PEG blend microparticle formulations of 0 (e) or 5 wt% (f) initial PEG content after 9 days of incubation at 37°C. 500X.
Figure 4-2a. Decrease of weight average molecular weight of PLGA 50:50 for PLGA/PEG blend microparticle formulations. Microparticle formulations of 0, 1, 2, and 5 wt% initial PEG content were incubated at 37°C. Error bars represent means ± SD for n=3.
Figure 4-2b. Linear regression of weight average molecular weight of PLGA 50:50 for PLGA/PEG blend microparticle formulations. Microparticle formulations of 0, 1, 2, and 5 wt% initial PEG content were incubation at 37°C. Error bars represent means ± SD for n=3.
4.4.3 *In Vitro* Release Studies

The cumulative mass and normalized mass release profiles for both compounds examined in this study depended on the PEG content of the microparticles (Figures 4-3 and 4-4). Microparticles prepared with FITC-IgG showed an initial release burst from 3.4 to 57.5% of loaded FITC-IgG over the first 24 hrs. while microparticles prepared with FITC-dextran showed a generally smaller release burst between 16.2 and 27.1% over the first 24 hrs. for initial PEG content of 0 and 5 wt% (Table 4-3). Much of the FITC-dextran was removed in the manufacturing process, as evidenced by lower loading (Table 4-1), and consequently less was available for release during the first 24 hrs of the *in vitro* release studies.

FITC-IgG and FITC-dextran release results for 0 wt% PEG formulations showed a small burst effect during the first 24 hrs. This was consistent with previous studies of PLGA microparticle formulations loaded with proteins (Cohen et al. 1991; Alonso et al. 1994). Microparticle carriers made of high molecular weight PLGA (ranging from 30,000 to 75,000) have exhibited a lag period after the initial burst of drug, during which the drug release rate is minimal. An enhanced release rate is observed after sufficient degradation of the polymer matrix which in turn facilitates the diffusion of entrapped compound (Sanders et al. 1986). The lag period may last several days to weeks and depends on the polymer molecular weight initially and the physicochemical properties of the drug. The microparticles prepared with 0 wt% PEG and loaded with FITC-IgG exhibited a lag period for the entire time course of investigation (22 days), as also shown in previous release studies (Iwata and McGinity 1993). However, the lag time for FITC-dextran was shorter because of its lower molecular weight.
Figure 4-3a. Cumulative release kinetics of FITC-IgG from PLGA/PEG blend microparticles. Microparticle formulations of 0, 1, 2, and 5 wt% initial PEG content were incubated at 37°C. Error bars represent means ± SD for n=3.
Figure 4-3b. Normalized cumulative release kinetics of FITC-IgG from PLGA/PEG blend microparticles. Microparticle formulations of 0, 1, 2, and 5 wt% initial PEG content were incubated at 37°C. Error bars represent means ± SD for n=3.
Figure 4-4a. Cumulative release kinetics of FITC-dextran from PLGA/PEG blend microparticles. Microparticle formulations of 0, 1, 2, and 5 wt% initial PEG content were incubated at 37°C. Error bars represent means ± SD for n=3.
Figure 4-4b. Normalized cumulative release kinetics of FITC-dextran from PLGA/PEG blend microparticles. Microparticle formulations of 0, 1, 2, and 5 wt% initial PEG content were incubated at 37°C. Error bars represent means ± SD for n=3.
Table 4-3. Burst effect of FITC-dextran and FITC-IgG for the first 24 hrs.

<table>
<thead>
<tr>
<th>Initial PEG Weight Percent (wt%)</th>
<th>Percent of Loading FITC-dextran</th>
<th>Percent of Loading FITC-IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.2 ± 1.9</td>
<td>3.4 ± 1.2</td>
</tr>
<tr>
<td>1</td>
<td>16.5 ± 0.4</td>
<td>15.5 ± 1.3§</td>
</tr>
<tr>
<td>2</td>
<td>27.4 ± 3.7§</td>
<td>54.0 ± 8.0§</td>
</tr>
<tr>
<td>5</td>
<td>27.1 ± 3.1</td>
<td>57.5 ± 3.8</td>
</tr>
</tbody>
</table>

§Statistically different from value corresponding to immediately smaller initial PEG content.
After the initial burst over the first 24 hrs, FITC-dextran loaded microparticles displayed a 12 day lag phase followed by a later release phase (Figure 4-4). A single factor ANOVA test showed that the release rates over the lag period were significantly different (p<0.05) for 0, 1, 2, and 5 wt% initial PEG content (Table 4-4). The FITC-dextran release rates were increased during the later phase (p<0.05) for all blend formulations until they became depleted (Figure 4-4b). Moreover, they were significantly different from each other as determined by a single factor ANOVA (p<0.05). The biphasic behavior of FITC-dextran release is likely related to the permeation of water into the microparticle. Following the initial release of readily solubilized FITC-dextran located at the surface of the microparticle, any further release occurs through the pores and channels formed by the dissolution of PEG (and FITC-dextran). Water permeation also contributes to the degradation of PLGA which in turn increases the release rate.

Microparticles containing FITC-IgG showed no lag phase over the time interval investigated. One of the most significant observations is that the release profiles appear to be linear after the initial burst for microparticles containing PEG and FITC-IgG. The correlation coefficients for all linear regressions were greater than 0.98. Furthermore, the rate constants increased significantly with increasing percentage of PEG (p<0.05). The FITC-IgG release rates were significantly lower than those of FITC-dextran during the lag phase for microparticles fabricated with 0 and 1 wt% initial PEG content, whereas the FITC-IgG release rates for 2 and 5 wt% PEG formulations were higher (p<0.05) (Table 4-4). In comparing later phases of release, FITC-dextran was released at a much greater rate for all formulations (p<0.05).
Table 4-4. Release rates for FITC-IgG and FITC-dextran from microparticles of varying initial PEG content calculated using linear regression.

<table>
<thead>
<tr>
<th>Release Rate</th>
<th>Initial PEG Weight Percent (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>FITC-IgG (μg/day/mg microparticles)</td>
<td>0.021</td>
</tr>
<tr>
<td>Days 1-28</td>
<td>±0.001</td>
</tr>
<tr>
<td>FITC-dextran (μg/day/mg microparticles)</td>
<td>0.082</td>
</tr>
<tr>
<td>Days 1-12</td>
<td>±0.018</td>
</tr>
<tr>
<td>FITC-dextran (μg/day/mg microparticles)</td>
<td>0.289</td>
</tr>
<tr>
<td>Days 12-28 *</td>
<td>±0.025</td>
</tr>
</tbody>
</table>

*Linear regression for 2 and 5 wt% initial PEG content was performed up to 22 days at which time at least 95% of FITC-dextran was released.
The release rate of entrapped model drugs was greater with increased PEG content. The leaching out of PEG (in addition to compounds) from the polymer blends would form molecular pores and channels within the polymer matrix which may enhance drug release. Microparticle formulations prepared with higher PEG weight percentages may have more molecular pores created during the manufacturing process that contribute to the initial release rate. They will also have more PEG remaining after manufacturing (Table 4-1) to form additional pores for release at later times. Moreover, the process of PEG solubilization and release may be influenced by polymer chain entanglements between PLGA and PEG, and hydrogen bonding between the PLGA carboxylic end groups and ether bonds of PEG (Park et al. 1992). The presence of PEG can further modify the emulsion process and, thus, the formed pore morphologies which would affect the drug release kinetics (Desai and Hubbell 1991). It is believed that degradation of PLGA plays a secondary role in controlling drug release because increasing the PEG content has no significant effect on PLGA half-life while increasing the rate of drug release.

A W/O/O technique has also been used to fabricate microparticles of PLGA/PEG blends (with blend ratios of 1:1 to 1:5) with diameter of 10 to 16 μm containing up to 50% ovalbumin (Yeh et al. 1995). These microparticles exhibited release rates ranging from 1 to 5 μg/day/mg microparticles which are much larger than those obtained in our study (Table 4-4). The PEG content and drug loading were much higher than those used in the W/O/W technique described in our study which explains the increased release rates. The different manufacturing processes, particle sizes, and model drugs used in the two studies also make any comparison of the release rates difficult.

The in vitro release study conducted here utilized a PLGA 50:50 copolymer and PEG with molecular weights of 42,500 and 4,600, respectively. Alterations in the release profile could be accomplished with different PLGA copolymer ratios and/or molecular
weights. Increasing the copolymer ratio or molecular weight may reduce the degradation rate significantly and the subsequent rate of drug release (Sanders et al. 1986; Alonso et al. 1994). Selection of PEG molecular weight in relation to drug molecular weight may also play an important role in determining the release profile and should also be considered. An alternate approach would be to use biodegradable copolymers of PLA and PEG which have been shown to have drug release profiles which depend on PEG copolymer composition (Zhu et al. 1990). Moreover, the physicochemical properties of the entrapped compound can have a dramatic effect on release as evidenced by the difference in release profiles obtained for FITC-IgG and FITC-dextran. Nevertheless, the use of blends of PLGA/PEG to create different release profiles provides a means to investigate effects of drug release kinetics on cellular response (Cleek et al. 1997).
CHAPTER 5

INHIBITION OF SMOOTH MUSCLE CELL GROWTH BY AN ANTISENSE OLIGODEOXYNUCLEOTIDE RELEASED FROM BLEND MICROPARTICLES

5.1 Introduction

Antisense oligodeoxynucleotides represent a potential therapeutic agent for inhibition of SMC proliferation and migration. AS-ODN effectiveness against SMC proliferation and migration has been demonstrated in vitro using c-myc AS-ODNs (Biro et al. 1993; Shi et al. 1993; Bennett et al. 1994). Furthermore, animal studies using rat, rabbit, and pig models have shown some efficacy of AS-ODNs in suppressing intimal thickening (Simons et al. 1992; Shi et al. 1994; Burgess et al. 1995).

In this chapter, we investigated the inhibition of SMC proliferation and migration in vitro by an AS-ODN targeted to the tenasin mRNA. We chose to use an AS-ODN to tenasin based on preliminary results showing inhibition of SMC proliferation (Denner et al. 1994). Tenasin is a large extracellular matrix glycoprotein secreted by SMCs that modulates SMC migration and differentiation in the vascular repair process (Hedin et al. 1991; Mackie et al. 1993). We sought to deliver a 24-mer ODN in a controlled manner using PLGA/PEG blend microparticles. Characterize inhibit of SMC proliferation and migration by an AS-ODN released from PLGA/PEG blend microparticles, and determine if inhibition of SMC proliferation is ODN sequence specific and dose dependent.
5.2 Materials

5.2.1 Polymers and Reagents

PLGA of 50:50 copolymer ratio of lactic to glycolic acid was purchased from Medisorb (Cincinnati, OH). The polymer weight average molecular weight (Mw) was measured by gel permeation chromatography as 45,000. Poly(ethylene glycol) (PEG) with a nominal Mw of 4,600 was purchased from Aldrich (Milwaukee, WI). Poly(vinyl alcohol) (PVA). 88% mole hydrolyzed, with a Mw of 25,000 was also supplied by Aldrich. All other reagents were of analytical grade. Distilled deionized water was utilized in all studies.

5.2.2 Oligodeoxynucleotides

Phosphorothioated ODNs were purchased from Oligos Etc., Inc. (Wilsonville, OR) and were shipped as lyophilized powder. The ODNs were resuspended in Tris-EDTA buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The AS-ODN sequence (5'ACCATG-GGGGCCCCTGCAGCTTGCTGA'3) was complementary to the translation initiation start site of the rat tenascin mRNA and inhibited SMC proliferation (Denner et al. 1994). The scrambled (SC) sequence (5'ATCAGCTCTGAGCGCAGCGCCAGCG3') was chosen to contain the same base composition as the AS-ODN sequence, but in a random order, and was screened through Genbank (Genetics Computer Group, Inc., Madison, WI) to ensure that the SC-ODN did not match rat mRNA for tenascin or other sequences.
5.3 Methods

5.3.1 Fabrication of ODN Loaded PLGA/PEG Microparticles

Microparticles were manufactured by a modification of a double-emulsion-solvent-extraction technique (Alonso et al. 1994) whereby 0.0025 g of ODN were dissolved in 50 µl water and injected into a flint glass test tube containing 245 mg PLGA and 2.5 mg PEG dissolved in 1 ml dichloromethane and emulsified with a vortexer for 30 sec. Blanks were prepared using 50 µl water. The solution was transferred to a 400 ml beaker, containing 98 ml 0.3% aqueous PVA. by vortexing 2 ml of 0.3% aqueous PVA in the test tube for not more than 30 sec and pouring the mixture into the beaker. The resulting solution was vigorously mixed using a 2 inch magnetic bar at 530 rpm. The re-emulsification resulted in a double emulsion to which 100 ml of 2% aqueous isopropanol was added. The extraction of the dichloromethane to the external alcoholic phase precipitated the dissolved polymer which in turn resulted in formation of microparticles. The system was stirred for 1 hr to assure total extraction of the solvent. Finally, the formed microparticles were finally sieved to sizes smaller than 100 µm. rinsed in water, and collected by centrifugation. All microparticles were sterilized by ethylene oxide before use.

The entrapment efficiency was determined by comparing the amount of starting ODN with the quantity actually entrapped. Approximately 5 mg of microparticles were added to 1.5 ml dichloromethane and allowed to dissolve over 6 hrs. The ODN was extracted by adding 1 ml water and periodically shaking over 24 hrs. then analyzed at 260 nm in a UV-VIS Beckman (Fullerton, CA) spectrophotometer.
5.3.2 ODN Release

A sample of 20 (±0.05) mg PLGA/PEG blend microparticles was placed into 1 ml water in 1.5 ml microvials, and maintained at 37°C on a shaker table (60 rpm). The water was collected and replaced at 6, 12, and 24 hours. daily thereafter until day 8, then every two days until day 20. The removed water was filtered and stored at 4°C until spectrophotometrically analyzed at 260 nm.

5.3.3 SMC Culture

Vascular SMCs were harvested from the carotid arteries of Sprague-Dawley rats by enzymatic digestion (Chamley-Campbell et al. 1981). Primary cells were cultured in Dulbecco’s Modified Eagle's Media (DMEM) supplemented with 20% fetal bovine serum (FBS), 1% glutamine, 100 U/ml penicillin, 100 mcg/ml streptomycin, and 50 mcg/ml neomycin. Cultures were maintained in a humidified incubator at 37°C with 5% CO2. All cells beyond the first passage were cultured in DMEM with 10% FBS, 1% glutamine, 100 U/ml penicillin, 100 mcg/ml streptomycin, and 50 mcg/ml neomycin.

5.3.4 SMC Proliferation

Second and third passage SMCs were seeded into 24-well cluster plates (Falcon, Lincoln Park, NJ) at a density of 30,000 cells per well (corresponding to 14,900 cells/cm²). Twenty-four hours after plating, the original medium was replaced with growth arrest medium (DMEM containing 0.1% FBS). After 72 hrs the arrest medium was replaced by 1 ml of medium containing 10% FBS. At that time, PLGA/PEG blend microparticles with or without ODN were also placed into the appropriate wells. After an additional 72 hrs, the cells were photographed, trypsinated, and counted on a Coulter Counter (Coulter, model ZF, Hialeah, FL). To achieve different dosages, 4 (±0.05), 8
(±0.05). 12 (±0.02), 16 (±0.04) and 20 (±0.07) mg of microparticles were used (n=6). Control wells contained DMEM/FBS alone.

5.3.5 SMC Migration

Third and fourth passage SMCs were seeded at 60,000 cell/well into 3/16 inch diameter Teflon fences (corresponding to 336,800 cells/cm²) in 6-well cluster plates and allowed to attach in 10% FBS containing DMEM. After 24 hrs. the Teflon fence was removed, the culture was rinsed 3 times with phosphate buffered saline (PBS) to remove any unattached cells, and 2 ml of fresh media (10% FBS) and 32 mg of microparticles (equivalent on concentration basis to 16 mg dose utilized in the proliferation study) were added to wells. SMCs were allowed to migrate over the surface. After 72 hrs. the cultures were rinsed with PBS, fixed with 10% neutral buffered formalin, and stained with toluidine blue-O (1% w/v). Morphometric analysis was performed to determine the area covered by the migrating and proliferating SMCs (Ishaug et al. 1996). Digitized images of the stained cultures were taken using a JVC Tk-10700 color video camera attached to the photographic port of an Askmina SMC4 (Jena, Micro-Tech Instruments, Dallas, TX) microscope and interfaced to a computer. The culture area occupied by SMCs was traced and calculated by calibrating the software (NIH Image 1.55) with a known standard.

5.3.6 Statistical Analysis

All samples were run in triplicate, except for dose response samples and control group in migration study (n=6), and expressed as mean ± standard deviation (SD). Single-factor analysis of variance (ANOVA) was employed to assess statistical significance of dose response data. If ANOVA was found to be significant by global F-test, Scheffe's test was performed to compare pairs of sample sets. Different groups were compared at a
given dosage using the unpaired two group t-test. A significance level of 0.05 was used in all of the statistical tests performed.

5.4 RESULTS

5.4.1 ODN Entrapment Efficiency and Release Kinetics

The entrapment efficiency of ODN loaded microparticles was 74% (± 4) for AS-ODN (n=6) and 77% (± 5) for SC-ODN (n=3). Therefore, the actual ODN loading of the microparticles was 0.74 and 0.77 wt% for AS-ODN and SC-ODN, respectively. The cumulative mass of AS-ODN released from 20 mg samples of PLGA/PEG blend microparticles exhibited a small burst effect at day 1 (17% of loaded drug) followed by a sigmoidal AS-ODN release profile with approximate release rates of 0.36 (± 0.02) and 0.09 (±0.01) μg/day/mg PLGA/PEG for days 1-5 and 5-20, respectively (Figure 5-1). After 20 days, 55% of total loaded AS-ODN was released. Release kinetics for SC-ODN were similar to AS-ODN (data not shown).

5.4.2 ODN Effects on SMC Proliferation

AS-ODN released from PLGA/PEG microparticles inhibited SMC proliferation in a dose dependent manner (Figure 5-2). Thus, SMCs exposed to 4, 8, 12, 16, and 20 mg of AS-ODN loaded microparticles exhibited decreases of 12, 36, 43, 61, 75%, respectively, relative to control wells which contained no microparticles. For 20 mg of PLGA/PEG blend microparticles loaded with AS-ODN, the final average cell count was not significantly lower than the attached cell number 24 hrs after plating. A single factor ANOVA test showed the dose responsiveness to be statistically significant (p<0.05) for 4, 8, 12, 16 and 20 mg dosages. The data were further analyzed with Scheffe's F-test to determine statistical significance of dosages from one another. All other dosages, except
the 8 mg compared to the 12 mg and 16 compared to 20 mg dose. were statistically significant from one another.

SC-ODN loaded microparticles also inhibited SMC proliferation in a dose dependent fashion (Figure 5-2) as determined by a single factor ANOVA involving all dosages (p<0.05). However, the inhibition was less than that seen with AS-ODN at all the doses tested (except at the 4 mg dose). PLGA/PEG blend microparticles without ODN (Figure 5-2) had no effect on SMC proliferation. The PLGA/PEG blend microparticles remained suspended in the medium. with no apparent cell attachment (Figure 5-3); they did not seem to interfere with the cultured SMCs.

5.4.3 ODN Effects on SMC Migration

A migration front was obtained by seeding SMCs into Teflon fences. Following removal of the fences 24 hrs after seeding. SMCs migrated radially and concurrently proliferated, resulting in an increased surface area (Ishaug et al. 1996).

Wells containing AS-ODN loaded PLGA/PEG blend microparticles showed smaller increases in cell surface area (Table 5-1) 72 hrs after fence removal (p<0.05) compared to wells containing no microparticles, SC-ODN loaded microparticles, or blank microparticles (0% ODN). The area covered by migrating SMCs in wells with AS-ODN loaded microparticles was 22% of the corresponding area in control wells (no microparticles). Moreover, the measured areas for wells with SC-ODN loaded microparticles and blank microparticles showed no significant difference from those for control wells containing only SMCs.
Figure 5-1. Cumulative mass and percent of AS-ODN released from PLGA/PEG blend microparticles. Microparticles contain 0.74 wt% AS-ODN. Error bars represent mean ± SD for n=3.
Figure 5-2. Dose-dependent growth inhibition of SMCs after administration of AS-ODN loaded (T72 AS-ODN), SC-ODN loaded (T72 SC-ODN), and blank (T72 Blank) PLGA/PEG blend microparticles. Cultures were serum stimulated (released from quiescence) over 72 hrs. Error bars represent mean ± SD for n=6. Statistical significance between microparticles containing AS-ODN and SC-ODN at a given dosage is indicated by (*), and ($) designates statistical significance between values containing either AS-ODN or SC-ODN and blank microparticles with no ODN (p<0.05). TP represents attached cell number 24 hrs after plating, and T72 represents cell number 72 hrs after serum stimulation in the absence of microparticles.
Figure 5-3. Light micrograph of cultured SMCs containing 12 mg blank microparticles with no ODN for 3 days (bar = 200 microns).
Table 5-1. Inhibition of SMC migration after administration of AS-ODN and SC-ODN loaded PLGA/PEG blend microparticles over 72 hrs. Cultures containing microparticles loaded with SC-ODN and no ODN (blank) showed fractional increases in cell population culture area not statistically different from those measured for controls with no microparticles (p>0.05). Statistical significance between microparticles containing AS-ODN and SC-ODN is indicated by (*). AS-ODN and blank by (§), and AS-ODN and control by (†) (p<0.05).

<table>
<thead>
<tr>
<th>SMC culture</th>
<th>Fractional increase in culture area ‡</th>
<th>% Area covered by migrating SMCs relative to control</th>
<th>Number of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.64 ± 0.19</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>Blank</td>
<td>1.81 ± 0.06</td>
<td>127</td>
<td>3</td>
</tr>
<tr>
<td>SC-ODN</td>
<td>1.70 ± 0.08</td>
<td>109</td>
<td>3</td>
</tr>
<tr>
<td>AS-ODN</td>
<td>1.14 ± 0.06 * § †</td>
<td>22</td>
<td>3</td>
</tr>
</tbody>
</table>

‡ Mean ± SD
5.5 DISCUSSION

ODN release from microparticles could provide an effective means of inhibiting SMC proliferation and migration *in vivo*. We showed that a 24-mer AS-ODN to the rat tenascin mRNA can be successfully incorporated into PLGA/PEG blend microparticles for controlled release, and that AS-ODN microparticles *in vitro* can inhibit SMC proliferation and migration.

The entrapment efficiency of our system, 74%, was slightly lower than that reported for protein loaded microparticle systems which varied from 80 to 90% (Alonso et al. 1994; Yan et al. 1994). The lower entrapment efficiency may be due to higher charge density and lower molecular weight of ODNs used in this study. The microparticle processing method was designed for the entrapment of proteins (Yan et al. 1994) and may need to be modified to increase the entrapment efficiency of ODNs.

ODN release results demonstrated a small burst effect during the first 24 hrs. This was consistent with microparticle systems containing proteins (Cohen et al. 1991; Alonso et al. 1994). Such a small burst effect may be advantageous, since it prevents sudden exposure of cells to a potentially toxic dose. Protein release from conventional microparticles made of high molecular weight PLGA (ranging from 30,000 to 75,000) has exhibited a lag time after the initial burst of drug. During the lag time, the microparticle release rate was minimal over several days before erosion of the polymer matrix became sufficient to release the entrapped compound, which resulted in an increased release rate (Sanders et al. 1986). Our ODN loaded microparticles, however, did not show this lag period and directly entered into a biphasic release pattern. This result deviates from previously reported conventional microparticle release kinetics (Iwata and McGinity 1993; Yan et al. 1994).
Inhibitors of SMC proliferation, including ODNs, have been delivered by a variety of polymer systems. Ethylene vinyl acetate copolymer, which is not biodegradable, has been utilized to deliver heparin (Edelman et al. 1990; Edelman and Karnovsky 1994) at zero order release rates of 0.0012 to 0.0024 μg/day/mg EVAc (Edelman et al. 1990) (release rate has been normalized by EVAc weight to allow comparison on per mass basis to PLGA/PEG blend microparticles). The EVAc device has also been used to incorporate an ODN sequence for controlled delivery and results suggest that it might be effective in vivo (Edelman et al. 1995) at inhibiting intimal thickening. Pluronic® gel has been used to deliver ODNs (Simons et al. 1992; Abe et al. 1994; Bennett et al. 1994; Edelman et al. 1995) and provided a relatively short (<3hr) bolus delivery of 200 (Bennett et al. 1994) to 1000 mg ODN (Edelman et al. 1995). The delivery rate for our microparticle system, on a per unit polymer mass basis, was significantly higher than that obtained with the EVAc matrix for heparin release (0.09 to 0.36 μg/day/mg PLGA).

The SC-ODN in this study served as a control for sequence specificity of the AS-ODN effect on SMC proliferation and migration. The SC-ODN had the same base composition as the AS-ODN, but in a randomized sequence such that it could not hybridize with tenascin or any other rat sequence contained within Genbank. The ability of the SC-ODN to inhibit SMC proliferation, although less effective than the AS-ODN, suggests that at least part of the inhibitory effect of the AS-ODN is not sequence specific, thus not an anti sense mechanism.

A true antisense sequence specificity is very difficult to demonstrate (Stein et al. 1993). In careful studies, AS-ODN to c-myb and c-myc have been shown to inhibit SMC proliferation in vitro and in vivo (Bennett et al. 1994). However, additional work has shown the inhibition of SMC by these AS-ODNs is due to the presence of four contiguous guanine residues within the sequences, which act by way of a non-antisense
mechanism to prevent the cells from progressing into S phase (Burgess et al. 1995). Other non-antisense ways that phosphorothioated ODNs can interfere with cell proliferation include their ability to bind and sequester basic fibroblast growth factor by virtue of their polyanionic nature (Guvakova et al. 1995): inhibition of DNA polymerase and RNAse H (Gao et al. 1992); and cytotoxic or cytostatic effects of mononucleotide degradation products.

The results presented in this study serve as a foundation for in vivo studies of microparticle based delivery of inhibitors of SMC proliferation and migration; this is one more step toward the develop a controlled release therapy for vascular proliferative disease.
CHAPTER 6
INHIBITION OF SMOOTH MUSCLE CELL PROLIFERATION BY AN ANTIBODY TO BASIC FIBROBLAST GROWTH FACTOR RELEASED FROM BLEND MICROPARTICLES

6.1 Introduction

In this study, we investigated the inhibition of SMC proliferation in vitro by an Ab targeted to bFGF following cardiovascular intervention. We chose to use an Ab for bFGF because we believe that therapeutic applications exist for compounds which can inhibit the inappropriate expression of bFGF. Injured SMCs are believed to release bFGF, which stimulates adjacent viable SMCs to proliferate excessively (Bikfalvi et al. 1997). This is supported by a number of in vivo studies (Lindner et al. 1991; Lindner and Reidy 1991) suggesting that inappropriate expression of bFGF may participate in this pathological condition. Systemic delivery of Ab-bFGF to injured arteries to reduce SMC replication has been proposed (Lindner et al. 1991; Lindner and Reidy 1991), however, improved antibody delivery systems will be necessary for monoclonal Ab therapy to reach its full potential. We therefore endeavored to deliver an Ab-bFGF in a controlled manner using PLGA/PEG blend microparticles to inhibit bFGF stimulated SMC proliferation. In addition, we sought to quantify the bioactivity of the Ab-bFGF released from PLGA/PEG blend microparticles.
6.2 Materials

6.2.1 Polymers and Reagents

Poly(DL-lactic-co-glycolic acid) (PLGA) of 50:50 copolymer ratio of lactic to glycolic acid was purchased from Medisorb (Cincinnati, OH). The polymer weight average molecular weight (Mw) was measured by gel permeation chromatography as 42,200. Poly(ethylene glycol) (PEG) with nominal Mw of 4,600 and poly(vinyl alcohol) (PVA). 88% mole hydrolyzed, with a Mw of 25,000 were purchased from Aldrich (Milwaukee, WI). Mouse gamma immunoglobulin was purchased from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit to measure the Ab-bFGF concentration in an aqueous solution was purchased from Pierce Chemical (Rockford, IL). Radiolabeled bFGF ([125]I bFGF) was purchased from Biomedical Technologies (Stoughton, MA).

6.2.2 Anti-bFGF Antibody

Female mice (8-week-old) BALB/c (Harlan Sprague Dawley, Indianapolis, IN) were immunized with human recombinant bFGF (hrbFGF) provided by Dr. J. Anthony Thompson of the University of Alabama (Birmingham, AL). Immunization was carried out by intraperitoneal and subcutaneous injection of 50 µg of hrbFGF in Complete Freund's Adjuvant (Sigma, St. Louis, MO). A booster immunization (50 µg of hrbFGF) was given a total of 3 times, 3 weeks apart in Incomplete Freund's Adjuvant. Two mice with the highest serum levels of anti-bFGF antibodies, as measured by ELISA, were given an additional 30 µg hrbFGF booster in phosphate buffered saline (PBS) intravenously 3 weeks after the last immunization, and spleen cells of the mice were harvested 3 days later for production of hybridomas. The monoclonal antibody against hrbFGF was produced by hybridomas using a modification of a previous technique
(Bjercke et al. 1986). The antibody was concentrated in Ultrafree centrifugal filters (Millipore. Bedford. MA) with a molecular weight cut off of 30,000.

6.3 Methods

6.3.1 Preparation and Characterization of Ab-bFGF Loaded Microparticles

Microparticles were manufactured by a modification of a double-emulsion-solvent-extraction technique (Cleek et al. 1997). Briefly, 125 µl of concentrated Ab-bFGF solution (2.5 mg/125 µl) was injected into a flint glass test tube containing 245 mg of PLGA and 2.5 mg of PEG dissolved in 1 ml dichloromethane and emulsified with a vortexer for 30 sec. The solution was transferred to a 400 ml beaker containing 98 ml 0.3% aqueous PVA. by vortexing 2 ml of 0.3% aqueous PVA in the test tube for not more than 30 sec and pouring the mixture into the beaker. The resulting solution was vigorously mixed using a 2 inch magnetic bar at 530 rpm. The re-emulsification resulted in a double emulsion to which 100 ml of 2% aqueous isopropanol was added. The extraction of the dichloromethane to the external alcoholic phase precipitated the dissolved polymer which in turn resulted in formation of microparticles: the system was stirred for 1 hr to extract the solvent. The formed microparticles were finally sieved to sizes smaller than 165 µm. rinsed in water and collected by centrifugation.

Size distribution of the microparticles was measured with a Coulter Multisizer (Coulter Electronics. Hialeah. FL) after microparticles were redispersed in Isoton II (Coulter Electronics). This analysis was carried out with a 200 µm aperture orifice tube and the results were reported as mean microparticle diameter.

The entrapment efficiency of the Ab was determined by comparing the amount of starting Ab-bFGF compound with the quantity actually entrapped. Approximately 10 mg
of microparticles were added to 1 ml of 1 N sodium hydroxide and shaken at room temperature for 40 min. The resultant hydrolyzed solution was then neutralized to pH of 7.4 by adding 500 μl of PBS. 190 μl of concentrated 5 N HCl. and a few drops of 0.01 N HCl. The concentration of Ab-bFGF compound in the aqueous phase was determined by the microplate BCA assay at 470 nm.

6.3.2 Ab-bFGF Release

A sample of 25 (± 0.09) mg PLGA 50:50 microparticles was placed into 1 ml PBS in 1.5 ml microvials. and maintained at 37°C. The fluid was collected and replaced at 6, 12, and 24 hours, daily thereafter until day 8, then every two days until day 30. The removed fluid was filtered and stored at 4°C until analyzed using microplate BCA assay at 470 nm.

6.3.3 Bioactivity of Microparticle Released Ab-bFGF

One mg of free Ab-bFGF and 50 mg of microparticles loaded with Ab-bFGF were suspended in 3 ml of PBS and separately dialyzed at 37°C against 1 l of PBS that was replenished at 7 days. At 1, 4, 7, and 14 days samples were taken from each dialysis bag. The microparticle-released samples were centrifuged and filtered before storage. All samples were stored at 4°C until further analysis. The total amount of Ab-bFGF present in each of the samples was determined by the microplate BCA assay. The amount of bioactive Ab-bFGF in the samples was determined by comparing the ability of each sample to inhibit [125]I bFGF binding to rat SMCs against a standard inhibition curve generated from known amounts of fresh Ab-bFGF. The relative bioactivity was calculated from the ratio of biologically active Ab-bFGF present to the total amount of Ab-bFGF in each sample.
For the binding assay, fourth and fifth passage SMCs were seeded into 24-well plates (Falcon, Lincoln Park, NJ) at a density of 30,000 cells per well and allowed to grow in 10% FBS for 3 days. To prepare standards, 30 μl of fresh Ab-bFGF was added in dilution series (1:10, 1:100, and 1:1000) to 300 μl of binding buffer (1 mg/ml BSA and 25 mM Hepes in PBS) containing 0.9 ng of [125]I bFGF and allowed to incubate at 37°C in 1.5 ml microvials. To prepare samples, 30 μl of microparticle-released Ab-bFGF solution and 30 μl of dilute free Ab-bFGF solution (1:10 dilution ratio) was treated in an identical fashion to standards as outlined above. After 30 min, the incubated solutions were added to cells which had been washed with PBS and aspirated. After 60 min at room temperature, the cells were washed with 500 μl of PBS. 500 μl of 250 μg/ml heparin (Elkins-sinn, Cherry Hill, NJ) in PBS, and again with 500 μl of PBS. A solution containing 1% Triton X-100 (Sigma, St. Louis, MO) in water was added to the cells for 15 min, and the lysate transferred to scintillation vials to which 5 ml of scintillation fluid (Packard Ultima Gold XR) was added. Samples were analyzed for gamma radiation levels on a Beckman LS 6000TA scintillation counter (Fullerton, CA).

6.3.4 SMC Culture

Vascular SMCs were harvested from the carotid arteries of Sprague-Dawley rats by enzymatic digestion (Chamley-Campbell et al. 1981). Primary cells were cultured in DMEM supplemented with 20% FBS, 1% glutamine, 100 U/ml penicillin, 100 mcg/ml streptomycin, and 50 mcg/ml neomycin. Cultures were maintained in a humidified incubator at 37°C with 5% CO₂. All cells beyond the first passage were cultured in DMEM with 10% FBS, 1% glutamine, 100 U/ml penicillin, 100 mcg/ml streptomycin, and 50 mcg/ml neomycin.
6.3.5 SMC Proliferation in Response to Free Ab-bFGF

Fifth and sixth passage SMCs were seeded into 48-well plates (Falcon, Lincoln Park, NJ) at a density of 9,000 cells per well. Twenty-four hours after plating, the original medium was replaced and DMEM containing bFGF (1 ng/ml) with or without Ab-bFGF was placed into the appropriate wells. After 96 hrs. the cells were trypsinized and counted on a Coulter Counter. To achieve different dosages, 5, 10, and 20 μg/ml of Ab-bFGF were used (n=3). Control wells contained DMEM/FBS alone and control IgG (mouse gamma immunoglobulin).

6.3.6 SMC Proliferation in Response to Ab-bFGF Loaded Microparticle

Fourth and fifth passage SMCs were seeded into 24-well plates (Falcon, Lincoln Park, NJ) at a density of 20,000 cells per well. Twenty-four hours after plating, the original medium was replaced and transwells containing PLGA/PEG microparticles with or without Ab were placed into the appropriate wells. After 24 hrs bFGF was added to the medium (1ng/ml) of the appropriate wells. After an additional 72 hrs, the cells were trypsinized and counted on a Coulter Counter. To achieve different dosages, 5 (±0.05), 15 (±0.05), and 30 (±0.08) mg of microparticles were used (n=3). Control wells contained DMEM/FBS alone. Parallel experiments were also conducted with SMCs not receiving bFGF stimulation.

6.3.7 Statistical Analysis

All samples were run in triplicate, except for microparticle dose response samples (n=6) and control groups in the same study containing no microparticles (n=9) or no microparticles or bFGF (n=12). All values were expressed as mean ± standard deviation (SD). Single-factor analysis of variance (ANOVA) was employed to assess statistical significance of dose response data. If ANOVA was found to be significant by global F-
test. Scheffe's test was performed to compare pairs of sample sets. A significance level of 0.05 was used in all of the statistical tests performed. The difference between two groups was compared at a given level using the parametric test on the difference of two means with a significance level of 0.05 in a two-tailed test. The bioactivity study was performed in duplicate (n=2) and values are expressed as mean ± range.

6.4 Results

6.4.1 Ab-bFGF Entrapment Efficiency and Release Kinetics

The average diameter of the microparticles was 81 (± 5) μm and the entrapment efficiency of Ab-bFGF loaded microparticles was 85 (± 2) %. The cumulative mass of Ab-bFGF released from 25 mg samples of PLGA/PEG microparticles exhibited a small burst effect over 24 hrs (15 ± 1 % of loaded compound) followed by a linear release profile (with correlation coefficient for linear regression of $r^2=0.984$) at a rate of 0.062 (± 0.03) μg/day/mg of PLGA/PEG microparticles for days 1 to 30 (Figure 6-1). After 30 days, 37 (± 2) % of total loaded Ab-bFGF was released.

6.4.2 Bioactivity of Released Ab-bFGF

The relative activities of microparticle-released and free Ab-bFGF dialyzed for 14 days are depicted in Figure 6-2. The activity of the microparticle-released Ab-bFGF decreased more rapidly than the free for the 1 and 4 day samples. However, after 4 days the microparticle-released Ab-bFGF maintained a relatively constant activity being 34% after 14 days, whereas the free Ab showed a precipitous drop after 4 days from a relative activity of 72% to 8% after 7 days.
Figure 6-1. Cumulative mass and percent of Ab-bFGF released from PLGA/PEG blend microparticles. Microparticles contain 0.85 (± 0.02) wt% Ab-bFGF. Error bars represent means ± SD for n=3.
Figure 6-2. Relative bioactivity of free Ab-bFGF and Ab-bFGF released from PLGA/PEG blend microparticles as compared to fresh Ab-bFGF. Error bars represent means ± range for n=2.
6.4.3 Free Ab-bFGF Inhibition

The bFGF-stimulated SMC proliferation depended on dose of free Ab-bFGF for 1, 5, 10, and 20 μg/ml (p<0.05) (Figure 6-3). The cultures with 1 and 5 μg/ml Ab-bFGF were not significantly inhibited relative to control IgG treated cultures, while 10 and 20 μg/ml Ab-bFGF significantly inhibited proliferation. Control IgG did not inhibit proliferation in bFGF-stimulated or unstimulated cultures. The 20 μg/ml Ab-bFGF dosage administered to non-bFGF-stimulated cultures showed a significantly lower average cell count compared to wells that had received control IgG.

6.4.4 Microparticle-Released Antibody Effects on SMC Proliferation

For the bFGF-stimulated SMC cultures, Ab-bFGF released from PLGA/PEG microparticles inhibited SMC proliferation in a dose dependent manner (Figure 6-4). For 30 mg of PLGA/PEG microparticles loaded with Ab-bFGF, the final cell counts were no different from the control wells containing no bFGF (p>0.05). A single factor ANOVA test showed the dose responsiveness to be statistically significant (p<0.05) for 5, 15 and 30 mg dosages. All dosages were statistically significant from one another as determined by Scheffé's F-test. Moreover, PLGA/PEG blank microparticles or microparticles loaded with control IgG had no effect on SMC proliferation. In addition, no toxicity effects were evident at any of the dosages.

Cultures that did not receive bFGF-stimulation showed significant inhibition only for the 15 and 30 mg dosages of Ab-bFGF loaded PLGA/PEG microparticles (Figure 6-5). Single factor ANOVA for Ab-bFGF loaded microparticles for cultures not receiving bFGF-stimulation showed no statistical significance for all dosages involved (p>0.05). For the 15 mg dosage, average cell counts were statistically different from the control IgG loaded and blank microparticles. At the 30 mg dosage, Ab-bFGF loaded
microparticles showed statistical difference only from the control IgG loaded microparticles.

6.5 Discussion

We showed that a monoclonal antibody to bFGF can be successfully incorporated into PLGA/PEG blend microparticles for controlled release, and that much of the bioactivity of the released antibody is retained for 14 days. Moreover, we demonstrated that Ab-bFGF released from microparticles can inhibit bFGF-stimulated SMC proliferation in vitro.

Ab-bFGF release showed a small burst effect during the first 24 hrs. consistent with previous studies of PLGA microparticle formulations loaded with proteins (Cohen et al. 1991). Previous studies with microparticle carriers made of 30,000 to 75,000 molecular weight PLGA exhibited a lag period of several days during which the release rate was minimal (Iwata and McGinity 1993; Yan et al. 1994), after the initial burst. This lag phase was not observed in our release studies and is attributed to the small weight fraction of PEG added to the microparticle formulation (Cleek et al. 1997). In addition, the release profile was linear after the initial burst.

A number of reports of sustained release of IgG from controlled release systems have been published. IgG released from EVAe showed a precipitous drop in the release rate after an initial burst making prolonged delivery problematic (Saltzman et al. 1993; Parkhurst and Saltzman 1996). Biodegradable polyanhydride copolymers were also shown to be diffusion controlled for early time release periods (Sherwood et al. 1992).
Figure 6-3. Dose-dependent inhibition of SMC proliferation after administration of free Ab-bFGF and control IgG over 72 hrs with bFGF (1 ng/ml) and without bFGF. Error bars represent means ± SD for n=3. Statistical significance between Ab-bFGF inhibited and control IgG cell counts at a given dosage is indicated by ($) (p<0.05).
Figure 6-4. Dose-dependent growth inhibition of bFGF stimulated SMCs after administration of Ab-bFGF loaded, control IgG loaded, and blank PLGA/PEG blend microparticles over 96 hrs. All SMCs were stimulated with the addition of 1 ng/ml of bFGF after 24 hrs incubation with microparticles, except for the SMC group designated by the horizontal line bar. Error bars represent mean ± SD for n=6 (wells containing microparticles), n=9 (bFGF and no microparticles), and n=12 (no bFGF nor microparticles). Statistical significance between microparticles containing Ab-bFGF and control IgG at a given dosage is indicated by ($), whereas (*) designates that there was no significant difference between the control containing no bFGF nor microparticles and the 30 mg Ab-bFGF loaded microparticle dosage (p<0.05).
Figure 6-5. Dose-dependent growth inhibition of SMCs not stimulated after administration of Ab-bFGF loaded, control IgG loaded, and blank PLGA/PEG blend microparticles over 96 hrs. SMCs were not stimulated with the addition of bFGF. Error bars represent mean ± SD for n=6. Statistical significance between microparticles containing Ab-bFGF and control IgG at a given dosage is indicated by ($$), whereas (*) designates significant difference as compared to the control containing no microparticles (p<0.05).
The average size of 81 (± 5) μm for our microparticles is important in preventing phagocytosis and promoting tissue immobilization. Microparticles slightly less than 100 μm were found to be too large to be phagocytosed (Tabata and Ikada 1988). Furthermore, for immobilization within tissue, microparticles should be larger than 5 μm, as evidenced by the immobilization of microparticles ranging from 5 to 15 μm to the arterial wall (Lincoff et al. 1993).

The activity of the microparticle-released Ab-bFGF decreased initially more rapidly than the free Ab-bFGF suggesting that the encapsulation procedure partially inactivated the Ab. Structural and conformational changes to the protein may have occurred during the microparticle fabrication process. Such steps as lyophilization (Pikal et al. 1991), emulsification (Tabata et al. 1993), and exposure to organic solvents (Edelman et al. 1991) have been shown to contribute to loss of bioactivity of proteins. Measures can be taken that may improve the activity through the addition of certain molecules to the Ab solution (Krewson et al. 1996). Furthermore, the protein microenvironment within the degrading polymer may also deactivate the protein through denaturation, aggregation, chemical degradation, or adsorption onto the polymer surface (Park et al. 1995), which may further explain the bioactivity difference between microparticle-released and free Ab-bFGF. However, some stabilization of the Ab-bFGF must have occurred because the relative activity of the microparticle-released Ab-bFGF remained relatively high over the fourteen day period.

Inhibitory differences between comparable dosages of Ab-bFGF released from the microparticles (Figure 6-4) and free Ab-bFGF (Figure 6-3) used to suppress bFGF stimulated SMC growth were noted. For example, the 15 mg Ab-bFGF loaded microparticle dosage, which over the first 24 hrs delivered an equivalent amount (19 ± 1.6 μg from Figure 6-1) to the 20 μg free dosage, showed significantly less inhibition (68% inhibition in cell proliferation relative to non-bFGF-stimulated growth) compared
to that for the free Ab-bFGF (99%). These differences between equivalent dosages of Ab-bFGF released from the microparticles and free Ab-bFGF at every time point may be attributed to a loss in bioactivity for Ab-bFGF released from microparticles.

Interestingly, the non-bFGF-stimulated cell cultures for both the free (Figure 6-3) and controlled released Ab-bFGF (Figure 6-5) showed minor but significant inhibition at the higher dosages. This was evident for the 20 μg free dosage and the 15 and 20 mg dosages of Ab-bFGF loaded microparticles. Such inhibitory effects may result from the suppression of self stimulating bFGF that is released from the SMCs in culture. SMCs synthesize and express bFGF (Lindner et al. 1991), however most of the bFGF remains inside the cell and only a small fraction is released extracellularly.
CHAPTER 7

EFFECT OF ANTIBODY TO BASIC FIBROBLAST GROWTH FACTOR RELEASED FROM BLEND MICROPARTICLES ON INTIMAL THICKENING: PRELIMINARY DATA

7.1 Introduction

We have shown in Chapter 6 that Ab-bFGF loaded PLGA/PEG blend microparticles can serve as carriers for delivery of Ab-bFGF with retention of bioactivity (30% of the initial value after 14 days). Furthermore, we have demonstrated that the extent of inhibition of SMC proliferation in vitro by Ab-bFGF released from PLGA/PEG blend microparticles was dose dependent for SMC cultures stimulated by bFGF.

In this feasibility study, we have investigated the inhibition of intimal hyperplasia in the rat carotid artery model by implanting Ab-bFGF loaded microparticles adventitiously after balloononing. In order to do this, we have had to first develop a method to immobilized PLGA/PEG blend microparticles adventitiously to the rat carotid artery. This is a preliminary study and represents a foundation for future work.

7.2 Materials and Methods

7.2.1 Fabrication of PLGA/PEG blend Microparticles

Three sets of microparticles were produced by a double-emulsion-solvent-extraction technique previously described in section 6.3.1. For immobilization studies, blank PLGA/PEG blend microparticles initially containing 5 wt% PEG were manufactured. For intimal thickening studies, Ab-bFGF and control IgG loaded microparticles were manufactured to initially contain 1 wt% theoretical loading Ab-bFGF.
or control IgG and 5 wt% PEG. The average size of the Ab-bFGF loaded microparticles was 67 ± 4 μm and the loading efficiency was determined to be 89 ± 3%.

7.2.2 Microparticle Immobilization Protocol

Sprague-Dawley rats (Sasco Laboratories, Houston, TX) weighing 350-400 g were used. All surgeries were performed under 1.5% halothane (Halocarbon, River Edge, NJ) and 98.5% oxygen gas continuously supplied at a rate of 1000 CC/min. 180.5 (± 0.8) mg of microparticles were mixed with 200 μl of Pluronic (F-127 formulation, BASF, Parsippany, NJ) and loaded into a 1 ml syringe ready for injection. The Pluronic solution was prepared by mixing 1.5 g of Pluronic with 5 ml water and storing over night at 4°C until needed. Microparticles were implanted by exposing the carotid, lifting it, inserting the syringe tip into the tissue below the carotid, and injecting the mixture. At 21 days after ballooning the animals were sacrificed by asphyxiation in a CO₂ rich environment. The animals were each perfused with 20 ml of heparin solution (500 ml of PBS containing 5 ml of heparin, Elkins-Sinn, Cherry Hill, NJ) and perfusion fixed with 30 ml of 10% buffered formalin acetate solution (Fisher Scientific). The carotid arteries were excised and immersed overnight in 10% buffered formalin acetate solution to assure fixation.

7.2.3 Ballooning and Microparticle Immobilization Protocol

All animals were anesthetized and Pluronic-microparticle mixtures were prepared according to section 7.2.2. Within 3 minutes after endothelial denudation by balloon catheterization with a 2F Fogarty balloon catheter (model 120602F, Baxter Heathcare Corp., Irvine, CA) drawn 3 time at 2.5 atm balloon pressure, the Ab-bFGF microparticles
were injected adventitiously (as outlined in section 7.2.2). Control animals were treated in an identical fashion with the exception that matching dosages of control IgG loaded microparticles were injected. At 14 days after ballooning the animals were sacrificed, perfused, and the carotid arteries excised according to section 7.2.2.

### 7.2.4 Histology

After 24 hr formalin fixation, a razor blade was used to cut two sections of approximately 4 mm in length from the central portion of the treated carotid arteries. Samples were dehydrated by sequentially increasing ethanol solutions to 100% ethanol followed by immersion in Hemo-De (Fisher Scientific, Pittsburgh, PA), paraffin saturated Hemo-De, and finally molten paraffin for sectioning. Tissue blocks were sectioned at 10 μm and stained by hematoxylin and eosin (H&E) for visualization of cells.

### 7.2.5 Morphometric Analysis

Morphometric analysis was performed on balloon injured arteries to determine the area of the carotid intima (I) and media (M) to obtain the I/M ratio. Digitized images of the stained and sectioned samples were taken using a Sony color video camera attached to the photographic port of an Olympus Bmax microscope interfaced to a computer. The image was captured using Snappy Video Snapshot (Play, Rancho Cordova, CA). The I and M perimeters were traced and the areas within calculated by calibrating the software (Sigma Scan, Jandel Scientific Software, San Rafael, CA) with a known standard. Determining the area of the I and M is analogous to determining the area of concentric rings. First the outer perimeter of the lumen (L) was traced, and then, the I and M were traced by hand with the aid of a mouse. These traces represent composite areas: the trace of the I perimeter represents the area of the I plus the L, while the M perimeter trace
results in an area that is the composite of the L, I. and M. To calculate the area of the I the value obtained from the I perimeter trace is subtracted from the L trace, and the M area is determined by subtracting the I perimeter trace from the M perimeter trace.

7.2.4 Statistics

All data were analyzed by using Student's t-test for comparing two means. Data were considered significant if $p < 0.05$.

7.3 Results

7.3.1 Implantation of microparticles

Inspection of the histological samples revealed that the blank PLGA/PEG microparticles had become immobilize adventitially (Figure 7-1) and were still present 21 days after implantation. It was also evident that fibroblasts had migrated and proliferated to fill the space between PLGA/PEG blend microparticles.

7.3.2 Ab-bFGF loaded microparticles

Ab-bFGF released from adventitially immobilized PLGA/PEG blend microparticles did not significantly reduce neointimal formation (Figure 7-2). as the average I/M ratio for control IgG treated arteries was $0.644 \pm 0.26$ and $0.606 \pm 0.24$ for Ab-bFGF treated arteries ($p>0.05$).
Figure 7-1. Cross section of rat common carotid artery showing adventitiously immobilized blank PLGA/PEG blend microparticles. Harvested 21 days after implantation. P = microparticle; L = lumen; M = media; A = adventitia.
Figure 7-2. Intimal thickening 14 days after balloon catheter denudation of common carotid artery and implantation of control IgG or Ab-bFGF loaded PLGA/PEG blend microparticles. Data represents mean ± SD for n = 4.
7.4 Discussion

Implantation studies showed that PLGA/PEG microparticles can be immobilized adventitiously and that they can remain in place for up to 21 days (Figure 7-1). This result lead to the implantation of Ab-bFGF loaded microparticles in ballooned carotid arteries to test our working hypothesis that arterial injury causes release of bFGF which then stimulates adjacent SMCs to proliferate. It was surprising to find that the neutralizing antibody-loaded microparticles to bFGF had no effect on the development of the arterial lesion (Figure 7-2).

One explanation for our inability to inhibit intimal thickening is that mass transfer limitations prevented the antibody from reaching the bFGF that is released immediately after ballooning. In fact, the diffusion limitations for the transport of the Ab-bFGF may result in the Ab-bFGF being present in the arterial wall only after many hours, or days, after implantation of the microparticles. Ideally, the antibody used to neutralize bFGF should be present in the arterial wall immediately after ballooning, since balloon catheterization has been shown to result in the immediate release of bFGF (Lindner and Reidy 1991). The problem may be further compounded in that release of the Ab-bFGF may be further delayed because of microparticle hydration: microparticles must imbibe water from their surroundings in order to release Ab-bFGF. This hydration could be a rate limiting step.

It may not be sufficient merely to deliver the Ab-bFGF immediately after ballooning. Researchers have shown that systemic delivery of an Ab-bFGF after ballooning was effective at reducing early SMC proliferation but did not significantly reduce the lesion developed within 8 days (Lindner and Reidy 1991). Release of bFGF
from the matrix over the period of several days after injury may be responsible for this result.

The kinetics of bFGF release from extracellular sites are largely unknown. *In vitro* studies have shown that bFGF bound to the matrix of endothelial and SMCs was released in a controlled fashion over the period of several days (Dinbergs et al. 1996). Furthermore, this release enhanced bFGF activity, *in vitro*, such that it was 86% more effective at increasing SMC proliferation than equal amounts of growth factor diluted from frozen stock. Such a scenario of controlled release of bFGF from arterial matrix after balloon injury would likely have a profound affect on lesion development.

Basic FGF seems to be a particularly difficult growth factor to target because of its early release. If Ab-bFGF loaded microparticles are going to be investigated further as a therapeutic system for inhibit intimal thickening, then the early release of bFGF needs to be neutralized. Possibly this could be done by a systemic injection of Ab-bFGF immediately before or after balloononing. Other scenarios would be to preimplant the microparticles 24 hrs before balloononing, or to load the Pluronic with Ab-bFGF for immediate release. Neutralizing any bFGF that might be released from the matrix over a period of several days, could be addressed by modifying the release rate of the antibody from the microparticles accordingly.
CHAPTER 8
CONCLUSIONS AND FUTURE WORK

We developed a method to fabricate PLGA/PEG blend microparticles that involves a double-emulsion-solvent-extraction technique for the controlled release of inhibitors of SMC growth. To characterize the microparticle delivery system, two model drugs (FITC-IgG and FITC-dextran) were entrapped using the double-emulsion-solvent-extraction technique with high efficiency. In vitro release studies showed that the initial burst effect was dependent on the PLGA/PEG blend ratio. Moreover, the release rate increased in direct relation to PEG content for up to 28 days. A linear release profile was obtained for microparticles loaded with FITC-IgG for initial PEG weight fractions up to 5 wt%, and a biphasic release profile was obtained for FITC-dextran loaded microparticles. Rates for both model compounds was dependent on the PEG content. These results demonstrate the feasibility of modulating the release profile of entrapped drug compounds in biodegradable microparticles by adjusting the PLGA/PEG blend ratio.

As a means of inhibiting SMC growth, we fabricated PLGA/PEG blend microparticles loaded with an AS-ODN against the rat tenascin mRNA, and determined the effect of the AS-ODN released on SMC proliferation and migration in vitro. AS-ODN was entrapped with high efficiency. Release of AS-ODN was characterized by a small initial burst effect followed by a period of controlled AS-ODN release for up to 20 days. The extent of inhibition of SMC proliferation in vitro by AS-ODN released from blend microparticles was ODN sequence specific and microparticle dose dependent. AS-ODN loaded microparticles produced the greatest inhibition. AS-ODN loaded microparticles inhibited SMC migration in vitro whereas the same dose of SC-ODN loaded
microparticles showed no inhibitory effect. These results serve as a foundation for the concept of microparticle based delivery of inhibitors of SMC proliferation and migration.

Blocking bFGF released after interventional cardiovascular procedures may be a means of inhibiting SMC proliferation. Using a double-emulsion-solvent-extraction technique we fabricated PLGA/PEG blend microparticles loaded with an Ab against bFGF and determined the effect of the Ab-bFGF released on SMC proliferation in vitro. The Ab-bFGF release profile exhibited a small burst effect followed by biphasic release kinetics. The extent of inhibition of SMC proliferation in vitro by Ab-bFGF released from PLGA/PEG microparticles was microparticle dose dependent. Furthermore, activity studies showed Ab-bFGF released from PLGA/PEG microparticles retained much of its activity after 2 weeks in the microparticle. These results demonstrate the feasibility of entrapping an Ab-bFGF into PLGA/PEG microparticles for controlled delivery to inhibit SMC proliferation.

In vivo experiments were conducted with Ab-bFGF loaded PLGA/PEG blend microparticles to inhibit intimal thickening in the balloononed rat model. For these experiments, rat carotid arteries were balloononed and Ab-bFGF loaded microparticles were implanted adventitially. Through our implantation procedure, the PLGA/PEG blend microparticles became immobilized adventitially. Surprisingly, the neutralizing antibody-loaded microparticles to bFGF had no effect on the development of the arterial lesion. The early release of bFGF after injury and the possible delayed release and permeation of the Ab-bFGF into the artery may be important factors responsible for the outcome observed in this study.

Our original hypothesis stated that the localized delivery of inhibitors of smooth muscle cell proliferation may reduce the rate of restenosis occurring after interventional cardiovascular procedures. This remains a valid concept for continued research with enormous potential. Future studies should include the investigation of the ability of large
molecules such as antibodies to penetrate the artery adventitiously. In order to deliver an effective dose, it may be necessary to fragment the Ab-bFGF used in these experiments to reduce diffusional resistances. Furthermore, any study should also include an attempt to correlate physicochemical parameters such as molecular weight and the hydrophilic nature of the compound to permeation and penetration of the artery. The effects of release profiles on intimal thickening would also be of interest.

These studies have shown that PLGA/PEG blend microparticles have great potential as a drug delivery platform for inhibitors of smooth muscle cell proliferation. The blend microparticles are able to modulate release, retain bioactivity of the drug, and can be immobilized adventitiously. Furthermore, AS-ODN and Ab-bFGF loaded blend microparticles have been determined to be potent inhibitors of SMC proliferation \textit{in vitro}. 
REFERENCES


