Specific and General Binding in Arterial Drug Delivery

by

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Abstract

Drug-eluting stents have emerged as the most effective method for treating restenosis following percutaneous coronary interventions. This thesis investigates how drugs with similar physiochemical properties but different specific binding targets yield drastically different tissue transport, retention and ultimately efficacy independent of their putative biological effects. Our central hypothesis is that both specific and general binding of drugs to tissue proteins, as mediated by drug-specific physiochemical properties, plays a central role in arterial transport and distribution. We define and compare the kinetic and transport properties of clinically implemented compounds with different binding modes. While hydrophilic compounds are rapidly cleared, hydrophobic ones are retained with an arterial transmural distribution dependent upon the distribution of specific and general binding sites. Common systemically administered cardiac drugs compete with locally delivered agents through displacement of general binding sites. Exploration of drug binding in thrombus indicates significant specific and general binding capacity. Stent-to-arterial wall drug transfer is acutely sensitive to stent strut position in clot relative to the wall due to thrombus binding capacity. A poorly controlled microthrombotic environment around a stent strut can drastically enhance systemic washout while reducing delivery to the tissue. Together this body of work implies that specific and general binding plays a critical role in the clinical efficacy of locally delivered drugs, and must be a consideration in the rational design of stent-based delivery devices.

Thesis Supervisor: Elazer R. Edelman
Thomas D. and Virginia W. Cabot Professor of Health Sciences and Technology

Thesis Committee: Martha L. Gray, Kimberly Hamad-Schifferli, Robert S. Langer

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For a journey where I have often felt lost and lonely, there have been so many along the way to help guide me…

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Chapter 1

Background and Significance

Overview of Thesis

Paclitaxel- and rapamycin-eluting stents have been overwhelmingly successful in the treatment of atherosclerosis. These devices reduce the expected intervention-induced tissue hyperplasia and clinical restenosis to an extent not seen with any other modality. Yet, for each of these successes there have been multiple failures and a central question has lingers: What distinguish the success of one drug over another in treating restenosis? Heparin, dexamethasone and actinomycin-D all showed great promise *in vitro* and, all failed in late stage clinical trials. One theory has been put forth that *in vivo*, compounds like paclitaxel and rapamycin have unique biologic effects, different from those therapies that prove ineffective. There is however little to no firm support for this possibility. In contrast, our central hypothesis is that physicochemical, not pharmacological, properties indeed can be used to distinguish drugs. In particular, we believe tissue retention to be a critical factor for tissue effect. Drugs that are rapidly cleared can not reside long enough to exert and effect. Compounds that bind to tissue elements can be in place for a sufficient amount of time to influence tissue events. Specific and general binding to target proteins allows certain drugs to remain in tissue and establish a meaningful biologic effect. When a compound weakly binds to tissue elements, as may be the case with unsuccessful treatments, it is too quickly cleared from the vessel before binding to its biologically relevant protein to inhibit smooth muscle cell growth.

This thesis consists of a series of studies which attempt to elucidate the role of specific and general binding in stent-based drug delivery. The specific aims are reflected in the individual core chapters:

- Chapter 2 characterizes the arterial transport and distribution properties of model hydrophilic and hydrophobic drugs with specific binding targets
- Chapter 3 defines specific and general binding in the context of local arterial drug delivery.

- Chapter 4 characterizes the role of binding to thrombus and its effects on arterial wall drug deposition

Through systematic study of physiochemical specific and general binding we hope to elucidate one of the fundamental mechanisms that make stent delivery so successful. Challenges remain in understanding how pathology changes the binding domains of tissue, especially in disease states such as diabetes where restenosis rates are significantly higher. The lessons of this work are generally applicable to fields of local delivery beyond the cardiovasculature, and the implications are presented for a broad audience.

1.1 Death and Dying from Heart Disease

In 2002 over one million Americans died of heart disease, accounting for 100 million hospital days and nearly half of all deaths recorded in the United States. One-third of the victims were under the age of 65. The American Heart Association estimates that over 12 million Americans suffer from obstructive coronary artery disease, principally from atherosclerosis. Ultimately, 90% of patients who die suddenly of heart disease have significant coronary artery obstruction[1].

1.2 Normal Arterial Anatomy

Familiarization with the architecture of a normal artery is essential as we consider effective treatments of coronary artery disease, stenting and arterial drug delivery. Arteries are composite structures consisting of elastin, collagen, smooth muscle cells and extracellular matrix arranged into three main layers, called tunics (figure 1). The *tunica intima* is closest to the lumen and blood flow and is composed of a monolayer of endothelial cells in animals and in humans vascular smooth muscle cells as well[2, 3]. Radial to, and supporting, the *tunica intima* lies the internal *elastic lamina* (IEL), a fenestrated connective tissue layer composed primarily of hydrophobic elastin. The *tunica media*, or middle layer, consists of concentrically arranged collagen and *elastin laminae*. Packets of smooth muscle cells interpose these laminae and are oriented orthogonally to the radial direction of the artery. The *media* is therefore ideally constructed to modulate vascular tone, modulating vessel diameter and perfusion to

downstream tissues. The smooth muscle cells contract and relax under rhythmic neurohormonal control and the *laminae* supply both cell support and elasticity. An extracellular matrix composed of reticular fibers and chondroitin sulfate glycosaminoglycans persists throughout the *tunica media*. Smaller muscular arteries contain less organized *elastic laminae*, forming a network of fibers surrounding the layers of smooth muscle cells[2, 3]. The external *elastic lamina* (EEL) separates the outermost *tunica adventitia* from the *tunica media* as a series of concentric rings. The adventitia is composed primarily of loose type I collagen and scattered fibroblasts, adipocytes and nerve fibers. The adventitia of larger arteries (wall thickness greater than 500μm) is permeated by a network of *vasa vasorum*, capillaries that help nourish the main vessel wall[2].

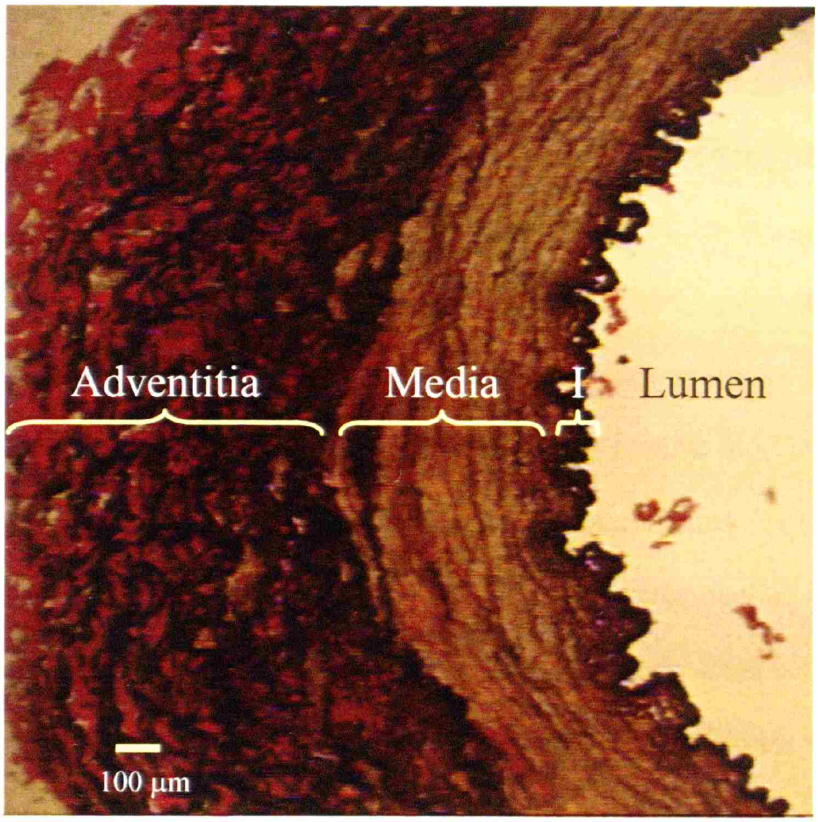

Figure 1.1: Normal Arterial Anatomy

verHoeff's stain of an internal carotid artery wall [4]. In this preparation, elastin is stained black, collagen is stained dark pink, and cellular material is stained yellow. "I" represents the intima.

1.3 Atherosclerosis Pathogenesis

Atherosclerosis is thought to involve a complex series of events that begins with endothelial injury and culminates in vascular obstruction. Thrombosis, inflammation, smooth muscle cell phenotypic transformation, migration and proliferation are accompanied by vascular remodeling[5-7]. Chronic hypercholesterolemia, diabetes, systemic hypertension, or even exogenous etiologies like chemicals in cigarette smoke have been associated with atherosclerosis. The evolving process begins with endothelial dysfunction or "injury" and continues to develop over a number of years. Over time the atherosclerotic lesion forms a thickened neointima on the luminal side of the internal *elastic laminae* as lipids accumulate in macrophages and smooth muscle cells within the new layer transforming these cells to foam cells. The accumulation of cells and extracellular matrix forms the atherosclerotic plaque. Death of the foam cells produces a necrotic environment rich in cholesterol esters lipids and potent cytokines which further prolong the chronic inflammatory reaction. Neointimal inflammation and proliferation can persist for years to decades culminating in the formation of a complex lesion, characterized by a severely narrowed and often asymmetric lumen with a fibrous tissue cap and a necrotic lipid core[7] (figure 2). These lesions are effectively scars from the healing process of the initial endothelial injury and can remain stable and asymptomatic for years. The neointima becomes vascularized over time where blood vessels, likely from the *vasa vasorum* in the adventitia, permeate the atherosclerotic core nourishing and promoting plaque progression. Myocardial infarctions, especially in the young, are often associated with plaque rupture and the acute and occlusive thrombotic response that

follows, rather than progressive luminal occlusion due to plaque formation that occurs with chronic disease in the older population.

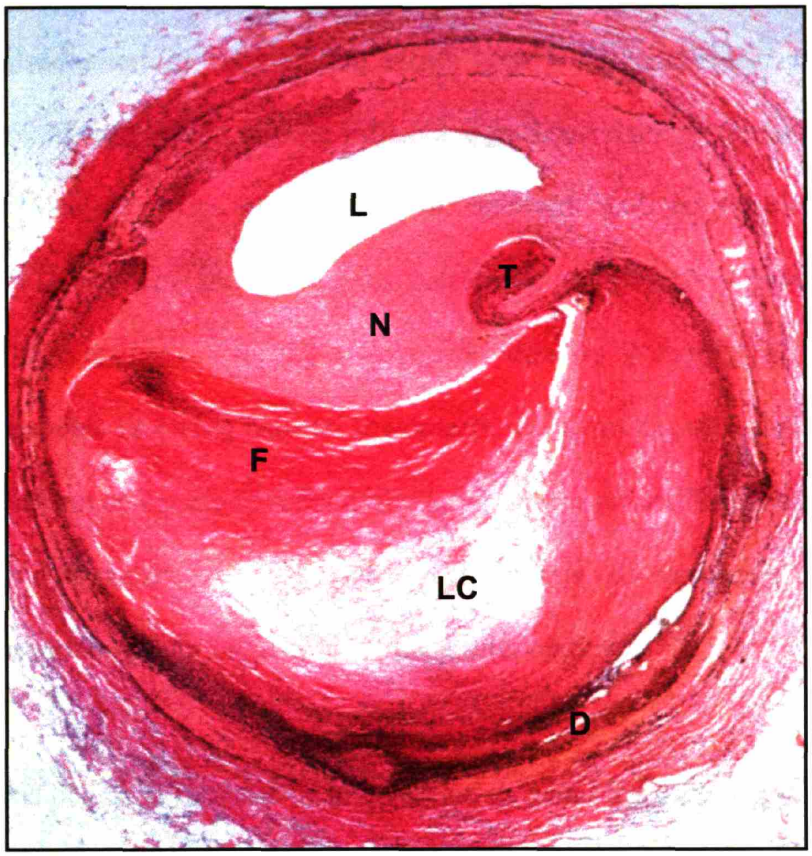

Figure 1.2: Complex Atherosclerotic Lesion

Complex atherosclerotic lesion showing the lumen (L), the neointima (N), fibrous tissue (F), a necrotic lipid core (LC), evidence for a previous thrombus (T) and mural dissection (D) [8].

1.4 Mechanical Interventions and Restenosis

Angioplasty, atherectomy and endovascular stenting prevail as the most frequent percutaneous coronary intervention therapies for symptomatic coronary atherosclerosis. Prior to the introduction of drug-eluting stents nearly all mechanical interventions designed to limit atherosclerotic disease inflicted significant damage to the very tissues they were intended to help, resulting in a renewed and accelerated occlusive vasculopathy[9-13]. In fact, these therapies accelerated the period of time to develop clinically significant occlusive vascular disease from decades to months[12, 13] (Figure 3). 30-40% of patients receiving balloon angioplasty treatment become obstructed in six months completely reversing the benefits of the initial intervention. This therapy-induced pathology, known as restenosis, is defined as a progressive re-narrowing of the blood vessel in response to the injury inflicted during the intervention.

Restenosis begins at the time of intervention as small tears develop in the arterial wall. An inflammatory cascade ensues as a natural wound healing response. Platelets and white blood cells from the blood adhere to the injured *intima* delivering power cytokines which signal smooth muscle cells to migrate and divide in an attempt to repair the injury. A new- or neointima of dividing smooth muscle cells forms, impeding blood flow through the lumen. Despite similar mechanisms of pathogenesis the initial acute insult of the restenotic lesions offer a separate and distinct disease from atherosclerosis. In atherosclerosis, initial endothelial insult may be non-denuding and subtle. While atherosclerotic lesions exhibit a protracted course, restenosis emerges in a period of

weeks to months. The restenotic neointima is primarily composed of smooth muscles cells while the atherosclerotic lesion is complex with high densities of macrophages and lipid deposits and, in advanced disease, calcification. Finally, the neointima of restenosis exhibits a dynamic advancing front that is usually more uniform and concentric, and completely envelopes the foreign body stent struts.

Ballon angioplasty- and endovascular stent-induced restenosis maintain different pathologic courses. In balloon-injured arteries, leukocyte recruitment is generally confined to early neutrophil infiltration with undetectable levels of pro-inflammatory cytokines such as IL-8 and MCP-1 after injury. In contrast, stented arteries also exhibit early neutrophil recruitment, but prolonged macrophage accumulation follows. Cytokine mRNA and protein levels can still remain detectable for at least several weeks post injury. The continued presence of macrophage maintains inflammatory cytokines levels in the vicinity of the foreign body stent struts prolonging the wound response including neutrophil recruitment with tissue remodeling[14].

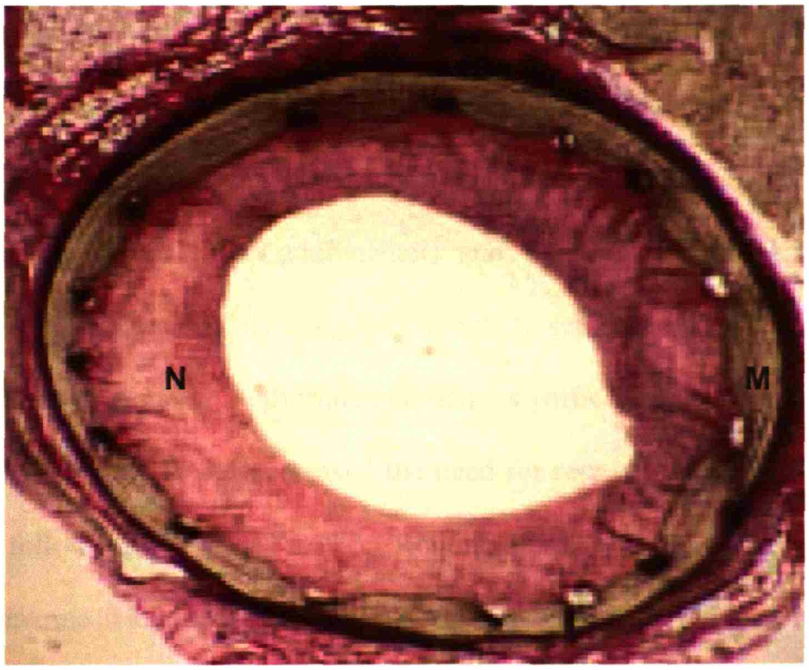

Figure 1.3: In-stent Restenosis

In-stent restenosis of a rabbit iliac artery 28 days post-intervention[4]. The black stent struts are clearly visible between the media (M) and the thick neointimal (N).

1.5 Previous Attempts at Treating Restenosis

Despite the extended inflammatory response, bare metal intracoronary stents significantly reduced the rate of restenosis compared to percutaneous balloon angioplasty (PTCA) alone. PTCA is now used in fewer than 10% of de novo procedures and only when stenting is not possible. Before the introduction of drug-eluting stents, two landmark studies established bare metal stenting as the preeminent first-line therapy for large vessel (3 mm or greater) coronary artery disease. The STRESS [15] (Stent Restenosis Study, United States multicenter) and BENESTENT [16] (Belgium and Netherlands Stent) trials randomly assigned patients to standard balloon PTCA or PTCA with elective stent placement. Both trials reported a significant reduction in angiographic restenosis (22 versus 32%) and decreased the need for repeat revascularization at six or seven month follow–up (17 versus 25%). Routine stenting unequivocally improves the in-hospital outcome when compared to PTCA alone. Stenting reduced the in-hospital mortality (0.3 versus 0.6% for PTCA) and the need for emergent coronary artery bypass grafting (CABG) (0.3 versus 0.7%) [17]. A retrospective review of 1240 stented patients and 4018 patients who underwent PTCA found that the risk of in-hospital CABG or death was reduced with stenting (3.1 versus 5.3 for no stent, adjusted odds ratio 0.63). The risk reduction was primarily associated with a 52% decrease in need for CABG or follow-up revascularization procedure (12.5 versus 17.6%, adjusted odds ratio 0.72) [18]. Stenting however had no effect on the six month incidence of MI or cardiac death[19].

Increasingly sophisticated techniques have not reduced restenosis rates relative to bare metal stents, and in some cases even performed more poorly. With excimer laser angioplasty, 50% of patients experienced angiographic evidence of restenosis at six months[20, 21]. Atherectomy devices show similar results[22, 23]. Bypass grafting yields a tenuous post-operative course with a seven year mean lifetime for a saphenous vein aorto-coronary interposition graft. 10% of grafts become occluded within the first two weeks after surgery, 20% within a year and 35% within five years[13, 24]. Fortunately, shared immunologic, metabolic and proliferative stimuli have been identified in all these settings, paving the way for the administration of vasoactive agents to combat restenosis.

Early advances in vascular biology suggested that potent vasoactive compounds might selectively target specific cellular events involved in the pathogenesis of restenosis. Numerous candidate drugs were tested with systemic administration in animal models including, heparin[25-36], anti-inflammatory drugs such as dexamethasone[37, 38], antimitotic inhibitors of tubulin polymerization or depolymerization such as colchicine[39] and paclitaxel[39-43], anti-platelet drugs such as forskolin[44, 45], antineoplastic agents such as actinomycin-D[46, 47] and antisense oligonucleotides directed against cell cycle proteins such as c-myc[48]. Many of these agents offered early promising results, but at therapeutic doses caused inadvertent injury to other tissues with rapidly dividing cells, including the gut epithelium, hair follicles and lymphoid cells.

With rapid drug degradation and denaturation for many of these compounds, profoundly toxic drug doses would be required to treat restenotic events.

1.6 Birth of Local Delivery

Local delivery offered a viable alternative to systemic methods, allowing for pharmacologic, nontoxic and targeted delivery. Heparin-eluting polymeric perivascular wraps in rats offered the first evidence of successful local delivery, out performing continuous pump infusion[49]. Pressurized drug infusion catheters were soon developed for high local dosing during interventions but unfortunately the candidate drugs including heparin and methotrexate showed little benefit in restenosis treatment[28, 50-52]. Additionally, upon removing the catheter, systemic drug wash out had adverse effects on non-target tissues.

Controlled-release devices allow for high and sustained levels of antiproliferative drugs over a period of days to weeks[53-55]. Controlled-release polymers have been engineered into endoluminal sheaths[56], perivascular wraps and beads[29, 57, 58] for sustained local delivery of antiproliferative agents designed to inhibit the pathophysiologic processes that lead to intimal hyperplasia and restenosis. Incorporating a polymeric coat directly onto an endovascular stent scaffolding has rapidly become the industry standard for treating restenosis[53, 54, 59] (Figure 4).

Despite demonstrated efficacy in tissue culture and animal models, early attempts at administration of forskolin, dexamethasone and actinomycin-D from stent platforms failed to decrease rates of clinical restenosis[38, 44, 45, 50, 59-61]. Paclitaxel and rapampycin, on the other hand, are now the most efficacious methods available for treating restenosis.

Figure 1.4: Drug-coated Stent

Paclitaxel-coated NIR stent (Medinol) in the collapsed state on the delivery catheter (above) and in the inflated state (below).

1.7 Drug-eluting Stent Trials

Numerous trials have been conducted comparing paclitaxel-eluting stents to bare metal stents, rapamycin-eluting stents to bare metal stents as well as head-to-head evaluations of paclitaxel and rapamycin stents performance. The TAXUS II trial enrolled 536 patients with low risk lesions, randomly assigning bare metal or paclitaxel-eluting stent treatments[62]. At six months, the paclitaxel stent showed significant reductions in both in-stent restenosis (3.5 versus 19.1%) and target lesion revascularization (3.9 versus 13.3%). Benefits of the paclitaxel stent were confirmed in the larger TAXUS IV trial where 1314 patients with previously untreated coronary stenoses were assigned paclitaxel-eluting or bare metal stents[63].

The RAVEL trial enrolled 238 patients for evaluation of the rapamycin-eluting stent versus the bare metal stent[64, 65]. At six months, with the metric of restenosis of more than 50% of the luminal diameter, rapamycin far outperformed the bare metal stent (zero versus 27%). The larger SIRIUS trial of 1058 patients evaluated overall performance and subcategorized patients with complex disease including long lesions (mean 14.4 mm), small target vessels (mean 2.8 mm), and patients with diabetes (26%)[66]. Clinical and angiographic follow-up at nine months demonstrated a significant benefit of rapamycin stents over bare metal stents for angiographic in-stent restenosis (3 versus 35%) and target lesion revascularization (4 versus 17%). These benefits were consistent across the subgroups, including longer lesions, smaller vessels and diabetic patients[67].

While paclitaxel and rapamycin stents certainly out perform bare metal stents and balloon angioplasty, emerging reports indicate significant clinical performance divergences between the two drug-eluting therapies. Several randomized trials have directly compared the rapamycin and paclitaxel stents. The TAXi trial randomly assigned 202 patients with de novo lesions to a rapamycin or paclitaxel stent[68]. Mean follow-up at seven months demonstrated statistically insignificant difference in rates of target vessel revascularization necessity (3 versus 1% for paclitaxel). Both stents also show similar rates of thrombotic occlusion at 1%[69]. More recently, the ISAR-Desire trial confirmed no statistical difference between restenosis rates in rapamycin- and paclitaxel-eluting stents after revascularizing for in-stent restenosis (14% vs. 22%, respectively, $p = 0.19$)[70]. However, the incidence of target vessel revascularization was significantly different for in-stent restenosis patients (8% for rapamycin and 19% for paclitaxel, $p = 0.02$). The most distinctive advantage of drug-eluting stents is the reduced need for target vessel revascularization. This clinically relevant performance metric highlights the need for further exploration of the functional characteristics of the various drug-eluting stent formulations.

1.8 Challenges in Treatment of Restenosis

When restenosis does occur with drug-coated stents, the poor performance has been attributed to a number of possible factors including extensive local vessel injury during the procedure, gaps in stent coverage or inadequate stent expansion[71, 72] and

even lesion complexity. The RESEARCH study of 238 patients found that in-segment restenosis occurred in 7.9% at six months. Several factors were identified as multivariate predictors of restenosis: treatment of in-stent restenosis (restenosis rate 19.6%), ostial lesion location (14.7), diabetes mellitus (14.3%), stented length >26 mm (13.9%), reference vessel diameter <2.17 mm (10.3%), vessel other than left anterior descending coronary artery (10.8%)[67]. Distinct performance characteristics of one eluted compound over another may tailor therapeutic choices for higher risk conditions.

Central to our understanding of drug-eluting stent therapy efficacy is the hypothesis that local drug concentration variations, even on the length scale of single cells, can affect the macroscopic outcome with regards to efficacy[73, 74]. Work by Lovich and Creel *et al.* showed that a hydrophobic drug like paclitaxel distributes inhomogenously across the arterial wall. Extensive work by Hwang *et al.* has shown that variability in clinical results might be explained by local drug distributions and the influence of local transport forces, local tissue structure and drug-tissue interactions ("local pharmacokinetics"). Their work showed that arterial wall uptake for hydrophilic drugs such as dextrans depend on vessel elastin content, such that arteries with greater elastin content have a higher partition coefficient than arteries with greater cellular content. They also found that geometric organization of the *elastic laminae* also causes significant anisotropy in transport where diffusivity in the planar direction exceeds that in the transmural direction by over an order of magnitude for hydrophilic drugs and for hydrophobic drugs which interact with serum carrier proteins like albumin.

This microscopic view of pharmacokinetics in addition to the traditional one is vital to understanding drug-eluting stent therapies. Unfortunately, tremendous drug concentration gradients are invariably established by transport forces such that drug concentrations in adjacent cells can differ significantly[75]. Local tissue ultrastructure, and the concentrations they enforce on the artery at a microscopic scale, become important considerations in the optimization of vascular drug delivery.

1.9 Definitions of Binding

Building on the fundamental tenets of local pharmacokinetics, this work will explore the roles of specific and general binding in local arterial drug delivery. Specific binding is defined as the association of a particular drug with its biologically relevant binding protein. Paclitaxel specifically binds to polymerized microtubules to inhibit tubulin depolymerization during cell DNA replication, prevent mitosis. Rapamycin specifically complexes with the FK506 binding protein (FKBP) and the mammalian target of rapamycin protein (mTOR). This heterotrimer prevents nuclear translocation of cell cycle critical protein, thereby inhibiting replication. General binding is defined as the association of drug with all tissue elements other than the therapeutically relevant binding protein.

1.10 Thesis Theme

With the overwhelming success of paclitaxel and rapamycin in the stent-based treatment of restenosis, a central question lingers: What physiochemical parameters distinguish the success of one drug over another? Where heparin, dexamethasone and actinomycin-D all showed early promise at inhibiting smooth muscle growth in culture and intimal hyperplasia in controlled animal trials, all failed in late stage clinical trials. One theory has been put forth that *in vivo*, compounds like paclitaxel and rapamycin have unique biologic effects, different from those therapies that prove ineffective. There is however little to no firm support for this possibility. In contrast, our central hypothesis is that physicochemical, not pharmacological, properties can be used to distinguish drugs. In particular, we believe tissue retention to be a critical factor for tissue effect. Drugs that are rapidly cleared can not reside long enough to exert and effect. Compounds that bind to tissue elements can be in place for sufficient time to influence tissue events. Specific and general binding to target proteins allows certain drugs to remain in tissue and establish a meaningful biologic effect. When a compound weakly binds to tissue elements, as may be the case with unsuccessful treatments, it is quickly cleared from the vessel and cannot inhibit growth. Challenges remain in understanding how pathology changes the binding domains of tissue, especially in disease states such as diabetes where restenosis rates are significantly higher. In other fields of local delivery, the lessons of this work are generally applicable and the implications are presented for a broad audience.

1.11 Thesis Organization

This thesis consists of a series of studies which attempt to elucidate the role of specific and general binding in stent-based drug delivery. The specific aims are:

- Chapter 2 characterizes the arterial transport and distribution properties of model hydrophilic and hydrophobic drugs with specific binding targets
- Chapter 3 defines specific and general binding in the context of local arterial drug delivery.
- Chapter 4 characterized the role of binding to thrombus and its effects on arterial wall drug deposition

Through systematic study of physiochemical specific and general binding we hope to elucidate one of the fundamental mechanisms that make stent delivery so successful.

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Chapter 2

Specific Binding in Arterial Tissue

Abstract

Endovascular stents eluting drugs have changed the practice of medicine and yet it is unclear how they achieve such effect and how to distinguish between the different formulations available. Biological drug potency is not the sole determinant of biological effect, physicochemical drug properties dominate as well. Hydrophobic drugs are retained within tissue and have dramatic effects while hydrophilic drugs are rapidly cleared and ineffective against restenosis. The question arises as to whether individual properties of different drugs beyond lipid avidity can further distinguish arterial transport and distribution. In bovine internal carotid arterial segments, tissue loading profiles for hydrophobic paclitaxel and rapamycin are indistinguishable, reaching load steady-state after 2 days. Hydrophilic dextran reaches equilibrium in several hours at levels no higher than surrounding solution concentrations. Both paclitaxel and rapamycin bind to the artery at 30-40 times bulk concentration. Competitive binding assays confirm binding to specific tissue elements. Most importantly, the transmural drug distribution profiles are markedly different for the two compounds, reflecting perhaps different modes of binding. Rapamycin, which binds specifically to the FKBP12 binding protein, distributes evenly through the artery while paclitaxel, which binds specifically to microtubules, remains primarily in the sub-intimal space. The data demonstrate that specific binding of rapamycin and paclitaxel to fixed tissue proteins plays an essential role in determining arterial transport and distribution and in distinguishing one compound from another. These results offer further insight into the mechanism of local drug delivery and the specific use of existing drug-eluting stent formulations.

2.1 Introduction

It now appears that the success of drug-eluting stents is predicated to as great a degree upon the extent of drug deposition and distribution through the arterial wall as virtually any other factor(1-5). The biological effects of a candidate drug are essential, but ultimately tissue residence time will determine effect and toxicity(6, 7). Fueled by its clinical relevance(8-11) a number of studies have been carried out to detect, model and predict the distribution of drugs within arterial segments beneath, adjacent to and a distance from stents(12-15). Drugs that are retained within the blood vessel are far more effective than those that are not. Heparin, is a perfect example of a compound whose ubiquitous biological potential is lost by virtue of its physicochemical properties. Heparin regulates virtually every aspect of the vascular response to injury(16). Yet heparin is so soluble and diffusible that it simply cannot stay in the artery for more than minutes after release and has no effect on intimal hyperplasia when eluted from a stent(4, 17, 18).

Paclitaxel in contradistinction is a far smaller compound with fewer effects specific to vascular biology, but paclitaxel is so hydrophobic and insoluble that it binds tenaciously to tissue protein elements and remains beneath stent struts long after release(13). The clinical efficacy of paclitaxel at reducing restenosis rates following elution from stents appears incontrovertible(8, 11). In addition to its hydrophobic general binding behavior, paclitaxel also binds to its protein target, polymerized microtubules(19), with nanomolar specificity. Analogously, rapamycin, also successful

after local vascular delivery(10), acts on a specific target through a series of events that requires binding to the specific binding proteins FKBP12 (FK506 binding protein 12) and FRAP (FKBP12-Rapamycin Associated Protein)(20, 21). Thus, we hypothesized that the tissue specific binding proteins can significantly influence arterial wall drug distribution and local pharmacological effects for compounds like paclitaxel and rapamycin above and beyond the influence of hydrophobic general interactions alone. The distribution of tissue binding proteins is not uniform in space or in time. For example, FKBP12 is found most abundantly in vascular smooth muscle cells at a concentration of 10^{-5}M(22). FKBP12 is upregulated with injury in neuronal systems(23) and likely after arterial injury as well. Microtubules have a similar cellular concentration of ~10^{-5}M(24). The non-uniform distribution of paclitaxel previously observed in the arterial wall may reflect an inhomogeneous distribution(13) of polymerized microtubules or carrier proteins(25). While regulated at a coarse level by transport forces and lipid avidity, the distribution of paclitaxel and rapamycin is also regulated at a fine level by the distribution and availability of their protein targets, a level of control not present for drugs like heparin that do not specifically bind to arterial wall proteins. With a more complete understanding of the role of specific binding in arterial drug distribution, stent design, drug composition and release formulation can be better optimized.

2.2 Methods

Arterial Loading and Elution

We defined the tissue pharmacokinetic profiles of dextran (10,000 Da), paclitaxel (853.9 Da), and rapamycin (914.2Da) in calf carotid arterial wall segments. Calf internal carotid arteries were harvested and transported in phosphate buffered saline with physiological calcium and magnesium added (PBS^{++}, 0.01 mol/L $CaCl_2$ and 0.01 mol/L $MgCl_2$, Sigma) at 4°C. Arteries were cleaned of excess fascia, opened longitudinally, cut into segments (400 to 600 mg), and placed in centrifuge tubes with 1.0 mL of [^{3}H]dextran, [^{3}H]paclitaxel or [^{14}C]rapamycin. All bulk solutions were made fresh immediately before experimentation.

For loading experiments, segments were removed in triplicate at indicated time points, briefly washed in PBS^{++} and blot-dried before being dissolved in Solvable (Packard-Canberra). Liquid scintillation cocktail (6 ml) was added to dissolved samples before counting with liquid scintillation spectroscopy (2500 TR Liquid Scintillation Analyzer, Packard-Canberra). For elution experiments, segments were allowed to equilibrate for 48 hours and were then placed in 50ml of PBS^{++} for the indicated time periods before being processed in triplicate as indicated with the loading experiments.

Measurements of Transmural and Planar Diffusion

Planar and transmural diffusivities were measured using diffusion cells. Planar diffusivities (diffusion in the plane of the elastin sheaths) were measured by mounting

opened arterial specimen between two glass slides and contacting the specimen's edge to a drug bath. After 25 minutes (a time determined to provide adequate diffusion and signal to noise ratio) specimens were processed for liquid scintillation counting. Planar diffusivities were measured in the longitudinal and circumferential direction. For transmural diffusivity, cleaned arteries were opened longitudinally and clamped in a standard Franz diffusion cell consisting of an upper sink compartment containing PBS^{++}, separated from a drug-containing lower compartment by an artery lying *en face*. The artery was thus exposed only to drug on the luminal side and only to buffer on the other. After 25 minutes of exposure to the drug, samples were processed for liquid scintillation counting.

The arterial wall is a highly heterogeneous structure composed of different tissue layers which impose individual effective diffusivities. Yet, there is a regularity from the alternating cylindrical bands of connective tissue and smooth muscle cells that permits use of a lumped effective diffusivity parameter to characterize bulk drug transport properties in the arterial wall(13). Lumped effective diffusivities can be calculated from the measured drug mass M in tissue, using the early time solution to the diffusion equation:

$$D = \frac{\pi}{t}\left(\frac{M}{2 \cdot A \cdot C \cdot k}\right)^2$$

where t is the time, A is the artery area exposed to drug, k is the binding capacity coefficient, C is the drug source concentration(3).

Drug-Tissue Binding Capacity and Distribution

We defined the bulk differential capacities of the arterial wall for dextran, paclitaxel and rapamycin at equilibrium. Arterial segments were weighed before being placed in drug bath solutions. Segments were allowed to equilibrate for 48 hours and were then processed for liquid scintillation counting. The drug concentration of each tissue sample was normalized by tissue mass and then by drug concentration in the bulk fluid during equilibrium incubation to determine the binding capacity.

Equilibrated transmural arterial drug distribution was measured through *en face* cryosectioning. Arterial segments were incubated in drug bath for 60 hours, then laid flat and snap frozen in a plastic encasement with OCT Embedding Medium (Tissue-Tek, Sakura Finetechnical Inc). Samples were stored in a –80°C freezer until sectioning parallel to the intima with a refrigerated microtome (Cryotome SME, Shandon, Inc.)(12, 26, 27). Upon sectioning, the segment length and width were measured with a caliper. 0.020-mm thick sections were cut parallel to the intima and the drug content of each sample was determined by liquid scintillation spectroscopy. Tissue drug concentration (c_T) at each transmural location was calculated as the mass of drug normalized by the measured tissue area and slice thickness. Tissue drug concentration was then normalized by the bulk fluid drug concentration during equilibrium incubation (c_{bulk}) to determine the binding capacity (k) at each transmural location x:

$$k(x) = c_T(x)/c_{bulk}$$

2.3 Results

Tissue Loading Kinetics

Arterial samples were incubated in [^{3}H]dextran, [^{3}H]paclitaxel or [^{14}C]rapamycin and harvested in triplicate over a period of 72 hours. As the drugs span a range of specific activities preventing use of equivalent initial bulk concentrations, loading data was normalized to an average peak value at 72 hours (Figure 2.1). While the hydrophilic dextran reached 80% of equilibrium value with in several hours, paclitaxel and rapamycin took nearly 24 hours to achieve the same level. The loading profiles for these two hydrophobic compounds are indistinguishable and approach steady state only after 48 hours.

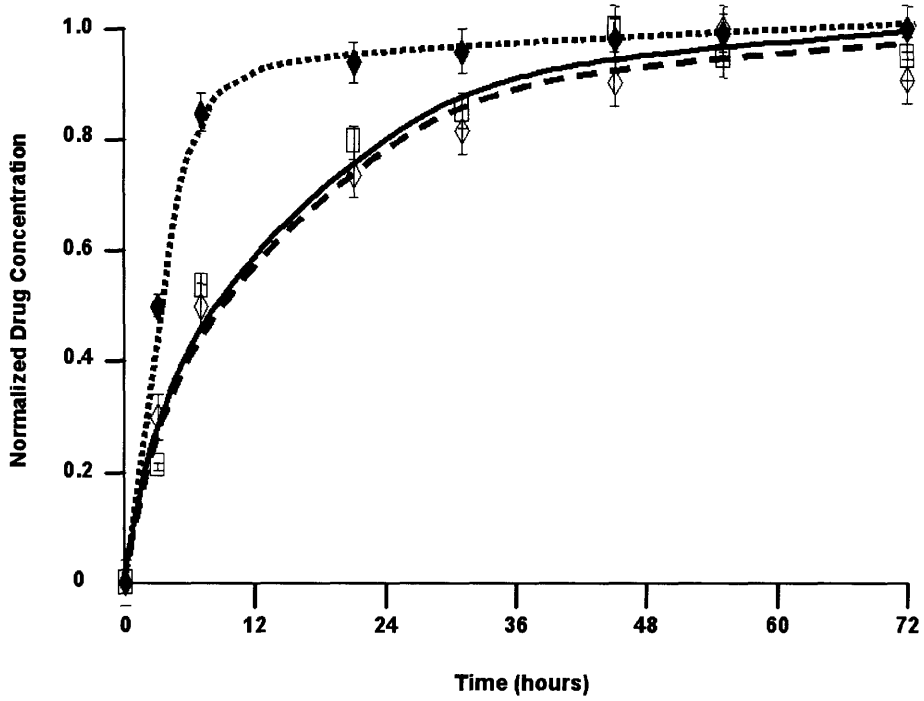

Figure 2.1: Tissue Loading

Pharmacokinetic tissue loading profile of radioactive dextran (♦), paclitaxel (□) and rapamycin (◊) in bovine internal carotid tissue segments.

Tissue Elution Kinetics

Arterial samples were pre-equilibrated in [^{3}H]dextran, [^{3}H]paclitaxel or [^{14}C]rapamycin for 60 hours and then placed in an elution sink of 50ml of PBS^{++}. Samples were processed in triplicate over the following 48 hours and data plotted as percentage of the pre-elution load value (Figure 2.2). Dextran elutes most rapidly, losing 90% of its equilibrated load within 2 hours and reaching a steady-state of ~10% of original material in less than 5 hours. The hydrophobic drugs take ~24 hours to reach an elution steady-state value. At 48 hours arterial segments loaded with rapamycin retain ~60% of their initial load, and paclitaxel 35%.

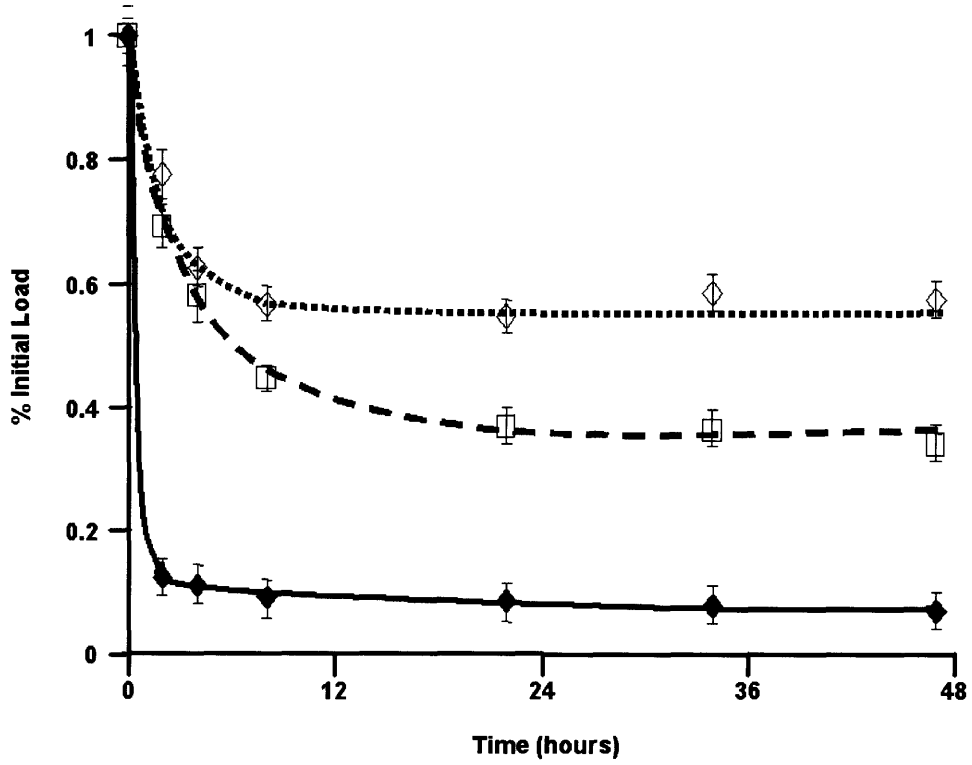

Figure 2.2: Tissue Elution

Pharmacokinetic tissue elution profile of radioactive dextran (♦), paclitaxel (□) and rapamycin (◊) in bovine internal carotid tissue segments normalized initial load values.

Bulk Diffusion Measurements

Effective planar and transmural diffusivities in the carotid artery were measured directly from the mass of drug transferred into the arterial wall (n=3) using the early-time solution of the diffusion equation(3) (Figure 2.3). Diffusivities in the circumferential and longitudinal direction were equivalent (data not shown). Planar coefficients represent measurements in the longitudinal direction. All compounds demonstrated anisotropic diffusivities, with more rapid planar than transmural diffusivity. Dextran diffusivity was two orders of magnitude greater than either of the hydrophobic compounds in both planar and transmural directions despite their 20-fold smaller size than the dextran. Anisotropic diffusion varies with molecular mass, invalidating the assumption that drug-tissue diffusion problems can assume a simple molecular conformation in a homogenous media.

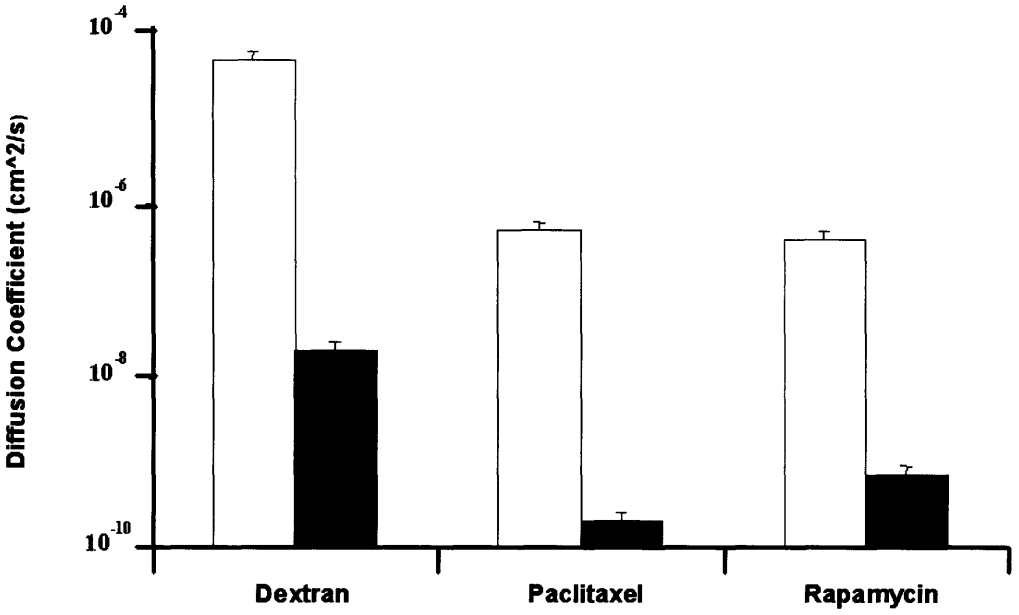

Figure 2.3: Arterial Wall Diffusion Coefficients

Planar (□) and transmural (■) diffusion coefficient of radioactive dextran, paclitaxel and rapamycin in bovine internal carotid tissue segments.

Tissue Binding Capacity (TBC) Coefficients

Tissue binding capacity (TBC) was defined as the tissue concentration (c_T) at equilibrium normalized by the bulk concentration at equilibrium (c_{Bulk}). Arterial segments were incubated in drug for 60 hours to allow for equilibration. Upon equilibration, bulk solutions were sampled and tissue samples were processed for liquid scintillation counting. Variation in initial bulk concentrations over an order of magnitude did not affect TBC. Dextran has no binding capacity in arterial tissue with a coefficient of ~0.60 (data not shown). As this value is similar to its physically accessible volume fraction of arterial tissue(17) dextran may not leave the extracellular space. Paclitaxel and rapamycin have binding coefficients greater than 1 indicating that general and/or specific binding interactions are sequestering these drugs in the tissue (Figures 2.4 and 2.5).

To assay for the binding specificity, experiments were conducted with mixtures of hot and cold drug. At a stock solution of 10^{-8} M paclitaxel TBC was 42 (Figure 2.4). As the amount of non-labeled drug was introduced in a solution of radiolabeled paclitaxel the TBC dropped as well indicating that paclitaxel binding sites were saturated by cold drug, excluding the hot. As a negative control, 10^{-8}M radiolabled paclitaxel was mixed with a 1000-fold molar excess of non-labeled rapamycin, which does not bind specifically to polymerized tubulin and the TBC decreased to 22 (data not shown) indicating that only half of the paclitaxel TBC was due to general binding, as rapamycin displaced paclitaxel involved in general protein interactions. A similar experiment was

conducted with radioactive rapamycin (Figure 2.5). However, because of differences in specific activity, we employed a labeled-rapamycin stock solution of 10^{-5}M, at nearly the level of FKBP saturation. At a 1:1 labeled to unlabeled rapamycin ratio the TBC dropped to 17, just above half of the control value of 30. When the supersaturated non-radiolabel rapamycin was in 100 fold excess the TBC dropped to 4. As a negative control hot rapamycin was mixed with a molar excess of cold paclitaxel and the TBC moved to 13 (data not shown), nearly half of the control value. These data suggest as well that slightly less than half of the rapamycin TBC is from general binding effects.

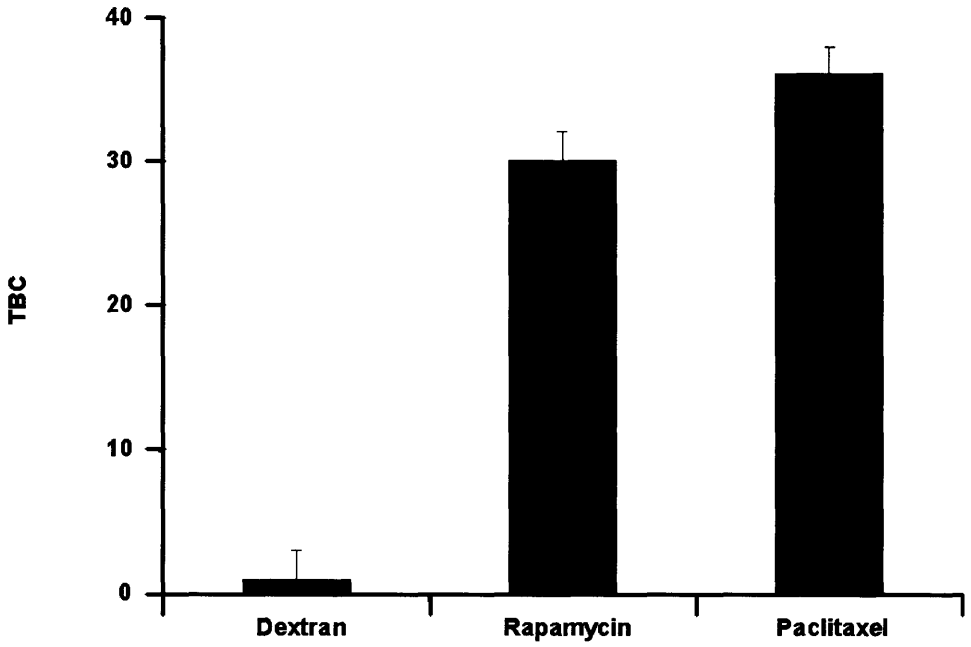

Figure 2.4: Tissue Binding Capacity

Tissue binding capacity of labeled dextran, rapamycin and paclitaxel in internal carotid tissue segments after 60 hours of equilibration.

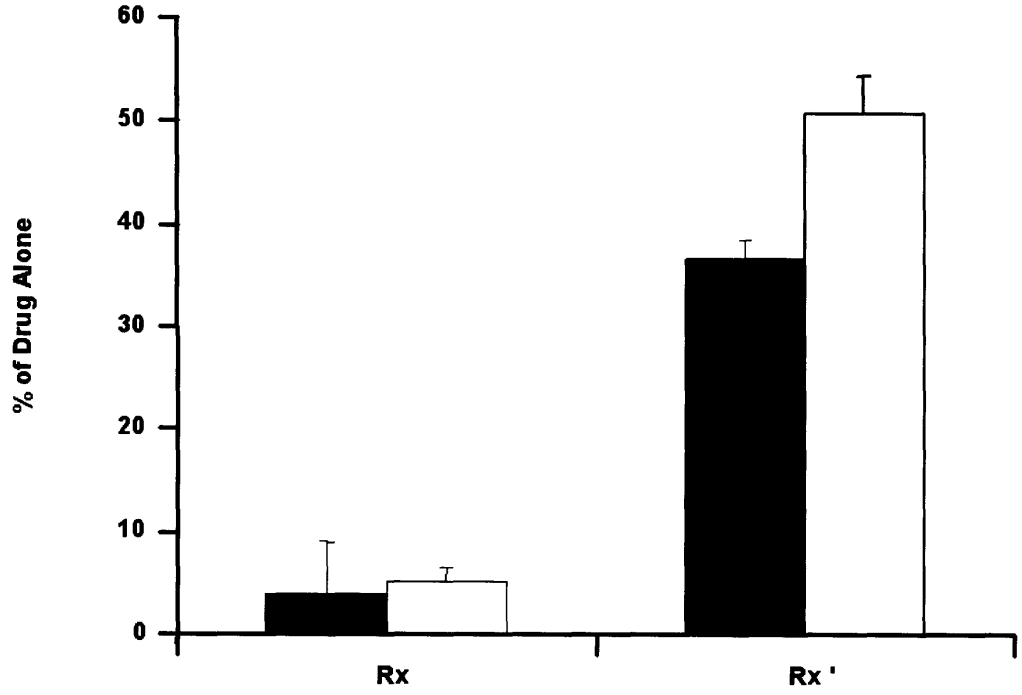

Figure 2.5: Specific Binding Capacity

Fractional tissue binding capacity of labeled paclitaxel (■) and labeled rapamycin (□) relative to pure labeled drug in internal carotid tissue segments after 60 hours of equilibration for the following conditions: (1) Rx, 10^{-6}M labeled drug plus 10^{-3}M of the same unlabeled drug. (2) Rx', 10^{-6}M labeled paclitaxel plus 10^{-3}M unlabeled rapamycin and 10^{-6}M labeled rapamycin plus 10^{-3}M unlabeled paclitaxel.

Transmural Drug Distribution

Arterial samples were incubated in [^{3}H]dextran, [^{3}H]paclitaxel or [^{14}C]rapamycin for 60 hours and then snap frozen for *en face* sectioning (Figure 2.6). Previous work with paclitaxel showed that TBC was maximal in the intima and declined precipitously within the most intimal regions of the arterial media to less than half the intimal level. At the outer edge of the media, the paclitaxel binding capacity increased gradually and peaked within the adventitia before falling off to near unity(13). These data have been recapitulated from Creel *et al* for comparison with dextran and rapamycin. Dextran again shows little binding capacity throughout the artery. Rapamycin shows a uniform transmural distribution, in stark contrast to the non-uniform distribution of paclitaxel. It is important to note the data from Creel *et al.* was conducted with paclitaxel concentrations near the polymerized tubulin saturation limit. When paclitaxel distribution experiments were repeated at lower sub-saturation concentrations, the TBC was higher though with a similarly heterogeneous transmural distribution (data not shown).

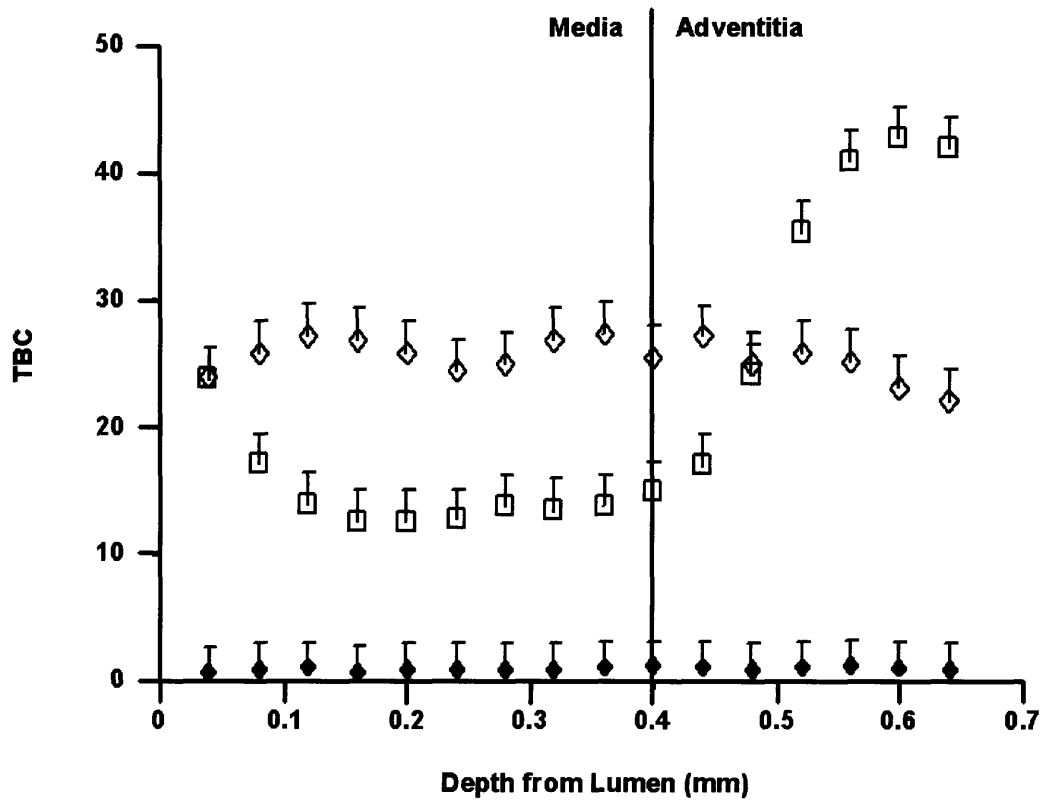

Figure 2.6: Transmural Equilibrium Drug Distributions

Transmural equilibrium distribution of labeled dextran (♦), paclitaxel (□) and rapamycin (◊) in 0.040mm thick bovine internal carotid tissue segments.

2.4 Discussion

Local drug delivery has great theoretical and practical appeal for vascular disease(28, 29). One important surprise is that biological potency is not the sole determinant of biological effect. Drug-specific physiochemical properties determine to a great degree whether concentrations sufficient for therapeutic activity can be sustained. Hydrophobic drugs, for example, are retained within tissue and have dramatic clinical effects; hydrophilic drugs are rapidly cleared and ineffective against restenosis. The data now suggest that specific binding also plays a critical role in determining drug distribution. While Creel *et al.*(13) showed that paclitaxel distributed heterogeneously through arterial tissue, our present data demonstrate that rapamycin distributes more uniformly through the media and adventitia. In addition, the tissue binding and diffusion results suggest that binding site availability and distribution regulate the fine structure of drug deposition beyond the coarse structure imposed by transport forces and lipid avidity. Ultimately, local tissue ultrastructure and the concentrations they enforce on the artery at a microscopic scale together with specific and general binding site distribution become critical considerations in the optimization of vascular drug delivery.

Specific and General Binding Determine Tissue Binding Capacity

Hydrophobic compounds must bind to proteins, fixed and soluble, to have a biological effect. This binding can take two forms; general interactions such as charge or water affinity, and specific binding idiosyncratic to the individual drug. Paclitaxel and rapamycin can both bind generally to serum proteins and hydrophobic tissue micro-

environments. Paclitaxel demonstrates nanomolar specificity to polymerized microtubules while rapamycin shows similar specific binding properties to FKBP12, a ryanodine receptor associated protein. Dextran, by virtue of its extreme hydrophilicity exhibited neither type of binding and accordingly its TBC was less than one, and its potential tissue level can never exceed what the concentration in bathing solution. In contrast, paclitaxel and rapamycin were deposited in the blood vessel at concentrations 30-40 fold higher than in surrounding bulk solution. Thus, tissue concentrations of hydrophobic paclitaxel and rapamycin can exceed the applied concentration several-fold establishing an effective volume of distribution within arteries larger than anticipated from surrounding solution concentration.

The specificity and potential of tissue binding was demonstrated by serial dilution experiments. The TBC for radiolabeled paclitaxel remained substantially greater than one until the non-radiolabeled drug was in 100-fold excess of the 10^{-8}M bulk solution and only fell significantly at 1,000-fold excess. Intriguingly, microtubules bind paclitaxel and reside in calf internal carotid segments at ~10^{-5}M(24) suggesting that microtubules account for a specific binding of paclitaxel in our system. Some of the displacement is general. When cold paclitaxel was substituted for a high concentration molar excess of rapamycin the TBC dropped nearly in half. Similar results were obtained when the same procedures were carried out with labeled rapamycin. As the specific activity of the radiolabel dictates a working concentration of 10^{-5}M, near the FKBP12 saturation limit, rapamycin TBC dropped in half upon mixing an equal amount of cold rapamycin and hot

rapamycin. As with paclitaxel when hot rapamycin was mixed with excess cold paclitaxel, the TBC also dropped in half. Thus, tissue distribution of both drugs are governed by almost equal parts of specific and general binding events.

Tissue Pharmacokinetics of Hydrophilic and Hydrophobic Compounds

It is expected that paclitaxel and rapamycin will have similar transport properties given that both compounds have solubilities of ~6ug/ml, molecular weights of less than 1 kD and nanomolar binding constants to their specific protein targets. Indeed, the compounds act quite similar when compared to dextran. While the latter takes several hours to reach a steady state tissue concentration lower than the surrounding media, paclitaxel and rapamycin reach near identical levels but after far greater than 1 day. Kinetics not concentration account for this effect as the time to reach steady state was independent of the concentration applied over a broad range. While net binding was identical distribution and tissue elution after binding and uptake were not. At steady state the artery retained only 10% of the applied hydrophilic dextran. Paclitaxel retention was 3.5-fold higher, and rapamycin almost twice paclitaxel levels. With similar elution kinetics, the paclitaxel-rapamycin disparity suggests that rapamycin has twice the number of specific binding sites relative to paclitaxel. Moreover, while paclitaxel remained in the subintimal space, rapamycin was evenly distributed throughout the arterial wall.

Movement of a molecule through a composite structure like a blood vessel wall is driven by a range of forces and phenomena. The influence of effective molecular radius

can dominate when all other factors are equal, but may recede in importance when other factors are present. For instance, despite being nearly 20-fold smaller than dextran, paclitaxel and rapamycin diffuse more slowly in both the transmural and planar directions. This difference may be attributed to the hydrophobicity of the compounds or possibly to the role of binding. While dextran has few binding sites, paclitaxel and rapamycin will repeatedly bind to and dissociate from their respective specific and general targets as they diffuse through tissue, in effect slowing the leading edge of the diffusion front.

We have previously shown that albumin and dextrans diffuse at least an order of magnitude faster in the planar than in the transmural direction(3). For paclitaxel and rapamycin the planar diffusivity exceeded transmural diffusivity by at least two orders of magnitude although for both drugs their respective diffusivities were two orders of magnitude smaller than those of dextran. These phenomena are likely governed by similar forces for all three drugs despite vastly different lipid avidities. The transport of hydrophilic compounds is enhanced in aqueous regimes of the vessel wall, but retarded by hydrophobic elastin layers. For hydrophobic compounds, these layers act in a reverse fashion; the movement of paclitaxel and rapamycin is likely impeded by the more water-rich regions of the blood vessel wall and aided by lipid pools or even the protein-studded elastin lamina. In both cases however, while individual layers might be isotropic, the greater composite of alternating layers of the arterial wall provide for planar diffusion that far exceeds transmural flux.

Conclusions

As the number of available drug-eluting devices increases, distinction and choice may reside not only in ease of use but in the physicochemical functionality of the drug-stent unit. Local drug distribution is modulated by transport and lipid avidity at a coarse level, but for clinically relevant compounds like rapamycin and paclitaxel, also by the distribution of their specific binding sites. Arterial ultrastructure also influences transport as alternating tissue layers of varying permeability result in anisotropic transport for both hydrophobic and hydrophilic drugs. Design and evaluation of a drug-eluting device thus requires a unified understanding of the drug, its physicochemical characteristics and its specific and general interactions with arterial structures.

2.5 Acknowledgments

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Chapter 3

Specific and General Binding Competition

Abstract

The efficacy of drug-eluting stents (DES) requires the delivery of potent compounds directly to underlying arterial tissue. The commercially available DES drugs rapamycin and paclitaxel interact with general protein elements in tissue as well as specific target sites. The question arises then as to whether other locally released compounds or systemically circulating drugs will alter tissue deposition to either of these sites and consequently change overall effect. The ability of either DES drugs to compete for specific protein binding and tissue deposition was assessed and compared to effects of other commonly administered cardiac drugs. Paclitaxel and ramaycin do not affect the other's binding to their biologically relevant specific protein targets, but can generally displace each other from tissue at three log order molar excess, decreasing arterials loads by greater than 50%. Specific and general binding sites for both drugs are distributed across the media and adventitia with higher specific binding associated with the higher specific binding site densities in the media. Drugs classically used to treat standard cardiovascular diseases, such as hypertension and hypercoaguability, also generally displace rapamycin and paclitaxel, possibly decreasing tissue reserve capacity for locally delivered drugs. Local competitive binding should not limit the placement of rapamycin and paclitaxel eluting stents in close proximity. Commonly administered systemic drugs can however compete with locally delivered rapamycin or paclitaxel to decrease tissue reserve capacity.

3.1 Introduction

The viability of candidate drug-eluting stents has been linked to the properties of the drugs they release. Successful devices elute compounds that penetrate and can be retained in tissue at high local concentrations. Failed devices seem to have considered drugs that are rapidly cleared from arterial tissue [1-4]. A hydrophilic compound like heparin cannot be made to stay in tissue to exert its numerous vasoactive effects over clinically viable time periods[5-7]. Rapamycin and paclitaxel have emerged as the two leading clinical therapies for stent delivery in major part because aside from their putative biological effects their physiochemical properties favor prolonged tissue retention. Paclitaxel binds specifically to a heterodimer of tubulin and in a more general manner to a range of plasma and tissue bound proteins. Rapamycin exhibits a similar effect as it specifically associates with the FK506 binding protein complex (FKBP) and binds generally to a wide range of nonspecific proteins. Neither rapamycin or paclitaxel are large compounds. They are both less than 1000 Da. Yet, their insolubility and the immediate impact of protein binding markedly reduces convection velocities compared to much larger, but much more readily diffusible and soluble compounds, like heparin. The confluence of these physical forces creates a relatively high overall partitioning within arterial tissues. Though more similar to each other than to other hydrophilic drugs the long term retention, elution from and distribution within arteries do differ significantly for these two compounds. It has been hypothesized that this difference derives in part from the dissimilar distribution in FKBP[8, 9] and polymerized microtubules [10].

As local binding is critical to local effect two questions arise with the increasing use of these devices. First, as the number of stents implanted per procedure and in a given artery rises concern has been raised as to the potential interactions of the same or different drugs eluted from multiple adjacent or over-lapping DES. Second, will the powerful circulating medications patients receive potentially change the binding of drugs eluted from the stents. In part these two questions pose the polar ends of a spectrum of issues. In the first case the question is whether high local tissue levels of drugs that have almost undetectable circulating concentration compete with each other when both are directed to the same defined target tissue, and the second is whether high steady state circulating levels of a systemically administered drug can compete with the tissue binding of a locally eluted compound. Accordingly, we examined the general and specific tissue binding of rapamycin and paclitaxel in the presence of added amounts of these compounds or common systemically administered cardiac drugs.

3.2 Methods

Tissue Binding Competition Assays

Rapamycin was generously donated by Johnson and Johnson/Cordis, radiolabeled Paclitaxel was provided by Vitrax and unlabeled Paclitaxel was from LC Laboratories. For all tissue experiments radiolabeled drugs were loaded at 10^{-6} M in $1cm^2$ fresh bovine calf carotid artery tissue. Rapamycin blocked general binding sites for paclitaxel tissue uptake, and *vice versa*, with each molecule serving as a competitive antagonists for the other's general binding sites. Specific binding was inhibited with FK506 (Eton Bioscience) and colchicine (Sigma). FK506 displaces rapamycin as it binds to the same site on the FKBP protein, and colchicine displaces paclitaxel binding by stabilizing depolymerized microtubules. All samples were processed and assayed using previously described standard liquid scintillation techniques[1]. Statistical significance was evaluated using a two-tailed Student's t-test with comparison to control cases.

Drug and binding site distribution radially through the arterial wall were correlated. Radiolabeled drug content in 40μm transmural sections was spatially mapped to tissue immunostained for the binding proteins - tubulin or FKBP (both antibodies from BD Bioscience, San Jose, CA). FK506, colchicine or the indicated non-labeled control competitor drugs (labeled rapamycin plus non-labeled paclitaxel and *vice versus*) were loaded in tissue at a three log order molar excess over the labeled drug concentration to maximize competitive displacement effects.

Specific Protein Binding Assays

Combinations of radiolabled rapamycin and human wild type FKBP protein (generously donated by Ariad, Cambridge, MA) at 1:1 molar ratios were equilibrated for one hour with competitor drugs at molar ratios ranging from 1:1000 to 1000:1. Solutions were purified through a lipophilic Sephadex column (Sigma-Aldrich, USA) to isolate labeled rapamycin bound to FKBP. Paclitaxel polymerization assays were performed using standard optical density spectrophotometry methods with measurements at 340nm and tubulin concentrations of 1mg/ml. In brief, purified tubulin (Cytoskeleton, Denver, CO) was mixed with GTP, paclitaxel and a competitor drug (with the exception of the control sample). Samples were allowed to polymerize for one hour at 32C with optical density measurements made at the beginning and end of the experiment. All data were corrected for absorbance of the competitor drugs by subtracting off the signal contributions of the individual drugs.

3.3 Results

Drug Competition in Local Delivery

Bovine carotid arterial tissue preparations were incubated for 24 hours in 10^{-6} M paclitaxel or rapamycin along with the indicated competitor drug at a three log order molar excess (Figure 1A). Rapamycin displaces 35% of the labeled paclitaxel from tissue specimens while paclitaxel blocks only 50% of rapamycin binding. FK506 has no significant effect on paclitaxel loading but eliminates 50% of the binding capacity for rapamycin, likely through occupying sites on the specific binding protein FKBP in the tissue. Similarly colchicine significantly decreases paclitaxel uptake by ~50% but had a statistically insignificant effect on rapamycin uptake.

To elucidate the mechanisms of displacement tissue samples were preloaded with only the competitor drugs for 24 hours. Following this incubation period, tissue was moved to a separate bath containing 10^{-6} M paclitaxel or rapamycin for an additional 24 hours. Only colchicine pre-incubation reduced paclitaxel binding, otherwise the tissue loading did not significantly differ from no-competition controls (Figure 1B). Previously we have shown that hydrophobic species such as paclitaxel and rapamycin, and hydrophilic dextrans [1] elute out of tissue to steady state levels over a period of approximately 12 hours. These results imply that the pre-incubated competitor drugs elute out of tissue and/or are displaced by paclitaxel and rapamycin, allowing the labeled drugs to achieve their full tissue loading potential. In the colchicine-paclitaxel case, it

appears that colchicine remains sufficiently associated with tissue proteins to reduce the overall paclitaxel tissue levels.

Dose response curves were constructed to evaluate local competition between paclitaxel and rapamycin. Paclitaxel (Figure 2A) and rapamycin (Figure 2B) at 10^{-6} M were both simultaneously incubated with varying doses of paclitaxel and rapamycin. When labeled paclitaxel is loaded with unlabeled paclitaxel, significant displacement of the labeled drug is noted at concentrations between 10^{-6}M and 10^{-5}M. For paclitaxel loading with rapamycin, significant displacement of drug does not occur until the concentration of rapamycin is between 10^{-4}M and 10^{-3}M. Similar results are observed when assaying rapamycin characteristics. For both cases, three log order molar excess of unmatched drug is required to reduce binding of the labeled drug significantly. Arterial concentrations from stent delivery in porcine models typically yield tissue concentrations between 10^{-8}M and 10^{-7}M. At 10^{-8}M loading of labeled drug, competition drug concentrations still must reach between 10^{-4}M and 10^{-3}M to displace significantly the labeled drug (data not shown). Tissue drug concentrations of 10^{-3}M have never been reported from paclitaxel or rapamycin-eluting stents.

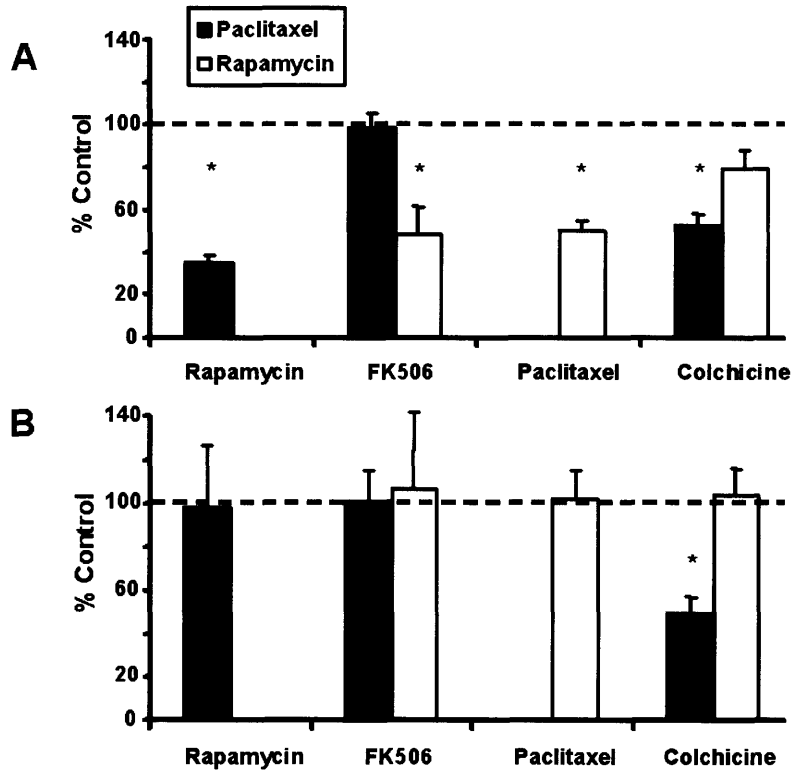

Figure 3.1: Specific and General Binding Competition

(A) Percentage of paclitaxel (■) and rapamycin (□) in tissue relative to no-competition controls after 24 hours when simultaneously loaded with various competitor drugs (indicated in the figure) and **(B)** when tissue was exposed to competitor drug for 24 hours followed by a separate 24 hour loading of the labeled drugs. (*) indicates a statistically significant difference ($p < 0.05$ with a two-tailed Student's t-test) from the no-competition control. Standard deviations are plotted with the data set. tissue loading profile of radioactive dextran (♦), paclitaxel (□) and rapamycin (◊) in bovine internal carotid tissue segments.

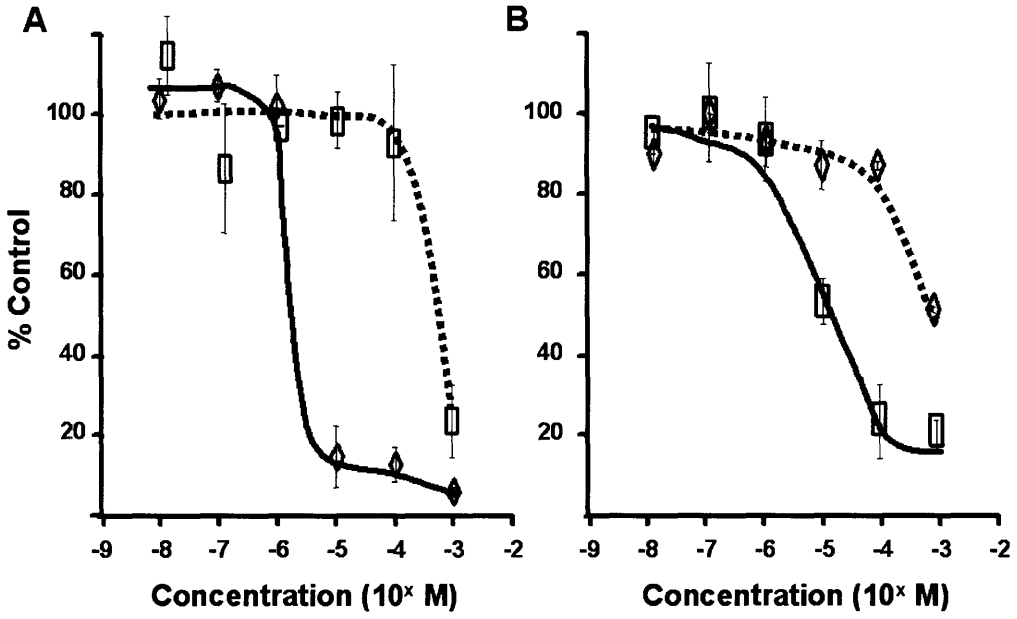

Figure 3.2: Paclitaxel and Rapamycin Tissue Binding Competition

(A) The percentage of control radiolabeled paclitaxel bound in tissue when in competition with unlabeled paclitaxel (□) and unlabeled rapamycin (◊). **(B)** The percentage of control radiolabeled rapamycin in tissue when in competition with unlabeled paclitaxel (□) and unlabeled rapamycin (◊).

Specific and General Binding Tissue Domains

Transmural distributions of rapamycin (Figure 3) and paclitaxel (Figure 4) under conditions of specific and general displacement were plotted over tissue sections immunohistochemically labeled for each drugs' specific binding target and normalized to their control profiles. We have previously shown that rapamycin maintains a homogenous transmural distribution across the width of arterial tissue while paclitaxel distributes more variably, with relatively higher sub-intimal and adventitial binding and lower medial deposition[1]. We now differentiate between specific and general tissue binding. Colchicine, reduced normalized paclitaxel content by ~ 50% in a uniform manner across the media and adventitia, despite a seemingly higher density of tubulin staining paclitaxel-binding sites in the media. In contrast, rapamycin forced the paclitaxel transmural profile to drop precipitously from 70% in the media to nearly 30% in the adventitia, indicating increased blockade of general binding sites by rapamycin in the outer vessel wall. The rapamycin profiles followed similar trends to those observed with paclitaxel. When FK506 inhibited specific binding of rapamycin to tissue, medial drug concentrations decreased as expected. Paclitaxel reduced general binding in the adventitia relative to the media. For both rapamycin and paclitaxel, specific displacement of the drug correlates with a higher density of the specific protein binding target in the media, while general displacement is stronger in the adventitia where the specific binding site concentration is decreased. Together these profiles indicate that both specific and general binding are found in the media and adventitia but with stronger specific binding trends in the media for both drugs.

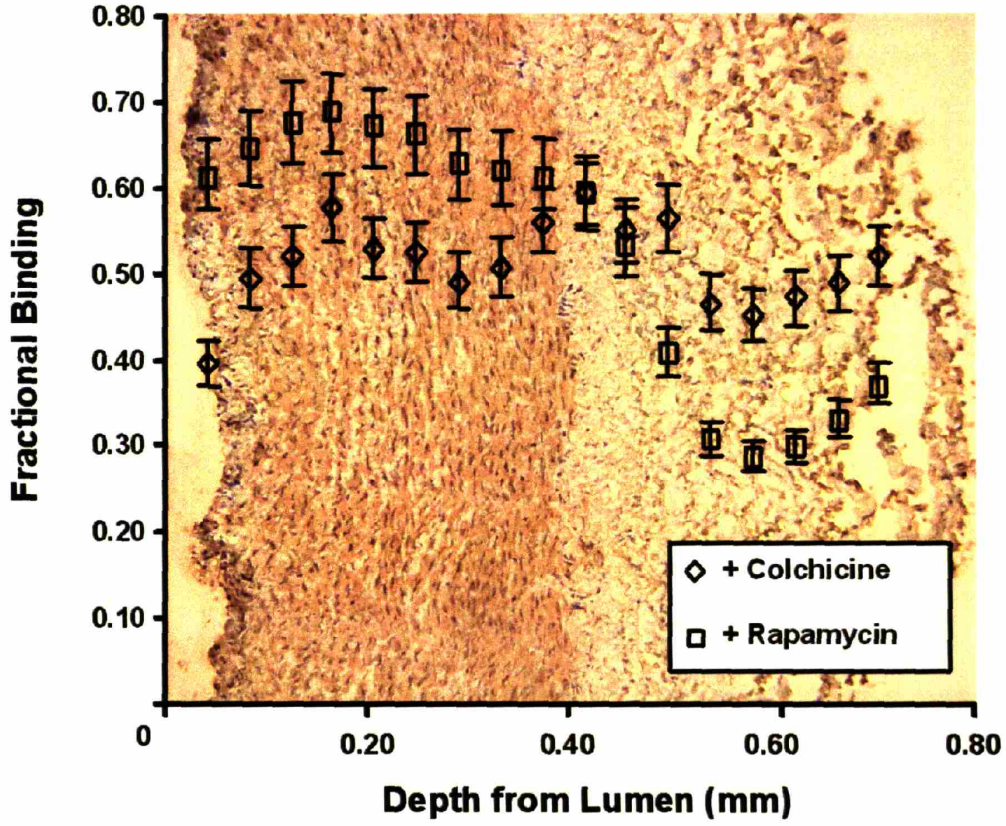

Figure 3.3: Transmural Paclitaxel Distribution with Competition

Transmural equilibrium distribution of radioactive paclitaxel plus colchicine (◊) or rapamycin (□) in 0.040mm thick bovine internal carotid tissue segments plotted over arterial tissue stained for tubulin protein (tubulin immunostain dilution 1:50). Each data point is normalized to no-competition profiles[1].

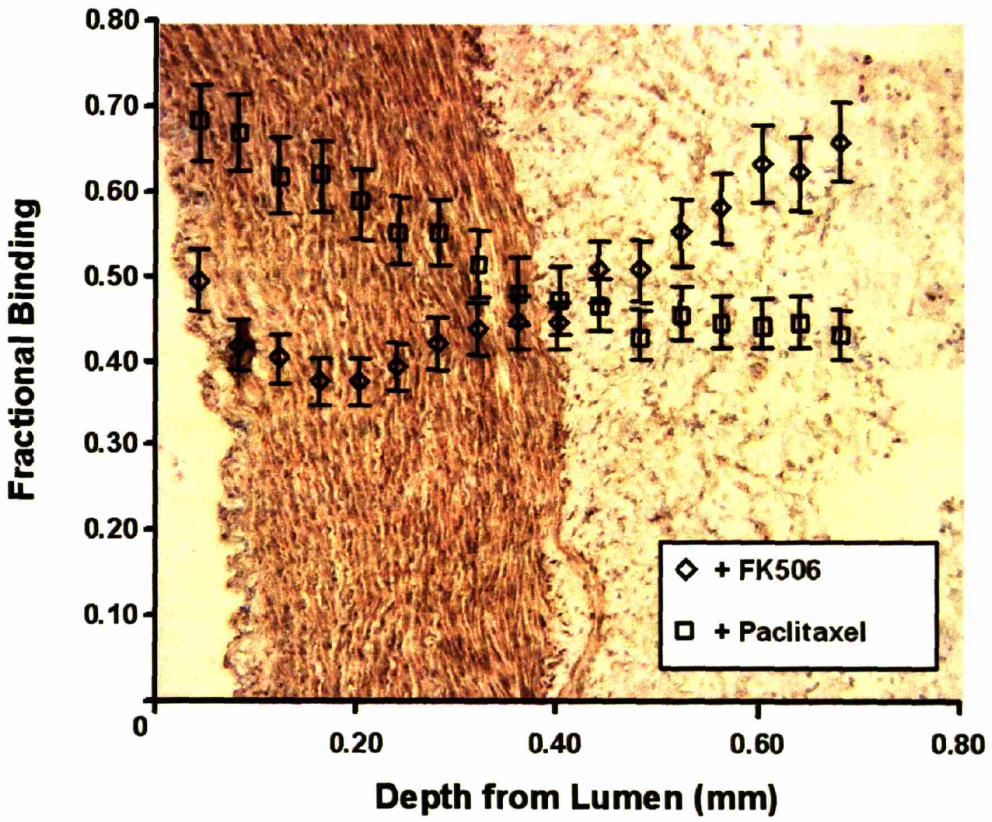

Figure 3.4: Transmural Rapamycin Distribution with Competition

Transmural equilibrium distribution of radioactive rapamycin plus FK506 (◊) or paclitaxel (□) in 0.040mm thick bovine internal carotid tissue segments plotted over arterial tissue stained for FKBP (FKBP immunostain dilution 1:10) Each data point is normalized to no-competition profiles[1].

Local Molecular Specificity

FKBP and tubulin binding assays were used to assess molecular binding specificity. Increasing concentrations of paclitaxel did not disrupt rapamycin binding to FKBP. These data imply that paclitaxel does not specifically bind to the rapamycin binding domain on FKBP and that paclitaxel and rapamycin do not associate with one another to decrease the amount of rapamycin available for binding (Figure 5). As expected unlabeled rapamycin and FK506, which share the same binding domain on FKBP, do have a competitive effect. Similarly only colchicine, a tubulin depolymerizing agent, alters the polymerization state of tubulin in the presence of paclitaxel while rapamycin has no effect (data not shown).

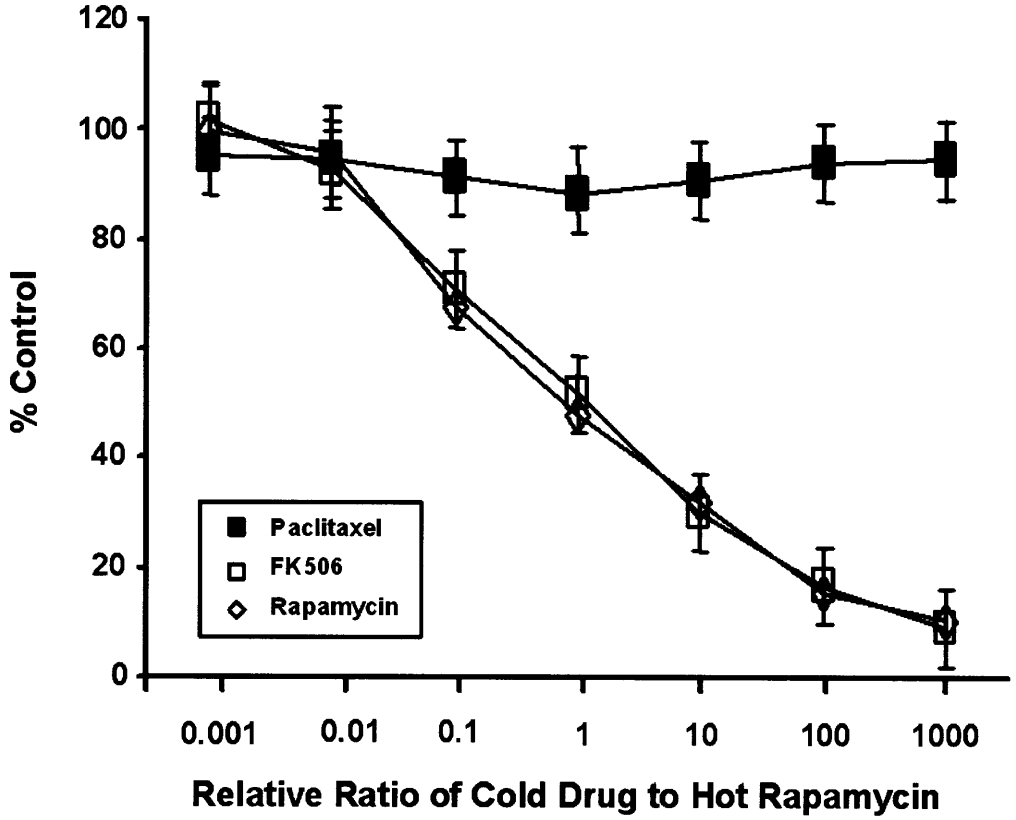

Figure 3.5: Molecular Specificity of Rapamycin Binding

Molecular specificity of FKBP binding. The percentage of radiolabeled rapamycin bound to human wild type FKBP plotted relative to no-competition controls for various unlabeled competitor drugs (indicated in the figure).

Systemic to Local Competition

A number of drugs commonly administered to patients with cardiovascular diseases were simultaneously loaded in tissue with labeled paclitaxel or rapamycin. Notably, insulin, captopril (ACE-inhibitor), atenolol and metoprolol (beta blockers) all significantly reduce arterial drug levels, likely by displacing labeled drug from general binding sites or offering alternative binding domains as may be the case with insulin. Salicyclic acid (aspirin), nifidipine (calcium channel blocker), hydrochlorothiazide (diuretic) and clopidogrel (anti-platelet) showed no significant reduction in drugs levels.

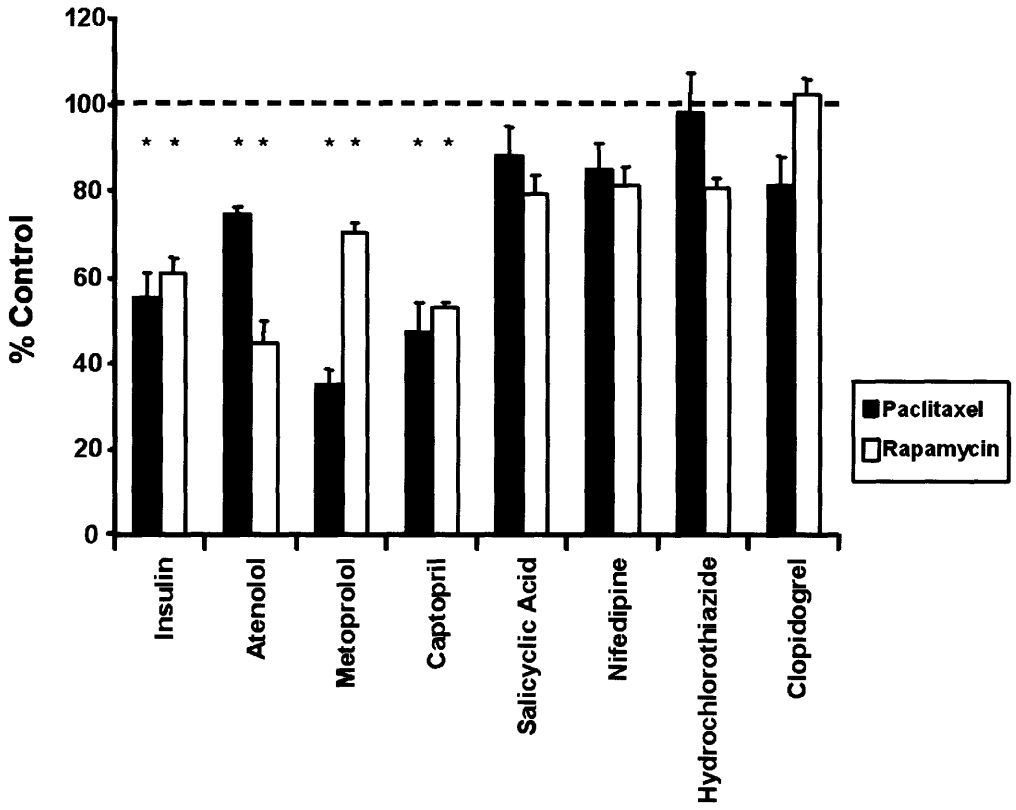

Figure 3.6: Systemically Delivered Drug Competition

(A) Percentage of radiolabeled paclitaxel (■) and rapamycin (□) relative to no-competition controls in tissue after 24 hours when simultaneously loaded with various unlabeled competitor drugs (indicated in the figure). (*) indicates a statistically significant difference ($p < 0.05$ with a two-tailed Student's t-test) from the no-competition control. Standard deviations are plotted with the data set.

3.4 Discussion

DES are now the clinically dominate intervention for treating occlusive coronary vascular pathologies, and yet full mechanistic definition of their success and shortcomings remain elusive. Specificity in binding has emerged as a vital component of DES functionality with competition for binding space a critical factor in arterial drug uptake. We now report the potential competitive effects on DES tissue deposition by other compounds released from adjacent stents or circulating after systemic administration.

Local/Local Competition

Paclitaxel does not interfere with rapamycin binding to FKBP, and rapamycin does not perturb paclitaxel associations with tubulin. Each drug can displace the other from tissue but only at surrounding concentrations on the order of 10^{-3} M. Such high molar concentrations of drug may be relevant for systemically delivered therapies, but are not likely observed after elution from multiple proximate stents even if all of the drug on the stents would pool locally. In the context of overlapping stents, the question of rapamycin-paclitaxel drug competition remains an open and clinically relevant question. In certain scenarios, placement of two stents with different drug formulations may be favored over placing two identical devices. Additionally, a multi-drug combination stent, *e.g.* paclitaxel-rapamycin, may prove more efficacious than single drug therapies. Our investigations show no physiochemical contraindication to placing a pair of dissimilar DES in close proximity or placing two different drugs on the same stent. As more

models of drug-eluting stents with different drug formulations emerge, these types of interactions may be therapeutically pertinent and should be considered in the evaluation of therapeutic viability.

Though DES has significantly reduced the number of restenosis cases, an appreciable proportion of patient population still manifest this condition[11-13]. No study has yet isolated the mechanism(s) of failure beyond identifying risk factors such as diabetes, previous interventions, lesion morphology, lesion dimension and vascular bed geometry[14]. Our models for specific protein binding inhibition with FK506 and colchicine demonstrate that variations in specific binding site availability significantly affects tissue uptake. Disease states such as diabetes or atherosclerosis may directly affect FKBP expression or tubulin polymerization, pathologically altering specific binding site availability and drug uptake capacity. In our model systems, blockade of general binding sites also reduces total tissue binding capacity. General binding sites throughout the tissue may serve as a reservoir for locally delivered drugs after the stent platform is depleted. Pathologic loss of general binding domains can also reduce overall tissue capacity.

Local/Systemic Competition

Systemically delivered compounds can maintain blood levels several log orders higher in concentrations than locally delivered drug tissue levels. The competitive displacing effects of systemically circulating drugs appear to be at the level of general

binding. Since general binding sites reside in both the media and adventitia, systemic drug competition may reduce drug distribution levels throughout the vessel wall. Heterogeneity exists between different screened compounds with regard to their overall effect on drug deposition and even between paclitaxel and rapamycin loading. The individual properties of each drug may have specific properties which favor general binding site displacement.

Conclusions

Local drug delivery maintains great appeal for many pathologic conditions. In vascular systems at the level of local/local competition, rapamycin and paclitaxel do not appear to interfere with the other's specific binding to the therapeutically relevant tissue proteins. However, systemic drugs can displace local stent-eluted compounds from general binding sites and decrease tissue reserve capacity. As the applications and combinations of drug formulations for local and systemic drug delivery expand, the competition implications of concomitant delivery must be consider to optimize delivery methodologies.

3.5 Acknowledgments

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Chapter 4

Binding, Microenvironment and Drug Delivery

Abstract

Drug-eluting stents deliver potent compounds directly to arterial segments but can become clot-laden when deployed. The question arises as to whether thrombi affect drug elution and arterial uptake. Paclitaxel transport and retention was assessed in clots of different blood components. Diffusivity, affected by clot organization, is fastest in fibrin (~ 347 $\mu m^2/s$), slower in fibrin-red cell clots (34.98 $\mu m^2/s$) and slowest in whole blood clots (3.55 $\mu m^2/s$). Blood cells bind and retain Paclitaxel such that levels in clot increase linearly with red cell fraction. At physiologic hematocrit, clot retains 3 times the amount of Paclitaxel in surrounding solutions. Computational models predict that the potential of thrombus to absorb, retain and release drug, or to act as a barrier to drug delivery depends on clot geometry and strut position in clot relative to the vessel wall. Clot between artery and stent can reduce uptake 10-fold, while clot overlying the stent can shield drug from washout, increasing uptake. Model assumptions were confirmed and predictions validated in a novel rat model that introduces thrombosis within stented aortae where non-occlusive thrombus acts as capacitive space for drug and shifts drug levels to decrease tissue uptake 2-fold. Thrombus apposed on stents creates large variations in drug uptake and can act to either increase or decrease wall deposition depending on the clot and stent geometry. Arterial deposition of drug from stents deployed in clots will be highly variable and unpredictable unless the clot can be adequately controlled or removed.

4.1 Introduction

The reduction of intimal hyperplasia in an arterial segment by drug-eluting stents [1-3] depends on appropriate drug dose distribution in the arterial wall over an extended period of time[4-7]. While several experimental and computational studies have examined the local pharmacokinetics of stent-based drug delivery[7, 8], none have considered how thrombus in the vicinity of the stent affects drug elution and subsequent tissue deposition. Stents are often directly deployed at sites of thrombosis[9], and clot inevitably develops after implantation once struts become coated with plasma proteins[10-13]. In most cases the thrombus is not angiographically apparent or clinically evident, as occlusive thrombosis rates for drug-eluting stents are low, approximately 1-4%[10, 13, 14]. Nevertheless, even a fine layer of material directly apposed to a stent strut can significantly transform the drug-eluting performance of the stent[15], and consequently alter the drug distribution in the arterial wall.

The influence of thrombus on the arterial distribution of Paclitaxel delivered continuously from a drug-eluting stent was considered by examining how the composition of clot affects drug transport. Since thrombus naturally remodels over time[10-12] we hypothesized that clot capacity and transport properties for Paclitaxel should evolve with the shift in clot organization and components. Additionally, we hypothesized that distribution of drug in the target blood vessel will depend on the geometry of the clot and the relative positioning of the overlying stent struts within any thrombus. Computational models simulating elution from a clot-covered stent revealed

complex behaviors. Given the vagaries of clot-strut positioning and clot composition, arterial levels of stent-eluted drug may fluctuate by orders of magnitude with variations in clot properties, implying an inherent uncertainty in predicting arterial drug levels if clot dimensions are not locally controlled. Clot carefully created over stents in a rat aorta verified model predictions by serving as a capacitive barrier, limiting uptake, and not simply as a passive conduit for drug.

Implantation of drug-eluting stents in the setting of thrombus presents a unique set of challenges as the irregularity of clots can cause large alterations in arterial drug uptake. Clot removal prior to stent deployment might reduce such variability and ensure greater control of the distribution of therapeutic drug levels within the arterial wall.

4.2 Methods

Preparation of Fibrin and Whole Blood Clots

Clots of varying compositions were prepared to examine drug transport through and binding to thrombus at different stages of development. Based on established methods[16], clots were prepared by mixing human fibrinogen (Calbiochem), thrombin (Calbiochem), and coagulation factor XIII (Calbiochem) with appropriate proportions of human packed red cells (Rhode Island Blood Center, Providence). These preparations allowed for measurement of diffusivities and drug capacity for different clot compositions. To create pure fibrin clots, 100 μL of stock 3 mg/mL fibrinogen were mixed with 100 μL of stock 6 U/mL thrombin and 100 μL of stock 0.27 U/mL Factor XIII, resulting in a final clot volume of 300 μL and final clot concentrations of 1 mg/mL fibrinogen, 2 U/mL thrombin and 0.09 U/mL Factor XIII. To create clots of different red cell volume fractions, more concentrated stock solutions of fibrinogen, thrombin and Factor XIII were used so that the final clot concentrations of these components did not vary after adding human packed red cells at 9%, 25% or 50% by volume. Each mixture was allowed to coagulate for at least 2 h at 37 °C before experimentation. For whole blood clots, 300 μL aliquots of fresh blood were drawn from the middle ear artery of New Zealand white rabbits (3-5 kg), placed into culture plate wells, and allowed to coagulate for at least 2 h at 37 °C before experimentation.

Transport Measurements

Paclitaxel transport in thrombus was characterized by a diffusivity and convective

velocity, and binding or retention capacity of tissue relative to clot, and of clot relative to surrounding solution (Figure 1).

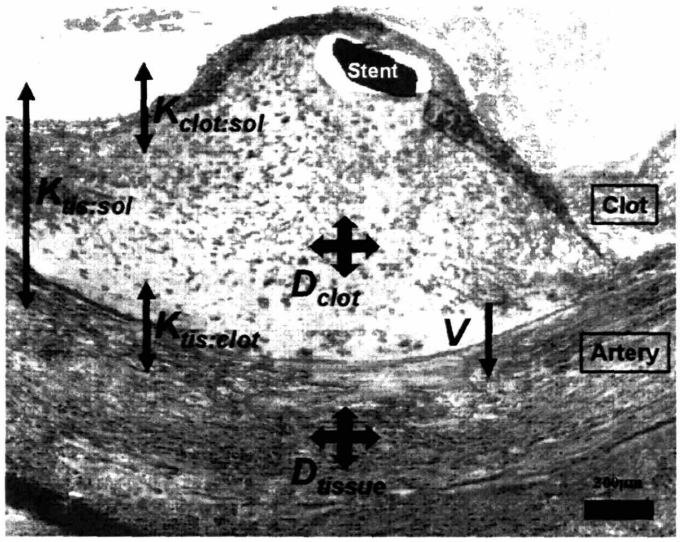

Figure 4.1: Transport Parameters

Transport parameters for a clotted stent strut: D_{clot} and D_{tiss} are drug diffusivities in clot and tissue, V is drug convective velocity, $K_{clot:sol}$ is drug capacity of clot relative to solution, $K_{tiss:sol}$ is drug capacity of arterial tissue relative to solution, and $K_{tiss:clot}$ is the drug capacity of arterial tissue relative to clot.

Drug Capacity

We determined the ratio of clot drug capacity relative to solution drug capacity ($K_{clot:solution}$) for different clot compositions. Whole blood and fibrin-red cell clots were prepared in 48-well tissue culture plate wells as described above and covered with 300 μL of [^{3}H]-Paclitaxel (Sigma) (1.06×10^{-8} mmol/mL) dissolved in 7.5 U/mL hirudin (Calbiochem) in phosphate-buffered saline (PBS, Sigma) to prevent coagulation of the drug solution. The potential effects of circulating protein drug adsorption on binding capacity was simulated with the addition of 4% bovine serum albumin (Sigma) to the drug solution. The clot was incubated with drug for 72 h, a period determined in separate experiments to be sufficient to approach tissue equilibrium. Clots were washed in PBS, dissolved for 48 h in 400 μL of Solvable (Packard-Canberra) and treated with 30% H_2O_2 (Mallincrokdt) in a 1:8 ratio. Drug content was measured using a liquid scintillation counter (2500 TR, Packard-Canberra) with 10 mL scintillation cocktail (Ultima Gold, Perkin-Elmer). Drug capacity of the clot was taken as the ratio of drug concentration in the clots referenced to final drug concentration in the source solution. Assuming specific and nonspecific drug binding interactions in tissue are independent of those in clot, we can estimate $K_{tissue:clot}$, the ratio of tissue drug capacity relative to clot drug capacity from $K_{clot:solution}$ as:

$$K_{tissue:clot} = K_{tissue:solution} / K_{clot:solution} \quad [1]$$

using previously reported values of $K_{tissue:solution}$ for Paclitaxel[17].

Drug Diffusivity

Fresh rabbit blood and fibrin-red cell mixtures were coagulated in 12-well tissue culture plates using a clot volume of 2 mL per well. Care was taken to minimize air bubbles, and larger well diameters were chosen to reduce the effects of meniscus formation. [^{3}H]-Paclitaxel (1.06×10^{-8} mmol/mL, 2 mL/well) in 7.5 U/mL hirudin was allowed to diffuse into the clot for up to 1 h. Following the diffusion phase, clots were washed in PBS and dissolved for 48 h in 2.1 mL of Solvable. After treatment with 30% H_2O_2, clot drug content was measured using a liquid scintillation counter with 10 mL scintillation cocktail. Drug diffusivity was determined by fitting the measured total clot drug content with the numerical solution of the diffusion equation[7, 18] for a constant drug source concentration boundary condition.

Drug Convective Velocity

While drug is efficiently transported through isolated clots[16] drug convective velocity in clots juxtaposed to the arterial wall is restricted at steady-state by continuity to match the slower transport through the arterial wall. Paclitaxel convective velocity was determined in 4-5 cm segments of bovine calf carotid arteries connected to tubing on one end and sealed on the other. [^{3}H]-Paclitaxel (1.06×10^{-8} mmol/mL) was infused at pressures of 60 and 90 mmHg. After 2 h, drug content in a 6 mm diameter biopsy punch of the arterial wall was measured. The source drug concentration, arterial drug content, convection time, biopsy punch dimensions, and tissue [^{3}H]-Paclitaxel diffusivity and capacity [17, 19] were fit to a drug diffusion-convection model [7, 18]. Convective

velocity increased three-fold, from 3.2 to 9.2 nm/s, for a 50% increase in transmural pressure from 60 to 90 mmHg.

Continuum Pharmacokinetics Modeling of Thrombus Transport

Drug release from a stent and transport within the thrombus and arterial wall were modeled using the diffusion-convection equation and a steady-release boundary condition (see appendix for code). We limited our analysis to a two-dimensional cross-section of the artery and clot, a 100 × 100 μm strut, and assumed rapid luminal washout, no endothelial resistance, and a perivascular sink. We used zero concentration boundary conditions on the endovascular and perivascular aspects of the artery, and symmetry boundary condition in the planar directions. We further assumed an arterial wall thickness of 800 μm and a strut-to-strut distance of 1000 μm. Simulations were performed in Cartesian coordinates. In such a system, transport of soluble drug is described as:

$$\frac{\partial U}{dt} = \frac{\partial}{\partial x}\left(D_x \cdot \frac{\partial U}{\partial x}\right) + \frac{\partial}{\partial y}\left(D_y \cdot \frac{\partial U}{\partial y}\right) - V\frac{\partial U}{\partial y} \qquad [2]$$

where U is free drug concentration, and V is the transmural convective velocity. D_x and D_y are diffusivities in the planar X and transmural Y directions, and within the arterial wall the former was three orders of magnitude larger than the latter (37.2 vs. 0.021 $\mu m^2/s$) [17, 19]. Free drug concentration is related to total concentration (C) by the tissue

capacity of the drug (K), so that $C = K \cdot U$. Because of the multi-component structure of the clot-artery system, K, D_x and D_y are experimentally determined for each component of the system. V was taken as 9.2 nm/s, corresponding to our measured drug convective velocity at 90 mm Hg of transmural pressure. Simultaneous transport and binding was implemented using operator splitting. During each time step, free drug released from the stent was allowed to diffuse and convect along with free drug already in the system. We thus first compute an intermediate post-transport free drug distribution (P) and then calculate the free (U) and total (C) drug concentrations at the start of the next computation cycle to account for differential drug capacity:

$$C(t + \Delta t) = C(t) + \Delta C = C(t) + (P - U(t)) = C(t) + P - C(t) / K \quad [3]$$

$$U(t + \Delta t) = U(t) + \Delta U = U(t) + \Delta C / K$$

$$= U(t) + (P - U(t)) / K = (C(t) + P) / K - C(t) / K^2 \quad [4]$$

We examined the theoretical total arterial drug content for a range of clot widths (100 to 900 μm), heights (50 to 450 μm), and stent strut distances from the arterial surface (0 to 350 μm). We considered clots composed of pure fibrin, 50% packed red cells or whole blood, with clot transport and drug uptake properties based on our experimental measurements for Paclitaxel. We further simulated clots with varying drug diffusivities and capacities to assess the specific effects of these parameters on arterial drug content.

Arterial drug content was expressed as an *arterial drug ratio*, defined as the total arterial drug deposition achieved by a specific stent-thrombus configuration normalized to the total arterial drug deposition achieved by a non-clotted drug-eluting stent directly apposed against the arterial wall. An *arterial drug ratio* greater than unity implies a greater amount of drug in the arterial wall relative to that achieved by a non-clotted drug-eluting stent. Simulations were run at a resolution of 50 μm per computational node. Finer mesh resolutions were tested for a number of cases and did not qualitatively change the simulation results. The model was run until steady-state drug levels were achieved both in the thrombus and in the arterial wall.

In vivo Thrombus Model

Paclitaxel uptake was evaluated in stented abdominal aortae of adult male Sprague-Dawley rats (500-700 mg, Charles River Laboratories) in the presence and absence of controlled induced mural thrombus. Procedures were in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care and the NIH. Under inhaled isofluorane anesthesia the right femoral artery was exposed, ligated and incised proximally to allow passage of a 0.014" angioplasty guide into the aorta. The abdominal aorta was exposed, and a 15mm segment above the origin of the renal arteries was ligated proximally and distally. Thrombus formed within 10 minutes in the isolated aortic segment, and the proximal aortic ligature was removed. A 2.625 mm steel stent (MULTI-LINK PIXEL, Guidant) was rapidly passed into the thrombosed segment and deployed for 15 seconds at 10 atm. The distal ligature was removed and

after visually insuring adequate blood flow in the aorta with no macroscopic evidence of aortic ischemia, the balloon and wire were removed, the femoral artery ligated proximal to the arteriotomy, and both incisions closed.

Control animals underwent abdominal aortic stenting utilizing a similar technique without aortic thrombus formation. Heparin (100 U/kg) was administered IV before stenting only to the control rats. Aspirin (5 mg/kg/day, per standard practice in this type of procedure) was added to drinking water immediately post surgery to all animals for the duration of the experiment. Paclitaxel was administered intra-peritoneally three times at 5 mg/kg every 12 hours with the first injection immediately after stenting. Animals were sacrificed 30 hours after stenting with inhaled CO_2. The aorta was pressure perfused with isotonic saline, cleaned of adherent fat and connective tissues and the stented segment detached. The stent was carefully removed and the tissue, stent and overlying thrombus were snap-frozen with liquid N_2. Paclitaxel in the arterial wall and excised thrombus was determined using a commercial immunoassay (Hawaii Biotech). Paclitaxel uptake in the stented region was normalized to uptake in a non-stented region and compared for cases with clot absent and clot present. Additionally, segments of the stented vessels were excised and histologically processed with verHoff's tissue elastin stain.

4.3 Results

Influence of Thrombus Geometry on Arterial Paclitaxel Uptake

Arterial Paclitaxel distribution is exquisitely sensitive to changes in the local geometry of the overlying thrombus. Strut position within the clot determines uptake (Figures 2A-D). When there is a greater mass of clot over the strut, the strut sits close to the wall and the overlying clot shields against systemic washout. Arterial drug uptake can rise 30-fold higher than if clot were not present. Conversely when the bulk of the clot is interposed between the strut and artery a barrier to transport is created that decreases arterial drug uptake. For some geometries these forces balance. Indeed the clot can grow in height, surface area or both to alter uptake. Arterial drug uptake peaks at a clot height to width ratio of ~ 0.3 when clot dimensions were varied and clot volume kept constant (Figure 2D). Radial and longitudinal washout increases at other ratios lowering drug uptake. Given the natural variability of thrombosis *in vivo* such sensitivity to geometry implies arterial drug uptake from drug-eluting stents deployed in clotted arterial segments may also be highly variable.

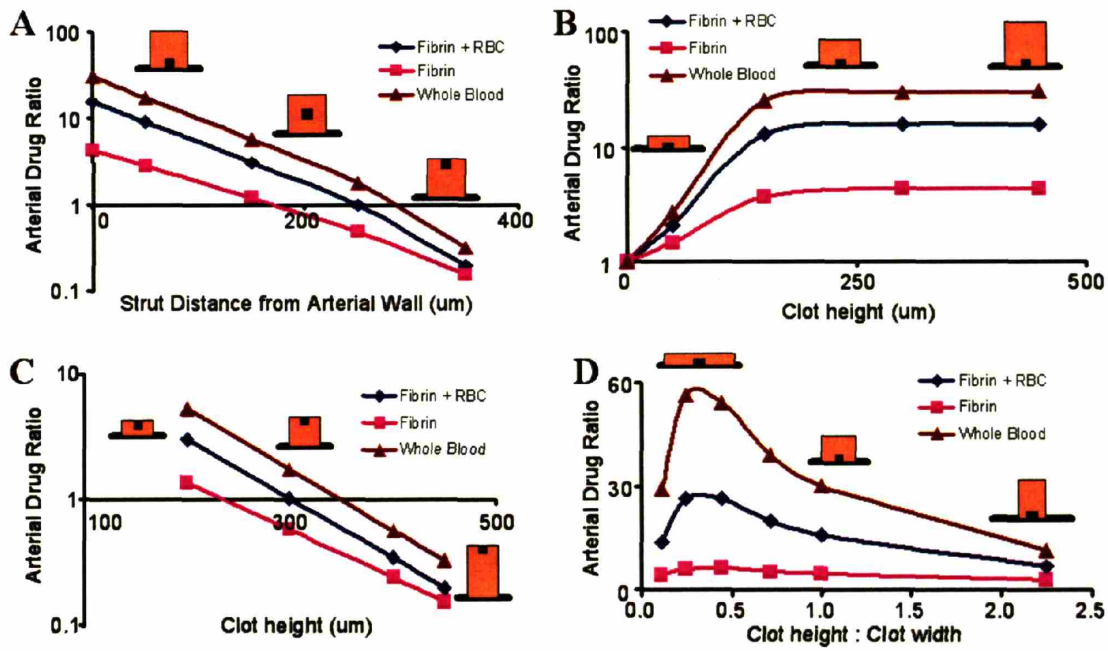

Figure 4.2: Clot and Stent Position in Drug Uptake

For 50% fibrin-red cell clots (▲), pure fibrin clots (♦), and whole blood clots (■), arterial drug ratios (total drug in artery from clotted stent *vs.* total drug in artery from non-clotted stent) were computed *vs.* changes in **(A)** strut-artery distance, with a clot of fixed dimensions, **(B)** clot height, with strut at base of clot, **(C)** clot height, with strut at top of clot, **(D)** clot thickness to width ratio, with constant clot size and strut position.

Paclitaxel Deposition and Transport in Fibrin, Fibrin-Red Cell and Whole Blood Clots

Paclitaxel diffusivity is retarded by fibrin clots once organized by addition of thrombin. Diffusivity decreases by half to 347±14 $\mu m^2/s$ when thrombin sufficient to induce crosslinking is added to fibrinogen and by an additional order of magnitude when red blood cells are present (Figure 3A). Until red blood cells are present the Paclitaxel capacity of fibrin clots is no different from the capacity of buffer solution ($K_{fibrin:solution}$ = 0.94±0.11), irrespective of thrombin concentration and degree of crosslinking thrombin induces (data not shown). Clots with 50% red cells retain nearly 3-fold more drug than pure fibrin clots ($K_{clot:solution}$ = 2.92±0.26, $p < 0.05$, Figure 3B). Paclitaxel capacity increases dramatically and in a linear fashion as the red cell fraction in the clot increases.

Mature or chronic thrombotic masses are more heterogeneous than fibrin-red cell clots. The fibrin meshwork in these clots contains platelets and other blood elements, adding further restrictions on drug transport. Paclitaxel diffusivity in whole blood clots is an order of magnitude lower than that in 50% fibrin-red cell clots ($D_{fibrin\text{-}red\ cell\ clot}$ = 34.98±10.3 $\mu m^2/s$ vs. $D_{whole\ blood\ clot}$ = 3.55±0.75 $\mu m^2/s$, Figures. 3A and 4A). The Paclitaxel capacity of whole blood clots is however remarkably close to that expected of a fibrin-red cell clot with physiologic hematocrit ($K_{clot:solution}$ = 2.63±0.17, Figure 4B). Thus, non-red cell components of blood delay drug transport but do not add substantially to the ability of fibrin and red cells to retain drug within clot. The protein binding nature of Paclitaxel has an effect as well. The presence of albumin in the drug solution reduced the binding of drug by red blood cells. Whole blood clot Paclitaxel diffusivity increased

when albumin was present (Figure 4A) while capacity was reduced to near unity (Figure 4B).

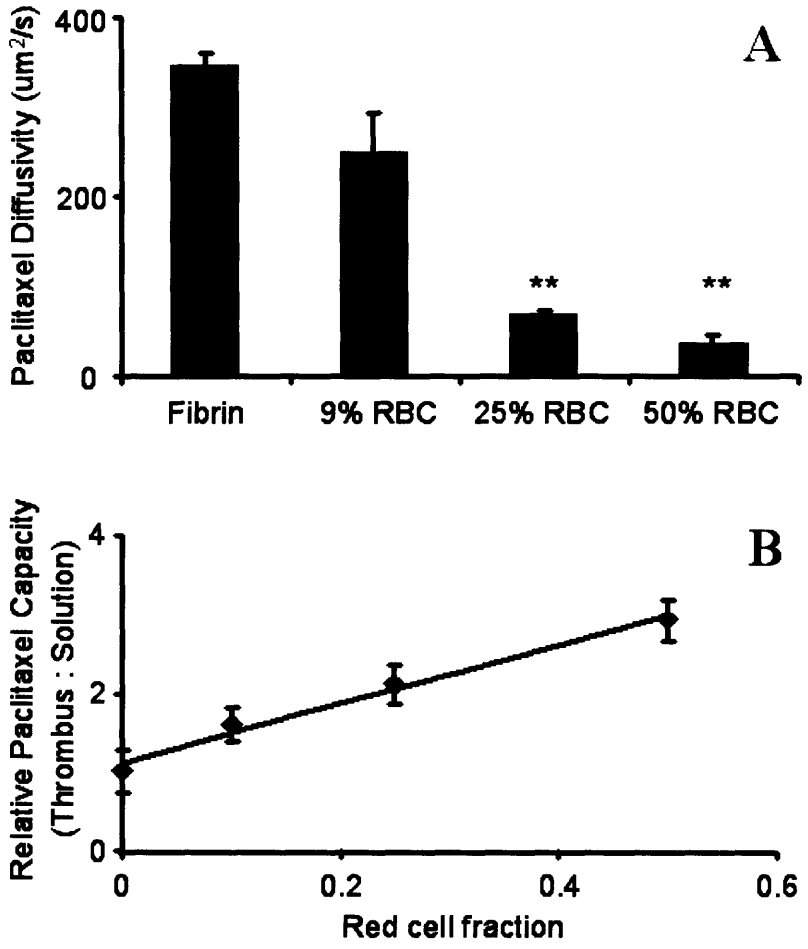

Figure 4.3: Paclitaxel Diffusivity and Capacity in Clot

(A) Paclitaxel diffusivity in fibrin-red cell clots *vs.* red cell fraction (** indicates statistically significant difference $p < 0.05$ compared to pure fibrin). **(B)** Paclitaxel capacity in fibrin-red cell clots relative to capacity in solution *vs.* clot red cell fraction.

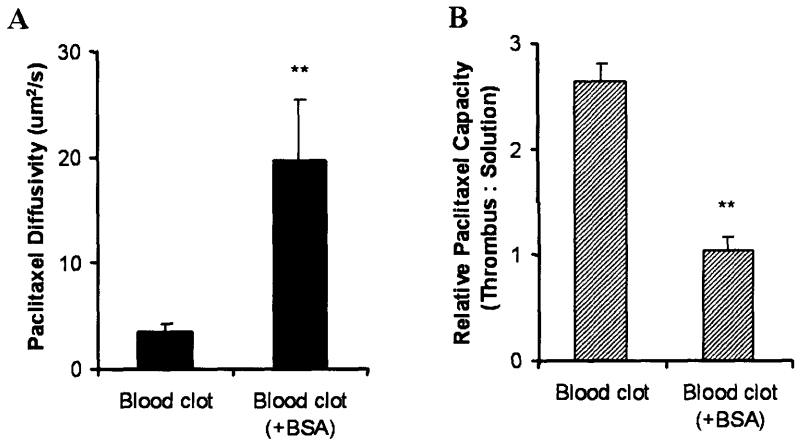

Figure 4.4: BSA and Paclitaxel Transport

(A) Paclitaxel capacity in whole blood clots relative to capacity in solution buffer with and without bovine serum albumin (BSA). **(B)** Paclitaxel diffusivity in whole blood clots with and without BSA in drug source. For both data sets in this figure, ** indicates statistically significant difference $p < 0.05$.

Influence of Clot Diffusivity and Capacity on Arterial Paclitaxel Uptake

Modeling allows us to investigate how drug retention capacity and diffusivity independently influence arterial drug uptake for stent struts adjacent to the arterial wall in the midline of an invariant clot. This type of analysis can be used to understand how drugs with transport and capacity characteristics different from Paclitaxel will behave. Drug levels are maximal when drug diffusivity in the clot is at, or below, transmural drug diffusivity in the arterial wall (0.021 $\mu m^2/s$ for Paclitaxel[19]). Arterial drug uptake will decrease in a sigmoidal fashion if drug can diffuse more freely in the clot (Figure 5A). This prediction is consistent with heightened arterial drug uptake for arteries embedded with stents surrounded by whole blood clots where diffusivity is lowest, and low uptake for stents surrounded by fibrin clots where diffusivity is maximized. Arterial drug loading is determined by more than diffusivity. For two drugs of identical clot diffusivities arterial uptake is delayed for the one which is more highly retained in the clot. Greater interactions of drug with clot components retard drug release from the clot (Figure 5B). However, with a continuous drug source from the stent, identical steady-state arterial drug content is eventually reached, independent of clot drug retention capacity, albeit at different time points.

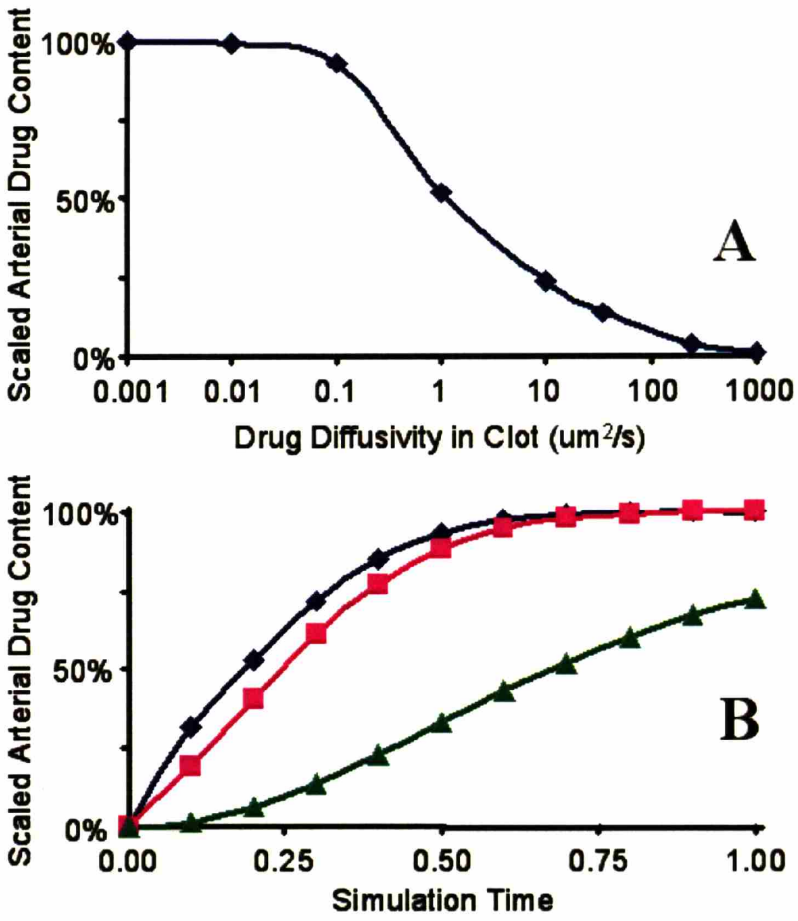

Figure 4.5: Variable Diffusivity and Capacity in Uptake

(A) Steady-state total arterial drug content (scaled to total drug content at D = 0.001 $\mu m^2/s$) as a function of clot drug diffusivity, with clot dimensions and capacities held invariant. **(B)** Normalized total arterial drug content *vs.* simulation time, for relative Paclitaxel capacities of $K_{clot:sol}$ = 3 (♦), $K_{clot:sol}$ = 100 (■) and $K_{clot:sol}$ = 300 (▲).

Thrombus and Paclitaxel Uptake In vivo

A novel model of controlled *in vivo* thrombus was developed for this study. These experiments in rats confirm that clot possesses capacitive properties for sequestering Paclitaxel. Clot does not simply pass drug through to the underlying artery after stent release, it alters arterial wall uptake. All stented aortas, control (Figure 6A) and clot-laden (Figure 6B), appeared viable with no evidence of necrosis or ischemia at sacrifice. Control animals had no notable thrombus in stented aortae at implantation, and upon device excision no clot present on the stent or the luminal surface of the vessel wall. Mural thrombus was present only after controlled induction, covering the stent struts without occluding the aortic lumen or affecting blood flow. A visible thrombotic mesh-like mass was attached to the stent struts and was excised with the stent. The thrombus was a heterogeneous composite of fibrin, platelets and red blood cells. The control and thrombosed stents were placed in a 20% ethanol solution with Tween 20 and vortexed for 15 minutes, a time period sufficient to dissolve the clot. The drug content on the thrombosed stents was significantly ($p < 0.05$) greater than the control devices (Figure 7A). This capacitive action of clot can both limit transport at the arterial wall interface and retard systemic washout depending on clot geometry. In our *in vivo* studies, arterial drug uptake in the presence of clot was significantly ($p < 0.05$) reduced by ~50% in stented vessels in comparison to the control devices (Figure 7B).

Systemic delivery via intraperitoneal administration is equivalent to a non-zero Dirichlet luminal loading condition, a situation most analogous to the computational

model with the stent strut adjacent to the lumen (Figure 2C). When the *in vivo* clot dimensions were determined and used as boundary conditions and input parameters the model predicted an arterial drug ratio of 0.56, strikingly close to the 50% decrease in arterial uptake seen in the animal model. In this case, drug recycles between the thrombus and the lumen more effectively than it passes from the clot to the tissue wall, reducing arterial drug uptake. As predicted, thrombus is not merely a passive medium, but rather a capacitive space to retain drug and shift the drug deposition distribution.

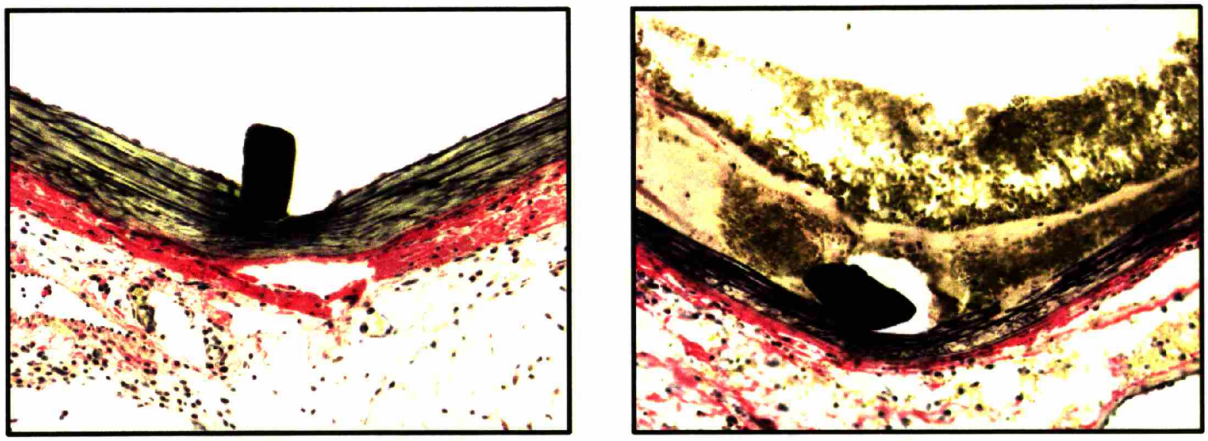

Figure 4.6: verHoff's Staining of Stented Rat Abdominal Aorta

(A) verHoff's staining of control stented rat abdominal aorta. **(B)** verHoff's stain of experimental mural thrombus in rat abdominal aorta. Thrombus (immediately adjacent to the darkly stained elastic laminae) is composed of fibrin, platelets and red blood cells, extends approximately 300μm in to the lumen and is non-occlusive.

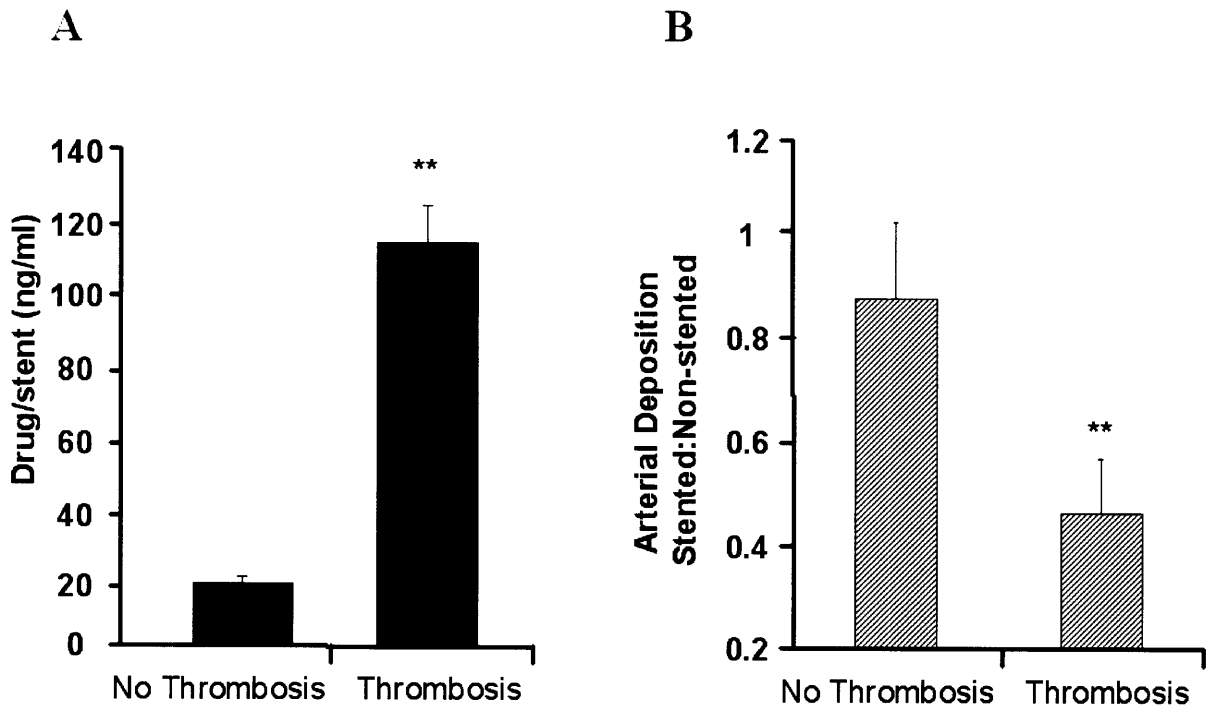

Figure 4.7: Paclitaxel Uptake and Thrombosis

(A) Paclitaxel eluted from stents in 1ml of 20% ethanol solution. **(B)** Paclitaxel deposition in stented rat abdominal aorta normalized to deposition in non-stented rat thoracic aorta. For both data sets in this figure, ** indicates statistically significant difference $p < 0.05$.

4.4 Discussion

Thrombosis is a feature of the acutely occluded artery and a catastrophic failure mode for endovascular devices. Drug elution increases the potential for subacute stent thrombosis. We now show that clot, even non-obstructing microthrombi, can affect drug deposition. Our physiologic and computational models demonstrate that clot changes the local environment of the stent strut and physiological transport forces[7, 8] to alter arterial wall drug uptake and retention[6, 17, 20]. Small amounts of local thrombus produce significant variations in arterial drug levels depending on clot geometry and composition. Because of the unpredictability of these factors in the clinical setting, deployment of a drug-eluting stent in a clot-laden arterial segment will inevitably lead to variability in arterial drug distribution, potentially affecting clinical outcome.

Transport Forces in the Clot Affect Arterial Drug Uptake

There exists a balance between the capacity of an artery to absorb a drug and the rate at which the drug is presented to the arterial tissue. Clot alters this balance by absorbing drug and retarding transport. Alterations in both capacity and diffusivity will likely change arterial drug levels during therapeutic delivery by modifying the amount and the rate at which drug can enter the vessel wall. Paclitaxel moves more slowly through clots with higher red cell content because of repeated binding and release from nonspecific and specific cellular components like tubulin[21] which are ubiquitous within tissue and red cells. Whole blood clots present a denser platelet-fibrin meshwork which further hampers drug transport. Diffusivities change with clot content and drug. The

steady-state arterial uptake of a given drug will be maximized when the drug diffusivity through a specific clot is slower than its diffusivity in arterial tissue. In this case clot retains drug within the local vicinity of the vessel wall allowing for increased contact time and potential infiltration and distribution through the arterial wall. At higher clot diffusivities drug is delivered to the artery more quickly than can be absorbed, and a greater fraction of the drug cannot be bound before it is lost to the circulation.

Clot is therefore a double-edged sword for drug-eluting stents. In some scenarios the capacitive and binding phenomena will increase tissue uptake and in others reduce it. The problem is that thrombosis is unpredictable and irregular. Indeed, the DELIVER trial of non-polymeric rapidly eluting Paclitaxel stents supports the potential beneficial impact of clot on drug-eluting stents. Patients who received Paclitaxel-eluting stents and glycoprotein-IIb/IIIa inhibitors had significantly higher rates of restenosis than those patients who received only the drug-eluting stent without the inhibitor[22]. It is possible that the glycoprotein inhibitor removed or reduced thrombus around the stent struts. In this specific case where the drug was so rapidly eluted off of the stent the capacitive-like properties of clot might serve as a secondary release platform. Here clot may reduce systemic washout and preserve drug for presentation to the arterial wall. In systems where polymeric coatings are designed specifically to elute drug into tissue over time, extended retention in clot may enhance systemic dilution. More consistent clinical results may thus be expected if clots are both removed from the target arterial segment before stent deployment and carefully regulated after interventions.

Drug Interactions with Clot

Just as arterial ultrastructure influences arterial drug uptake[6, 17], the organization of thrombus affects the capacity to store and release Paclitaxel to the arterial wall. Clots with more red cells absorb greater amounts of drug as a result of an increase in both nonspecific and specific interactions. These same interactions however also retard arterial drug uptake kinetics. Most of the Paclitaxel in high capacity clots is bound, and since only free drug can diffuse[7, 19], delivery to the artery slows when drug passes through clot before it contacts the arterial wall. Drug capacity in clot thus helps determine drug uptake kinetics in arteries. The rate of drug transport in clot influences the extent to which the clot can retard systemic drug washout. Effective drug transport distances are determined both by the drug transport coefficients within the clot and by the geometric dimensions of the clot. Net arterial drug uptake is governed by a balance of drug retention in the clot, drug transport from the clot to the systemic circulation and from the clot into the artery. Clot-stent geometry determines which of these competing processes dominate. This fundamental mechanism must be considered for optimizing drug-eluting stent therapies.

The importance of binding was demonstrated further by the competition established by BSA. High levels of circulating proteins can act as an enhanced sink for drug that does not interact with the arterial wall beneath a strut or with clot. The competition for drug between blood and blood vessel is amplified *in vivo* and the ability

of clot to retain and retard drug is all the more critical for protein binding compounds. In this regard specific and nonspecific binding in arterial systems are major determinants of tissue transport and uptake for both Paclitaxel and Rapamycin[20]. Polymerized microtubules in red blood cells and smooth muscles bind Paclitaxel with nanomolar specificity. At similar concentrations in the same cell types, the FK506 binding protein also shows nanomolar binding specificity to rapamycin. With such similar cellular concentrations of binding proteins, and nearly identical pharmacokinetic and physicochemical properties[20], Rapamycin-eluting stents will likely behave very similarly to Paclitaxel in the setting of thrombus.

Drug Uptake and Stent Positioning

Our simulations demonstrate intriguing relationships between stent positioning and arterial Paclitaxel uptake. The effects of positioning are primarily mediated by the local transport forces which drive the migration of drug through the clot to the arterial wall[7]. Macromolecular transport in thrombus has been a fertile area of research in thrombolytic therapy, as researchers have sought ways to enhance clot uptake of thrombolytic drugs and enzymes to maximize clot dissolution[16, 23, 24]. Controlling the thrombus is a delicate issue in stent-based drug delivery as efforts to modulate uptake must be balanced with concern for creating local zones of toxic or sub-therapeutic drug levels[7]. In general, arterial drug uptake is decreased for clot configurations with larger luminal surface area, stent positioning which increase the strut-to-intima distance and for narrow clots which allow for a steep stent-lumen concentration gradient that washes drug

out to the circulation. In some instances, even changes in stent positioning of just tens of microns have dramatic effects in raising or lowering arterial drug uptake. Deploying a stent in a pre-existing thrombus or thrombus that develops after implantation are both cases where these effects may be realized.

Conclusions

Clots can modulate stent-based drug elution to alter significantly arterial drug levels and potentially efficacy. Thrombus composition influences uptake kinetics through changes in its retention capacity for drug. Clot geometry mediates arterial drug levels through the balance of local transport forces. The exquisite sensitivity of tissue uptake to geometry and composition implies that drug deposition will be highly variable and difficult to predict in a thrombotic micro-environment. As such, the full power of drug-eluting stents in clinical practice may not be entirely realized until local thrombosis is tightly controlled.

4.5 Acknowledgments

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APPENDIX A

Simulation Code

```
% 2D Thrombus Transport Program 1.0, v 505n
%
% 1. Stent release 1 unit of drug per 100*100 um^2 per sec
% 2. Partitioning referenced to Lumen only.
%
% NEUMANN STENT RELEASE CONDITION

clear all; close all;

v = '505n';    % Version #
disp(['2D Thrombus Transport 1.0, v ' v ': NEUMANN RELEASE']);
disp(['Last modified by Chao-Wei Hwang']);

% TRANSPORT CONSTANTS ----------------
% Constants in um's and sec's (or HRS where indicated)
umV = 0.01;
umDTisX = 37.2;
umDTisY = 0.073;
umDClot = 250;
umDLumn = 250;
KLumnLumn = 1;
KClotLumn = 1;
KTissLumn = 30;
KTissClot = KTissLumn/KClotLumn; % All K will be represented relative to lumen (1) -
        -> assume linear K relationship;
STENTRELEASERATE = 1;  % units/100*100 um^2/sec
totalhrs = 1000;
% ----------------------------------------

% GEOMETRY CONSTANTS ----------------------
CLOTWIDTH = 300; % microns
CLOTHEIGHT = 300; % microns
STENTDIST = 0; % stent distance from intima in microns
STENTWIDTH = 100; % microns
STENTHEIGHT = 100; % microns
TISSUEWIDTH = 1000; % microns
TISSUEHEIGHT = 800; % microns
LUMENHEIGHT = 500; % microns;
% ----------------------------------------
```

```
% SCALING FACTORS ----------
% To ensure stability in FD, can either scale space or time, but not both.
pix2um = 50;    % 1 pixel = 50 um
V = umV/pix2um;
DTisX = umDTisX/pix2um/pix2um;
DTisY = umDTisY/pix2um/pix2um;
DClot = umDClot/pix2um/pix2um;
DLumn = umDLumn/pix2um/pix2um;
dx = 1; % in pix
dt = dx^2/(2*max([DTisX DTisY DClot DLumn]))*0.99;  % time in seconds
simulcycles = totalhrs*3600/dt;
% ------------------------------------

% LOCATION OF TISSUE, STENT, CLOT, LUMEN MATRICES ----------
n = ceil((TISSUEHEIGHT+LUMENHEIGHT)/pix2um)+2;  % Number of pixels in Y
        direction necessary to simulate 1500 um (deep into artery)
m = ceil(TISSUEWIDTH/pix2um)+2;  % Number of pixels in X direction

% Denote location of tissue, stent, clot, lumen by 1's.
Tiss = zeros(m,n);
TissTop = n-ceil(TISSUEHEIGHT/pix2um);
Tiss(2:m-1,TissTop:n-1) = 1;
Stnt = zeros(m,n);
StntBot = TissTop-ceil(STENTDIST/pix2um)-1;
StntTop = StntBot-ceil(STENTHEIGHT/pix2um)+1;
StntLft = floor((m/2)-(STENTWIDTH/2)/pix2um)+1;
StntRgt = floor((m/2)+(STENTWIDTH/2)/pix2um);
Stnt(StntLft:StntRgt,StntTop:StntBot) = 1;
Clot = zeros(m,n);
ClotBot = TissTop-1;
ClotTop = ClotBot-ceil(CLOTHEIGHT/pix2um)+1;
ClotLft = floor((m/2)-(CLOTWIDTH/2)/pix2um)+1;
ClotRgt = floor((m/2)+(CLOTWIDTH/2)/pix2um);
Clot(ClotLft:ClotRgt,ClotTop:ClotBot) = 1;
Clot = Clot-Stnt;
Lumn = zeros(m,n);
Lumn(2:m-1,2:n-1) = 1 - Tiss(2:m-1,2:n-1) - Clot(2:m-1,2:n-1) - Stnt(2:m-1,2:n-1);
% ------------------------------------

% INITIALIZE SIMULATION MATRICES ------
C = zeros(m,n);  % Tissue concentration
U = zeros(m,n);  % Working Bulk concentration
CF = zeros(m,n); % Free portion of tissue concentration
```

```
NF = zeros(m,n); % Fraction of new drug concentration that should remain free after
        transport
DX = DTisX*Tiss+DClot*Clot+DClot*Stnt+DLumn*Lumn;
DY = DTisY*Tiss+DClot*Clot+DClot*Stnt+DLumn*Lumn;
K = zeros(m,n) + Tiss*KTissLumn + Clot*KClotLumn + Stnt*KClotLumn +
        Lumn*KLumnLumn;
% ------------------------------------

% COMPUTATIONAL MATRICES and VARIABLES
rD = dt/(2*dx^2);  rV = dt*V/dx;
DX1=zeros(m,n); DX2=zeros(m,n); DX3=zeros(m,n); DY1=zeros(m,n);
        DY2=zeros(m,n); DY3=zeros(m,n);
ChangeXandY=zeros(m,n); ChangeV=zeros(m,n);
DX1(2:m-1,2:n-1) = DX(1:m-2,2:n-1)+DX(2:m-1,2:n-1);
DX2(2:m-1,2:n-1) = DX(1:m-2,2:n-1)+2*DX(2:m-1,2:n-1)+DX(3:m,2:n-1);
DX3(2:m-1,2:n-1) = DX(2:m-1,2:n-1)+DX(3:m,2:n-1);
DY1(2:m-1,2:n-1) = DY(2:m-1,1:n-2)+DY(2:m-1,2:n-1);
DY2(2:m-1,2:n-1) = DY(2:m-1,1:n-2)+2*DY(2:m-1,2:n-1)+DY(2:m-1,3:n);
DY3(2:m-1,2:n-1) = DY(2:m-1,2:n-1)+DY(2:m-1,3:n);
% ------------------------------------

displaypercent = 1;  % Display every displaypercent% of completion

tic;
elapsedtime = 0;

% MAIN SIMULATION LOOP ----------
% Works with free drug diffusion and convection only

% Release first batch of drug
StentReleaseMatrix = zeros(m,n);
StentReleaseMatrix(2:m-1,2:n-1) = (Stnt(2:m-1,2:n-
        1)*STENTRELEASERATE/(100*100/pix2um/pix2um)*dt);

for t = 1:simulcycles

   % STEP 1: Release drug, and impose boundary conditions
   U = U + StentReleaseMatrix;
   U(1,:)=U(2,:);  U(m,:)=U(m-1,:);

   % STEP 2: Compute diffusion and convection of free drug + newly released drug
   ChangeXandY(2:m-1,2:n-1) = rD*(DX1(2:m-1,2:n-1).*U(1:m-2,2:n-1) +  DY1(2:m-
        1,2:n-1).*U(2:m-1,1:n-2) -...
     (DX2(2:m-1,2:n-1)+DY2(2:m-1,2:n-1)).*U(2:m-1,2:n-1) + ...
```

```
    DX3(2:m-1,2:n-1).*U(3:m,2:n-1) + DY3(2:m-1,2:n-1).*U(2:m-1,3:n));
  ChangeV(2:m-1,2:n-1) = rV*(U(2:m-1,1:n-2)-U(2:m-1,2:n-1));
  U = (U+ChangeXandY+ChangeV) .* (1-Lumn);

  % STEP 3: From the post-transport drug distribution, calculate fraction of
  % newly released drug that should remain free  (NF)
  CF(2:m-1,2:n-1) = C(2:m-1,2:n-1) ./ K(2:m-1,2:n-1);
  NF(2:m-1,2:n-1) = (U(2:m-1,2:n-1)-CF(2:m-1,2:n-1)) ./ K(2:m-1,2:n-1);

  % STEP 4: Calculate new tissue concentration
  C(2:m-1,2:n-1) = C(2:m-1,2:n-1) + U(2:m-1,2:n-1) - CF(2:m-1,2:n-1);

  % STEP 5: Calculate total amount of drug in remaining in bulk phase
  U(2:m-1,2:n-1) = CF(2:m-1,2:n-1) + NF(2:m-1,2:n-1);

  if (mod(t,round(displaypercent/100*simulcycles)) == 0)

    simtime = t*dt; % Actual simulation time in seconds
    disp([' ']);
    disp(['--> Simulating ' num2str(simtime/3600) ' hours, tissue drug = '
      num2str(sum(sum(C.*Tiss)))]);
    subplot(1,2,1);
    pcolor(2*Tiss(2:m-1,2:n-1)+Lumn(2:m-1,2:n-1)+3*Clot(2:m-1,2:n-1)+4*Stnt(2:m-
      1,2:n-1)), view(90,90), shading faceted, axis equal, axis tight, axis off;
    title('Simulation configuration');
    subplot(1,2,2);
    pcolor(C(2:m-1,2:n-1)), view(90,90), shading faceted, axis equal, axis tight, axis off;
    title(['Time: ' num2str(round(simtime/3600)) ' hrs.']);
    drawnow;

    if(min(min(C)) < 0)
      error('Numerical instability.');
      % If C < 0, washout too quick due to discretization, so becomes negative?
      % Can also make 0 all C < 0 by ==> C = (C >= 0) .* C;
    end;

    elapsedtime = elapsedtime + toc;
    disp(['Computation time: ' num2str(elapsedtime/60) ' mn, speed '
      num2str(round((dt*t)/elapsedtime)) 'X, est ' num2str((simulcycles-
      t)/(t/elapsedtime)/60) ' mn left.']);
    tic;
  end;

end;
```

```
% ------------------------------------

simtime = t*dt; % Actual simulation time in seconds
disp([' ']);
disp(['--> Simulating ' num2str(simtime/3600) ' hours, tissue drug = '
        num2str(sum(sum(C.*Tiss)))]);
subplot(1,2,1);
pcolor(2*Tiss(2:m-1,2:n-1)+Lumn(2:m-1,2:n-1)+3*Clot(2:m-1,2:n-1)+4*Stnt(2:m-1,2:n-
        1)), view(90,90), shading faceted, axis equal, axis tight, axis off;
title('Simulation configuration');
subplot(1,2,2);
pcolor(C(2:m-1,2:n-1)), view(90,90), shading faceted, axis equal, axis tight, axis off;
title(['Time: ' num2str(round(simtime/3600)) ' hrs.']);
drawnow;

elapsedtime = elapsedtime + toc;
disp([' ']);
disp(['Total computation time: ' num2str(elapsedtime/60) ' mn, speed '
        num2str(round((dt*t)/elapsedtime)) 'X, est ' num2str((simulcycles-
        t)/(t/elapsedtime)/60) ' mn left.']);
```

Chapter 5

Conclusion

Thesis Summary

After great trial and error the elution of specific drugs from endovascular stents now appears to be the most effective long term therapy for obstructive coronary artery disease. In a fascinating turn the rational selection of drugs on the basis of biological activity failed. Powerful vasoactive agents such as heparin regulate each of the individual events in the vascular response to stenting and fail to affect the ultimate development of restenosis. It appears that the physico-chemical properties of the drugs take far greater precedence. Hydrophilic compounds, like heparin, are rapidly cleared from vascular tissue and are consequently therapeutically ineffective for DES. Hydrophobic species with high tissue binding capacity enter and are cleared from tissue much more slowly, increasing overall residence time. The two compounds currently clinically approved for use have specific and general means of tissue binding. While specific binding is a seemingly obvious attribute of these compounds the general binding events may be equally important. Rapamycin's interaction with the FKBP protein and paclitaxel's association with polymerized microtubules are responsible for their biological activity. But, the general binding of these drugs to other tissue elements also plays a major role in determining the prolonged tissue retention and overall pharmacokinetics. Physiochemical specific and general binding has emerged as one of the fundamental mechanisms contributing to the success of DES, and must be a consideration in the rational design of local delivery devices.

5.1 Specific Findings

This thesis consists of a series of experiments designed to explore the roles of specific and general binding in local arterial drug delivery.

In chapter 2, we defined the pharmacokinetic implications of specific binding to arterial drug transport. Characteristics of hydrophobic, and clinically relevant, rapamycin and paclitaxel were compared with the model hydrophilic compound, dextran. We found that hydrophobic compounds enter and elute out of arterial tissues much more slowly than hydrophilic ones. Hydrophobic diffusion coefficients are two log orders slower than hydrophilic compounds like dextran and, diffusivity of all drugs in the planar direction exceeds that of the transmural direction by two to three orders of magnitude. Further, paclitaxel and rapamycin maintain tissue concentrations 30-40 times that of dextran, with specific binding to biologically relevant protein targets accounting for 35% and 50% of binding for paclitaxel and rapamycin, respectively. Finally we discovered that paclitaxel and rapamycin distribute transmurally in arterial tissue with starkly different profiles.

In chapter 3, we investigated the roles of specific and general binding by defining the contribution of each in arterial transport and distribution. Using specific and general blocking agents we found that both types of displacement can decrease tissue binding capacity by near 50%. We saw that specific binding has higher contributions in the media where target protein density is greater. Similarly, general binding contributions

localized preferentially to the adventitia. We also focused on the question of paclitaxel's and rapamycin's abilities to displace one another from specific and general binding sites. Molecular and tissue assays confirmed that each drug does not displace the other from specific binding sites but can perturb general binding at three log order concentration excess. Such concentrations gradients may be relevant for competition scenarios of systemically delivered compounds over local therapeutics. A screen of several commonly administered cardiac drugs found a heterogeneity in general binding competition characteristics.

In chapter 4, we applied our knowledge of specific and general binding properties to the clinically important problem of stent deployment in a thrombotic environment. After measuring a series of binding capacity, diffusion and convection parameters in fibrin, red blood cells and whole blood we developed a finite difference model which allowed us to study tissue uptake of drug for various stent and thrombus geometries. The results showed that thrombus can act as both a protective cap to increase drug uptake or a vehicle to enhance elution from the stent strut to the vessel lumen. Thrombus composition influences uptake kinetics through changes in its retention capacity for drug while clot geometry mediates arterial drug levels through the balance of local transport forces. The exquisite sensitivity of tissue uptake to thrombus geometry and composition implies that drug deposition will be highly variable and difficult to predict in a thrombotic micro-environment.

This thesis has identified and characterized several vital mechanistic factors which derive the functionality of local drug delivery devices. While this work has focused on arterial drug systems, the fundamental tenants are generalizable to many delivery systems where specific and general binding will determine efficacy. We hope that comprehension and application of these important factors will help optimize local drug delivery therapeutics.

5.2 Future Directions

Binding and Transport in Pathological Arteries

Vascular disease can significantly increase the complexity of arterial drug transport and distribution, and possibly the binding site availability and distribution. While animal models of neointimal, lipid-laden and stent-induced pathologies have long existed[1, 2], the resulting lesions are highly variable and as a result, drug transport studies in these lesions has remained difficult to assess experimentally. Nevertheless, our studies of native arteries have defined general transport and binding baseline properties for future comparisons to disease models. It has already been observed that a hyperplastic neointimal layer has little, if any, distinct geometric orientation[3] while containing high concentrations of microtubules and FKBP. Since the diffusive anisotropy in the arterial wall is a product of the geometric orientation of smooth muscle cells, the regular layering of tissues of differing permeabilities and binding site availability, the new intima may yield isotropic transport behavior. Compared to the hyperplastic neointima, lipid-laden atherosclerotic plaques might be expected to offer increased general binding capacity for more highly hydrophobic drugs. Site-specific pathology offers another opportunity for investigations; vascular branching points prove technically difficult to stent and show reduced responsiveness to DES therapy. Finally, stented arteries with highly compressed media may actually exhibit even higher diffusive anisotropy than native arteries since medial compression tightly packs arterial tissue layers together[4], further limiting transmural transport compared to planar transport. As diseased arteries are the ultimate target of vascular drug delivery, the impact of

pathological arterial states on tissue structure and drug binding and transport clearly merits further experimental study.

Diabetes

While DES has made significant advances in the treatment of coronary artery disease, the problem is far from solved. Restenosis still persists in some patients, especially those with diabetes, for yet unexplained reasons. In some studies, using DES in diabetic patients shows little benefit over bare metal stents. It is not yet clear whether diabetic resistance to drug-eluting stent therapy is a result of alterations in binding site availability, transport or signaling mechanisms. A major challenge in studying vascular disease and diabetes is the lack of a good animal model. Rat vasculature does not respond to injury in the same manner has human tissue. Pig diabetic models more accurately recapitulate atherosclerotic pathology but inducing the model in these animals is very expensive and requires extremely talented husbandry. Nonetheless, with type II diabetes on the rise in most of the western world, mastery of the limitations in treating this population is a must.

Cocktails

Despite, or perhaps because of, the clear complexity underlying human physiology, the medical community is often cautious to embrace combination therapies. The HIV triple cocktail is now a well accepted therapy. Regiments for treating heart disease come in combinations beyond triples addressing hypertension, cholesterol,

platelet activity, etc. Local stent delivery offers a unique opportunity to explore combination therapies while limiting the complicated nature of evaluating secondary systemic effects. Rapamycin and paclitaxel act through two entirely unique mechanism to inhibit smooth growth and would likely be a more efficacious combination than either drug alone.

More Vessels

As DES stent treatment move to other vessels such as the renal and carotid arteries a number of important considerations arise. As discussed previously, the composition of the target tissue will change the diffusive, convective and binding properties of the eluted drug. For example, the carotid arteries are relatively large bore with high elastin contents. Additionally, luminal washout may take on increased importance when stenting non-coronary vessels. Stenting in the coronaries has the distinct advantage of rapid systemic dilution of washed out drug. Stenting immediately proximal to the kidneys or the brain may increase the toxic sensitivity of these organs.

5.3 Generalizing the Lessons of Local Arterial Transport

Delivery

Drug distribution within tissue is a function of the interplay between local physiological transport forces and the physical characteristics of the target tissue[5, 6]. This statement is generalizable to any local drug delivery system but will be summarized here in terms of local arterial drug delivery. The potential of local drug delivery is tremendous once adequate targeting is achieved, and thus, a rational approach to the design of local delivery devices requires careful consideration of the interplay of drug with arterial tissue organization and composition, as well as factors that influence the devices ability to interact with target tissue.

Basic Transport Principles

Local drug distribution is influenced by ultrastructure both through the geometric organization and through the variable avidity for drug uptake of the component arterial tissues. Drugs generally do not distribute evenly throughout blood vessels. Drug transport is highly dependent upon direction and plane of the tissue, and their deposition and uptake are determined by tissue binding. Animal studies have confirmed that drug delivery performed in one arterial bed cannot be directly applied to other arterial beds without factoring in the underlying variations in tissue composition and organization.

Specific and General Binding

While we have previously observed that dextrans and heparin naturally sequester into specific connective tissue layers within the arterial wall[7, 8], hydrophobic compounds like rapamycin and paclitaxel bind tightly to their target proteins in addition to general associations. These binding interactions determine diffusive, distributive and tissue residence time properties which dominant the efficacy of these treatments.

Microenvironment and DES

Variation in the local microenvironment immediately adjacent to the delivery device can significantly impact device performance. Clots immediately adjacent to stent struts can modulate the DES properties to significantly alter arterial drug levels and potentially efficacy of treatment. Thrombus composition influences uptake kinetics through changes in its retention capacity for drug while clot geometry mediates arterial drug levels through the balance of local transport forces. The exquisite sensitivity of tissue uptake to thrombus geometry and composition implies that drug deposition will be highly variable and difficult to predict in a thrombotic micro-environment.

Rational Local Delivery Design

Incorporation of all these concepts into local drug delivery design is essential to developing successful therapeutics. While no simple formula exists to integrate the multifaceted considerations, care must be taken to evaluate each parameter to achieve optimal delivery.

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