ENDOTHELIAL CELLS AND BASEMENT MEMBRANE: A CO-REGULATORY UNIT FOR FIBROBLAST GROWTH FACTOR-2 IN HYPERGLYCEMIC STRESS

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ABSTRACT

Endothelial cells and basement membrane interact as a biochemical and mechanical co-regulatory unit. The wide spectrum of manifestations of diabetic vascular disease could be related to altered kinetics of vasoactive compounds within this regulatory unit. We hypothesized that hyperglycemic stress mediates storage, release, and function of fibroblast growth factor-2 (FGF-2) through changes in interaction between endothelial cells and basement membrane.

We discovered that basement membrane associated FGF-2 increased linearly with culture glucose concentration. Using novel assays, we demonstrated that FGF-2 binding kinetics were surprisingly unchanged over a range of basement membrane culture glucose. Instead, the combination of increased endothelial cell apoptosis-associated FGF-2 release and enhanced endothelial cell permeability allowed more FGF-2 to bind into the basement membrane. Such high levels of basement membrane FGF-2 abrogated the effects of hyperglycemia on proliferation but not apoptosis. An FGF-2 stimulus returned endothelial cell proliferation close to euglycemic levels, but increased apoptosis was still evident as FGF-2 signaling down an intracellular survival pathway was inhibited by glucose. These same findings were confirmed in vivo where FGF-2 levels were elevated in the aortic subendothelial space of diabetic animals.

This thesis suggests a new paradigm for active cellular control of basement membrane and indicates the complexities of growth factor signaling in endothelial cells. Characterization of the interaction between endothelial cells and basement membrane in health and disease may advance our understanding of diabetic vascular disease and lead to development of novel biomimetic materials for therapeutic intervention.

Thesis supervisor: Elazer R. Edelman, M.D., Ph.D.
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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Thesis Overview

Diabetes is reaching epidemic prevalence worldwide. With cardiovascular disease as the major cause of morbidity and mortality in diabetics, a mechanistic understanding of diabetic vascular dysfunction is critical to preventing a subsequent cardiovascular disease epidemic. Hyperglycemia, the hallmark of diabetes, has been linked to vascular abnormalities, but hyperglycemia alone does not correlate with diabetic morbidity and mortality. While glucose is known to induce osmotic effects on a timescale of hours and protein glycation on a timescale of weeks, the effect of physiologic glucose fluctuations on the vasculature over the course of days—such as what might occur even in a patient with tight glucose control—is unknown.

Diabetes and cardiovascular disease intersect at the endothelium—the single cell layer lining blood vessels. While the endothelium was once thought to be vascular "cellophane," we now know that endothelial cells perform a delicate balancing act by sensing signals from the bloodstream and communicating them into vessel functional alterations (1). Diabetes is a complex disease with a plethora of biochemical abnormalities, but altered glucose metabolism alone is sufficient to cause endothelial cell dysfunction.

Diabetic vascular dysfunction takes varied forms in different vascular beds, but since much of the dysfunction involves disordered vascular remodeling, angiogenic growth factors are prime candidates for dysregulation. Fibroblast growth factor-2 (FGF-2) in particular has key implicating features, including a lack of release mechanism other than endothelial cell injury and a storage reservoir in basement membrane, the protein matrix that supports endothelial cells both physically and biochemically. By perturbing endothelial cells and proteins with an

environmental stress such as hyperglycemia, we can learn not only about diabetic vascular dysfunction but also about cooperative FGF-2 regulation by endothelial cells and basement membrane.

This thesis investigates how external stress modulates vasoactive compound kinetics within the endothelial cell – basement membrane co-regulatory unit. We hypothesize that a physiologic glucose range mediates altered storage and release of FGF-2, as well as altered FGF-2 functional effects through changes in endothelial cell – basement membrane interaction. Understanding FGF-2 regulation by cells above and basement membrane below may shed additional light on diabetic vascular disease.

In support of these concepts, this thesis consists of a series of studies to elucidate the role of FGF-2 in glucose-induced vascular dysfunction, in particular as FGF-2 is cooperatively stored, released, and metabolized by endothelial cells and basement membrane. The specific aims of this work are:

- **Chapter 2:** Develop novel methods to examine FGF-2 binding kinetics with isolated basement membrane *in vitro*.
- **Chapter 3:** Investigate the effect of glucose on basement membrane FGF-2 storage and binding kinetics (capacity, association, and dissociation).
- **Chapter 4:** Define the role of altered endothelial cell function (FGF-2 release, apoptosis, and permeability) with glucose in controlling FGF-2 basement membrane storage.
- **Chapter 5:** Examine how FGF-2 released from basement membrane affects endothelial cell proliferation and survival with glucose.

This thesis provides both an improved understanding of unregulated vascular remodeling in diabetes and a clearer picture of interaction between endothelial cells and basement membrane in vascular homeostasis. These systematic studies lead towards a quantitative model of the endothelial cell – basement membrane co-regulatory unit, which in turn will help us develop novel biomaterials and basement membrane therapies.

1.1 Clinical Background

1.1.1 Epidemiology of diabetes

Public health improvements have drastically reduced mortality from communicable diseases. At the same time, technological advances have created modern conveniences, which lead to sedentary lifestyle and poor nutrition. With human progress, globalization, and unprecedented control over our environment has come a growing diabetes epidemic (2). The worldwide diabetes prevalence is estimated to rise from the current figure of 150 million affected to 300 million affected over the next twenty years. The majority of new cases will be type 2 diabetes, which is strongly associated with a sedentary lifestyle and obesity (3, 4).

In the United States, according to national health survey data analyses from 1999 to 2003 by the American Diabetes Association, 20.8 million children and adults—around 7% of the population—have diabetes. This figure includes 14.6 million with diagnosed disease and 6.2 million who are undiagnosed but does not account for 41 million in a pre-diabetic state. These astonishing figures continue to grow. In 2005, 1.5 million new diabetes cases were diagnosed in people aged 20 years or older.

Diabetes is a deadly disease. While improvements in health care have reduced death rates due to heart disease, stroke, and cancer, the diabetic death rate has risen by 45% since 1987. Diabetes ranks as the fifth-deadliest disease in the United States, but it is widely considered underreported on death certificates, especially in persons with multiple chronic conditions. The diabetic death toll is likely significantly higher than officially reported. Diabetes also does not strike equally across racial and social backgrounds. Asians, blacks, Native Americans, and Hispanics are 1.5 to 1.8 times as likely to have diabetes as non-Hispanic whites.

1.1.2 Diabetic biochemistry

Diabetes is defined as a heterogeneous group of disorders with impaired glucose tolerance and subsequent hyperglycemia in common (5). Type 1 diabetes (insulin dependent, juvenile onset) accounts for ~10% of the total, whereas type 2 diabetes (non-insulin dependent, adult onset) accounts for 80-90% of diabetics. The third

diabetes class, maturity-onset diabetes of the young (MODY), is caused by a genetic defect and accounts for the remaining ~5%.

Type 1 diabetes, marked by an absolute lack of insulin, is caused by destruction of pancreatic islets where insulin is produced (5). While no clear event sequence leading to this destruction has been identified, current theory postulates that people are genetically predisposed to type 1 diabetes. Disease is then triggered by an environmental insult, leading to autoimmune islet destruction. Interestingly, children only become symptomatic after 90% of their islet mass has been destroyed.

Type 2 diabetes, on the other hand, is caused by both derangement of insulin secretion and decreased peripheral tissue response to insulin (5). Insulin resistance results in a lack of directed glucose deposition, and hence a persistent hyperglycemia following a glucose load. Type 2 diabetes has an even stronger genetic predisposition, but rather than being linked to a single gene, a collection of defects or polymorphisms exists, each with its own modifiable environmental risk.

Diabetic biochemistry revolves around glucose and insulin. Glucose homeostasis is regulated by several mechanisms, including hepatic glucose production, glucose metabolism by peripheral tissues, and insulin secretion. Insulin is produced in pancreatic islet β cells. After insulin gene expression, preproinsulin is synthesized in rough endoplasmic reticulum and delivered to the Golgi apparatus, where proteolytic cleavage generates mature insulin and C peptide (6). Both are stored in secretory granules and secreted together when extracellular glucose rises and more glucose is brought into the cell through membrane glucose transporters, in particular GLUT-2.

Insulin is important for hepatic and muscular glycogen formation as well as nucleic acid and protein synthesis, but its primary function is transmembrane glucose transport induction. Insulin particularly increases glucose transport into striated muscle cells, fibroblasts, and fat cells which constitute two-thirds of body weight. Insulin binds its receptor, composed of two glycoprotein subunits, and activates intracellular response cascades to translocate glucose transport proteins (primarily GLUT-4) from the Golgi apparatus to the plasma membrane.

Data suggest that primary diabetic complications are associated with hyperglycemia, in particular because strict glucose control decreases both prevalence

and severity of these complications (7). In tissues that do not require insulin for glucose transport, which include nerves, lens, kidneys, and blood vessels, plasma hyperglycemia in diabetes causes increased intracellular glucose as well. High intracellular glucose leads to both nonenzymatic glycosylation and glucose metabolism pathway abnormalities, both of which are examined in greater detail when the glucose effect on endothelial cells is discussed.

1.1.3 Vascular morbidity and mortality in diabetes

1.1.3.1 Accelerated atherosclerosis

Diabetics suffer from accelerated atherosclerosis affecting vessels ranging in size from the aorta to capillaries (8). Diabetic atherosclerosis is indistinguishable from that of non-diabetics, and while the reason for the accelerated process is unclear, it is likely related to a combination of blood lipid alterations, hypertension, and increased lipoprotein deposition in and platelet adherence to the vessel wall. Diabetic atherosclerosis is so severe that myocardial infarction is the leading cause of death in diabetics. According to the American Diabetes Association, heart disease and stroke combined account for ~65% of diabetic deaths, two to four times higher than for non-diabetic adults. Of modifiable risk factors for cardiovascular disease, including hypertension, hypercholesterolemia, smoking, and diabetes, only diabetes prevalence continues to rise.

1.1.3.2 Diabetic retinopathy

Diabetic retinopathy causes 12,000 to 24,000 new blindness cases each year, making diabetes the leading cause of new onset blindness in adults aged 20-74 years. Early background retinopathy is characterized by capillary basement membrane thickening, capillary microaneurysms and hemorrhage, loss of endothelial cells and pericytes, and microvascular obstructions that can lead to tissue hypoxia. This background, or non-proliferative, retinopathy is often succeeded by a proliferative retinopathy characterized by neovascularization. The shift from non-proliferative to proliferative retinopathy is thought to occur in response to vascular endothelial growth factor (VEGF) produced by pericytes in response to retinal ischemia and hypoxia (9).

1.1.3.3 Diabetic nephropathy

Diabetes is the leading cause of kidney failure. In the United States in 2002, 153,730 people with end stage renal disease due to diabetes were living on chronic dialysis or with a kidney transplant. Glomerular lesions include capillary membrane thickening, diffuse glomerulosclerosis, and nodular glomerulosclerosis. Both glomerulosclerosis forms involve increased mesangial matrix deposits, leading to renal ischemia and scarring. Basement membrane changes show more type IV collagen and fibronectin and less heparan sulfate proteoglycan, which are thought to contribute to glomerular filtration function loss and eventual kidney failure (7).

1.1.3.4 Microangiopathy

More than 60% of nontraumatic lower-limb amputations occur in people with diabetes, with the diabetic amputation rate ten times higher than for non-diabetics. Amputations are generally related to infection and gangrene of chronic wounds resulting from diabetic microangiopathy. In diabetic microangiopathy, diffuse basement membrane thickening primarily from increased type IV collagen makes capillaries leakier and inhibits angiogenesis and healing response. Diabetic microangiopathy is indistinguishable, although more severe, from similar alterations with aging (10).

1.1.3.5 Role of hyperglycemia

A prospective study of the effect of strict glucose control on type 1 diabetics showed that intensive insulin therapy delays onset and slows progression of diabetic retinopathy, nephropathy, and neuropathy (11). However, hyperglycemia, which was considered the hallmark of diabetes, cannot singlehandedly account for diabetic morbidity and mortality. Certainly other biochemical alterations contribute, including variable insulin and lipoprotein levels.

A question that remains unanswered is the effect of physiologic fluctuations in blood glucose that occur on the timescale of days—even in a person with tight glucose control. Extreme hyperglycemia can lead to osmotic effects in just hours, and chronic hyperglycemia can lead to protein glycation over the course of weeks. However,

supposedly benign physiologic fluctuations that last for around a day may contribute significantly to vascular dysfunction over the course of a lifetime.

1.2 Basement Membrane

1.2.1 Normal vascular anatomy

Large blood vessels are composed of three layers, or tunics. The innermost layer, in direct contact with the bloodstream, is the tunica intima. This layer consists of an endothelial cell monolayer resting on a protein network called basement membrane. The tunica intima is separated from the middle blood vessel layer, the tunica media, by a fenestrated elastin sheath called the internal elastic lamina. The tunica media itself consists of circumferentially oriented smooth muscle cells embedded in an extracellular matrix of collagen, elastin, and proteoglycans. This layer is separated from the outermost vessel layer by another elastin sheath, the external elastic lamina. The tunica adventitia, which includes collagenous tissue, vascular nerves, vasa vasorum (small blood vessels that feed outer large blood vessel layers), and fibroblasts, forms the outer vessel support (5).

Blood vessel composition varies depending on vessel size and function. Large veins have the same three layer structure as large arteries, but since they operate in a low pressure environment, the layers are less distinct and the tunica media significantly thinner. Capillaries and postcapillary venules, whose main function is nutrient diffusion across blood vessels, are tubes with a single endothelial cell layer on a basement membrane and pericytes adherent to the outside.

1.2.2 Endothelium

The endothelium is a confluent monolayer of thin cells lining the intimal surface of all blood vessels. Until the late 1960s, the endothelium was considered a relatively inert nonthrombogenic cellular barrier. However, in 1966 Florey challenged these beliefs, stating that endothelium was more than a nucleated cellophane sheet (1). In thirty years since endothelial cells were first cultured as a homogeneous population, endothelial

cells have been shown to sense mechanical, chemical, and humoral stimuli, process these signals, and respond by synthesizing and releasing a myriad of factors (12, 13).

The endothelium maintains a delicate balance between influx and efflux from the vasculature, growth promotion and inhibition, vasoconstriction and vasodilation, blood cell adherence and nonadherence, and anticoagulation and procoagulation. In this way, endothelium modulates vascular permeability, angiogenesis, vasomotor tone, inflammatory response, and hemostasis. The endothelium is strategically situated between the bloodstream and tunica media to sense changes in humoral substances as well as blood flow and vascular mechanics, responding through vasoactive factor production and release (14).

The blood vessel wall forms a selective permeability barrier, with an estimated area of 350 square meters, for molecule transport between blood and tissue (15). The endothelium maintains this barrier through cell-cell linkages via two main intercellular junctions: tight junctions and adherens junctions (16). In both junction types, adhesion is mediated by homophilic protein interactions with adjacent cells, resulting in a dynamic pericellular zipper (17).

Tight junctions are formed of transmembrane proteins called claudins and occludins (18). In extracellular space, these proteins from adjacent cells join together to form sealing strands. Intracellularly, claudins and occludins anchor the actin cytoskeleton via ZO proteins (19). Endothelial cell adherens junctions are formed primarily of the transmembrane protein vascular endothelial cadherin (Ve-cadherin), which connects to intracellular anchor proteins such as catenins, vinculin, and α-actinin that then connect to actin filaments (20). Both junctions require association with cytoskeletal actin for dynamic regulation of junction opening and closure. In some cellular systems, adherens junctions are required for tight junction formation.

Endothelial cell permeability changes in response to cytokines or permeability regulators such as VEGF are associated with occludin and cadherin redistribution (21). Permeability changes lead to both tissue edema and blood protein and lipoprotein deposition in the vascular wall (22, 23). Often such permeability changes are associated with inflammation or abnormal blood flow (23, 24).

Endothelial cells respond to injury by inducing blood vessel growth, therefore they must maintain their cell division capacity. In the normal state, cells divide slowly but can rapidly proliferate and even recruit precursor cells from the blood stream when needed. Angiogenesis is defined as sprouting of new vessels from existing vessels. This process is governed by endothelial cells, which produce proteases to digest basement membrane, migrate towards a tissue hypoxia-induced VEGF signal, proliferate, form tubes, and finally differentiate (25, 26).

In 1980, vascular smooth muscle relaxation in response to acetylcholine was demonstrated to be dependent on an intact endothelium (27). Several years later, endothelial cell derived relaxing factor was identified as nitric oxide (NO) (28). NO, in conjunction with a second endothelial cell-produced vasodilator prostacyclin (PGI_2), regulates not only stimulus-induced vascular dilation but also basal motor tone (29). As part of the counterregulatory balance endothelial cells produce proendothelin, which is cleaved extracellularly to form the long-lasting potent vasoconstrictor endothelin. Many factors which induce endothelin synthesis similarly induce NO or PGI_2, demonstrating the complex, multifaceted balance of constriction and dilation in endothelial cell vasomotor tone control.

Endothelial cells respond to inflammatory signals from cytokines such as lipopolysaccharide (LPS), tumor necrosis factor-α (TNFα), or interleukin-1 (IL-1) by producing their own chemokines and cytokines as well as upregulating cell adhesion molecules (30). Endothelial cell-derived cytokines recruit leukocytes and cell surface selectins modulate leukocyte rolling, after which adhesion molecules such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) mediate firm adhesion and eventual transmigration through the endothelial cell layer (31, 32). Endothelial cells tightly tune the inflammatory response since inappropriate, excessive, or chronic inflammation leads to pathological conditions (33).

The healthy endothelial surface is anticoagulatory and antithrombotic. Vasodilators PGI_2 and NO are constitutively released to prevent platelet adherence to the vessel wall, and the intact endothelial cell surface is rich in proteoglycans that inactivate thrombin to prevent blood clot formation (34). Vessel damage or cytokine exposure shifts endothelium toward a procoagulant phenotype, leading to release of

platelet activating factor, von Willebrand factor, and tissue factor which promote platelet adhesion and thrombus formation (35-37).

1.2.3 Endothelial alterations in diabetes

Just as normal endothelial cell function is critical to healthy vascular regulation, diabetic endothelial cell dysfunction plays an important role in vascular dysregulation. Endothelial cells do not require insulin for glucose uptake and are therefore particularly susceptible to intracellular hyperglycemia in a high glucose environment. Endothelial cells appear to downregulate glucose transporters in hyperglycemia, but this reaction takes 24 to 48 hours to fully manifest, therefore endothelial cells are particularly susceptible to short term glucose loads (38). Each critical endothelial cell function discussed previously is altered in diabetics, and the combination likely leads to diabetic vascular morbidity and mortality.

The endothelial cell permeability barrier is compromised in diabetes. In retinal microvasculature, fluid and proteins pass through capillary walls causing increased pressure (39). In glomerular capillaries, endothelial cell and basement membrane defects lead to filtration function loss, albuminuria, and eventual kidney failure (7). In macrovasculature, increased permeability sites due to flow abnormalities allow plasma protein and lipoprotein deposition in the vascular wall, and a similar mechanism is suspected in diabetes (40, 41).

Angiogenesis is markedly disordered in diabetics. Endothelial cells have been reported to suffer from both reduced proliferation and increased apoptosis, yet angiogenesis can be magnified or diminished depending on vascular bed (42, 43). Renal filtration function loss in diabetic nephropathy follows glomerular capillary hypertrophy, and fragile new vessels formed by excessive angiogenesis create a unique retinopathy (44). In contrast, reduced angiogenesis in extremities contributes to poor wound healing (45). Vascular growth factors, including VEGF and FGF-2, have been implicated in these conditions but with no consensus as to their absolute role (45, 46).

Glucose-induced alterations in endothelial cell vasomotor tone control vary with exposure time. Early in diabetes, blood flow preferentially increases to certain areas, in particular to kidneys resulting in damaging hyperfiltration. Early flow abnormalities are

thought to be related to glucose-induced alterations in polyol pathway, protein kinase C (PKC), VEGF, and NO (47-50). Later in diabetes, vasomotor tone balance tips in favor of vasoconstrictors (51). Hyperglycemia in particular leads to a reduced endothelial cell response to NO, decreased available NO, and increased endothelin-1 (52, 53). This imbalance could contribute to diabetic vascular disease and mortality.

Finally, in both inflammatory response as well as hemostasis, endothelial cells present a more adherent surface for leukocytes, platelets, and thrombi. Hyperglycemia induces abnormal leukocyte adhesion molecule expression, with increased selectins, ICAM, and VCAM, and spontaneous platelet aggregation due to tissue plasminogen activator also occurs (54, 55). Leukocyte and platelet adhesion could cause vessel occlusion, leading to downstream hypoxia, which could in turn trigger compensatory angiogenesis.

While diabetes is a complex disease with many biochemical abnormalities, hyperglycemia in particular has been linked to the described endothelial cell dysfunctions. Three main mechanisms have been proposed for how hyperglycemia leads to diabetic complications: increased polyol pathway flux, increased advanced glycation end product (AGE) formation, and PKC activation (56). Only recently has a hypothesis been suggested that may unify these three mechanisms.

The polyol pathway, which begins with the enzyme aldose reductase, rarely metabolizes glucose in normal conditions. However in hyperglycemia, as much as 33% of glucose is reduced through the polyol pathway. While the mechanism by which increased polyol flux leads to endothelial cell damage is unclear, aldose reductase inhibitor clinical trials have proven effective in decreasing diabetic neuropathy (57). Since animal trials showed no effect on retinopathy or capillary basement membrane thickening, results are inconsistent and suggest that the polyol pathway does not encompass the entire mechanism of hyperglycemia-induced cellular damage (58).

In nonenzymatic glycosylation, also referred to as glycation, glucose chemically attaches to a protein amino group without aid of enzymes. While the process starts out as reversible, eventually protein and glucose rearrange to become irreversible advanced glycation end products (AGE). AGE form crosslinks between collagen peptides, trapping plasma proteins such as low density lipoprotein and albumin in the

vessel wall and impairing proteolytic extracellular matrix digestion. For several cell types, including endothelial cells, AGE binds to a specific AGE receptor to induce cytokine and growth factor release, increase permeability, enhance proliferation, and increase extracellular matrix synthesis (59).

AGE were originally thought to form through nonenzymatic extracellular reactions between proteins and glucose, but they now seem more likely to occur intracellularly because extracellular sugars such as glucose glycate proteins at a much slower rate than intracellular sugars such as fructose (60). AGE induce endothelial cell damage via alteration of both intracellular and extracellular matrix protein functions, and AGE bound to their endothelial cell receptors induce intracellular reactive oxygen species production. Increased AGE are found in diabetic retinal vessels and renal glomeruli, and AGE inhibitors block vascular hyperglycemic complications both in animal models and in humans (61, 62).

Intracellular hyperglycemia increases the lipid second messenger diacyl glycerol (DAG), which in turn activates many PKC family members (63). PKC activation mediates blood flow abnormalities, endothelial cell permeability, and altered matrix accumulation (48, 64, 65). Treatment of animals with a PKC inhibitor partially normalizes glomerular filtration rate, glomerular mesangial expansion, and urinary albumin excretion (66).

Although inhibition of each pathway blocks some diabetic complications, only recently has superoxide overproduction by the mitochondrial electron-transport chain been proposed as the common element (67). Hyperglycemia increases the inner mitochondrial membrane proton gradient above a threshold value due to electron donor overproduction by the TCA cycle. This causes the lifetime of superoxide-generating electron-transport intermediates to be prolonged, consequently leading to a marked increase in endothelial cell superoxide production (68). Overexpression of manganese superoxide dismutase, the mitochondrial specific superoxide dismutase, not only abolished reactive oxygen species but also prevented increased polyol pathway flux, AGE formation, and PKC activation (67). While specific mitochondrial superoxide inhibition is challenging, a common hyperglycemic pathway opens new avenues for research and therapeutics.

1.2.4 Basement membrane composition and function

The vascular basement membrane is a 40 to 120 nm thick protein network basolateral to endothelium (6). It is synthesized primarily by endothelial cells and contains type IV collagen, perlecan, laminin, and entactin/nidogen as its major components. The basement membrane has important structural and filtering roles, along with determining cell polarity, influencing cell metabolism, organizing adjacent plasma membrane proteins, promoting cell survival, proliferation, or differentiation, and serving as the highway for cell migration.

The basement membrane is formed by interconnected networks of type IV collagen and laminin (6). Type IV collagen, a triple stranded helix more flexible than fibrillar collagen, interacts with its own uncleaved terminal domains to assemble extracellularly. Laminin, a large three chain glycoprotein shaped like an asymmetric cross held together by disulfide bonds, self-assembles into a sheet via interactions between neighboring laminin arms. Nidogen and perlecan bind to both type IV collagen and laminin and are thought to hold the two sheets together.

Perlecan is a large basement membrane heparan sulfate proteoglycan formed of glycosaminoglycans (GAGs) covalently linked to a core protein (6). GAGs are repeating disaccharide chains, most of which have sulfate or carboxyl groups. For this reason, GAGs are highly negatively charged, strongly hydrophilic, and can take up a large volume for a relatively small mass. Proteoglycans, in addition to being critical for compressive force resistance in tissue such as cartilage, provide important filtration and binding functions in vascular basement membrane.

Historically, vascular basement membrane was viewed purely from a structural perspective as a platform on which endothelial cells function. More recently, it has become clear that basement membrane supports a plethora of biochemical activities. Endothelial cells interact with basement membrane through integrins, transmembrane proteins composed of two noncovalently associated glycoprotein subunits. Integrins connect intracellularly to actin filaments and extracellularly to basement membrane, coupling cell to matrix for both structural support and signaling capabilities. Integrins demonstrate low affinity binding to basement membrane proteins, allowing cells to

migrate and proliferate rather than become permanently glued to a surface. Many cell types, including endothelial cells, are anchorage dependent and require integrin attachment to basement membrane for growth, survival, and proliferation (69, 70).

In addition to mechanically and biochemically supporting cells through integrins, basement membrane is a reservoir for local vasoactive factor delivery. Proteoglycans bind cytokines and growth factors, potentially restricting their range of action, temporarily blocking their activity, providing a storage reservoir, and protecting proteins from degradation. VEGF, FGF-2, interleukins, and interferon-γ all bind to and are released from basement membrane, which extends their lifetime and efficacy in the vasculature (71-74). Even in degradation, basement membrane produces protein fragments that promote or inhibit angiogenesis (75, 76).

1.2.5 Basement membrane alterations in diabetes

Alterations in vascular basement membrane may help unify the diabetic vascular disease spectrum. Throughout varied vascular beds, from kidney to eye to aorta, basement membrane becomes consistently thicker over the course of months to years (44). Increased membrane size results from a combination of increased synthesis and decreased degradation (77-80). The relative quantity of major protein components also changes, with increases in type IV collagen and fibronectin and decreases in laminin and heparan sulfate proteoglycans (44, 80-82). While no single biochemical diabetic alteration has been identified as the cause of these changes, hyperglycemia has been implicated (83, 84). *In vitro*, high glucose increases basement membrane protein production and leads to glycation of these proteins, altering both matrix-matrix and matrix-cell interactions (85-87).

Glucose-induced changes in basement membrane present an interesting hypothesis for the cause of hyperglycemic memory. In animal models, restoration of euglycemia after a hyperglycemic period does not reverse or even prevent diabetic vascular complication progression, in particular retinopathy (88, 89). Instead, disease progression appears to depend on the hyperglycemic period length (88, 90). This phenomenon has similarly been observed in human trials. In multiple cohorts that received either intensive therapy or normal therapy and then were followed for up to

eight years after returning to the same therapy (with statistically similar blood glucose levels), both retinopathy and nephropathy rates were consistently significantly lower in the intensive therapy group (91, 92). Furthermore, a transient worsening of retinopathy with intensive glucose therapy was observed (11). While other mechanisms, such as AGE and mitochondrial DNA mutations due to superoxide, have been suggested, it is equally possible that basement membrane changes would persist long after euglycemia is reinstated (56, 59, 93).

1.3 Fibroblast Growth Factor-2

1.3.1 Biological significance

Fibroblast growth factor-2 (FGF-2) is an extensively studied member of a large heparin binding protein family. FGF-2 is a pleiotropic factor which affects multiple cell types and induces a multiplicity of differential gene expression. In endothelial cells in particular, FGF-2 influences cell proliferation, survival, migration, and differentiation.

FGF-2, which is highly conserved among species, was originally isolated from the pituitary and is translated as a 155 amino acid protein (94). Longer and shorter FGF-2 versions are formed in different tissues by means of alternative translation and proteolytic degradation respectively (95, 96). The three dimensional FGF-2 structure consists of 12 antiparallel β-strands, six in a β-barrel closed at one end by remaining β-strands. The barrel core includes hydrophobic amino acid side chains, and the barrel surface has charged amino acids (97).

Scientists agree that FGF-2 is released from endothelial cells, stored in basement membrane or extracellular matrix, and then released from basement membrane to bind to endothelial cell receptors. Upon binding, FGF-2 is re-internalized by the cell and finally delivered to the nucleus (97, 98). Initiation of this process is a mystery, as FGF-2 has no signal sequence for secretion yet appears in abundance both inside and outside cells. It is known that FGF-2 does not progress through the endoplasmic reticulum, Golgi apparatus, and on through the plasma membrane through the normal secretory pathway (99). It has been proposed, therefore, that FGF-2 is

released either through an alternative secretory pathway or during cell damage and non-lethal plasma membrane disruptions (100, 101).

FGF-2 interaction with heparin, and in particular with heparan sulfate proteoglycans, plays a critical role in FGF-2 biology. FGF-2 binds heparan sulfate proteoglycans both in basement membrane and on the cell surface which allows growth factor storage and mediates binding to cell surface receptors. FGF-2 bound to heparin or heparan sulfate is also resistant to denaturation by acid or heat, attack by proteases such as thrombin or trypsin, as well as nonenzymatic glycation by high intracellular glucose (102, 103).

The pleiotropic nature of FGF-2 makes it important in diverse organ and biological processes, from the more obvious blood vessel genesis to more subtle neuronal development in brain and spermatogenesis in the reproductive system (97). In vasculature in particular, FGF-2 is critical to both angiogenesis and smooth muscle cell growth (104, 105). *In vivo*, FGF-2 administration has been demonstrated to initiate angiogenesis in the chick embryo, avascular mouse cornea, and subcutaneous Matrigel (106-108).

FGF-2 has varied effects on endothelial cells in particular, most of which relate to angiogenesis or vascular wound healing. When FGF-2 binds to its receptor, receptor autophosphorylation is followed by recruitment of adaptor molecules Shc, FRS2, and Crk which lead to MAP-kinase signaling pathway activation and proliferation (109). PKC is also needed for a full mitogenic response (110). Via the same MAP-kinase and PKC-dependent pathway, FGF-2 stimulates chemotaxis and migration of endothelial cells (111-113). In order for cells to proliferate and move, FGF-2 must alter cell to cell binding via cadherins and cell to matrix binding via integrins (114, 115). FGF-2 regulation of matrix – endothelial cell interaction is complex, as FGF-2 both promotes matrix destruction to initiate angiogenesis and later promotes matrix rebuilding to strengthen the new vessel (116). Finally, FGF-2 alters endothelial cell morphology and phenotype from a quiescent to an angiogenic state.

1.3.2 Binding and signaling

FGF-2 binds heparan sulfate proteoglycans both in basement membrane and on the cell surface, as well as specific cell surface FGF receptors. The basement membrane serves as an FGF-2 reservoir, storing FGF-2 released from endothelial cells, protecting it from degradation by coupling it to heparan sulfate proteoglycans, and then releasing it later for use by endothelial cells (72, 117). FGF-2 is released from storage in basement membrane by heparin, thrombin, and several other mechanisms, which may be natural pathways for FGF-2 to manifest its paracrine activity (71, 118).

FGF-2 cell surface binding sites include high affinity FGF receptors and low affinity heparan sulfate proteoglycans. The latter are in far greater abundance than the former, and together they create a complex means of local retention and release (119). There are four FGF receptor (FGFR) types, which increase in expression with an FGF-2 stimulus, but endothelial cells primarily express FGFR1 (120, 121). All FGF receptors have an N-terminal extracellular region with immunoglobulin-like domains, a single transmembrane domain, and an intracellular tyrosine kinase domain. Heparan sulfate proteoglycans, which bind both FGF-2 and FGF receptors, are required for maximal binding and internalization but receptors can still internalize FGF-2 without them (122).

When FGF-2 binds cell surface receptors and heparan sulfate proteoglycans, a receptor dimer is formed. Intracellular receptor tyrosine kinases are activated, resulting in tyrosine residue phosphorylation on receptors. These then serve as docking sites for adaptor proteins or signaling enzymes. In particular, FRS2 phosphorylation allows a signaling complex of Shp2, Grb2, and GAB1 to form. The FRS2 complex recruits guanidine nucleotide exchange factor SOS via Grb2, which in turn causes MAP-kinase pathway activation (123). MAP-kinase activation could take the form of Erk leading to proliferation or p38/JNK leading to an inflammatory or stress response (124). While p38/JNK activation is generally considered pro-apoptotic, this depends highly on cell type. Alternatively, FRS2 through GAB1 can activate Akt, providing an anti-apoptotic signal (125). Finally, receptor tyrosine residue phosphorylation can induce PLCγ to form DAG, activating PKC and calcium/calmodulin dependent protein kinases (125). FGF-2 can also translocate to the cell nucleus where it appears to initiate DNA synthesis and proliferation.

The differential intracellular response to a homogeneous extracellular FGF-2 signal may be mediated by binding modalities. While FGF-2 can be internalized on its receptor or heparan sulfate proteoglycan, it only stimulates DNA synthesis and proliferation when internalized with the FGF receptor (126). Alternatively, different receptor subtypes may result in variable downstream signaling. The presence or absence of intracellular secondary molecules could enhance or inactivate certain pathways, as could signaling molecule abundance. Finally, response of a particular cell to FGF-2 stimulation could be regulated at the transcriptional level.

1.3.3 Implications in diabetes

Both FGF-2 and VEGF have been implicated in dysregulated angiogenesis in diabetes, but with no consensus as to their absolute role. VEGF, which is upregulated in hypoxia, has been reported at abnormal levels in aberrant diabetic angiogenesis (45). Patients with proliferative diabetic retinopathy have elevated vitreous VEGF, whereas ocular VEGF is normal in controls and diabetic patients without retinopathy (46, 127). Renal VEGF and VEGF receptor are increased in rat diabetic models (128, 129). A VEGF antibody decreased hyperfiltration, albuminuria, and glomerular hypertrophy in streptozotocin-induced diabetic rats (130). Alternatively, decreased VEGF has been reported in both human and animal wounding models (131-133).

Data for FGF-2 are similar to VEGF but not as extensive. Patients with diabetic retinopathy have elevated FGF-2, and in type 2 diabetics, high plasma FGF-2 correlated with poor glycemic control, diabetic retinopathy, and albuminuria (134, 135). In contrast, gastric wounds in diabetic rats showed impaired healing which was ameliorated by exogenous FGF-2 addition (136). As FGF-2 binds to basement membrane and is stored there for long time periods, FGF-2 is potentially related to the diabetic memory effect.

1.4 Central Hypothesis

Questions abound regarding the vascular effects of diabetes. How can diabetes lead to vastly different vascular dysfunction across vascular beds? Why do effects from hyperglycemia persist long after euglycemia is restored? What role do growth factors

play in disordered angiogenesis? This thesis leverages loss of vascular homeostasis in diabetes to study the co-regulatory system of endothelial cells, basement membrane, and FGF-2. By perturbing the system with an environmental stress, we can uncover fundamental properties which govern complex interactions in the vessel wall.

This thesis investigates how external stress modulates vasoactive compound kinetics within the endothelial cell – basement membrane co-regulatory unit. We hypothesize that a physiologic glucose range mediates altered storage, release, and function of FGF-2 through changes in endothelial cell – basement membrane interaction. Understanding FGF-2 regulation by cells above and basement membrane below may shed additional light on diabetic vascular disease.

1.5 Thesis Organization

In support of these concepts, this thesis consists of a series of studies which attempt to elucidate the role of FGF-2 in glucose-induced vascular dysfunction, in particular as FGF-2 is cooperatively stored, released, and metabolized by endothelial cells and basement membrane. The specific aims of this work are:

- **Chapter 2:** Develop novel methods to examine FGF-2 binding kinetics with isolated basement membrane *in vitro*.
- **Chapter 3:** Investigate the glucose effect on basement membrane FGF-2 storage and binding kinetics (capacity, association, and dissociation).
- **Chapter 4:** Define the role of endothelial cells (FGF-2 release, apoptosis, and permeability) in controlling FGF-2 basement membrane storage with glucose.
- **Chapter 5:** Examine how FGF-2 released from basement membrane affects endothelial cell proliferation and survival with glucose.

This thesis provides both an improved understanding of unregulated vascular remodeling in diabetes and a clearer picture of interaction between endothelial cells and basement membrane in vascular homeostasis. These systematic studies lead towards a quantitative model of the endothelial cell-basement membrane co-regulatory unit, which in turn will help us develop novel biomaterials and basement membrane therapies.

1.6 Chapter References

1. Florey. 1966. The endothelial cell. *Br Med J* 5512:487-490.
2. Zimmet, P., Alberti, K., and Shaw, J. 2001. Global and societal implications of the diabetes epidemic. *Nature* 414:782-787.
3. King, H., Aubert, R.E., and Herman, W.H. 1998. Global burden of diabetes, 1995-2025 - Prevalence, numerical estimates, and projections. *Diabetes Care* 21:1414-1431.
4. Zimmet, P.Z. 1999. Diabetes epidemiology as a tool to trigger diabetes research and care. *Diabetologia* 42:499-518.
5. Cotran, R., Kumar, V., Collins, T., and Robbins, S. 1999. *Robbins Pathological Basis of Disease*: W. B. Saunders Company. 1425 pp.
6. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. 2002. *Molecular Biology of the Cell*: Garland. 1616 pp.
7. Ritz, E., and Orth, S.R. 1999. Nephropathy in patients with type 2 diabetes mellitus. *New England Journal Of Medicine* 341:1127-1133.
8. Beckman, J.A., Creager, M.A., and Libby, P. Diabetes and atherosclerosis - Epidemiology, pathophysiology, and management. *Jama-Journal Of The American Medical Association*:2570-2581.
9. Aiello, L.P., Gardner, T.W., King, G.L., Blankenship, G., Cavallerano, J.D., Ferris, F.L., and Klein, R. 1998. Diabetic retinopathy. *Diabetes Care* 21:143-156.
10. Falanga, V. 2005. Wound healing and its impairment in the diabetic foot. *Lancet* 366:1736-1743.
11. Shamoon, H., Duffy, H., Fleischer, N., Engel, S., Saenger, P., Strelzyn, M., Litwak, M., Wylierosett, J., Farkash, A., Geiger, D., et al. 1993. The Effect Of Intensive Treatment Of Diabetes On The Development And Progression Of Long-Term Complications In Insulin-Dependent Diabetes-Mellitus. *New England Journal Of Medicine* 329:977-986.
12. Jaffe, E.A., Nachman, R.L., Becker, C.G., and Minick, C.R. 1973. Culture Of Human Endothelial Cells Derived From Umbilical Veins - Identification By Morphologic And Immunological Criteria. *Journal Of Clinical Investigation* 52:2745-2756.
13. Michiels, C. 2003. Endothelial cell functions. *Journal Of Cellular Physiology* 196:430-443.
14. Inagami, T., Naruse, M., and Hoover, R. 1995. Endothelium - As An Endocrine Organ. *Annual Review Of Physiology* 57:171-189.
15. Pries, A.R., Secomb, T.W., and Gaehtgens, P. 2000. The endothelial surface layer. *Pflugers Archiv-European Journal Of Physiology* 440:653-666.
16. Dejana, E., Corada, M., and Lampugnani, M.G. 1995. Endothelial Cell-To-Cell Junctions. *Faseb Journal* 9:910-918.
17. Dejana, E. 2004. Endothelial cell-cell junctions: Happy together. *Nature Reviews Molecular Cell Biology* 5:261-270.
18. Nitta, T., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimoto, N., Furuse, M., and Tsukita, S. 2003. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *Journal Of Cell Biology* 161:653-660.

19. Matter, K., and Balda, M.S. 2003. Signalling to and from tight junctions. *Nature Reviews Molecular Cell Biology* 4:225-236.

20. Dejana, E., Bazzoni, G., and Lampugnani, M.G. 1999. Vascular endothelial (VE)-cadherin: Only an intercellular glue? *Experimental Cell Research* 252:13-19.

21. Kevil, C.G., Payne, D.K., Mire, E., and Alexander, J.S. 1998. Vascular permeability factor/vascular endothelial cell growth factor-mediated permeability occurs through disorganization of endothelial junctional proteins. *Journal Of Biological Chemistry* 273:15099-15103.

22. Bell, F.P., Adamson, I.L., and Schwartz, C.J. 1974. Aortic Endothelial Permeability To Albumin - Focal And Regional Patterns Of Uptake And Transmural Distribution Of I-131 Albumin In Young Pig. *Experimental And Molecular Pathology* 20:57-68.

23. Feldman, D.L., Hoff, H.F., and Gerrity, R.G. 1984. Immunohistochemical Localization Of Apoprotein-B In Aortas From Hyperlipemic Swine - Preferential Accumulation In Lesion-Prone Areas. *Archives Of Pathology & Laboratory Medicine* 108:817-822.

24. Wojciak-Stothard, B., Entwistle, A., Garg, R., and Ridley, A.J. 1998. Regulation of TNF-alpha-induced reorganization of the actin cytoskeleton and cell-cell junctions by Rho, Rac, and Cdc42 in human endothelial cells. *Journal Of Cellular Physiology* 176:150-165.

25. Carmeliet, P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nature Medicine* 6:389-395.

26. Liekens, S., De Clercq, E., and Neyts, J. 2001. Angiogenesis: regulators and clinical applications. *Biochemical Pharmacology* 61:253-270.

27. Furchgott, R.F., and Zawadzki, J.V. 1980. The Obligatory Role Of Endothelial-Cells In The Relaxation Of Arterial Smooth-Muscle By Acetylcholine. *Nature* 288:373-376.

28. Palmer, R.M.J., Ferrige, A.G., and Moncada, S. 1987. Nitric-Oxide Release Accounts For The Biological-Activity Of Endothelium-Derived Relaxing Factor. *Nature* 327:524-526.

29. Topper, J.N., Cai, J.X., Falb, D., and Gimbrone, M.A. 1996. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: Cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 93:10417-10422.

30. Muller, W.A. 2002. Leukocyte-endothelial cell interactions in the inflammatory response. *Laboratory Investigation* 82:521-533.

31. Ley, K. 2002. Integration of inflammatory signals by rolling neutrophils. *Immunological Reviews* 186:8-18.

32. Scholz, D., Devaux, B., Hirche, A., Potzsch, B., Kropp, B., Schaper, W., and Schaper, J. 1996. Expression of adhesion molecules is specific and time-dependent in cytokine-stimulated endothelial cells in culture. *Cell And Tissue Research* 284:415-423.

33. Nathan, C. 2002. Points of control in inflammation. *Nature* 420:846-852.

34. Cines, D.B., Pollak, E.S., Buck, C.A., Loscalzo, J., Zimmerman, G.A., McEver, R.P., Pober, J.S., Wick, T.M., Konkle, B.A., Schwartz, B.S., et al. 1998. Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* 91:3527-3561.

35. Zimmerman, G.A., McIntyre, T.M., Mehra, M., and Prescott, S.M. 1990. Endothelial Cell-Associated Platelet-Activating Factor - A Novel Mechanism For Signaling Intercellular-Adhesion. *Journal Of Cell Biology* 110:529-540.

36. Denis, C.V. 2002. Molecular and cellular biology of von Willebrand factor. *International Journal Of Hematology* 75:3-8.

37. Mackman, N. 1997. Regulation of the tissue factor gene. *Thrombosis And Haemostasis* 78:747-754.

38. Alpert, E., Gruzman, A., Riahi, Y., Blejter, R., Aharoni, P., Weisinger, G., Eckel, J., Kaiser, N., and Sasson, S. 2005. Delayed autoregulation of glucose transport in vascular endothelial cells. *Diabetologia* 48:752-755.

39. Murata, T., Ishibashi, T., Khalil, A., Hata, Y., Yoshikawa, H., and Inomata, H. 1995. Vascular Endothelial Growth-Factor Plays A Role In Hyperpermeability Of Diabetic Retinal-Vessels. *Ophthalmic Research* 27:48-52.

40. Laight, D.W., Carrier, M.J., and Anggard, E.E. 1999. Endothelial cell dysfunction and the pathogenesis of diabetic macroangiopathy. *Diabetes-Metabolism Research And Reviews* 15:274-282.

41. Haller, H. 1997. Endothelial function - General considerations. *Drugs* 53:1-10.

42. Lorenzi, M., Cagliero, E., and Toledo, S. 1985. Glucose Toxicity For Human-Endothelial Cells In Culture - Delayed Replication, Disturbed Cell-Cycle, And Accelerated Death. *Diabetes* 34:621-627.

43. Baumgartner-Parzer, S.M., Wagner, L., Pettermann, M., Grillari, J., Gessl, A., and Waldhausl, W. 1995. High-Glucose-Triggered Apoptosis In Cultured Endothelial-Cells. *Diabetes* 44:1323-1327.

44. Tsilibary, E. 2003. Microvascular basement membranes in diabetes mellitus. *J Pathol* 200:537-547.

45. Martin, A., Komada, M.R., and Sane, D.C. Abnormal angiogenesis in diabetes mellitus. *Medicinal Research Reviews*:117-145.

46. Duh, E., and Aiello, L.P. Vascular endothelial growth factor and diabetes - The agonist versus antagonist paradox. *Diabetes*:1899-1906.

47. Tilton, R.G., Chang, K., Pugliese, G., Eades, D.M., Province, M.A., Sherman, W.R., Kilo, C., and Williamson, J.R. 1989. Prevention Of Hemodynamic And Vascular Albumin Filtration Changes In Diabetic Rats By Aldose Reductase Inhibitors. *Diabetes* 38:1258-1270.

48. Ishii, H., Jirousek, M.R., Koya, D., Takagi, C., Xia, P., Clermont, A., Bursell, S.E., Kern, T.S., Ballas, L.M., Heath, W.F., et al. 1996. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science* 272:728-731.

49. Hovind, P., Tarnow, L., Oestergaard, P.B., and Parving, H.H. 2000. Elevated vascular endothelial growth factor in type 1 diabetic patients with diabetic nephropathy. *Kidney International* 57:S56-S61.

50. Veelken, R., Hilgers, K.F., Hartner, A., Haas, A., Bohmer, K.P., and Sterzel, R.B. 2000. Nitric oxide synthase isoforms and glomerular hyperfiltration in early diabetic nephropathy. *Journal Of The American Society Of Nephrology* 11:71-79.

51. Hopfner, R.L., and Gopalakrishnan, V. 1999. Endothelin: emerging role in diabetic vascular complications. *Diabetologia* 42:1383-1394.

52. Chakravarthy, U., Hayes, R.G., Stitt, A.W., McAuley, E., and Archer, D.B. 1998. Constitutive nitric oxide synthase expression in retinal vascular endothelial cells is suppressed by high glucose and advanced glycation end products. *Diabetes* 47:945-952.

53. Park, J.Y., Takahara, N., Gabriele, A., Chou, E., Naruse, K., Suzuma, K., Yamauchi, T., Ha, S.W., Meier, M., Rhodes, C.J., et al. 2000. Induction of endothelin-1 expression by glucose - An effect of protein kinase C activation. *Diabetes* 49:1239-1248.

54. Morigi, M., Angioletti, S., Imberti, B., Donadelli, R., Micheletti, G., Figliuzzi, M., Remuzzi, A., Zoja, C., and Remuzzi, G. 1998. Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-kappa B-dependent fashion. *Journal Of Clinical Investigation* 101:1905-1915.

55. Meigs, J.B., Mittleman, M.A., Nathan, D.M., Tofler, G.H., Singer, D.E., Murphy-Sheehy, P.M., Lipinsky, I., D'Angostino, R., and Wilson, P.W.F. 2000. Hyperinsulinemia, hyperglucemia, and impaired hemostasis - The Framingham offspring study. *Jama-Journal Of The American Medical Association* 283:221-228.

56. Brownlee, M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820.

57. Greene, D.A., Arezzo, J.C., and Brown, M.B. 1999. Effect of aldose reductase inhibition on nerve conduction and morphometry in diabetic neuropathy. *Neurology* 53:580-591.

58. Engerman, R.L., Kern, T.S., and Larson, M.E. 1994. Nerve-Conduction And Aldose Reductase Inhibition During 5 Years Of Diabetes Or Galactosemia In Dogs. *Diabetologia* 37:141-144.

59. Degenhardt, T.P., Thorpe, S.R., and Baynes, J.W. 1998. Chemical modification of proteins by methylglyoxal. *Cellular And Molecular Biology* 44:1139-1145.

60. Nakamura, S., Makita, Z., Ishikawa, S., Yasumura, K., Fujii, W., Yanagisawa, K., Kawata, T., and Koike, T. 1997. Progression of nephropathy in spontaneous diabetic rats is prevented by OPB-9195, a novel inhibitor of advanced glycation. *Diabetes* 46:895-899.

61. Hammes, H.P., Martin, S., Federlin, K., Geisen, K., and Brownlee, M. 1991. Aminoguanidine Treatment Inhibits The Development Of Experimental Diabetic-Retinopathy. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 88:11555-11558.

62. Koya, D., and King, G.L. 1998. Protein kinase C activation and the development of diabetic complications. *Diabetes* 47:859-866.

63. Hempel, A., Maasch, C., Heintze, U., Lindschau, C., Dietz, R., Luft, F.C., and Haller, H. 1997. High glucose concentrations increase endothelial cell permeability via activation of protein kinase C alpha. *Circulation Research* 81:363-371.

64. Koya, D., Jirousek, M.R., Lin, Y.W., Ishii, H., Kuboki, K., and King, G.L. 1997. Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *Journal Of Clinical Investigation* 100:115-126.

65. Koya, D., Haneda, M., Nakagawa, H., Isshiki, K., Sato, H., Maeda, S., Sugimoto, T., Yasuda, H., Kashiwagi, A., Ways, D.K., et al. 2000. Amelioration of accelerated diabetic

mesangial expansion by treatment with a PKC beta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. *Faseb Journal* 14:439-447.

66. Nishikawa, T., Edelstein, D., Du, X.L., Yamagishi, S., Matsumura, T., Kaneda, Y., Yorek, M.A., Beebe, D., Oates, P.J., Hammes, H.P., et al. 2000. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787-790.

67. Korshunov, S.S., Skulachev, V.P., and Starkov, A.A. 1997. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *Febs Letters* 416:15-18.

68. Danen, E.H.J., and Yamada, K.M. 2001. Fibronectin, integrins, and growth control. *Journal Of Cellular Physiology* 189:1-13.

69. Giancotti, F.G., and Ruoslahti, E. 1999. Transduction - Integrin signaling. *Science* 285:1028-1032.

70. Bashkin, P., Doctor, S., Klagsbrun, M., Svahn, C.M., Folkman, J., Vlodavsky, I. 1989. Basic Fibroblast Growth Factor Binds to Subendothelial Extracellular Matrix and Is Released by Heparitinase and Heparin-like Molecules. *Biochemistry* 28:1737-1743.

71. Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., Klagsbrun, M. 1987. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *PNAS* 84:2292-2296.

72. Gibbs, R.V. 2003. Cytokines and glycosaminoglycans (GAGs). In *Glycobiology And Medicine*. 125-143.

73. Park, J.E., Keller, G.A., and Ferrara, N. 1993. Vascular Endothelial Growth-Factor (Vegf) Isoforms - Differential Deposition Into The Subepithelial Extracellular-Matrix And Bioactivity Of Extracellular Matrix-Bound Vegf. *Molecular Biology Of The Cell* 4:1317-1326.

74. Kalluri, R. 2003. Basement membranes: structure, assembly, and role in tumour angiogenesis. *Nature Reviews: Cancer* 3:422-433.

75. Stamenkovic, F.T.B.a.I. 2003. Functional structure and composition of the extracellular matrix. *Journal of Pathology* 200:423-428.

76. Fukui, M., Nakamura, T., Ebihara, I., Shirato, I., Tomino, Y., and Koide, H. 1992. Ecm Gene-Expression And Its Modulation By Insulin In Diabetic Rats. *Diabetes* 41:1520-1527.

77. Pugliese, G., Pricci, F., Romeo, G., Pugliese, F., Mene, P., Giannini, S., Cresci, B., Galli, G., Rotella, C.M., Vlassara, H., et al. 1997. Upregulation of mesangial growth factor and extracellular matrix synthesis by advanced glycation end products via a receptor-mediated mechanism. *Diabetes* 46:1881-1887.

78. Reckelhoff, J.F., Tygart, V.L., Mitias, M.M., and Walcott, J.L. 1993. Stz-Induced Diabetes Results In Decreased Activity Of Glomerular Cathepsin And Metalloprotease In Rats. *Diabetes* 42:1425-1432.

79. Roy, S., Cagliero, E., and Lorenzi, M. 1996. Fibronectin overexpression in retinal microvessels of patients with diabetes. *Investigative Ophthalmology & Visual Science* 37:258-266.

80. Cohen, M.P., and Khalifa, A. 1977. Renal Glomerular Collagen-Synthesis In Streptozotocin Diabetes - Reversal Of Increased Basement-Membrane Synthesis With Insulin Therapy. *Biochimica Et Biophysica Acta* 500:395-404.

81. Shimomura, H., and Spiro, R.G. 1987. Studies On Macromolecular Components Of Human Glomerular-Basement-Membrane And Alterations In Diabetes - Decreased Levels Of Heparan-Sulfate Proteoglycan And Laminin. *Diabetes* 36:374-381.

82. Bangstad, H.J., Osterby, R., Dahljorgensen, K., Berg, K.J., Hartmann, A., and Hanssen, K.F. Improvement Of Blood-Glucose Control In Iddm Patients Retards The Progression Of Morphological-Changes In Early Diabetic Nephropathy. *Diabetologia*:483-490.

83. Cagliero, E., Roth, T., Roy, S., and Lorenzi, M. Characteristics And Mechanisms Of High-Glucose Induced Overexpression Of Basement-Membrane Components In Cultured Human Endothelial-Cells. *Diabetes*:102-110.

84. Haitoglou, C.S., Tsilibary, E.C., Brownlee, M., and Charonis, A.S. 1992. Altered Cellular Interactions Between Endothelial-Cells And Nonenzymatically Glucosylated Laminin Type-Iv Collagen. *Journal Of Biological Chemistry* 267:12404-12407.

85. Charonis, A.S., Reger, L.A., Dege, J.E., Kouzikoliakos, K., Furcht, L.T., Wohlhueter, R.M., and Tsilibary, E.C. 1990. Laminin Alterations After Invitro Nonenzymatic Glycosylation. *Diabetes* 39:807-814.

86. Tsilibary, E.C., Charonis, A.S., Reger, L.A., Wohlhueter, R.M., and Furcht, L.T. 1988. The Effect Of Nonenzymatic Glucosylation On The Binding Of The Main Noncollagenous Nc1 Domain To Type-Iv Collagen. *Journal Of Biological Chemistry* 263:4302-4308.

87. Kowluru, R.A. 2003. Effect of reinstitution of good glycemic control on retinal oxidative stress and nitrative stress in diabetic rats. *Diabetes* 52:818-823.

88. Engerman, R.L., and Kern, T.S. 1987. Progression Of Incipient Diabetic-Retinopathy During Good Glycemic Control. *Diabetes* 36:808-812.

89. Kador, P.F., Takahashi, Y., Akagi, Y., Neuenschwander, H., Greentree, W., Lackner, P., Blessing, K., and Wyman, M. 2002. Effect of galactose diet removal on the progression of retinal vessel changes in galactose-fed dogs. *Investigative Ophthalmology & Visual Science* 43:1916-1921.

90. Shannon, H., Duffy, H., Dahms, W., Mayer, L., Brillion, D., Lackaye, M., Whitehouse, F., Kruger, D., Bergenstal, R., Johnson, M., et al. 2000. Retinopathy and nephropathy in patients with type 1 diabetes four years after a trial of intensive therapy. *New England Journal Of Medicine* 342:381-389.

91. Steffes, M.W., Chavers, B.M., Molitch, M.E., Cleary, P.A., Lachin, J.M., Genuth, S., Nathan, D.M., Genuth, S., Nathan, D., Engel, S., et al. 2003. Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy - The Epidemiology of Diabetes Interventions and Complications (EDIC) study. *Jama-Journal Of The American Medical Association* 290:2159-2167.

92. Brownlee, M. 1992. Glycation Products And The Pathogenesis Of Diabetic Complications. *Diabetes Care* 15:1835-1843.

93. Takenaka, K., Yamagishi, S., Matsui, T., Nakamura, K., and Imaizumi, T. 2006. Role of Advanced Glycation End Products (AGEs) in Thrombogenic Abnormalities in Diabetes. *Curr Neurovasc Res* 3:73-77.

94. Bohlen, P., Baird, A., Esch, F., Ling, N., and Gospodarowicz, D. 1984. Isolation And Partial Molecular Characterization Of Pituitary Fibroblast Growth-Factor. *Proceedings Of The National Academy Of Sciences Of The United States Of America-Biological Sciences* 81:5364-5368.

95. Prats, H., Kaghad, M., Prats, A.C., Klagsbrun, M., Lelias, J.M., Liauzun, P., Chalon, P., Tauber, J.P., Amalric, F., Smith, J.A., et al. 1989. High Molecular Mass Forms Of Basic Fibroblast Growth-Factor Are Initiated By Alternative Cug Codons. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 86:1836-1840.

96. Klagsbrun, M., Smith, S., Sullivan, R., Shing, Y., Davidson, S., Smith, J.A., and Sasse, J. 1987. Multiple Forms Of Basic Fibroblast Growth-Factor - Amino-Terminal Cleavages By Tumor Cell-Derived And Brain Cell-Derived Acid Proteinases. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 84:1839-1843.

97. Bikfalvi, A., Klein, S., Pintucci, G., and Rifkin, D.B. 1997. Biological roles of fibroblast growth factor-2. *Endocrine Reviews* 18:26-45.

98. Nugent, M.A., and Iozzo, R.V. 2000. Fibroblast growth factor-2. *International Journal Of Biochemistry & Cell Biology* 32:115-120.

99. Mignatti, P., Morimoto, T., and Rifkin, D.B. 1992. Basic Fibroblast Growth-Factor, A Protein Devoid Of Secretory Signal Sequence, Is Released By Cells Via A Pathway Independent Of The Endoplasmic-Reticulum Golgi-Complex. *Journal Of Cellular Physiology* 151:81-93.

100. Brooks, R.A., Burrin, J.M., and Kohner, E.M. 1991. Characterization Of Release Of Basic Fibroblast Growth-Factor From Bovine Retinal Endothelial-Cells In Monolayer-Cultures. *Biochemical Journal* 276:113-120.

101. Muthukrishnan, L., Warder, E., and McNeil, P.L. 1991. Basic Fibroblast Growth-Factor Is Efficiently Released From A Cytolsolic Storage Site Through Plasma-Membrane Disruptions Of Endothelial-Cells. *Journal Of Cellular Physiology* 148:1-16.

102. Sommer, A., and Rifkin, D.B. 1989. Interaction Of Heparin With Human Basic Fibroblast Growth-Factor - Protection Of The Angiogenic Protein From Proteolytic Degradation By A Glycosaminoglycan. *Journal Of Cellular Physiology* 138:215-220.

103. Nissen, N.N., Shankar, R., Gamelli, R.L., Singh, A., and DiPietro, L.A. 1999. Heparin and heparan sulphate protect basic fibroblast growth factor from non-enzymic glycosylation. *Biochemical Journal* 338:637-642.

104. Lindner, V., and Reidy, M.A. 1991. Proliferation Of Smooth-Muscle Cells After Vascular Injury Is Inhibited By An Antibody Against Basic Fibroblast Growth-Factor. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 88:3739-3743.

105. Presta, M., Dell'Era, P., Mitola, S., Moroni, E., Ronca, R., and Rusnati, M. 2005. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine & Growth Factor Reviews* 16:159-178.

106. Ribatti, D., Urbinati, C., Nico, B., Rusnati, M., Roncali, L., and Presta, M. 1995. Endogenous Basic Fibroblast Growth-Factor Is Implicated In The Vascularization Of The Chick-Embryo Chorioallantoic Membrane. *Developmental Biology* 170:39-49.

107. Seghezzi, G., Patel, S., Ren, C.J., Gualandris, A., Pintucci, G., Robbins, E.S., Shapiro, R.L., Galloway, A.C., Rifkin, D.B., and Mignatti, P. 1998. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial

cells of forming capillaries: An autocrine mechanism contributing to angiogenesis. *Journal Of Cell Biology* 141:1659-1673.

108. Passaniti, A., Taylor, R.M., Pili, R., Guo, Y., Long, P.V., Haney, J.A., Pauly, R.R., Grant, D.S., and Martin, G.R. 1992. Methods In Laboratory Investigation - A Simple, Quantitative Method For Assessing Angiogenesis And Antiangiogenic Agents Using Reconstituted Basement-Membrane, Heparin, And Fibroblast Growth-Factor. *Laboratory Investigation* 67:519-528.

109. Cross, M.J., and Claesson-Welsh, L. 2001. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends In Pharmacological Sciences* 22:201-207.

110. Presta, M., Tiberio, L., Rusnati, M., Dellera, P., and Ragnotti, G. 1991. Basic Fibroblast Growth-Factor Requires A Long-Lasting Activation Of Protein-Kinase-C To Induce Cell-Proliferation In Transformed Fetal Bovine Aortic Endothelial-Cells. *Cell Regulation* 2:719-726.

111. Terranova, V.P., Diflorio, R., Lyall, R.M., Hic, S., Friesel, R., and Maciag, T. 1985. Human-Endothelial Cells Are Chemotactic To Endothelial-Cell Growth-Factor And Heparin. *Journal Of Cell Biology* 101:2330-2334.

112. Shono, T., Kanetake, H., and Kanda, S. 2001. The role of mitogen-activated protein kinase activation within focal adhesions in chemotaxis toward FGF-2 by murine brain capillary endothelial cells. *Experimental Cell Research* 264:275-283.

113. Daviet, I., Herbert, J.M., and Maffrand, J.P. 1990. Involvement Of Protein Kinase-C In The Mitogenic And Chemotaxis Effects Of Basic Fibroblast Growth-Factor On Bovine Cerebral-Cortex Capillary Endothelial-Cells. *Febs Letters* 259:315-317.

114. Klein, S., Giancotti, F.G., Presta, M., Albelda, S.M., Buck, C.A., and Rifkin, D.B. 1993. Basic Fibroblast Growth-Factor Modulates Integrin Expression In Microvascular Endothelial-Cells. *Molecular Biology Of The Cell* 4:973-982.

115. Yang, S., Graham, J., Kahn, J.W., Schwartz, E.A., and Gerritsen, M.E. 1999. Functional roles for PECAM-1 (CD31) and VE-cadherin (CD144) in tube assembly and lumen formation in three-dimensional collagen gels. *American Journal Of Pathology* 155:887-895.

116. Mignatti, P., and Rifkin, D.B. 2000. Nonenzymatic interactions between proteinases and the cell surface: Novel roles in normal and malignant cell physiology. In *Advances In Cancer Research, Vol 78*. 103-157.

117. Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D., and Vlodavsky, I. 1988. A Heparin-Binding Angiogenic Protein - Basic Fibroblast Growth-Factor - Is Stored Within Basement-Membrane. *American Journal Of Pathology* 130:393-400.

118. Benezra, M., Vlodavsky, I., Ishaimichaeli, R., Neufeld, G., and Barshavit, R. 1993. Thrombin-Induced Release Of Active Basic Fibroblast Growth Factor-Heparan Sulfate Complexes From Subendothelial Extracellular-Matrix. *Blood* 81:3324-3331.

119. Nugent, M.A., and Edelman, E.R. 1992. Kinetics Of Basic Fibroblast Growth-Factor Binding To Its Receptor And Heparan-Sulfate Proteoglycan - A Mechanism For Cooperativity. *Biochemistry* 31:8876-8883.

120. Bastaki, M., Nelli, E.E., DellEra, P., Rusnati, M., MolinariTosatti, M.P., Parolini, S., Auerbach, R., Ruco, L.P., Possati, L., and Presta, M. 1997. Basic fibroblast growth factor-induced angiogenic phenotype in mouse endothelium - A study of aortic and

microvascular endothelial cell lines. *Arteriosclerosis Thrombosis And Vascular Biology* 17:454-464.

121. Javerzat, S., Auguste, P., and Bikfalvi, A. 2002. The role of fibroblast growth factors in vascular development. *Trends In Molecular Medicine* 8:483-489.

122. Fannon, M., and Nugent, M.A. 1996. Basic fibroblast growth factor binds its receptors, is internalized, and stimulates DNA synthesis in Balb/c3T3 cells in the absence of heparan sulfate. *Journal Of Biological Chemistry* 271:17949-17956.

123. Kouhara, H., Hadari, Y.R., SpivakKroizman, T., Schilling, J., BarSagi, D., Lax, I., and Schlessinger, J. 1997. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell* 89:693-702.

124. Johnson, G. 2002. Signal transduction - Scaffolding proteins - More than meets the eye. *Science* 295:1249-1250.

125. Schlessinger, J. 2000. Cell signaling by receptor tyrosine kinases. *Cell* 103:211-225.

126. Quarto, N., and Amalric, F. 1994. Heparan-Sulfate Proteoglycans As Transducers Of Fgf-2 Signaling. *Journal Of Cell Science* 107:3201-3212.

127. Aiello, L.P., Avery, R.L., Arrigg, P.G., Keyt, B.A., Jampel, H.D., Shah, S.T., Pasquale, L.R., Thieme, H., Iwamoto, M.A., Park, J.E., et al. 1994. Vascular Endothelial Growth-Factor In Ocular Fluid Of Patients With Diabetic-Retinopathy And Other Retinal Disorders. *New England Journal Of Medicine* 331:1480-1487.

128. Cooper, M.E., Vranes, D., Youssef, S., Stacker, S.A., Cox, A.J., Rizkalla, B., Casley, D.J., Bach, L.A., Kelly, D.J., and Gilbert, R.E. 1999. Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes. *Diabetes* 48:2229-2239.

129. Tsuchida, K., Makita, Z., Yamagishi, S., Atsumi, T., Miyoshi, H., Obara, S., Ishida, M., Ishikawa, S., Yasumura, K., and Koike, T. 1999. Suppression of transforming growth factor beta and vascular endothelial growth factor in diabetic nephropathy in rats by a novel advanced glycation end product inhibitor, OPB-9195. *Diabetologia* 42:579-588.

130. De Vriese, A., Tilton, R.G., Elger, M., Stephan, C.C., Kriz, W., and Lameire, N.H. 2001. Antibodies against vascular endothelial growth factor improve early renal dysfunction in experimental diabetes. *Journal Of The American Society Of Nephrology* 12:993-1000.

131. Kampfer, H., Pfeilschifter, J., and Frank, S. 2001. Expressional regulation of angiopoietin-1 and-2 and the tie-1 and-2 receptor tyrosine kinases during cutaneous wound healing: A comparative study of normal and impaired repair. *Laboratory Investigation* 81:361-373.

132. Frank, S., Hubner, G., Breier, G., Longaker, M.T., Greenhalgh, D.G., and Werner, S. 1995. Regulation Of Vascular Endothelial Growth-Factor Expression In Cultured Keratinocytes - Implications For Normal And Impaired Wound-Healing. *Journal Of Biological Chemistry* 270:12607-12613.

133. Bitar, M.S., and Labbad, Z.N. 1996. Transforming growth factor-beta and insulin-like growth factor-1 in relation to diabetes-induced impairment of wound healing. *Journal Of Surgical Research* 61:113-119.

134. Zimering, M.B., and Eng, J. 1996. Increased basic fibroblast growth factor-like substance in plasma from a subset of middle-aged or elderly male diabetic patients with microalbuminuria or proteinuria. *Journal Of Clinical Endocrinology And Metabolism* 81:4446-4452.

135. Schultz, G.S., and Grant, M.B. 1991. Neovascular growth factors. *Eye* 5 (Pt 2):170-180.

136. Takeuchi, K., Takehara, K., Tajima, K., Kato, S., and Hirata, T. 1997. Impaired healing of gastric lesions in streptozotocin-induced diabetic rats: Effect of basic fibroblast growth factor. *Journal Of Pharmacology And Experimental Therapeutics* 281:200-207.

CHAPTER 2

MEASURING GROWTH FACTORS IN THE BASEMENT MEMBRANE

Abstract

Investigation of the endothelial cell – basement membrane unit begins with development of an interaction model. Porcine aortic endothelial cells were selected for the *in vitro* hyperglycemic model because they retain their phenotype in culture up to 30 mM glucose and produce a basement membrane that remains adherent to tissue culture plates. While binding kinetics protocols designed for endothelial cells were robust when applied to basement membrane, results were confounded by FGF-2 binding to tissue culture polystyrene in a manner strikingly similar to basement membrane. A technique was developed to account for polystyrene FGF-2 binding by subtracting FGF-2 bound to exposed polystyrene from basement membrane experiments, and the method was validated using bacteriologic plates. The validated *in vitro* hyperglycemic cell culture model created in this chapter provides the basis for the remainder of the thesis.

2.1 Introduction

Endothelial cells, due to their unique role in controlling vascular homeostasis, have been implicated in diabetic vascular complications. For this reason, endothelial cell reaction to high glucose has been extensively studied (40-43, 45, 56). However, *in vitro* hyperglycemic cell culture systems use a wide range of glucose levels, include or omit insulin, and evaluate a variety of effects in and on endothelial cells from different species and vascular beds. This diversity highlights the importance of carefully constructing a hyperglycemic culture model appropriate for a given cell type so glucose effects can be studied while maintaining otherwise healthy cells.

Beyond hyperglycemic endothelial cell culture itself, the cell culture model must produce a basement membrane that remains attached to tissue culture plates when endothelial cells are removed. Similar to endothelial cell culture in high glucose, endothelial cell basement membrane has been isolated using a variety of cell lysis techniques on a range of endothelial cell types (137, 138). As corneal and porcine aortic endothelial cells are reported to produce the highest quality basement membrane in culture, we selected porcine aortic cells as the basis of our cell culture model. We tested different published and novel cell removal methods to isolate a basement membrane closest to that on which endothelial cells naturally reside.

Vlodavsky et al. first showed that endothelial cell basement membrane is a potential storage site for growth factors (72). Specifically, basement membrane heparan sulfate proteoglycans bind and release fibroblast growth factor-2 (FGF-2) produced by endothelial cells. While previous studies on basement membrane and growth factors examined binding sites, release mechanisms, and transport through tissues, none examined the unique growth factor binding kinetics to basement membrane binding sites (71, 118, 139). We now demonstrate new methods for measuring basement membrane FGF-2 binding kinetics, based on proven techniques used to study endothelial cell FGF-2 binding kinetics.

This chapter details logistics of establishing a new cell culture model, validates a method for isolating basement membrane from that model, and identifies techniques

and challenges involved in studying basement membrane FGF-2 binding kinetics *in vitro*. These initial experiments lay the groundwork for the remainder of the thesis.

2.2 Materials and Methods

2.2.1 Cell isolation and culture

Porcine aortic endothelial cells (PAEC) were isolated from porcine aortae by the collagenase dispersion method and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2% glutamine (140). All cell culture reagents, unless otherwise specified, were from Gibco. Culture media was changed every 48 hours, and cells were used between passages four and nine. High glucose media was produced by adding D-glucose to supplemented low glucose DMEM (5 mM, 90 mg/dL) to a final 30 mM concentration (530 mg/dL). For osmotic controls, mannitol or L-glucose was added to base media to achieve a 30 mM osmolar solution. To culture cells for basement membrane, PAEC were seeded near confluence and grown for four days in multiwell tissue culture polystyrene plates (6, 12, 24, or 96 well; Corning). 4% w/v 40 kDa dextran (Sigma) was added to media for the last two days to increase cellular basement membrane production (141).

2.2.2 Fluorescent microscopy

PAEC cultured on cover slips were washed with PBS and fixed in 4% w/v paraformaldehyde (pH 7.4; Sigma). Fixed cells were then thoroughly washed in PBS to remove residual paraformaldehyde, after which cells were incubated with a primary antibody to platelet endothelial cell adhesion molecule (Pecam) (1:100, MCA1746, Serotec) for 60 minutes at room temperature. After three phosphate buffered saline (PBS) washes, fixed cells were incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (1:100, A11017, Molecular Probes) with Hoescht nuclear stain (1:1000) for 60 minutes at room temperature. Dil-Ac-LDL, acetylated low density lipoprotein labeled with 1,1'-dioctadecyl – 3,3,3',3'-tetramethyl-indocarbocyanine perchlorate, was used to phenotypically label endothelial cells. Lysosomal enzymes

found specifically in vascular endothelial cells degrade DiI-Ac-LDL, and the DiI fluorescent probe accumulates in intracellular membranes, whereas other vascular cells (fibroblasts, smooth muscle, pericytes, epithelial cells) are not labeled (142). DiI-Ac-LDL (10 μg/ml; Biomedical Technologies) was added to PAEC in culture for four hours, after which cells were fixed as described previously. Cover slips were mounted onto microscope slides with 1:1 glycerol-PBS and stored at 4 °C.

2.2.3 Cell removal methods

After four days in culture, PAEC were removed by four different methods to determine which best produced an intact basement membrane free from cellular debris. The first two methods used a combination of detergent Triton X-100 and base ammonium hydroxide (NH_4OH) to lyse cells and remove them from basement membrane (143). The second method additionally included Complete Protease Inhibitor (Roche) and DNAse (Invitrogen) to ensure that basement membrane proteins were not degraded in the lysis process and that any remaining DNA was removed from basement membrane. The third tested method used 4% sodium deoxycholate as an alternative detergent treatment. The last method tested whether cell lysis with water alone was adequate to isolate basement membrane. Isolated basement membrane was stored at 4 °C in sterile PBS for up to one week.

2.2.4 Sample preparation for scanning electron microscopy

PAEC were cultured on glass cover slips (VWR) as described previously in preparation for scanning electron microscopy (SEM). After basement membrane isolation, samples were dehydrated in solutions of 70%, 80%, 90%, and three times 100% ethanol for 10 to 30 minutes each. Samples were then transferred to 50% ethanol – 50% hexamethyldisilazane (HMDS, Sigma) for thirty minutes, followed by two times 100% HMDS. HMDS was evaporated overnight in a fume hood, and basement membrane samples were stored in a desiccator for a minimum of three days to ensure complete dehydration. HMDS, with its low surface tension and protein cross-linking properties, is as effective at preserving biological SEM sample structure as critical point drying with no required equipment and lower time and cost (144, 145). After drying,

samples were mounted on stubs and coated with 20 nm gold to allow electron conduction during imaging. Samples were examined with a JEOL 5910 SEM.

2.2.5 Basement membrane extraction and measurement

After thorough PBS washing of each sample, basement membrane FGF-2 was extracted using a salt buffer (2 M NaCl, 20 mM Hepes, pH 7.4) for 10 minutes with gentle shaking (146). This incubation was deemed sufficient after subsequent extractions failed to produce additional FGF-2. Basement membrane FGF-2 was quantified using an FGF ELISA (R&D Systems). Extraction buffer alone did not alter FGF ELISA accuracy. Since each ELISA varied slightly, and FGF-2 degraded quickly in salt buffer, FGF-2 for each experiment was quantified immediately using a single ELISA whenever possible.

2.2.6 FGF-2 binding kinetics

FGF-2 basement membrane binding kinetics were evaluated using a modification of the method of Nugent and Edelman for FGF-2 binding kinetics to endothelial cells (119). To determine FGF-2 equilibrium binding capacity, recombinant human FGF-2 (Peprotech) in binding buffer (25 mM Hepes, 0.05% w/v gelatin, pH 7.4; Sigma) was added to isolated basement membrane at concentrations from 0 to 1 μg/ml. Equilibrium, defined as time at which association and dissociation occur at equal rates resulting in no change in FGF-2 bound to basement membrane, occurred approximately three hours after growth factor addition. The FGF-2 solution was aspirated, basement membrane washed quickly in binding buffer to remove unbound FGF-2, and bound FGF-2 extracted as previously described. Basement membrane FGF-2 association was measured by adding 5 ng/ml FGF-2 in binding buffer to isolated basement membrane for 0 to 360 minutes. This concentration (5 ng/ml) is well within the linear binding range and results in physiologically relevant bound FGF-2 levels. After the incubation period, FGF-2 was aspirated, basement membrane washed quickly in binding buffer, and bound FGF-2 extracted as described above.

FGF-2 basement membrane dissociation kinetics were determined by incubating isolated basement membrane with 5 ng/ml ^{125}I-FGF-2 (Perkin Elmer) in binding buffer to

equilibrium (3 hours). The ^{125}I-FGF-2 solution was removed, followed by three quick washes in binding buffer. Binding buffer containing unlabeled FGF-2 (1 μg/ml) was added to each well for 0 to 360 minutes. Unlabeled FGF-2 was included in dissociation buffer to decrease rebinding of released ^{125}I-FGF-2 to basement membrane. After the dissociation period, dissociation buffer was removed and basement membrane bound ^{125}I-FGF-2 was extracted. ^{125}I-FGF-2 in dissociation buffer and salt extraction buffer was quantified in a gamma counter (Packard).

2.2.7 Statistics

All statistical analyses were performed with Prism software (Graphpad). Data were normally distributed and expressed as mean ± standard deviation. Comparisons between two groups were analyzed by Student's t test, and comparisons between more than two groups were analyzed by ANOVA. A value of $p < 0.05$ was considered statistically significant and is indicated in the text as such or in figures with a pound sign (#). A value of $p < .01$ is indicated with an asterisk (*). If no statistical significance is reported, none was observed.

2.3 Results

2.3.1 Hyperglycemic cell culture model

PAEC maintained uniform DiI-Ac-LDL labeling at all glucose levels, indicating retention of their unique phenotype throughout various glucose conditions (FIGURE 2.1). PAEC proliferation was consistent from 5 to 30 mM glucose media at day 3, but as cells approached confluence, cell number decreased with increasing culture glucose (23% less in 17.5 mM glucose, 35% less in 30 mM glucose as compared to 5 mM glucose) (FIGURE 2.2). When cells were visualized using Pecam labeling to outline cell borders, high glucose PAEC had larger intracellular area at confluence (3260 ± 153 to 5033 ± 310 μm^2, $p < .0001$; FIGURE 2.3, FIGURE 2.4). High glucose cells were faster to differentiate and begin to form vessels than low glucose cells once confluence was achieved.

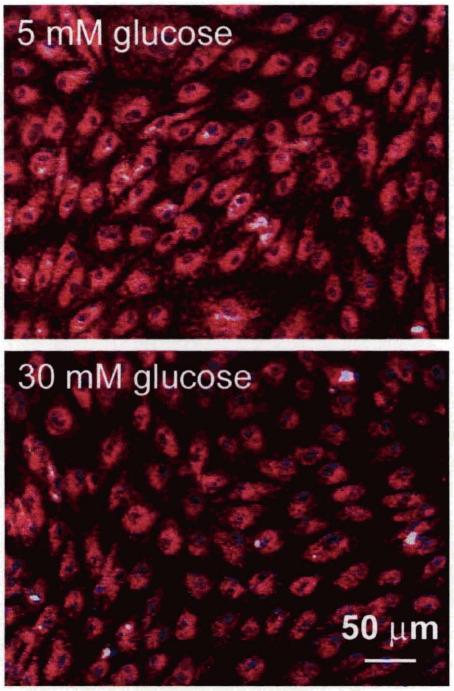


FIGURE 2.1: ENDOTHELIAL CELLS MAINTAIN PHENOTYPE IN HIGH GLUCOSE CULTURE. Porcine aortic endothelial cells were incubated with dil-Ac-LDLfor 4 hours. Endothelial cells are unique in their ability to degrade dil-Ac-LDL to release the DiI fluorescent probe (red).

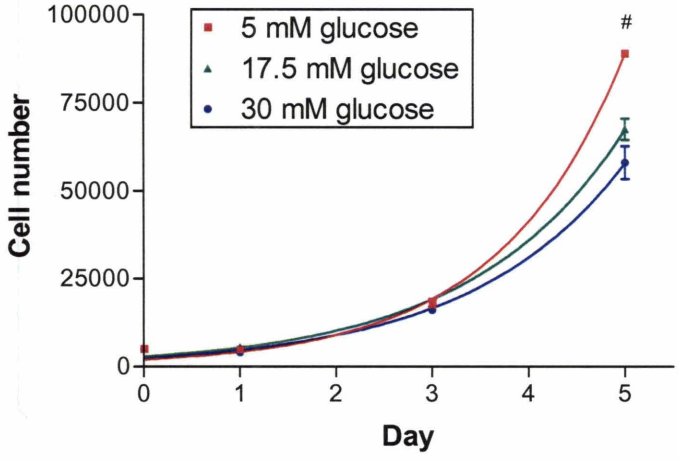


FIGURE 2.2: ENDOTHELIAL CELLS PROLIFERATE AT SIMILAR RATES IN LOW AND HIGH GLUCOSE. Porcine aortic endothelial cells cultured in 5, 17.5, or 30 mM glucose growth medium show similar proliferation rates, but cell number at confluence decreases with increasing glucose concentration. (#) $p < .05$

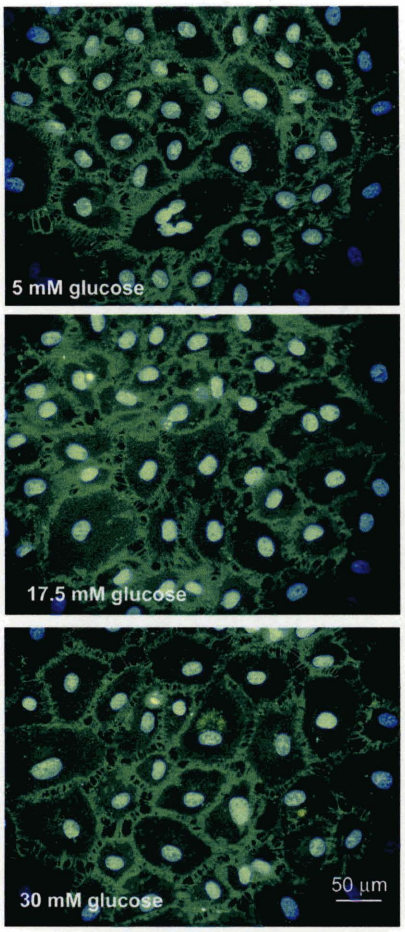


FIGURE 2.3: ENDOTHELIAL CELL INTRACELLULAR AREA INCREASES WITH GLUCOSE CONCENTRATION. After four days in culture, porcine aortic endothelial cells were labeled with Pecam (green) to outline cell borders and Hoescht nuclear stain (blue). Intra- and intercellular area increase with culture glucose concentration.

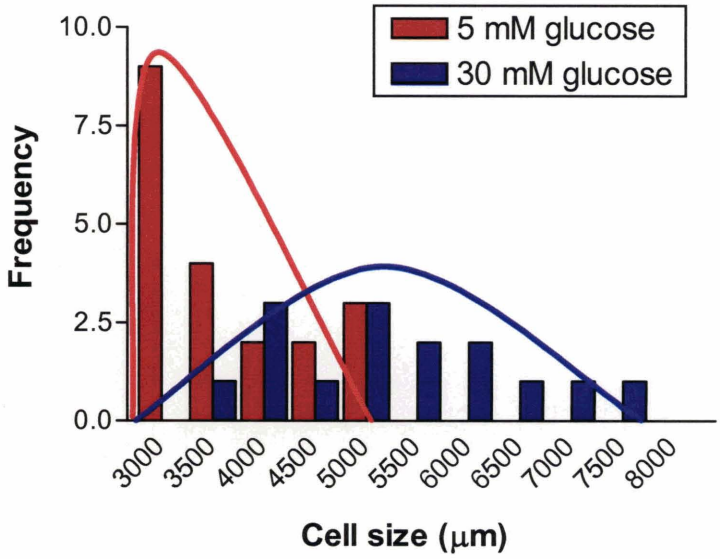


FIGURE 2.4: CELL SIZE HISTOGRAM SHOWS INCREASED INTRACELLULAR AREA WITH GLUCOSE CONCENTRATION. Cell size was quantified from PECAM images (Figure 2.3) by tracing cell borders in Adobe Photoshop and measuring intracellular area.

2.3.2 Basement membrane isolation

Four endothelial cell removal methods were tested to determine which method best produced an intact basement membrane devoid of cellular debris. When examined by scanning electron microscopy, basement membrane resulting from three detergent lysis buffers similarly showed a dense protein network with little to no cellular debris (FIGURE 2.5). There was no significant qualitative difference among the three methods based on SEM micrographs alone. However, when cells were lysed with water, no fibrillar protein mesh was observed on the cover slip. Additional functional tests showed no significant differences in native FGF-2 or endothelial cell ability to re-seed and

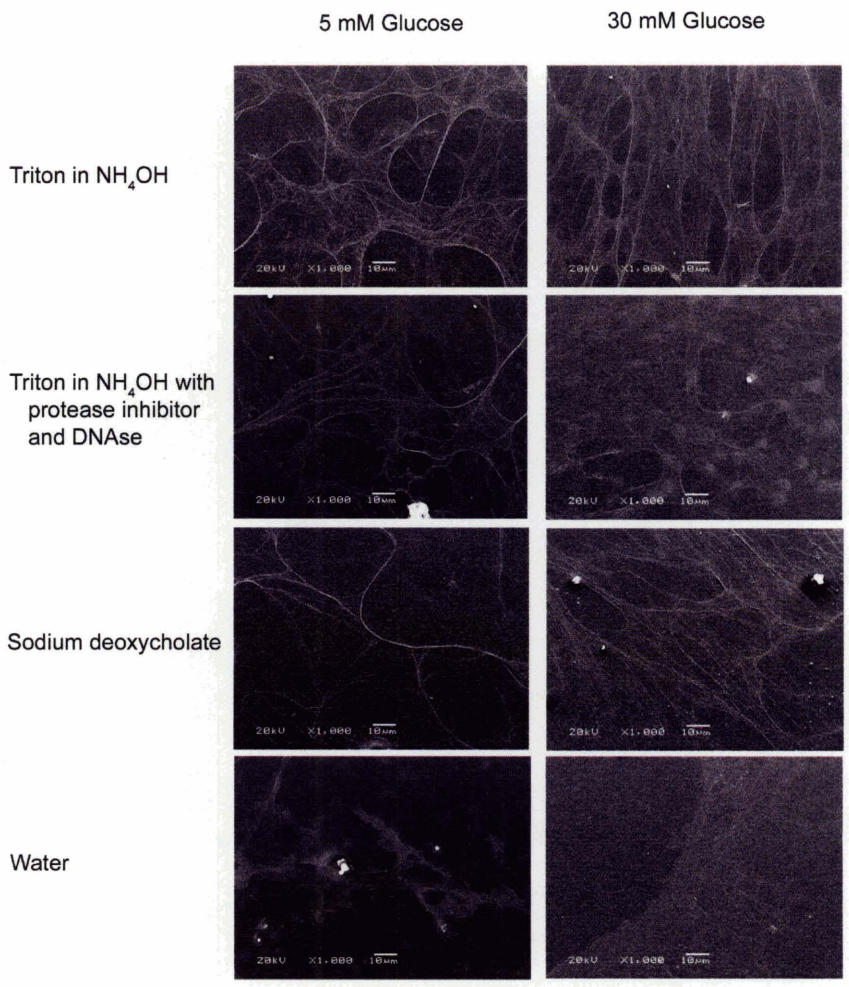


FIGURE 2.5: TRITON-NH_4OH LYSIS BUFFER PRODUCES AN INTACT BASEMENT MEMBRANE. Scanning electron micrographs show isolated basement membrane after porcine aortic endothelial cells (four days in culture) were lysed and removed using different buffers.

proliferate on basement membrane isolated by different methods.

2.3.3 Basement membrane FGF-2 binding kinetics

The methodology for measuring endothelial cell FGF-2 binding kinetics was modified to allow measurement of basement membrane FGF-2 equilibrium capacity, association, and dissociation. However, in each case, basement membrane FGF-2 binding kinetics were confounded by FGF-2 binding to tissue culture polystyrene underneath basement membrane.

Basement membrane equilibrium FGF-2 binding capacity increased linearly over a large FGF-2 range, from 0 to 2500 ng/ml, and reached a maximum at around 5000 ng/ml (FIGURE 2.6). Similarly, FGF-2 bound to tissue culture polystyrene at equilibrium increased linearly with added FGF-2 over the same range and reached equilibrium at around the same point, albeit at a slightly lower value. On average, FGF-2 bound to basement membrane on tissue culture polystyrene was 25% higher than FGF-2 bound to the same area of tissue culture polystyrene alone.

FGF-2 association to basement membrane and tissue culture polystyrene had similar equilibrium dissociation constants (3.07×10^{-11} M vs. 3.08×10^{-11} M, TABLE 2.1), both increasing rapidly to equilibrium at approximately 60 minutes (FIGURE 2.7). FGF-2 bound to basement membrane on tissue culture polystyrene at equilibrium was about 15% higher than that bound to the same area of tissue culture polystyrene alone ($p < .01$). While less FGF-2 associated with polystyrene, more FGF-2 dissociated from polystyrene as opposed to basement membrane (FIGURE 2.8). FGF-2 rapidly dissociated from basement membrane and polystyrene up to ~ 60 minutes, but rather than reach equilibrium, FGF-2 continued to dissociate at a slow rate for up to six hours. FGF-2 dissociated from basement membrane was ~ 50% of that dissociated from polystyrene.

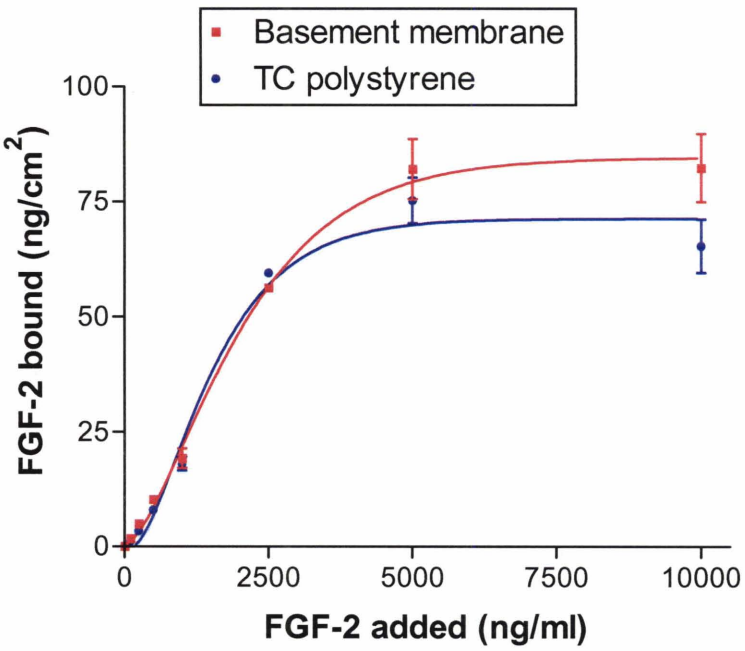


FIGURE 2.6: BASEMENT MEMBRANE FGF-2 BINDING CAPACITY IS HIGHER THAN TISSUE CULTURE POLYSTYRENE. FGF-2 was added to tissue culture polystyrene with or without basement membrane for three hours in binding buffer, after which the FGF-2 solution was removed. Bound FGF-2 was extracted with 2M NaCl and quantified via FGF ELISA. ($p < .05$)

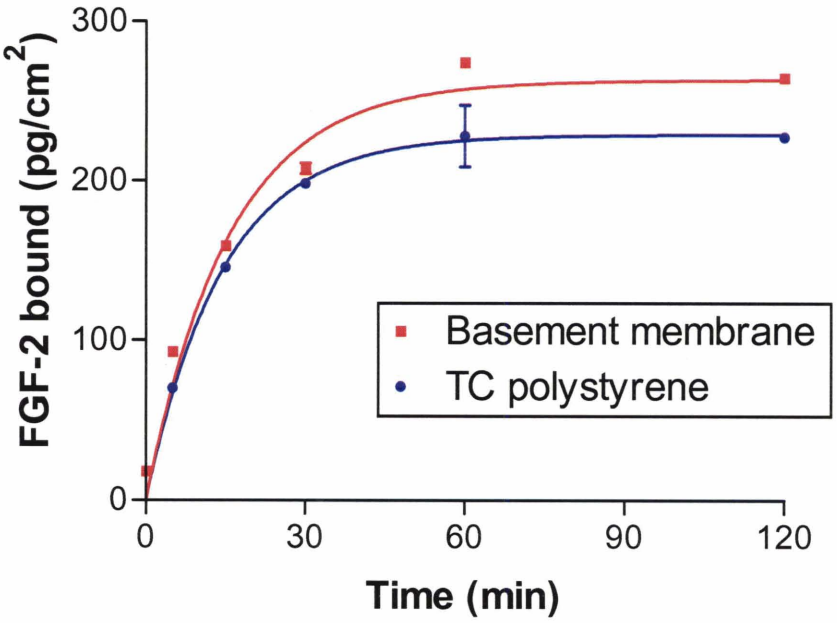


FIGURE 2.7: FGF-2 ASSOCIATION TO BASEMENT MEMBRANE IS GREATER THAN TO TISSUE CULTURE POLYSTYRENE. 5 ng/ml FGF-2 was added to tissue culture polystyrene with or without basement membrane in binding buffer for various time points. The FGF-2 solution was removed after which bound FGF-2 was extracted with 2M NaCl and quantified via FGF ELISA. ($p < .01$).

While similarities in binding kinetics between basement membrane and tissue culture polystyrene were interesting, we wanted to study basement membrane FGF-2 binding alone. Therefore we attempted to block FGF-2 binding to polystyrene, as well as modify extraction timing to decrease FGF-2 component from polystyrene. However, we were unable to block FGF-2 binding using media proteins or gelatin or by trying to remove binding sites using a detergent or heparinase polystyrene pre-treatment (FIGURE 2.9). Decreased extraction time also did not alter extracted FGF-2 from polystyrene instead of basement membrane.

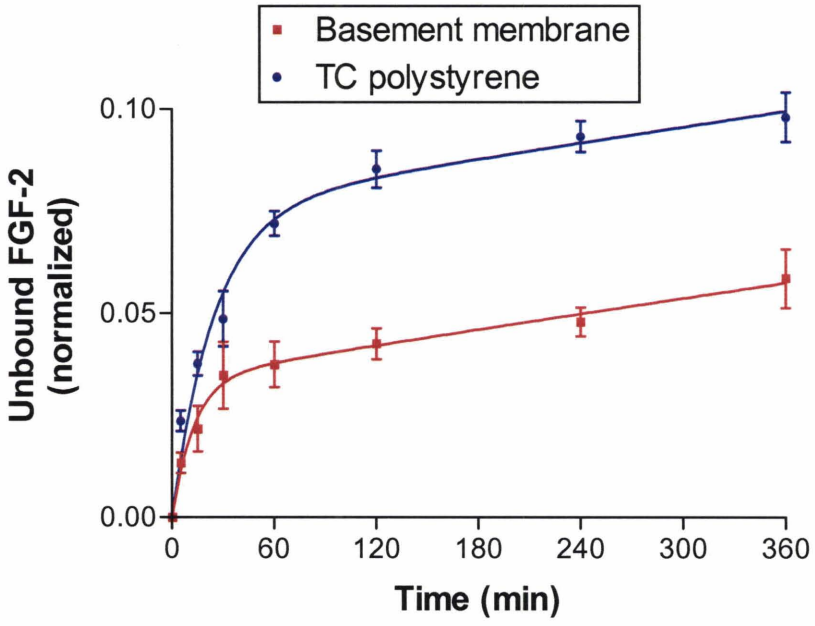


FIGURE 2.8: FGF-2 DISSOCIATION FROM BASEMENT MEMBRANE IS LESS THAN THAT FROM TISSUE CULTURE POLYSTYRENE. 5 ng/ml ^{125}I-FGF-2 was added to tissue culture polystyrene with or without basement membrane for 3 hours in binding buffer, after which the ^{125}I-FGF-2 solution was removed. Fresh binding buffer was added for various time periods, and dissociated ^{125}I-FGF-2 was quantified in a gamma counter. ($p < .01$)

	Basement membrane	Tissue culture polystyrene
k_{off}	.00338 ± .0014 min^{-1}	.00367 ± .0020 min^{-1}
k_{obs}	.06376 ± .0073 min^{-1}	.06905 ± .0036 min^{-1}
k_{on}	1.10 x 10^{8} M^{-1} min^{-1}	1.19 x 10^{8} M^{-1} min^{-1}
K_d	3.07 x 10^{-11} M	3.08 x 10^{-11} M

TABLE 2.1: EQUILIBRIUM DISSOCIATION CONSTANTS ARE SIMILAR FOR BASEMENT MEMBRANE AND TISSUE CULTURE POLYSTYRENE. On and off rate constants (k_{on} and k_{off}) were calculated from association and dissociation experiments, and then used to calculate the equilibrium dissociation constant (K_d).

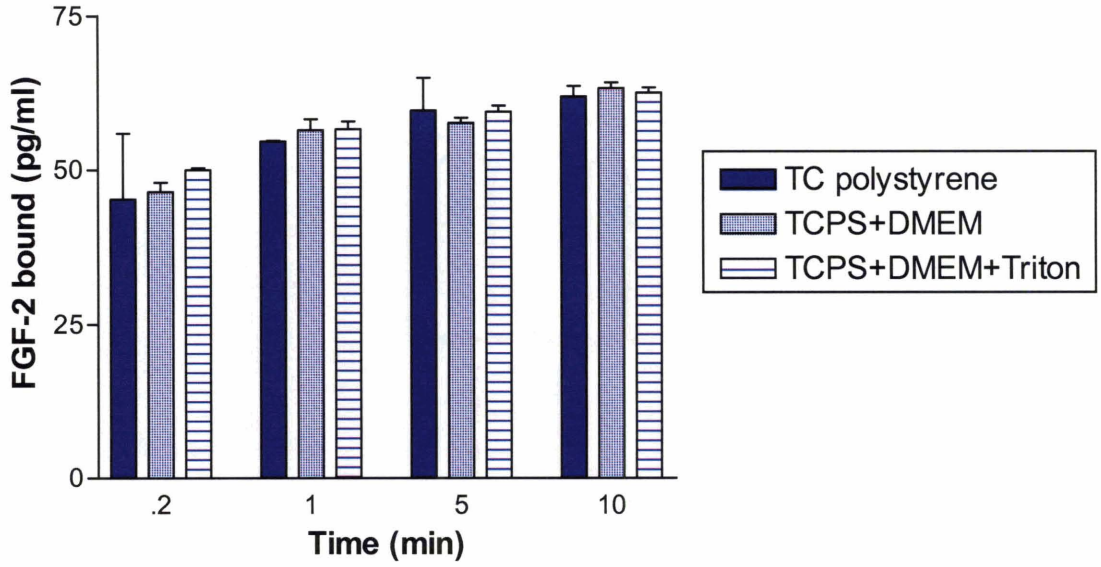


FIGURE 2.9: FGF-2 BINDING TO POLYSTYRENE COULD NOT BE BLOCKED. Tissue culture polystyrene was incubated in media or media followed by Triton X-100 for three hours, but there was no significant difference in FGF-2 that could be extracted from the polystyrene plate.

2.3.4 Correction for substrate binding

Since FGF-2 tissue culture polystyrene binding could not be blocked, we created a model to account for it in basement membrane FGF-2 binding kinetics calculations. First we converted SEM basement membrane images to black and white to roughly calculate (Adobe Photoshop) tissue culture polystyrene area exposed underneath basement membrane proteins (FIGURE 2.10). On average, 30% of polystyrene area on the bottom of the tissue culture well was not covered by basement membrane. We then used this figure, in addition to calculating exposed polystyrene on the side of the well, to subtract FGF-2 bound to exposed polystyrene.

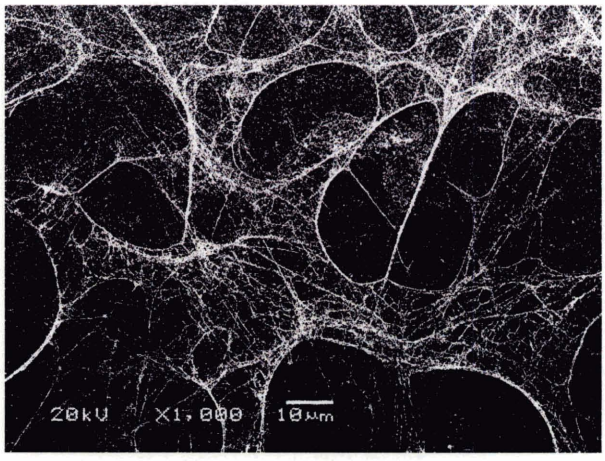


FIGURE 2.10: BLACK AND WHITE SCANNING ELECTRON MICROGRAPH OF BASEMENT MEMBRANE. The percentage black space was used to calculate exposed polystyrene in Adobe Photoshop after image conversion to black and white.

For example, at a radius of 0.32 cm, 96 well plates have an area of 0.32 cm^2. The FGF-2 volume added was $Volume_{FGF-2} = .1ml = .1cm^3$. This FGF-2 volume contacted not only the bottom but also the sides of the well, therefore the area of the sides of the well exposed to FGF-2 was:

$$Area_{side} = height_{FGF-2} \times circumference_{well} = \frac{Volume_{FGF-2}}{Area_{well}} \times circumference_{well} = .626cm^2$$

The total contact area between added FGF-2 solution and tissue culture polystyrene without basement membrane was:

$$Area_{contact-BM} = Area_{well} + Area_{side} = .946cm^2$$

The total contact area between added FGF-2 solution and tissue culture polystyrene with attached basement membrane was:

$$Area_{contact+BM} = .33 \times Area_{well} + Area_{side} = .733cm^2$$

Then corrected FGF-2 basement membrane binding capacity was determined by measuring FGF-2 bound to basement membrane on polystyrene ($FGF2_{BM+TCPS}$) and FGF-2 bound to polystyrene alone ($FGF2_{TCPS}$). FGF-2 bound to polystyrene when basement membrane was present was calculated using the ratio of polystyrene areas with and without basement membrane. This was then subtracted from measured FGF-2 binding to basement membrane on polystyrene and divided by area only of the bottom of the well:

$$FGF-2_{BM} = \frac{FGF2_{BM+TCPS} - FGF2_{TCPS}\left(\frac{Area_{contact+BM}}{Area_{contact-BM}}\right)}{Area_{bottom}}$$

This methodology was validated by growing basement membrane on bacteriologic plates, which hardly bind FGF-2 at all. When the correction methodology was used, calculated FGF-2 bound to basement membrane on tissue culture polystyrene was very close to the amount bound to basement membrane on bacteriologic plates (FIGURE 2.11).

The same method was applied to correct basement membrane FGF-2 equilibrium capacity, association, and dissociation data for polystyrene. In each case, the correction lowered absolute bound FGF-2 values but did not change the shape of

the curves, indicating that relationships for FGF-2 basement membrane binding kinetics were correct despite additional FGF-2 binding to polystyrene (FIGURE 2.12, FIGURE 2.13).

The importance of correction is evident in basement membrane FGF-2 binding with glucose in binding buffer (FIGURE 2.14). Without the correction, high binding buffer glucose appeared to decrease FGF-2 binding to basement membrane. However, high binding buffer glucose actually decreased FGF-2 binding to tissue culture polystyrene (FIGURE 2.15), and when FGF-2 binding to polystyrene was subtracted from FGF-2 bound to basement membrane, the glucose effect disappeared.

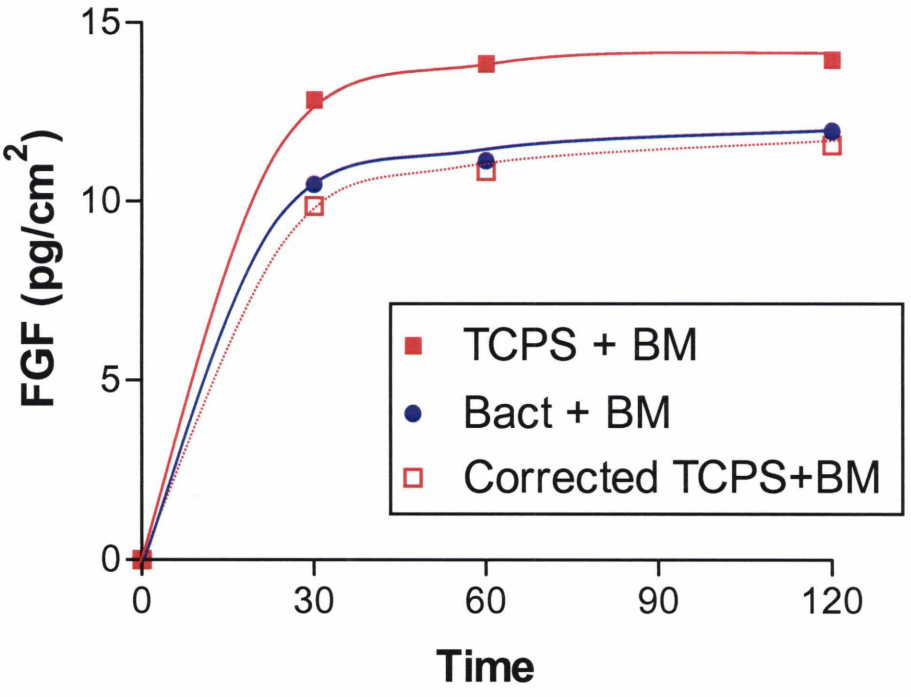


FIGURE 2.11: CORRECTED FGF-2 ASSOCIATION TO BASEMENT MEMBRANE ON POLYSTYRENE IS SIMILAR TO BACTERIOLOGIC PLATES. FGF-2 association with time was measured on bacteriologic plates and tissue culture polystyrene plates with basement membrane. The tissue culture polystyrene results were then corrected using the methodology presented above ($p > .05$)

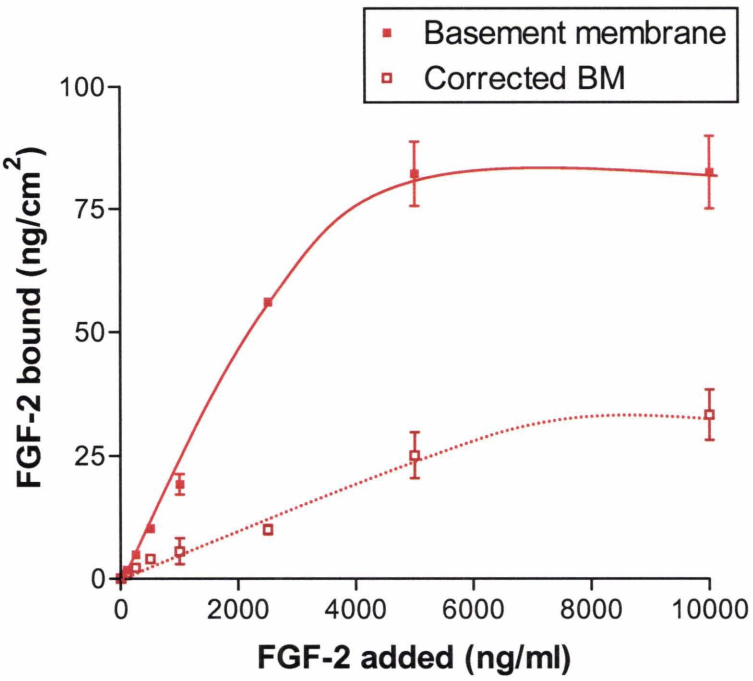


FIGURE 2.12: CORRECTED BASEMENT MEMBRANE FGF-2 CAPACITY IS LOWER THAN UNCORRECTED VALUES. Correction for FGF-2 binding to tissue culture polystyrene decreases measured basement membrane FGF-2 binding capacity. ($p < .01$)

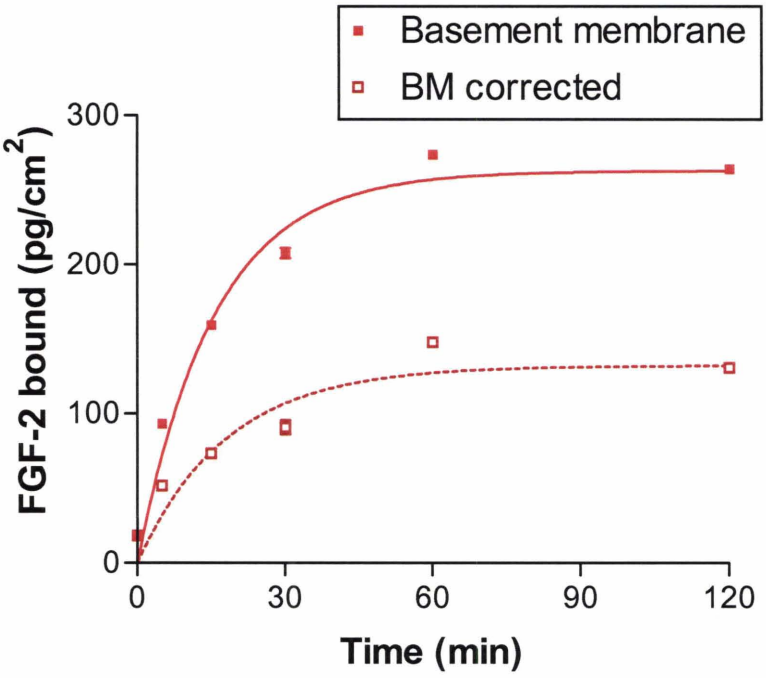


FIGURE 2.13: CORRECTED BASEMENT MEMBRANE ASSOCIATION IS LOWER THAN UNCORRECTED VALUES. Basement membrane association follows the same time course but reaches equilibrium at a lower value after the correction is applied ($p < .01$).

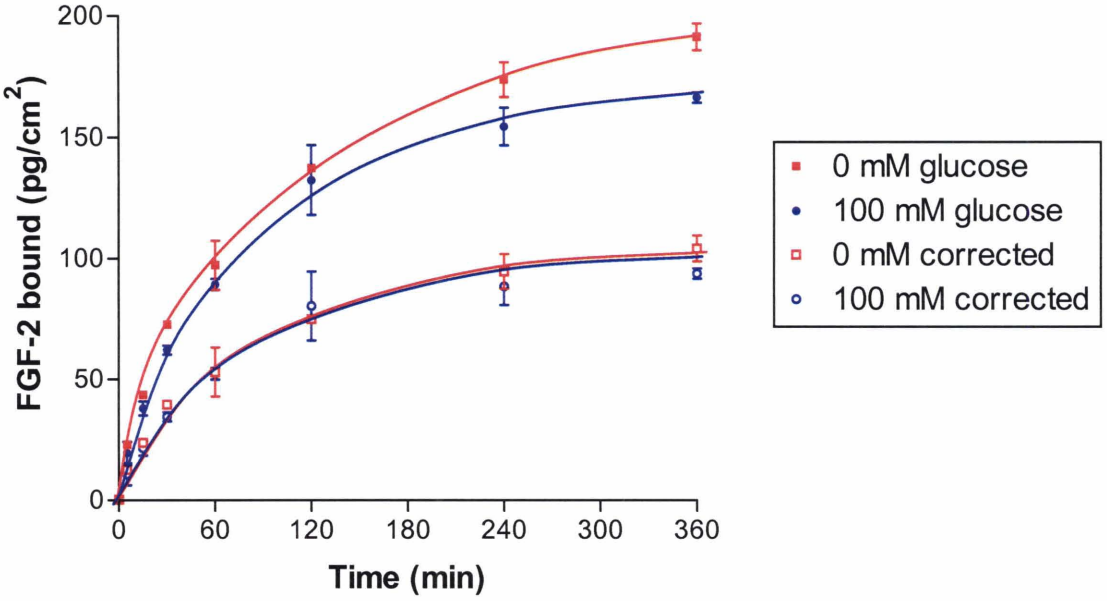


FIGURE 2.14: DECREASED BASEMENT MEMBRANE ASSOCIATION WITH GLUCOSE IS DUE TO TISSUE CULTURE POLYSTYRENE. When basement membrane association with glucose ($p < .01$) is corrected, the glucose effect is no longer significant ($p > .05$).

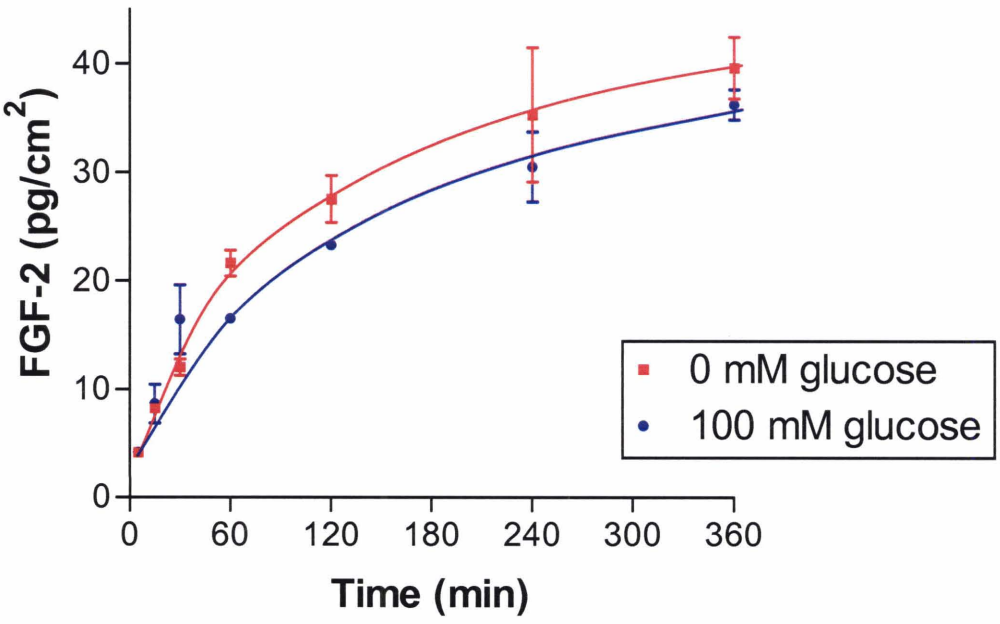


FIGURE 2.15: ASSOCIATION TO TISSUE CULTURE POLYSTYRENE IS LOWER WITH GLUCOSE. 5 ng/ml FGF-2 was added to tissue culture polystyrene in 0 or 100 mM glucose binding buffer, and bound FGF-2 with time was measured as described previously. ($p < .05$).

FGF-2 binding and release from polystyrene also made it similar to basement membrane in its ability to support endothelial cell proliferation. By incubating polystyrene with FGF-2 prior to seeding PAEC, we increased cellular proliferation to be nearly equivalent to basement membrane (FIGURE 2.16).

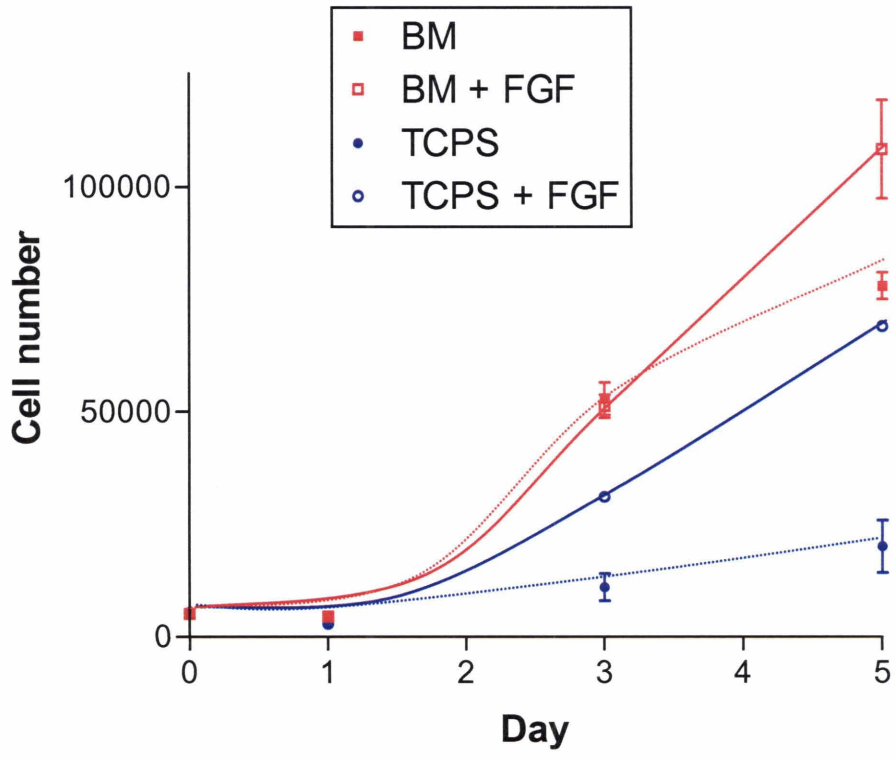


FIGURE 2.16: ENDOTHELIAL CELL GROWTH INCREASES ON BASEMENT MEMBRANE OR TISSUE CULTURE POLYSTYRENE WITH ADDED FGF-2. Tissue culture polystyrene with or without basement membrane was incubated with 100 ng/ml FGF-2 for one hour prior to cell seeding. Endothelial cell number was measured by trypsinizing cells and counting with a Coulter counter ($p < .01$).

2.4 Discussion

Study of endothelial cells and basement membrane in hyperglycemic conditions required method development both to culture cells in high glucose and isolate basement membrane. Existing methods for endothelial cell FGF-2 binding kinetics were also modified to account for unique basement membrane properties.

Porcine aortic endothelial cells maintain both their phenotype and proliferative capacity in high glucose culture, metabolizing LDL and multiplying at a rate similar to low glucose cells. High glucose culture was maintained at 30 mM glucose for all experiments, but successful PAEC culture was achieved as high as 50 mM glucose without noticeable change in endothelial cell phenotype. 30 mM glucose was used as the high glucose point for the majority of experiments as it defines the high range of physiologic values; 50 mM glucose culture was only used to clarify linearity of critical effects.

High glucose cells appear larger than low glucose cells by microscopy, and upon cell count, consistently show a lower cell number at confluence. This phenomena has been cited in the past and attributed to increased intracellular metabolism in high glucose, however we found that PAEC grown under osmotic controls such as mannitol similarly show decreased cell number at confluence. Therefore, we believe that the increase in cell size and decrease in cell number at confluence is an osmotic rather than a metabolic effect. However, without confocal microscopy, it remains unclear if the cell size increase holds for cell volume or just for cell area. Another significant difference between low and high glucose cells is a greater difficulty in maintaining cells more than a few days beyond confluence. This limited long term study of hyperglycemic conditions and will be discussed in greater detail in Chapter 3.

Detergent buffers are commonly used to perforate and solubilize cell membranes. Both Triton X-100 and sodium dexoycholate remove endothelial cells and leave an intact basement membrane adherent to the tissue culture plate. However, sodium deoxycholate is an ionic detergent which modifies protein structure to a greater extent than non-ionic detergents such as Triton X-100. Additionally, ionic detergents are more sensitive to pH, which might be an issue when a base such as ammonium

hydroxide is used in conjunction with detergent to aid in quickly lysing cells. Because of its effectiveness in thoroughly removing cells while causing minimal apparent damage to basement membrane, we decided to use the Triton X-100 NH_4OH solution to isolate basement membrane throughout thesis experiments. As protease inhibitor and DNAse addition did not significantly alter growth factor in basement membrane or cellular debris on basement membrane, these additional reagents were not included in the cell lysis buffer. While the basic Triton solution was the best lysis solution we tested, it could still cause damage to basement membrane or incorporate cellular contents into basement membrane when cells are lysed. We did test a urea cell removal method that avoided cell lysis altogether and did not observe a significant change in basement membrane characteristics.

To study basement membrane FGF-2 binding kinetics, modifications were made to a previously developed methodology for endothelial cell FGF-2 binding kinetics. Many modifications are actually simplifications. For example, binding temperature was relatively unimportant as there was no requirement for cell viability maintenance and cellular growth factor metabolism was not an issue. Basement membrane also only has one specific FGF-2 binding site, heparan sulfate proteoglycans, whereas cells have both heparan sulfate proteoglycans and cell surface FGF receptors. This allows basement membrane FGF-2 extraction using only high salt buffer as salt conditions isolate heparan sulfate proteoglycans.

However, a unique complication does arise in basement membrane FGF-2 binding kinetic measurements. Tissue culture polystyrene, which is exposed underneath the fibrillar basement membrane, binds and releases FGF-2 in a manner similar to basement membrane. We accounted for this effect by determining exposed polystyrene area and subtracting FGF-2 bound to that area from the total amount bound to basement membrane on polystyrene. Through this calculation, we show that FGF-2 bound to basement membrane is significantly lower at equilibrium, but the shapes of the equilibrium capacity, association, and dissociation curves are similar.

This correction methodology is limited by our ability to determine how much tissue culture polystyrene is exposed. The basement membrane is fibrillar and porous, complicating the situation significantly. While we validated our method with bacteriologic

plates which bind little to no FGF-2, it remains unclear if we have accurately corrected for FGF-2 binding to polystyrene. Because binding relationships themselves are unchanged, we restrict use of the correction to this chapter. FGF-2 binding to tissue culture polystyrene was determined for all conditions presented in the thesis, and the only case in which it was significant to binding kinetics is with buffer glucose.

The fact that FGF-2 binds and releases in a controlled manner from tissue culture polystyrene likely contributes to polystyrene's effectiveness as a cell culture substrate. FGF-2 is present in media supplemented with FBS, therefore when cells are seeded, some FGF-2 binds to polystyrene. This FGF-2 is then slowly released as soluble FGF-2 stores are consumed by cells. This polystyrene property can be taken advantage of further by simply incubating tissue culture polystyrene plates with FGF-2 prior to cell seeding. Polystyrene plate coating with FGF-2 achieves long term improved proliferation comparable to coating a plate with basement membrane or a matrix derivative.

2.5 Conclusions

Porcine aortic endothelial cells maintain their phenotype at glucose levels up to 30 mM and additionally produce a high quality basement membrane that remains adherent to the culture plate. Based on these two factors, PAEC were selected as the cell choice for the *in vitro* hyperglycemic system. Basement membrane binding kinetics measurement was confounded by FGF-2 binding to tissue culture polystyrene in a way that is strikingly similar to binding to basement membrane. However, FGF-2 bound to basement membrane could be calculated by subtracting off FGF-2 bound to exposed polystyrene, as was confirmed by studies on bacteriologic plates. Overall, basement membrane FGF-2 binding kinetics protocols proved to be robust throughout glucose conditions.

2.6 Chapter References

1. Laight, D.W., Carrier, M.J., and Anggard, E.E. 1999. Endothelial cell dysfunction and the pathogenesis of diabetic macroangiopathy. *Diabetes-Metabolism Research And Reviews* 15:274-282.
2. Haller, H. 1997. Endothelial function - General considerations. *Drugs* 53:1-10.
3. Lorenzi, M., Cagliero, E., and Toledo, S. 1985. Glucose Toxicity For Human-Endothelial Cells In Culture - Delayed Replication, Disturbed Cell-Cycle, And Accelerated Death. *Diabetes* 34:621-627.
4. Baumgartner-Parzer, S.M., Wagner, L., Pettermann, M., Grillari, J., Gessl, A., and Waldhausl, W. 1995. High-Glucose-Triggered Apoptosis In Cultured Endothelial-Cells. *Diabetes* 44:1323-1327.
5. Martin, A., Komada, M.R., and Sane, D.C. Abnormal angiogenesis in diabetes mellitus. *Medicinal Research Reviews*:117-145.
6. Brownlee, M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820.
7. Gospodarowicz, D., Lepine, J., Massoglia, S., and Wood, I. 1984. Comparison Of The Ability Of Basement-Membranes Produced By Corneal Endothelial And Mouse-Derived Endodermal Pf-Hr-9 Cells To Support The Proliferation And Differentiation Of Bovine Kidney Tubule Epithelial-Cells Invitro. *Journal Of Cell Biology* 99:947-961.
8. Gospodarowicz, D., Gonzalez, R., and Fujii, D.K. 1983. Are Factors Originating From Serum, Plasma, Or Cultured-Cells Involved In The Growth-Promoting Effect Of The Extracellular-Matrix Produced By Cultured Bovine Corneal Endothelial-Cells. *Journal Of Cellular Physiology* 114:191-202.
9. Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., Klagsbrun, M. 1987. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *PNAS* 84:2292-2296.
10. Bashkin, P., Doctor, S., Klagsbrun, M., Svahn, C.M., Folkman, J., Vlodavsky, I. 1989. Basic Fibroblast Growth Factor Binds to Subendothelial Extracellular Matrix and Is Released by Heparitinase and Heparin-like Molecules. *Biochemistry* 28:1737-1743.
11. Benezra, M., Vlodavsky, I., Ishaimichaeli, R., Neufeld, G., and Barshavit, R. 1993. Thrombin-Induced Release Of Active Basic Fibroblast Growth Factor-Heparan Sulfate Complexes From Subendothelial Extracellular-Matrix. *Blood* 81:3324-3331.
12. Dowd, C.J., Cooney, C.L., and Nugent, M.A. 1999. Heparan sulfate mediates bFGF transport through basement membrane by diffusion with rapid reversible binding. *Journal Of Biological Chemistry* 274:5236-5244.
13. Wong, M.K.K., and Gotlieb, A.I. 1984. Invitro Reendothelialization Of A Single-Cell Wound - Role Of Microfilament Bundles In Rapid Lamellipodia-Mediated Wound Closure. *Laboratory Investigation* 51:75-81.
14. Gospodarowicz, D., Delgado, D., and Vlodavsky, I. 1980. Permissive Effect Of The Extracellular-Matrix On Cell-Proliferation Invitro. *Proceedings Of The National Academy Of Sciences Of The United States Of America-Biological Sciences* 77:4094-4098.

15. Voyta, J.C., Via, D.P., Butterfield, C.E., and Zetter, B.R. 1984. Identification And Isolation Of Endothelial-Cells Based On Their Increased Uptake Of Acetylated-Low Density Lipoprotein. *Journal Of Cell Biology* 99:2034-2040.

16. Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishaimichaeli, R., Sasse, J., and Klagsbrun, M. 1987. Endothelial Cell-Derived Basic Fibroblast Growth-Factor - Synthesis And Deposition Into Subendothelial Extracellular-Matrix. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 84:2292-2296.

17. Braet, F., deZanger, R., and Wisse, E. 1997. Drying cells for SEM, AFM and TEM by hexamethyldisilazane: A study on hepatic endothelial cells. *Journal Of Microscopy-Oxford* 186:84-87.

18. Nation, J.L. 1983. A New Method Using Hexamethyldisilazane For Preparation Of Soft Insect Tissues For Scanning Electron-Microscopy. *Stain Technology* 58:347-351.

19. Moscatelli, D. 1987. High And Low Affinity Binding-Sites For Basic Fibroblast Growth-Factor On Cultured-Cells - Absence Of A Role For Low Affinity Binding In The Stimulation Of Plasminogen-Activator Production By Bovine Capillary Endothelial-Cells. *Journal Of Cellular Physiology* 131:123-130.

20. Nugent, M.A., and Edelman, E.R. 1992. Kinetics Of Basic Fibroblast Growth-Factor Binding To Its Receptor And Heparan-Sulfate Proteoglycan - A Mechanism For Cooperativity. *Biochemistry* 31:8876-8883.

CHAPTER 3

BASEMENT MEMBRANE FGF-2 CHANGES IN HYPERGLYCEMIA

Abstract

Disordered angiogenesis in diabetic vascular disease points to dysregulation of angiogenic growth factors such as FGF-2. We therefore examined FGF-2 storage in the endothelial cell basement membrane. Our data suggest for the first time that basement membrane FGF-2 increases with glucose over the course of mere days of exposure to high but still physiologic glucose levels. Surprisingly, basement membrane FGF-2 binding kinetics are unchanged, indicating that the alteration in FGF-2 storage occurs on a timescale faster than that of significant basement membrane changes. Aortic tissue harvested from a porcine diabetic model was used to validate the FGF-2 increase *in vivo*.

3.1 Introduction

Vascular dysfunction in diabetes takes varied forms in different vascular beds. While excessive angiogenesis in the retina and kidney leads to retinopathy and nephropathy respectively, insufficient angiogenesis in peripheral vasculature is associated with poor wound healing (44, 45). In macrovasculature, diabetics experience accelerated atherosclerosis, rapid and more extensive restenosis following endovascular intervention, and decreased collateral vessel formation around blockages (8, 147). The complex range of vascular morbidity can perhaps be summarized as disordered angiogenesis through endothelial cell dysfunction.

Angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) have been implicated as being in excess or in deficiency in these varied conditions, but no clear causative mechanistic role for these growth factors has been established. VEGF concentration in ocular fluid of diabetics with active proliferative retinopathy is higher than that of non-diabetics, and VEGF blockade with monoclonal antibodies reduced hyperfiltration, albuminuria, and glomerular hypertrophy in streptozotocin-induced diabetic rats (127, 130). However, initially low VEGF mRNA in diabetic mouse keratinocytes decreased further upon wounding, in contrast to the elevation in VEGF with wounding found in normal mice (132). For FGF-2, increased plasma FGF-2 has been reported in diabetic patients with microalbuminuria or proteinuria, yet in a gastric wound model, streptozotocin-induced diabetic rats showed decreased FGF-2 and delayed lesion healing which improved upon exogenous FGF-2 administration (134, 136). Interestingly, these animal models have also shown continued vascular dysfunction after restoration of normoglycemia (148, 149).

The vascular basement membrane, in particular in its role in growth factor storage and release, may help unify the spectrum of diabetic vascular disease. In both micro- and macrovasculature, basement membrane changes in size and composition in diabetes. Throughout varied vascular beds, from kidney to retina to aorta, basement membrane becomes consistently thicker (44). In addition, relative quantities of major protein components change, with increases in type IV collagen and fibronectin and

decreases in laminin and heparan sulfate proteoglycans (44, 80-82). Large scale changes in basement membrane occur over the course of weeks and months rather than days. While no single biochemical diabetic alteration has been identified as the cause of these changes, hyperglycemia has been implicated (83, 84). *In vitro*, high glucose affects basement membrane protein production and leads to glycation of these proteins, altering both matrix-matrix and matrix-cell interactions (85-87).

Since it was first shown that cell-derived growth factors such as FGF-2 are stored in basement membrane for later release, the effect of disease states on basement membrane growth factors has been left largely unexplored (72). We examined the hypothesis that glucose modulates FGF-2 storage in basement membrane. Basement membrane FGF-2 was measured in increasing glucose conditions and validated using osmotic controls. We then examined the role of basement membrane FGF-2 binding kinetics in altered FGF-2 storage, including specific binding to heparin moieties. Finally, these findings were correlated with *in vivo* data to confirm physiological relevance. In this chapter, we show for the first time that basement membrane FGF-2 increases with glucose *in vitro* and *in vivo*, but without alterations in binding kinetics.

3.2 Materials and Methods

3.2.1 Basement membrane growth

To measure native basement membrane FGF-2, porcine aortic endothelial cells (PAEC) were cultured for two, four, or six days in 12 well tissue culture plates at 5, 17.5, or 30 mM glucose as described previously (Chapter 2). Mannitol and L-glucose were used as osmotic controls. After two days, media was changed to growth media with 4% w/v 40 kDa dextran. Dextran increases media viscosity, which is thought to induce endothelial cells to produce more basement membrane. Basement membrane for binding kinetics studies was grown by seeding PAEC near confluence and culturing them for four days at varying glucose, with the last two days in growth media with dextran. Basement membrane was isolated using NH_4OH-Triton X-100 lysis buffer described in Chapter 2 and thoroughly washed with PBS. Samples were stored in PBS at 4 °C and used within several days.

3.2.2 FGF-2 and total protein assays

FGF-2 was extracted from basement membrane using 2 M NaCl in 20 mM Hepes (pH 7.4) for 10 minutes with gentle shaking. The extraction buffer was then collected and stored at -20 ºC until use. As FGF-2 degraded quickly in salt buffer, extracted FGF-2 was quantified immediately via FGF ELISA whenever possible. Total protein in extraction buffer was measured using the BCA Total Protein Assay (BioRad) 96 well plate procedure. Each sample was measured in triplicate and compared to bovine serum albumin standards.

3.2.3 FGF-2 binding kinetics

Basement membrane FGF-2 binding kinetics were investigated as described in Chapter 2. Due to well-documented variability among FGF-2 samples, expected and significant FGF-2 integrity loss in storage, and variability among FGF ELISAs, comparable experiments were performed at the same time and measured in the same ELISA whenever possible. When this was not possible, results were normalized to the FGF-2 level at equilibrium. For experiments determining the glucose effect on FGF-2 association to or dissociation from basement membrane, 100 mM glucose was added to either association or dissociation buffer respectively. Glycated FGF-2 was prepared by incubating 1 μg/ml FGF-2 in 0.25 M fructose for 24 hours at 37 ºC (150).

For basement membrane heparan sulfate proteoglycan measurement, endothelial cells were grown for four days in 5, 17.5, or 30 mM glucose. On the fourth day, growth medium was removed, cells were washed with PBS, and labeling medium with 100 μCi/ml ^{35}S (Perkin Elmer) was added for 24 hours. After media removal, cells were washed once with ice cold wash buffer (25 mM Tris, 0.15 M NaCl). Ice cold scraping buffer (10 mM Tris, 1 M Urea, 1 mM DTT, 10 mM EDTA, 2 mM PMSF, pH 7.4) was added, and cells and basement membrane were immediately scraped from the dish and transferred to a microcentrifuge tube. After a 10 minute incubation on ice, tubes were centrifuged to pellet cells, and basement membrane protein supernatant was collected and transferred to a new tube. Samples were stored at -20 ºC until analysis.

Samples were analyzed using the Bio-Dot Microfiltration System (BioRad). A Zeta-Probe membrane (BioRad) was prepared by soaking in tris-buffered saline (TBS) for 30 minutes. The membrane was then placed into the Bio-Dot apparatus, and each well was washed thoroughly with TBS. 200 μl of basement membrane extract was added to wells in quadruplicate and pulled through the membrane with vacuum. The membrane was again thoroughly washed, after which it was removed from the Bio-Dot apparatus and rinsed in TBS followed by ddH_2O. The membrane was dipped in 95% ethanol and air dried. When the membrane was dry, dots for each well were separated, placed into scintillation vials with UltimaGold scintillation fluid (PerkinElmer), and measured in a liquid scintillation counter (Packard).

3.2.4 Heparin column

1 ml of 0.5 mg/ml FGF-2 in sodium phosphate buffer (10 mM sodium phosphate, pH 7.0) was applied to a 1 ml Heparin HiTrap HP column (Amersham) prepacked with heparin sepharose. After the column was thoroughly washed with phosphate buffer, bound FGF-2 was eluted from the column using a 0 to 2 M NaCl linear gradient in sodium phosphate buffer at a 1 ml/min flow rate. 1 ml fractions were collected in an automated fraction collector, and FGF-2 in each fraction was quantified via FGF ELISA. All chromatography was performed using an FPLC system equipped with a conductivity meter and UV monitor, set at 280 nm, under computer control using the Director software package (Pharmacia Biotech).

3.2.5 Porcine diabetic model

Male domestic pigs, initially 27 to 29 kg, were assigned to a control (n=2) or diabetic group (n=4). Prior to the study a vascular access port (VAP, Access Technologies) was surgically implanted into each pig via left external jugular vein catheterization (151). All pigs received intramuscular buprenorphine (0.03 mg/kg) on the day of surgery. Anesthesia was induced with intramuscular xylazine (2 mg/kg), atropine (0.04 mg/kg), butorphanol (0.55 mg/kg) and telazol (6.6 mg/kg). The pigs were intubated and anesthesia was maintained with isoflurane inhalant (0.5-1.5%) via an endotracheal tube.

Diabetes was induced by streptozotocin injection (STZ, 50mg/kg in 0.1 mol/l Na-citrate, ph 4.5) each day for three days (152). All animals were fed a normal diet. Fasting blood glucose concentrations were measured once a week using a standard, portable glucometer. Blood glucose concentrations were maintained at 200 to 250 mg/dl by adjusting daily insulin injections for the duration of the 9 week study period (151). Serum lipids were measured at endpoint in a routine diagnostic analyzer using enzymatic colorimetric assays. Insulin therapy consisted of a mixture containing regular and NPH insulin (Eli Lilly). At study completion, the animals were anesthetized and euthanized with KCl 40 mEq IV. An abdominal aortic segment was rapidly excised, washed thoroughly in PBS, and frozen in liquid nitrogen. Aortic samples were stored at -80 °C until use.

3.2.6 Immunohistochemistry

Immunohistochemical analysis was performed on frozen sections (8 μm). Sections were acetone fixed and air-dried for 24 hours. Endogenous peroxidase activity was first quenched by incubating sections with peroxidase block, after which sections were incubated with mouse monoclonal antibody to FGF-2 (1:250, MC-GF1, Abcam). Mouse nonspecific IgG was used as negative control. The sections were incubated with a peroxidase labeled dextran polymer conjugated to goat anti-mouse IgG (Dako Cytomation). Staining was completed by incubation with 3,3'-diaminobenzidine (DAB)+ substrate-chromogen, which results in a brown-colored precipitate at FGF-2 sites. Slides were counterstained with hematoxylin and mounted.

3.2.7 Statistics

All statistical analyses were performed with Prism software (Graphpad). Data were normally distributed and expressed as mean ± standard deviation. Comparisons between two groups were analyzed by Student's t test, and comparisons between more than two groups were analyzed by ANOVA. A value of $p < 0.05$ was considered statistically significant and is indicated in the text as such or in figures with a pound sign (#). A value of $p < .01$ is indicated with an asterisk (*). If no statistical significance is reported, none was observed.

3.3 Results

3.3.1 Native FGF-2 extracted from basement membrane

PAEC basement membrane associated FGF-2 increases with time and with culture glucose concentration (FIGURE 3.1). After only four days in culture, basement membrane cultured in 30 mM glucose stored two to three fold more FGF-2 than that from cells cultured in 5 mM glucose ($p < .01$). The difference between basement membrane FGF-2 in low and high glucose culture increased up to six days, however culture beyond that point was inhibited by endothelial cell differentiation, in particular for high glucose cells. At four days, measurement of additional glucose gradations shows that basement membrane FGF-2 increased linearly from 5 mM up to as high as 50 mM glucose ($p < .01$) (FIGURE 3.2). Only D-glucose increased basement membrane FGF-2; osmotic controls mannitol and L-glucose did not (FIGURE 3.3). Total protein in the extraction buffer ranged from 30 to 70 pg/ml and was not significantly different for low or high glucose basement membrane or any osmotic controls (FIGURE 3.4).

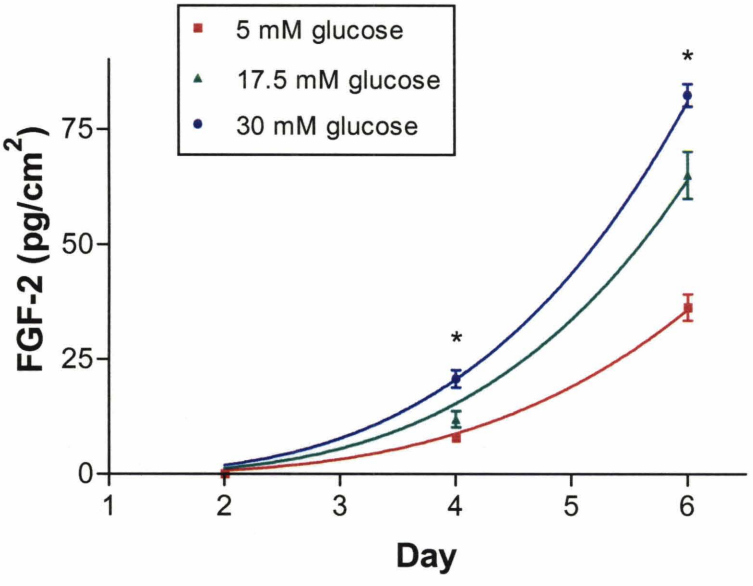


FIGURE 3.1: BASEMENT MEMBRANE FGF-2 INCREASES WITH TIME AND CULTURE GLUCOSE. Porcine aortic endothelial cells were cultured for two, four, or six days in supplemented media of 5, 17.5, or 30 mM glucose. At the end of each time period, cells were removed, basement membrane FGF-2 extracted, and measured via FGF ELISA. $p < .0001$ (ANOVA), (*) $p < .01$ for time point.

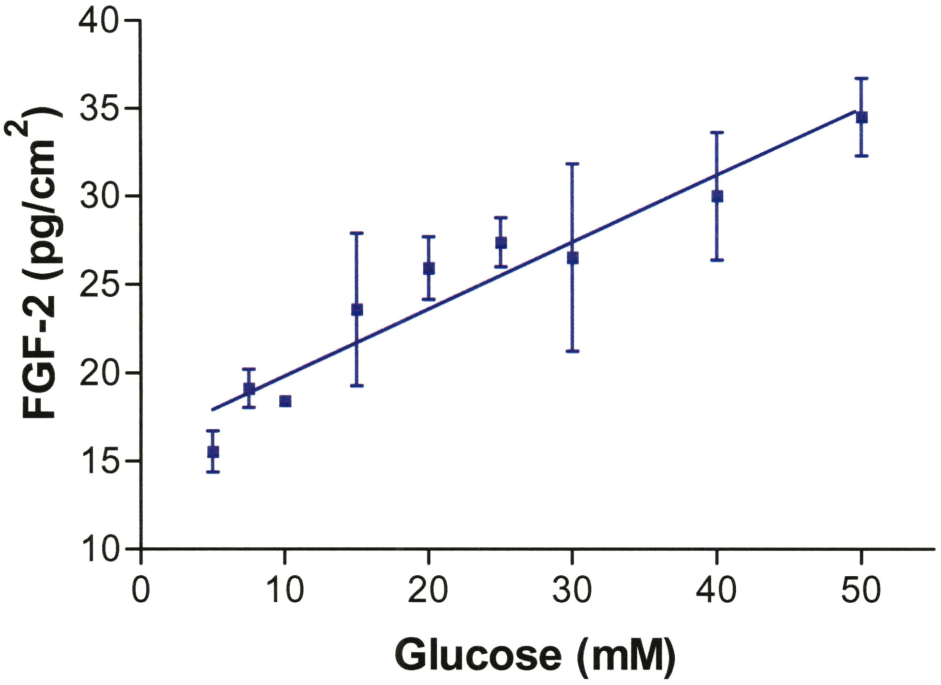


FIGURE 3.2: BASEMENT MEMBRANE FGF-2 INCREASES LINEARLY WITH CULTURE GLUCOSE. Porcine aortic endothelial cells were cultured for four days in supplemented media with glucose ranging from 5-50 mM. After four days, cells were removed, FGF-2 extracted from basement membrane, and measured via FGF ELISA. $p < .0001$ (ANOVA).

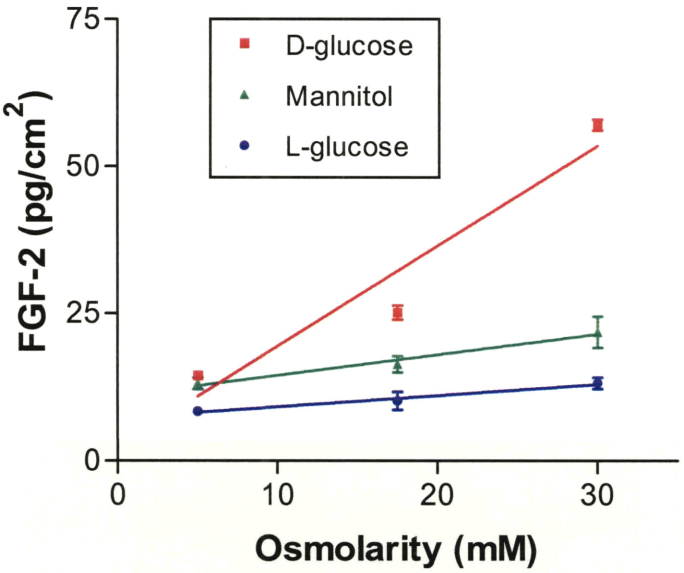


FIGURE 3.3: INCREASED BASEMENT MEMBRANE FGF-2 IS SPECIFIC TO GLUCOSE. Endothelial cells were cultured in 5 mM glucose supplemented media, and D-glucose, mannitol, and L-glucose were added to increase media osmolarity. Cells were removed at four days, FGF-2 extracted from basement membrane, and measured via FGF ELISA. ($p < .01$).

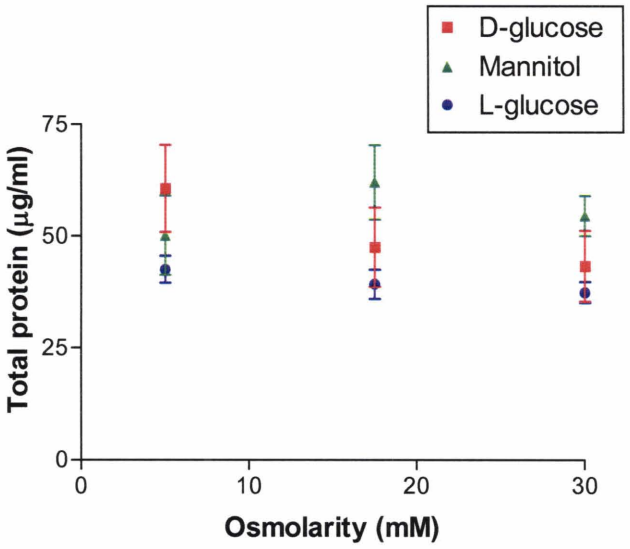


FIGURE 3.4: INCREASED EXTRACTED BASEMENT MEMBRANE FGF-2 IS INDEPENDENT OF MEMBRANE BREAKDOWN. Total protein extracted from basement membrane with salt buffer was quantified and shown not to significantly change with glucose or osmotic controls.

3.3.2 Basement membrane FGF-2 association and dissociation kinetics

Since basement membrane protein content and structure are known to change in hyperglycemia, we investigated whether the increase in FGF-2 extracted from high glucose basement membrane was related to altered basement membrane FGF-2 binding kinetics. The increased native FGF-2 could result from increased basement membrane amount, capacity, or association, or decreased dissociation. As previously described in Chapter 2, FGF-2 bound to basement membrane at equilibrium increased linearly from 0 ng/ml to 5000 ng/ml FGF-2 added, after which bound FGF-2 reached a maximum around 10,000 ng/ml FGF-2 added. For basement membrane isolated from cells cultured at 5, 17.5, or 30 mM glucose, both the linear section and maxima of the binding capacity relationship were similar in values. Since physiologic basement membrane FGF-2 levels are well within the linear range, we examined this portion of the equilibrium capacity curve in greater detail (FIGURE 3.5). Independent of glucose, basement membrane FGF-2 equilibrium binding capacity was linear with a slope of 1 ng/cm2 FGF-2 bound : 40 ng/ml FGF-2 added. No significant difference existed in equilibrium binding capacity for any basement membrane glucose concentration.

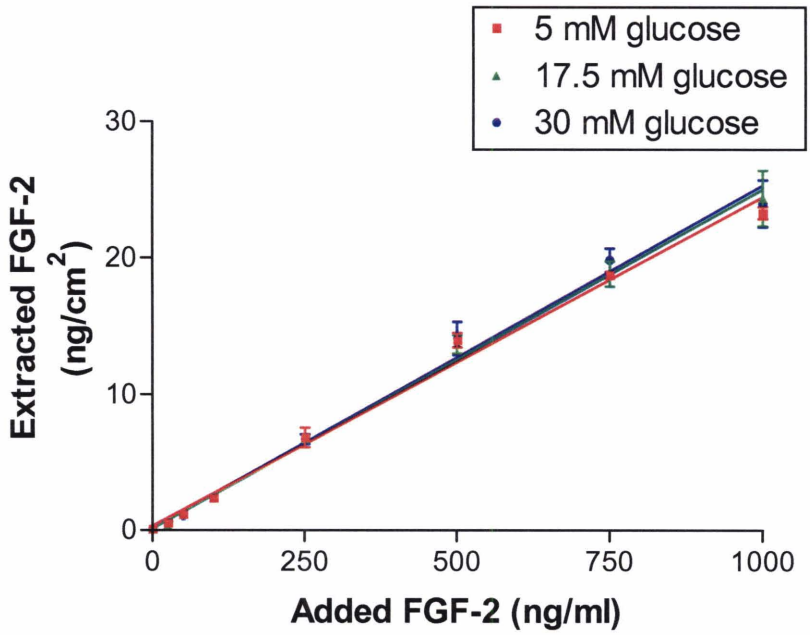


FIGURE 3.5: BASEMENT MEMBRANE FGF-2 EQUILIBRIUM BINDING CAPACITY IS UNCHANGED FOR DIFFERENT GLUCOSE CULTURE CONDITIONS. Endothelial cells were grown in 5, 17.5, or 30 mM glucose supplemented media for four days, after which cells were lysed to leave an intact basement membrane. FGF-2 was added to basement membrane in binding buffer to equilibrium (three hours), after which bound FGF-2 was extracted and quantified via ELISA. ($p > .05$).

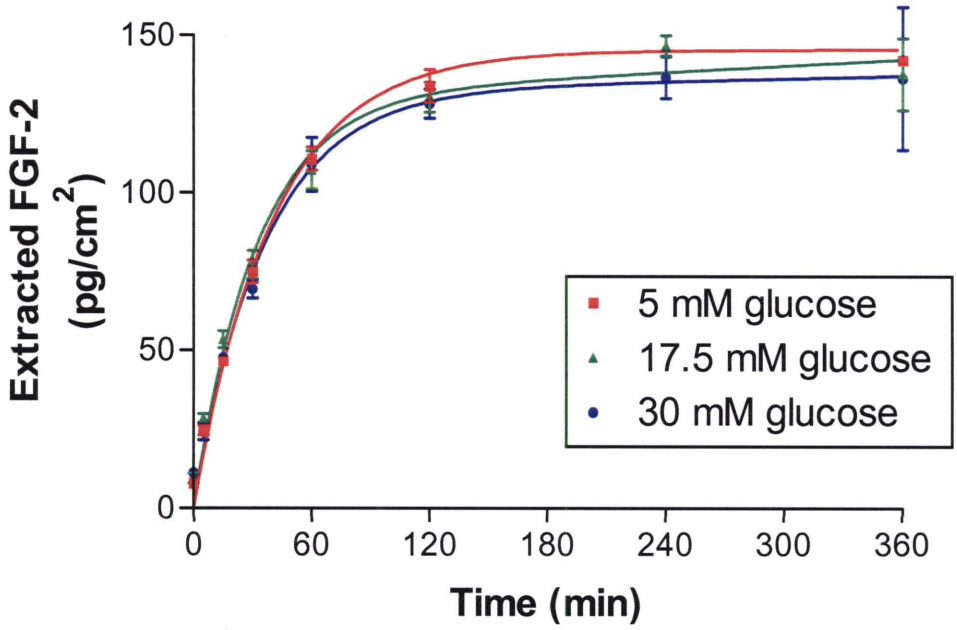


FIGURE 3.6: BASEMENT MEMBRANE FGF-2 ASSOCIATION IS UNCHANGED FOR DIFFERENT GLUCOSE CULTURE CONDITIONS. Endothelial cells were grown in 5, 17.5, or 30 mM glucose supplemented media for four days, after which cells were lysed to leave an intact basement membrane. 5 ng/ml FGF-2 was added to basement membrane in binding buffer for increasing times, after which bound FGF-2 was extracted from basement membrane and quantified via ELISA ($p > .05$).

Basement membrane grown at different glucose concentrations showed strikingly similar association kinetics (FIGURE 3.6). FGF-2 basement membrane association increased rapidly for the first 60 minutes, leveling off between 60 and 360 minutes at approximately 1 ng/cm^2 bound : 40 ng/ml added. The addition of 100 mM glucose to binding buffer at first appeared to slightly decrease FGF-2 association with basement membrane, however this was discovered to be a confounding effect of FGF-2 association to tissue culture polystyrene (FIGURE 3.7). As detailed in Chapter 2, when FGF-2 binding to polystyrene was taken into account, the slight difference in association with glucose in binding buffer was eliminated.

Just as FGF-2 association to basement membrane was unchanged for basement membrane grown at different glucose concentrations, FGF-2 dissociation from basement membrane was also unchanged. FGF-2 dissociated from basement membrane, whether from basement membrane grown under different glucose conditions or with glucose in dissociation buffer, increased rapidly up to 60 minutes, and then equilibrated between 60 and 360 minutes (FIGURE 3.8, FIGURE 3.9). FGF-2 that did

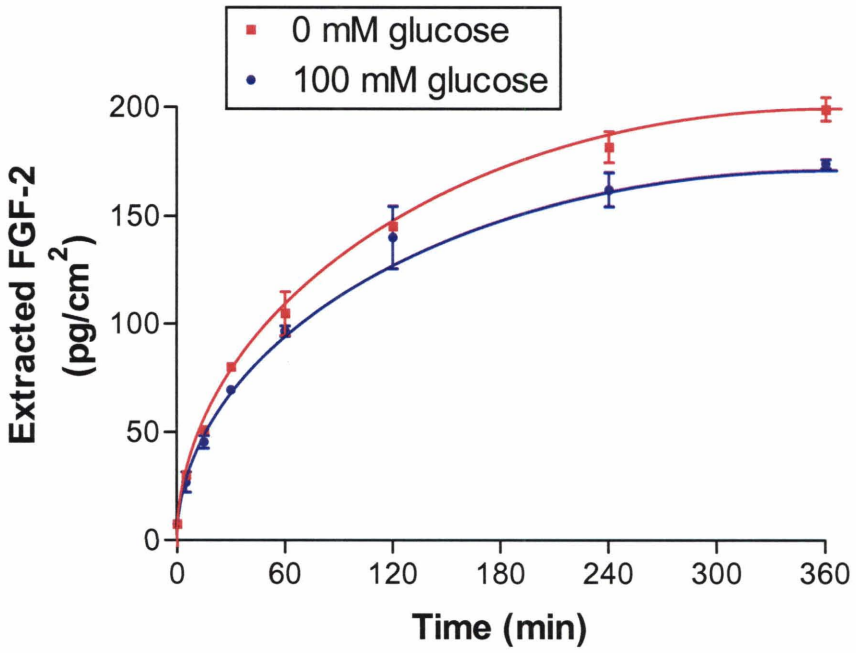


FIGURE 3.7: BASEMENT MEMBRANE FGF-2 ASSOCIATION IS DECREASED WITH GLUCOSE IN BINDING BUFFER. 5 ng/ml FGF-2 was added to 5 mM isolated basement membrane with either 0 or 100 mM glucose in the binding buffer. Association of FGF-2 to basement membrane with time was slightly lower in 100 mM glucose ($p < .01$), but this is an artifact of the glucose effect on FGF-2 binding to polystyrene as discussed in Chapter 1.

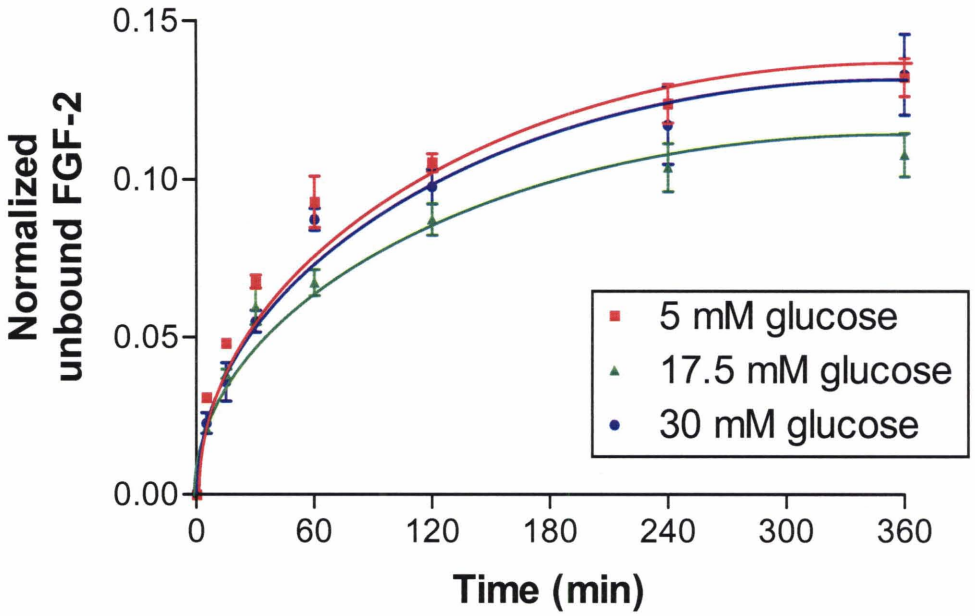


FIGURE 3.8: BASEMENT MEMBRANE FGF-2 DISSOCIATION IS UNCHANGED FOR DIFFERENT GLUCOSE CULTURE CONDITIONS. Endothelial cells were grown in 5, 17.5, or 30 mM glucose supplemented media for four days, after which cells were lysed to leave an intact basement membrane. 5 ng/ml ^{125}I-FGF-2 was added to basement membrane in binding buffer to equilibrium (3 hours). Fresh binding buffer was added, and dissociated FGF-2 was measured in a gamma counter ($p > .05$).

not dissociate was also measured and showed no significant change with glucose. Differences in equilibrium dissociation constants for 5, 17.5, and 30 mM glucose (8.63 x 10-11 M, 2.86 x 10-10 M, 2.034 x 10-10 M; TABLE 3.1), as well as 0 and 100 mM binding buffer glucose (4.01 x 10-10 M, 1.26 x 10-10 M; TABLE 3.2) were not statistically significant.

The only case in which FGF-2 basement membrane binding kinetics were altered by glucose was when FGF-2 was glycated (FIGURE 3.10). The shape of the FGF-2 association curve was significantly flattened, and glycated FGF-2 bound to basement membrane at equilibrium was only about 20% of non-glycated FGF-2. Excepting FGF-2 glycation, FGF-2 basement membrane association and dissociation kinetics were unchanged despite decreased heparan sulfate proteoglycans in basement membrane with increasing culture glucose (FIGURE 3.11).

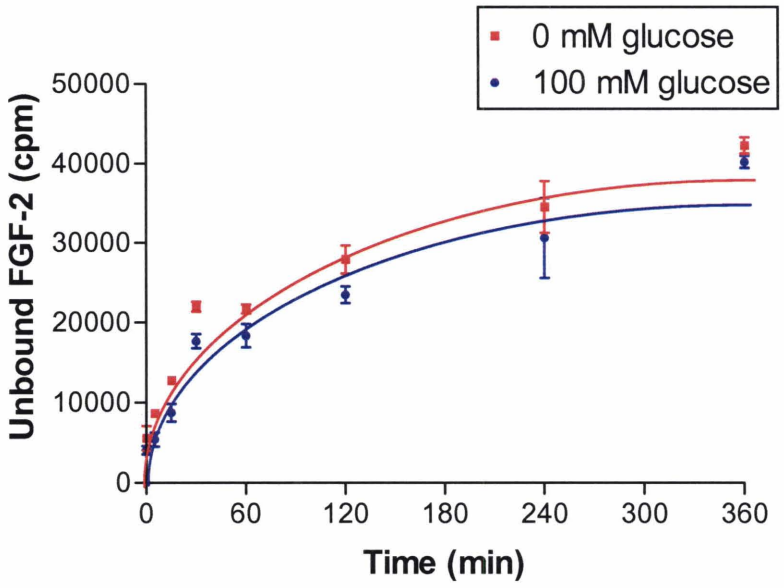


FIGURE 3.9: BASEMENT MEMBRANE FGF-2 DISSOCIATION IS UNCHANGED WITH GLUCOSE IN UNBINDING BUFFER. 5 ng/ml ^{125}I-FGF-2 was added to 5 mM isolated basement membrane to equilibrium. Fresh binding buffer with either 0 or 100 mM glucose was then added, and dissociated FGF-2 was quantified in a gamma counter ($p > .05$).

	5 mM glucose	17.5 mM glucose	30 mM glucose
k_{off}	.00334 ± .0013 min^{-1}	.00956 ± .0053 min^{-1}	.00714 ± .0043 min^{-1}
k_{obs}	.02457 ± .0014 min^{-1}	.02791 ± .0021 min^{-1}	.02646 ± .0023 min^{-1}
k_{on}	3.87 x 10^{7} M^{-1} min^{-1}	3.34 x 10^{7} M^{-1} min^{-1}	3.51 x 10^{7} M^{-1} min^{-1}
K_d	8.63 x 10^{-11} M	2.86 x 10^{-10} M	2.034 x 10^{-10} M

TABLE 3.1: EQUILIBRIUM DISSOCIATION CONSTANTS ARE SIMILAR FOR 5, 17.5, AND 30 MM BASEMENT MEMBRANE. On and off rate constants (k_{on} and k_{off}) were calculated from association and dissociation experiments, and then used to calculate the equilibrium dissociation constant (K_d).

	0 mM glucose	100 mM glucose
k_{off}	.00640 ± .0035 min^{-1}	.00303 ± .0024 min^{-1}
k_{obs}	.01507 ± .0013 min^{-1}	.01630 ± .0012 min^{-1}
k_{on}	1.58 x 10^{7} M^{-1} min^{-1}	2.41 x 10^{7} M^{-1} min^{-1}
K_d	4.05 x 10^{-10} M	1.26 x 10^{-10} M

TABLE 3.2: EQUILIBRIUM DISSOCIATION CONSTANTS ARE SIMILAR FOR BINDING BUFFER GLUCOSE. On and off rate constants (k_{on} and k_{off}) were calculated from association and dissociation experiments, and then used to calculate the equilibrium dissociation constant (K_d).

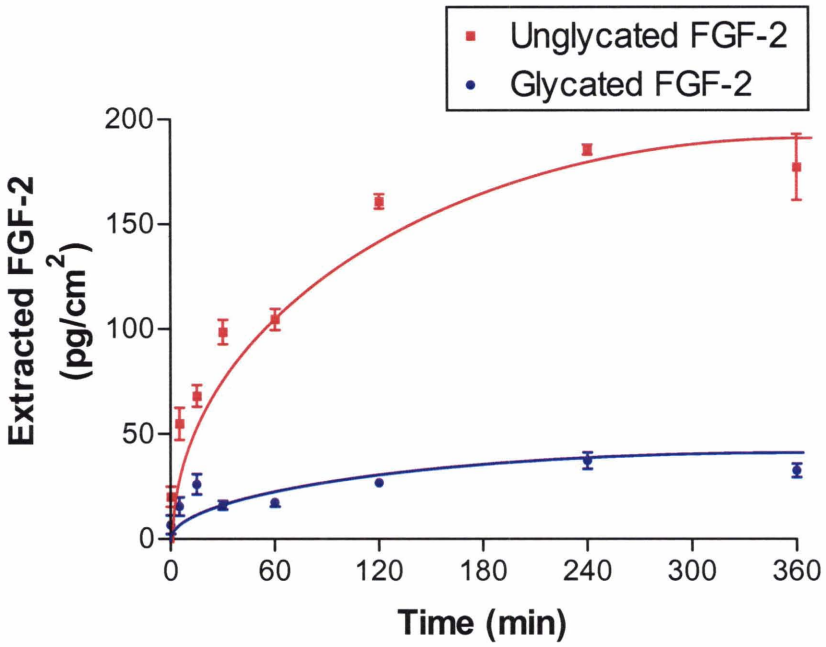


FIGURE 3.10: GLYCATED FGF-2 ASSOCIATION WITH BASEMENT MEMBRANE IS SIGNIFICANTLY LOWER THAN NON-GLYCATED FGF-2. FGF-2 was glycated by 24 hour incubation in 0.25 M fructose at 37 ºC. Association experiments as described previously were done on 5 mM glucose basement membrane with glycated and unglycated FGF-2 ($p < .01$).

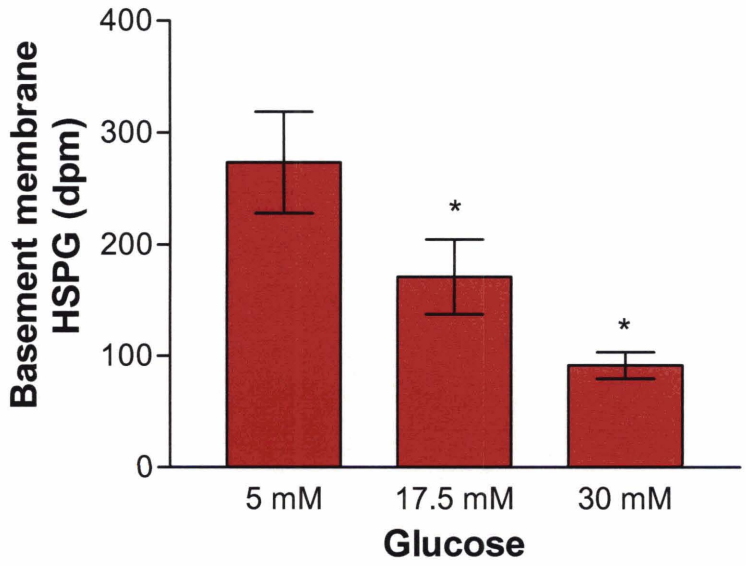


FIGURE 3.11: BASEMENT MEMBRANE HEPARAN SULFATE PROTEOGLYCANS DECREASE WITH CULTURE GLUCOSE. Endothelial cells were cultured for four days in 5, 17.5, or 30 mM glucose media. On the fourth day, cells were switched to labeling media with 100 μCi/ml ^{35}S for 24 hours. Cells were scraped, the basement membrane portion isolated, and sulfation quantified using a Bio-Dot Microfiltration system and a liquid scintillation counter. (*) $p < .01$

3.3.3 FGF-2 heparin binding kinetics

The basement membrane is a complex protein mesh, with potential for nonspecific FGF-2 binding to either basement membrane itself or tissue culture polystyrene underneath basement membrane. We simulated FGF-2 binding specifically to heparan sulfate proteoglycans using a heparin Sepharose column and determined that this specific binding was not altered by glucose in binding buffer.

FGF-2 that passed through the heparin column is indicated by the peak location at fractions 1 to 5 (FIGURE 3.12, INSET A). Little FGF-2 passed directly through the heparin column with either 0 or 100 mM buffer glucose, indicating that glucose does not significantly decrease FGF-2 binding to heparin. The large peak at fractions 45 to 50 shows FGF-2 dissociated from heparin at a given salt gradient. With either 0 or 100 mM buffer glucose, FGF-2 began to dissociate from heparin at 1.3 M NaCl and peaks at 1.55 M NaCl. Less than 1% of FGF-2 dissociated from heparin below 1 M NaCl with or without buffer glucose (FIGURE 3.12, INSET B).

When the same experiment was repeated with glycated FGF-2, glycated FGF-2 that passed directly through the heparin column was more than six times greater than unglycated FGF-2 (FIGURE 3.13, INSET A). The major peak for glycated FGF-2 dissociation still occurred at 1.55 M NaCl, however nearly 10% of glycated FGF-2 dissociated below 1 M NaCl (FIGURE 3.13, INSET B).

3.3.4 *In vivo* correlate

We measured FGF-2 in aortas from control and streptozotocin-treated pigs to validate that the increase in basement membrane FGF-2 was not restricted to endothelial cells in culture but was a real and valid effect in intact vessels *in vivo*. On average, the fasting blood glucose of pigs treated with streptozotocin was 267±100 mg/dl vs. 68±4 mg/dl in control animals. Porcine aortic tissue immunohistochemical analysis for FGF-2 showed greater FGF-2 throughout the arterial wall in hyperglycemic animals but particularly in the subendothelial layer (FIGURE 3.14). When FGF-2 was measured in tissue extracts, FGF-2 normalized to total protein was 44% higher in diabetic animal tissue (FIGURE 3.15).

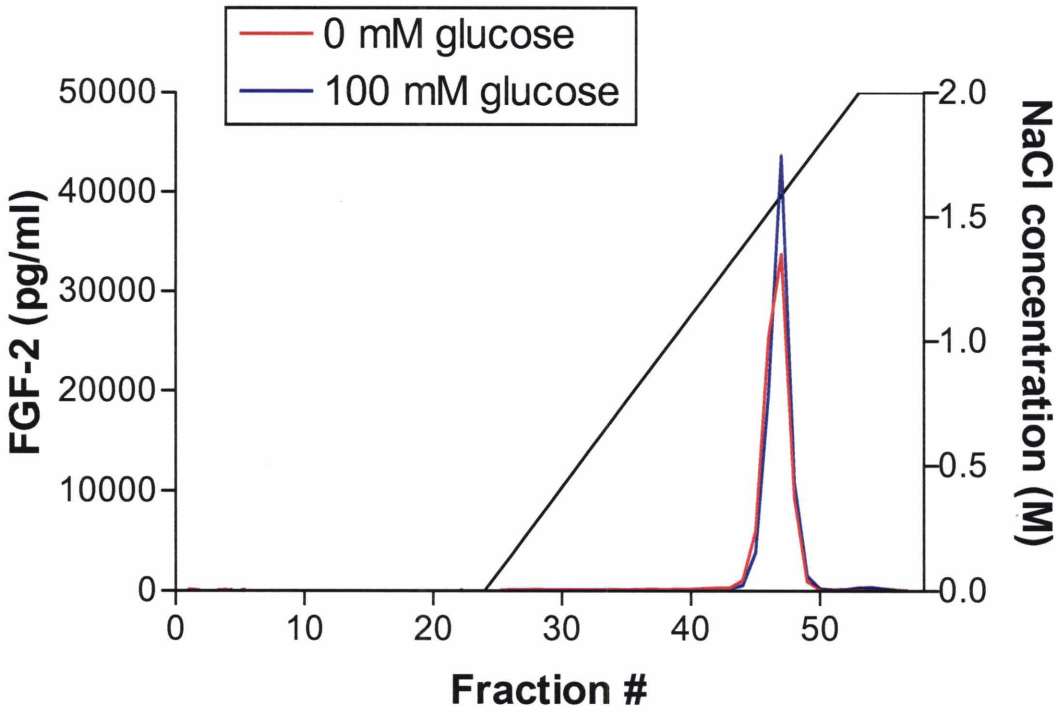


FIGURE 3.12: ELUTION OF FGF-2 FROM A HEPARIN COLUMN WITH DOES NOT CHANGE WITH GLUCOSE IN THE BUFFER. 1 ml of 0.5 μg/ml FGF-2 solution was added to heparin Sepharose column in either 0 or 100 mM glucose buffer. FGF-2 was eluted with a salt gradient from 0 to 2 M, and FGF-2 in each fraction was quantified via FGF ELISA.

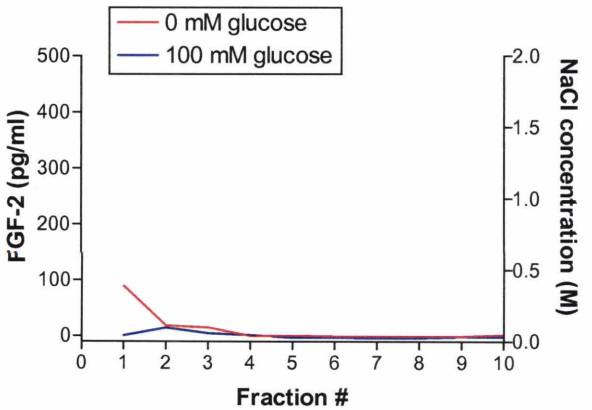


FIGURE 3.12: INSET A (MAGNIFICATION OF FRACTIONS 0 – 10) SHOWING FGF-2 PASSING THROUGH THE COLUMN.

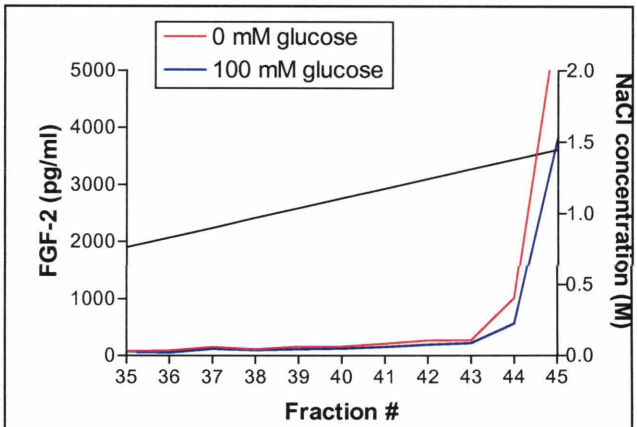


FIGURE 3.12: INSET B (MAGNIFICATION OF FRACTIONS 35-45) SHOWING FGF-2 ELUTION AT LESS THAN 1.5 M NACL.

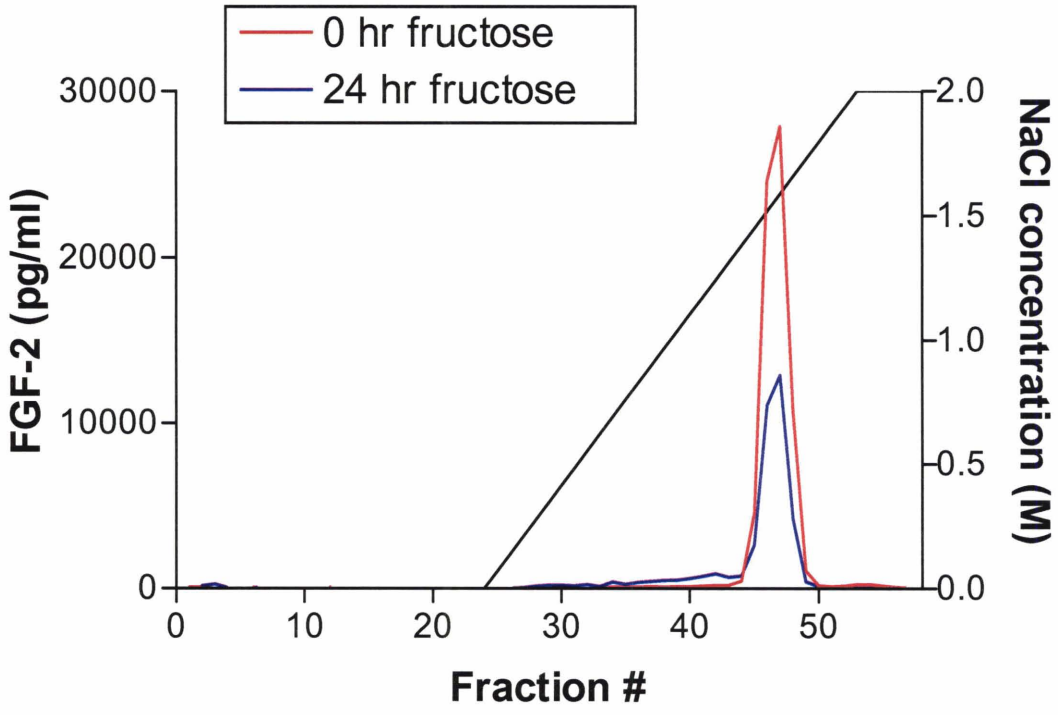


FIGURE 3.13: GLYCATED FGF-2 DEMONSTRATES LOWER AFFINITY FOR HEPARIN SEPHAROSE COLUMN. 0.5 μg/ml FGF-2 either in 0.25 M fructose or incubated for 24 hours in 0.25 M fructose was added to a heparin Sepharose column. FGF-2 was eluted with a salt gradient from 0 to 2 M, and FGF-2 in each fraction was quantified via FGF ELISA. More glycated FGF-2 passes through the heparin column and glycated FGF-2 elutes from a heparin column at a lower salt concentration.

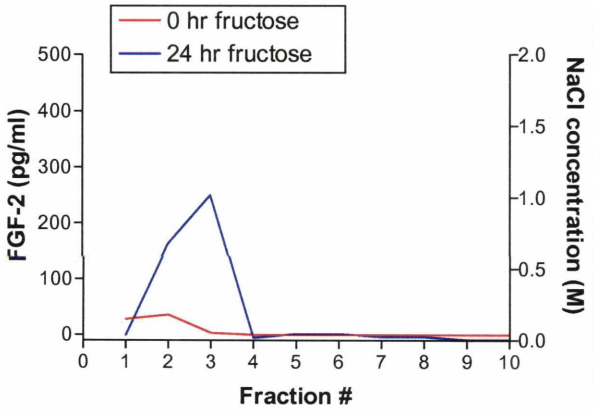


FIGURE 3.13: INSET A (MAGNIFICATION OF FRACTIONS 0-10) SHOWING INCREASED GLYCATED FGF-2 PASSING THROUGH THE COLUMN.

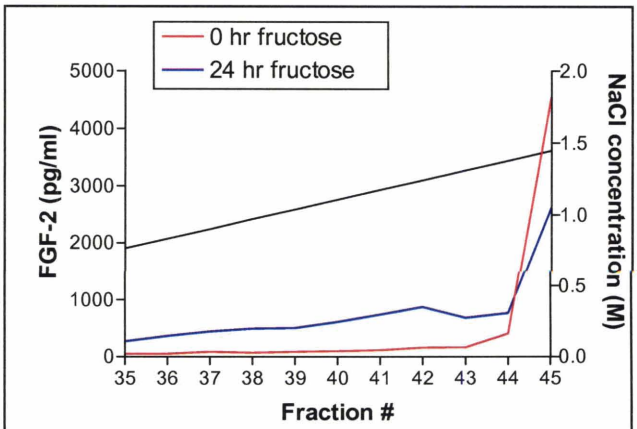


FIGURE 3.13: INSET B (MAGNIFICATION OF FRACTIONS 35-45) SHOWING GLYCATED FGF-2 ELUTION AT LESS THAN 1.5 M NACL.

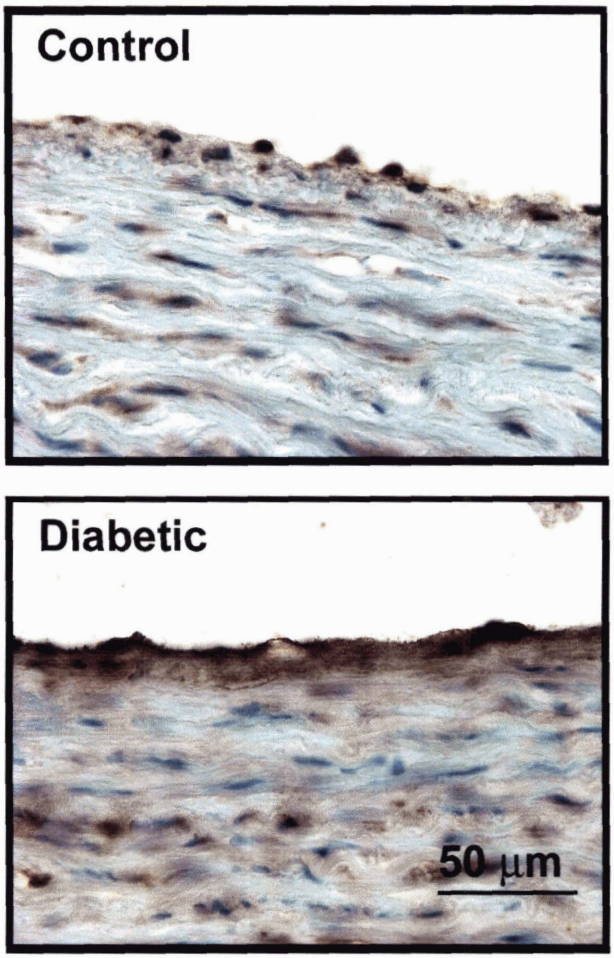


FIGURE 3.14: DIABETIC PORCINE AORTIC TISSUE HAS HIGHER FGF-2 THAN CONTROLS. Diabetes was induced in male domestic swine by streptozotocin injection. Blood glucose concentrations were maintained at 200-250 mg/dl by adjusting daily insulin injections for the duration of the nine week study period. At the end of the study, animals were euthanized, aortic tissue extracted, and rapidly frozen. Frozen sections were labeled with a monoclonal antibody for FGF-2 (brown). Higher levels of FGF-2 are evident in the sub-endothelial space and throughout the vessel thickness of diabetic samples.

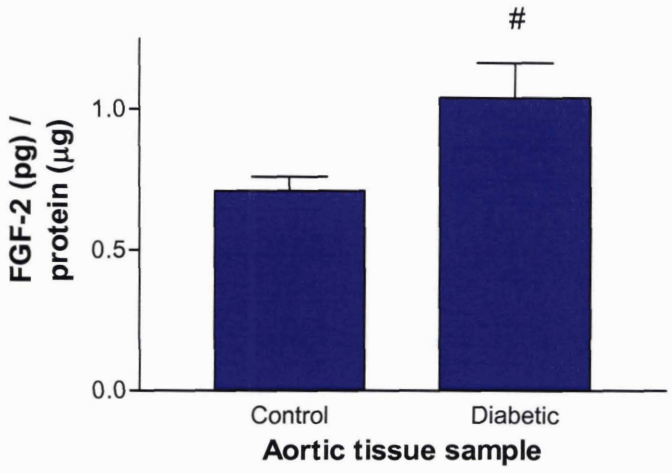


FIGURE 3.15: EXTRACTS FROM DIABETIC PORCINE AORTIC TISSUE SHOW INCREASED FGF-2 COMPARED TO CONTROLS. 3x3 mm porcine aortic tissue sections were homogenized in lysis buffer with protease inhibitors. After centrifugation to remove insoluble material, FGF-2 levels were measured in the extracts via FGF ELISA. (#) $p < .05$ compared to control (Student's t-test).

3.4 Discussion

Our data show that basement membrane FGF-2 storage increases with glucose but not osmotic controls. While others have demonstrated altered protein levels in basement membrane with diabetes or hyperglycemia, this is the first time that a vasoactive factor has been shown to increase (80-82). Our initial hypothesis that inherent changes in basement membrane composition and structure would be linked to altered FGF-2 binding kinetics proved false, despite measured changes in heparan sulfate proteoglycans.

3.4.1 FGF-2 in the media and basement membrane

From two to six days, basement membrane FGF-2 increases exponentially with time across glucose culture conditions. As cells grow and proliferate, increased basement membrane production may combine with increased basement membrane exposure time to FGF-2 to raise basement membrane FGF-2. Similarly, *in vivo*, increased vascular basement membrane thickness in disease states such as diabetes could increase overall basement membrane FGF-2 capacity by increasing total binding sites. Even sites far from endothelial cells could contribute as FGF-2 can diffuse through basement membrane with rapid reversible binding (139). The increase in basement membrane FGF-2 with time should reach equilibrium, as in our association experiments; however we were unable to investigate long term hyperglycemic effects on basement membrane FGF-2 because of endothelial cell differentiation. After six days in culture, cells lost their cobblestone appearance, especially in high glucose conditions. Any difference in basement membrane FGF-2 beyond six days could be attributed to endothelial cell phenotypic change. We therefore turned to an *in vivo* model for long term results.

Basement membrane FGF-2 increases insignificantly with osmotic controls mannitol and L-glucose, indicating that the increase in basement membrane FGF-2 is primarily associated with glucose rather than osmotic pressure. The linear increase in basement membrane FGF-2 with glucose is likely related to either an inherent change in basement membrane that facilitates increased storage or an alteration in endothelial

cell function that allows increased basement membrane exposure to soluble FGF-2. Before investigating these possibilities, we measured total protein in FGF-2 extraction buffer to ensure that the increase in basement membrane FGF-2 with glucose is not an artifact of increased hyperglycemic basement membrane breakdown. Total protein was in fact not significantly different with glucose or osmotic controls.

Study of basement membrane FGF-2 could be confounded by basement membrane exposure to supplemental, exogenous FGF-2 from FBS in the culture media. Porcine aortic endothelial cells used in these experiments required a minimum of 5% v/v FBS for growth. At a measured value of 50 pg/ml FGF-2 in FBS, supplemented media contains 2.5 pg/ml exogenous FGF-2. The total exogenous FGF-2 to which cultured endothelial cells are exposed over four days is 2 pg/cm^2. As only 1/40th of soluble FGF-2 is bound by basement membrane at equilibrium, the maximum FGF-2 that FBS might contribute to the basement membrane is 0.05 pg/cm^2. This value is less than 0.7% of total FGF-2 extracted from basement membrane, indicating that exogenous FGF-2 contribution to basement membrane FGF-2 is minor compared to endothelial cell-derived FGF-2.

3.4.2 FGF-2 basement membrane binding kinetics

Our results indicate that FGF-2 binding kinetics, including equilibrium capacity, association, and dissociation, are unchanged for basement membrane grown under increasing glucose concentrations. This is surprising given the extensive alterations in diabetic vascular basement membrane composition and structure, in particular the reported decrease in heparan sulfate proteoglycans to which FGF-2 binds (82). Vogl-Willis et al. measured small decreases in sulfation of basement membrane heparan sulfate proteoglycans in short term hyperglycemic culture of both bovine and human endothelial cells (153). We similarly show a decrease in basement membrane sulfation with glucose, but this appears to have no effect on FGF-2 binding kinetics. When we drastically decreased basement membrane heparan sulfate proteoglycans by growing cells with sodium chlorate, binding kinetics did change. Therefore, we believe that changes in basement membrane heparan sulfate proteoglycans with glucose are not large enough to significantly affect FGF-2 binding kinetics.

We further demonstrate that glucose in either binding or dissociation buffer does not alter basement membrane FGF-2 association or dissociation respectively. We had hypothesized that, because heparan sulfate and glucose are both polysaccharides, glucose itself might displace FGF-2 from heparan sulfate proteoglycans in basement membrane. However, extremely high glucose does not alter FGF-2 association to or dissociation from basement membrane, or FGF-2 binding specifically to heparin in a heparin Sepharose column.

Glucose modifies basement membrane FGF-2 binding kinetics only through FGF-2 glycation, which decreases binding to both basement membrane and specifically to heparin in a heparin Sepharose column. This agrees well with the literature showing that glycation decreases FGF-2 activity and affinity for heparan sulfate proteoglycans (150). Heparan sulfate protects FGF-2 from glycation, implying that the increased basement membrane FGF-2 store should remain unglycated and therefore active over long hyperglycemic episodes (103). Since glycation would decrease basement membrane FGF-2 in high glucose, and our samples were exposed to glucose for a relatively short time period, FGF-2 glycation does not appear to be significant in basement membrane FGF-2 modulation with glucose in our experiments.

3.4.3 *In vivo* model

Immunohistochemical porcine aortic tissue analysis confirms that FGF-2 in the vessel wall increases in hyperglycemic states. The *in vivo* model is not without limitations, as these animals were exposed to far greater stress than hyperglycemia alone. For example, treated animals were periodically injected with exogenous insulin, showed increased triglycerides, and decreased weight at the study endpoint. Nonetheless, the *in vivo* data do extend the *in vitro* findings by suggesting that long term hyperglycemia can lead to increased FGF-2 vascular storage. Immunohistochemical identification of increased FGF-2 was noted in the subendothelial layer and throughout the aortic media. This could result from FGF-2 transport through the arterial wall through reversible binding to heparan sulfate proteoglycans (139).

3.4.4 Limitations

While these data suggest that basement membrane FGF-2 storage increases without a change in basement membrane FGF-2 binding kinetics, there are limitations to these studies. Alterations in basement membrane FGF-2 could be an artifact of the cell removal process, although we achieved similar results by removing cells without lysis. Similarly, increased FGF-2 could be a phenomenon only at the basement membrane surface, since the salt buffer likely only removes FGF-2 from accessible surface binding sites. Even if this is the case, our data show that the available surface fraction of basement membrane FGF-2 is increased.

Basement membrane binding kinetics experiments were performed entirely on a macro size scale. There could possibly be differences, either in timing or quantity of bound FGF-2 that could not be detected at this level. Additionally, some dissociation data were collected on separate days and therefore had to be normalized to account for changes in radiolabeled FGF-2 over time. This limited our ability to detect small changes in FGF-2 basement membrane dissociation. We were able to show decreased glycated FGF-2 association to basement membrane, however our heparin column experiments showed a smaller loss of FGF-2 affinity for heparin with glycation. This could be related to either incomplete FGF-2 glycation, or early dissociation of glycated FGF-2 from the heparin perhaps in unmeasured column washes.

As with any *in vitro* experiments, extension of tissue culture findings to *in vivo* situations is limited. It is nearly impossible to create an *in vivo* model that replicates *in vitro* conditions without additional environmental influences. The porcine diabetic model limitations were discussed above, but there are also limitations to our aortic tissue processing. We were unable to extract or label just endothelial cell basement membrane FGF-2, therefore these data represent FGF-2 in the entire vessel wall including intracellular stores. Our *in vitro* data suggests that intracellular FGF-2 is not increased, and we normalized FGF-2 to total protein to account for changes in total cell number and increased intracellular size, but it is possible that increased measured FGF-2 in diabetic porcine aorta is an artifact of cellular changes.

3.5 Conclusions

Our data show that glucose mediates a linear increase in basement membrane FGF-2. Surprisingly, this change occurs without an alteration in basement membrane FGF-2 binding kinetics. Only FGF-2 glycation, which might occur intracellularly over the course of weeks, decreases FGF-2 association with heparin moieties in basement membrane. This chapter suggests that the increase in basement membrane FGF-2 occurs on a time scale that is faster than other basement membrane changes, and therefore is more likely to be mediated by cellular dysfunctions that occur with glucose on a shorter time scale.

3.6 Chapter References

1. Tsilibary, E. 2003. Microvascular basement membranes in diabetes mellitus. *J Pathol* 200:537-547.

2. Martin, A., Komada, M.R., and Sane, D.C. Abnormal angiogenesis in diabetes mellitus. *Medicinal Research Reviews*:117-145.

3. Beckman, J.A., Creager, M.A., and Libby, P. Diabetes and atherosclerosis - Epidemiology, pathophysiology, and management. *Jama-Journal Of The American Medical Association*:2570-2581.

4. Carrozza, J.P., Kuntz, R.E., Fishman, R.F., and Baim, D.S. Restenosis After Arterial Injury Caused By Coronary Stenting In Patients With Diabetes-Mellitus. *Annals Of Internal Medicine*:344-349.

5. Aiello, L.P., Avery, R.L., Arrigg, P.G., Keyt, B.A., Jampel, H.D., Shah, S.T., Pasquale, L.R., Thieme, H., Iwamoto, M.A., Park, J.E., et al. 1994. Vascular Endothelial Growth-Factor In Ocular Fluid Of Patients With Diabetic-Retinopathy And Other Retinal Disorders. *New England Journal Of Medicine* 331:1480-1487.

6. De Vriese, A., Tilton, R.G., Elger, M., Stephan, C.C., Kriz, W., and Lameire, N.H. 2001. Antibodies against vascular endothelial growth factor improve early renal dysfunction in experimental diabetes. *Journal Of The American Society Of Nephrology* 12:993-1000.

7. Frank, S., Hubner, G., Breier, G., Longaker, M.T., Greenhalgh, D.G., and Werner, S. 1995. Regulation Of Vascular Endothelial Growth-Factor Expression In Cultured Keratinocytes - Implications For Normal And Impaired Wound-Healing. *Journal Of Biological Chemistry* 270:12607-12613.

8. Zimering, M.B., and Eng, J. 1996. Increased basic fibroblast growth factor-like substance in plasma from a subset of middle-aged or elderly male diabetic patients with microalbuminuria or proteinuria. *Journal Of Clinical Endocrinology And Metabolism* 81:4446-4452.

9. Takeuchi, K., Takehara, K., Tajima, K., Kato, S., and Hirata, T. 1997. Impaired healing of gastric lesions in streptozotocin-induced diabetic rats: Effect of basic fibroblast growth factor. *Journal Of Pharmacology And Experimental Therapeutics* 281:200-207.

10. Kowluru, R.A., and Koppolu, P. 2002. Termination of experimental galactosemia in rats, and progression of retinal metabolic abnormalities. *Investigative Ophthalmology & Visual Science* 43:3287-3291.

11. Engerman, R.L., and Kern, T.S. 1995. Retinopathy In Galactosemic Dogs Continues To Progress After Cessation Of Galactosemia. *Archives Of Ophthalmology* 113:355-358.

12. Roy, S., Cagliero, E., and Lorenzi, M. 1996. Fibronectin overexpression in retinal microvessels of patients with diabetes. *Investigative Ophthalmology & Visual Science* 37:258-266.

13. Cohen, M.P., and Khalifa, A. 1977. Renal Glomerular Collagen-Synthesis In Streptozotocin Diabetes - Reversal Of Increased Basement-Membrane Synthesis With Insulin Therapy. *Biochimica Et Biophysica Acta* 500:395-404.

14. Shimomura, H., and Spiro, R.G. 1987. Studies On Macromolecular Components Of Human Glomerular-Basement-Membrane And Alterations In Diabetes - Decreased Levels Of Heparan-Sulfate Proteoglycan And Laminin. *Diabetes* 36:374-381.

15. Bangstad, H.J., Osterby, R., Dahljorgensen, K., Berg, K.J., Hartmann, A., and Hanssen, K.F. Improvement Of Blood-Glucose Control In Iddm Patients Retards The Progression Of Morphological-Changes In Early Diabetic Nephropathy. *Diabetologia*:483-490.

16. Cagliero, E., Roth, T., Roy, S., and Lorenzi, M. Characteristics And Mechanisms Of High-Glucose Induced Overexpression Of Basement-Membrane Components In Cultured Human Endothelial-Cells. *Diabetes*:102-110.

17. Haitoglou, C.S., Tsilibary, E.C., Brownlee, M., and Charonis, A.S. 1992. Altered Cellular Interactions Between Endothelial-Cells And Nonenzymatically Glucosylated Laminin Type-Iv Collagen. *Journal Of Biological Chemistry* 267:12404-12407.

18. Charonis, A.S., Reger, L.A., Dege, J.E., Kouzikoliakos, K., Furcht, L.T., Wohlhueter, R.M., and Tsilibary, E.C. 1990. Laminin Alterations After Invitro Nonenzymatic Glycosylation. *Diabetes* 39:807-814.

19. Tsilibary, E.C., Charonis, A.S., Reger, L.A., Wohlhueter, R.M., and Furcht, L.T. 1988. The Effect Of Nonenzymatic Glucosylation On The Binding Of The Main Noncollagenous Nc1 Domain To Type-Iv Collagen. *Journal Of Biological Chemistry* 263:4302-4308.

20. Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., Klagsbrun, M. 1987. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *PNAS* 84:2292-2296.

21. Giardino, I., Edelstein, D., and Brownlee, M. 1994. Nonenzymatic Glycosylation In-Vitro And In Bovine Endothelial-Cells Alters Basic Fibroblast Growth-Factor Activity - A Model For Intracellular Glycosylation In Diabetes. *Journal Of Clinical Investigation* 94:110-117.

22. Boullion, R.D., Mokelke, E.A., Wamhoff, B.R., Otis, C.R., Wenzel, J., Dixon, J.L., and Sturek, M. 2003. Porcine model of diabetic dyslipidemia: Insulin and feed algorithms for mimicking diabetes mellitus in humans. *Comparative Medicine* 53:42-52.

23. Gerrity, R.G., Natarajan, R., Nadler, J.L., and Kimsey, T. 2001. Diabetes-induced accelerated atherosclerosis in swine. *Diabetes* 50:1654-1665.

24. Dowd, C.J., Cooney, C.L., and Nugent, M.A. 1999. Heparan sulfate mediates bFGF transport through basement membrane by diffusion with rapid reversible binding. *Journal Of Biological Chemistry* 274:5236-5244.

25. Vogl-Willis, C.A., and Edwards, I.J. 2004. High-glucose-induced structural changes in the heparan sulfate proteoglycan, perlecan, of cultured human aortic endothelial cells. *Biochimica Et Biophysica Acta-General Subjects* 1672:36-45.

26. Nissen, N.N., Shankar, R., Gamelli, R.L., Singh, A., and DiPietro, L.A. 1999. Heparin and heparan sulphate protect basic fibroblast growth factor from non-enzymic glycosylation. *Biochemical Journal* 338:637-642.

CHAPTER 4

ENDOTHELIAL CELL INFLUENCE ON BASEMENT MEMBRANE FGF-2

Abstract

The endothelial cell – basement membrane co-regulatory unit produces, releases, stores, and metabolizes FGF-2. In chapter 3, we demonstrated that the increase in basement membrane FGF-2 with glucose was not related to inherent changes in basement membrane FGF-2 binding kinetics. We now show for the first time that endothelial cells actively control basement membrane FGF-2 through FGF-2 release and cell permeability. These two changes in cell function with glucose occur on a timescale of hours to days, which correlates well with the rapid increase in basement membrane FGF-2. This cell-mediated dysregulation of FGF-2 storage in the endothelial cell – basement membrane unit could contribute to vascular dysfunction.

4.1 Introduction

Basement membrane FGF-2 storage increases with culture glucose concentration, but basement membrane FGF-2 binding kinetics are unchanged with glucose. While this was surprising given the reported extensive alterations in basement membrane structure and protein composition, it is likely that large changes in basement membrane composition occur over the course of weeks rather than days. We now hypothesize that changes in endothelial cell function that occur on a timescale of hours to days could lead to altered basement membrane FGF-2.

Through equilibrium capacity studies (Chapter 3), we showed that FGF-2 bound to basement membrane increases linearly with available soluble FGF-2. Based on this model, if endothelial cells released more FGF-2 into the media, more FGF-2 would bind to basement membrane. However these capacity studies were performed on bare basement membrane, whereas our native basement membrane is grown beneath an endothelial cell layer. Not only does more FGF-2 need to be released by cells, but cells also must allow FGF-2 through to bind to basement membrane. In this chapter, we explore how FGF-2 release from endothelial cells and endothelial cell permeability could lead to increased basement membrane FGF-2 with glucose, focusing in the end on glucose-induced stress mechanisms.

The FGF-2 release mechanism remains an intriguing mystery. FGF-2 does not have a signal sequence for secretion, and therefore is not released through the classic signal sequence pathway (97). The primary current hypothesis is that FGF-2 is released from endothelial cells at times of sub-lethal injury or cell death. Cellular stresses such as UV irradiation, mechanical wounding, and shear stress have been observed to result in FGF-2 release (154-156). An alternative hypothesis asserts that a non-classical release pathway exists, but no specific pathway has yet been identified.

Cell injury or death could lead to FGF-2 release through the apoptotic pathway. The term apoptosis was first coined in 1972 to describe a form of cell death morphologically distinct from necrosis (157). Apoptotic morphological changes include cell rounding, chromatin condensation, nuclear fragmentation, and plasma membrane blebbing. Most of these changes are attributed to activation of caspases, cysteine

proteases specific to apoptosis (158). Because caspases, and in particular caspase 3, are a common mediator across the spectrum of stimuli for apoptotic cell death, caspase blockade is an attractive means of controlling apoptosis.

Endothelial cell apoptosis increases in high glucose conditions. Human umbilical vein endothelial cells showed 1.5 fold greater apoptosis in 30 mM than 5 mM glucose culture, and intermittent high and low glucose proved even more damaging than constant hyperglycemia (159, 160). Inhibitors of this effect have been investigated and several pathways suggested, but a common apoptotic pathway clearly leads through caspase 3 (161-164). As apoptosis is linked to both angiogenesis and atherosclerosis, there is strong potential for a link between glucose-induced endothelial cell apoptosis and vascular dysfunction in diabetes (165, 166).

In addition to increasing endothelial cell apoptosis and potentially FGF-2 release, glucose increases endothelial cell permeability, in particular to inert proteins such as albumin and dextran (167). This change has further been linked to aldose reductase or protein kinase C (PKC) activation, which provide potential pathways for glucose-induced cellular dysfunction as described later (64, 167). This increased permeability has been demonstrated *in vitro* and *in vivo* but data are limited to inert, rather than vasoactive, proteins. Altered endothelial cell permeability is linked to retinopathy, nephropathy, and atherosclerosis *in vivo*, providing yet another potential relationship between glucose-induced endothelial cell dysfunction and vascular morbidities in diabetes (22, 168, 169).

Glucose induces endothelial cell dysfunction such as apoptosis and altered permeability through three seemingly independent pathways: PKC activation, advanced glycation end product (AGE) formation, and increased flux through the aldose reductase pathway. Suppression of these pathways can enhance glomerular filtration and albumin secretion in the kidney, suppress normally accelerated atherosclerosis, and improve microvascular function amongst a spectrum of effects (48, 170, 171). More recently, Nishikawa et al. showed that all three pathways of glucose-induced cellular stress could be blocked through normalization of mitochondrial superoxide species, either by inhibiting the electron transport chain, uncoupling oxidative phosphorylation, or directly inhibiting reactive oxygen species through manganese superoxide dismutase, (67). Many other environmental stressors similarly cause an increase in reactive oxygen

species, pointing to oxidative stress as a potential common mediator for endothelial cell dysfunction.

In this chapter, we explore endothelial cell effects on basement membrane FGF-2. We show that endothelial cell FGF-2 release increases with glucose, and this increase occurs concomitant with an increase in apoptosis. Our data also indicate that this released FGF-2 has greater access to basement membrane binding sites due to enhanced endothelial cell permeability, extending the concept of glucose-induced cell permeability to include vasoactive proteins. While attempts to block caspases, PKC, and reactive oxygen species did not abrogate the increase in basement membrane FGF-2 with glucose, they did elucidate potential mechanisms by which the increase could occur. Finally, reactive oxygen species were identified as a potential common mediator for endothelial cell dysfunction that leads to increased FGF-2 release and permeability.

4.2 Materials and Methods

4.2.1 Endothelial cell culture

Porcine aortic endothelial cells (PAEC) were cultured in growth media with 5, 17.5, or 30 mM D-glucose as described, with mannitol as osmotic control. Porcine tumor necrosis factor-α (TNFα; R&D Systems), a potent inflammatory cytokine, and tert-butyl hydroperoxide (tBHP; Sigma), which is broken down intracellularly to produce reactive oxygen species, were used as alternative methods to induce environmental stress. TNFα was added for 24 hours and tBHP for 48 hours.

For PKC inhibition, PAEC were exposed to 1.0 nM bisindolylmaleimide (Calbiochem), which binds to the catalytic domain to inhibit PKC α-, β_I-, β_{II}-, γ-, δ-, and ε-isoforms, for one hour before glucose-replete media addition. For caspase inhibition, either Z-VAD-FMK (25 μM;Calbiochem), a general caspase inhibitor, or Ac-DEVD-CHO (100 μM; Calbiochem), a specific caspase 3 inhibitor, was added to media 30 minutes prior to high glucose exposure. A neutralizing VEGF antibody (AF-293-NA; R&D Systems), with a reported ND_{50} of 0.01 μg/ml, was used at 0.1 μg/ml for two days. Superoxide dismutase, either from horseradish (Sigma) or a cell-permeable mimetic (Calbiochem) was used to block reactive oxygen species formation. Each inhibitor was tested to ensure that it adequately blocked the appropriate factor.

4.2.2 FGF-2 quantification

To measure intracellular FGF-2, endothelial cells were trypsinized, washed, pelleted, and frozen overnight at -80 °C. The following morning, cells were resuspended in PBS with complete protease inhibitor and homogenized. Samples were centrifuged to pellet insoluble cell debris, and the supernatant was stored at 4 °C until use. Total cell extract protein showed no significant difference between 5, 17.5, and 30 mM glucose samples. FGF-2 released from PAEC was collected in cell culture media and centrifuged to remove cellular debris. The conditioning media contained FGF-2 from FBS, but this was quantified at 2.5 pg/ml, which is less than 1% of measured values of released FGF-2. The conditioning media with FGF-2 was stored at 4 °C until use, and

all samples were quantified using an FGF ELISA (R&D Systems). While FGF-2 was more stable in cell extracts and conditioning media, samples were analyzed immediately using a single ELISA whenever possible and always within 24 hours.

4.2.3 Apoptosis

PAEC apoptosis was measured using four complementary methods: cell counts, annexin V – propidium iodide, caspase 3 activation, and terminal deoxynucleotidyl-transferase dUTP nick end labeling (TUNEL). Optimal cell exposure to environmental stress for apoptosis was determined for glucose, TNFα, and tBHP individually as their cell kinetic effects differ greatly. For example, cells exposed to TNFα showed a rapid apoptotic response within 24 hours (FIGURE 4.1). Cells exposed to high glucose, on the other hand, required at least 48 hours of exposure before a significant difference in apoptosis could be measured. Apoptosis was no different after eight days of exposure than after four days, likely because culture media with floating cells, of which the majority are apoptotic, was entirely removed every 48 hours (FIGURE 4.2). Therefore cells were exposed to glucose for four days, TNFα for 24 hours, and tBHP for 48 hours. Cell counts were performed by trypsinizing cells and counting with a Coulter counter (Beckman Coulter).

The annexin V – propidium iodide assay binds annexin V to phosphatidylserine which is translocated from inner to outer cell membrane in apoptosis. Cells are also identified to be alive using the vital dye propidium iodide. Therefore, cells in early apoptosis are classified as annexin V positive and propidium iodide negative. Several cell removal methods, including scraping, ethyl diamine tetraacetic acid (EDTA), and trypsin were compared to determine how to remove attached cells with the least damage (FIGURE 4.3). Scraping produced a wide cell size array, shown by extensive distribution along the forward scatter axis (FSC-H), indicating many fragmented or clumped cells. Scraping also produced extensive cell damage as seen by the high cell fragment concentration, indicated by the yellow color in the lower left corner of the plot. Cells removed by EDTA still had a large size distribution but showed less fragmentation. Trypsin produced the least cell fragmentation with the most uniform cell size, as indicated by the high cell concentration focused in one plot area (FIGURE 4.3, yellow).

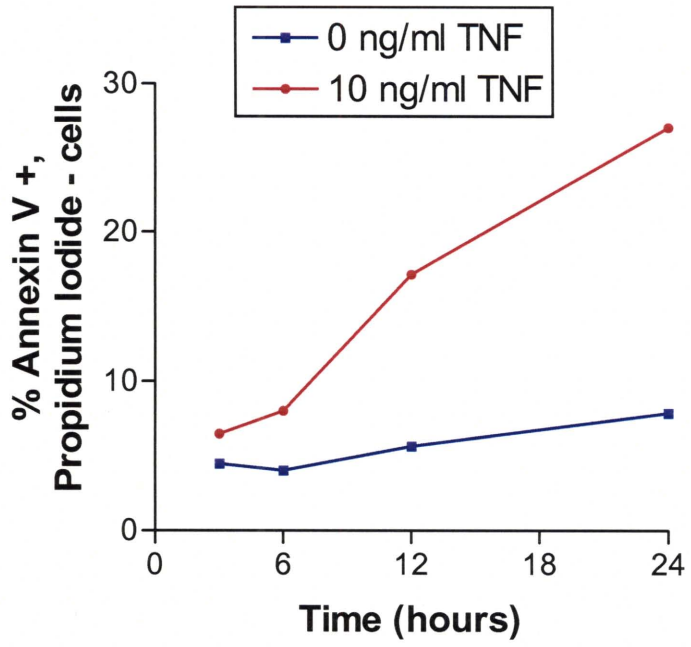


FIGURE 4.1: ANNEXIN V – PROPIDIUM IODIDE ASSAY INCREASES UP TO 24 HOURS FOR TNFα EXPOSURE. Endothelial cells grown for four days in 5 mM glucose growth media were exposed to 0 or 10 ng/ml TNFα. At the end of each time period, cells were trypsinized, labeled with Annexin V and propidium iodide, and analyzed by flow cytometry.

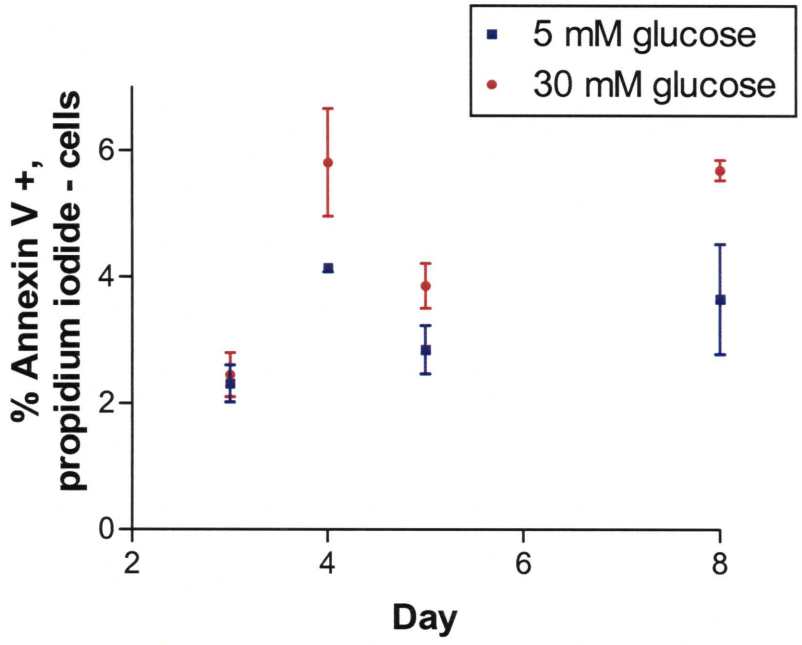


FIGURE 4.2: ANNEXIN V – PROPIDIUM IODIDE ASSAY PEAKS ON EVEN DAYS FOR GLUCOSE EXPOSURE. Endothelial cells were grown in 5 or 30 mM glucose growth media. At the end of each time period, cells were trypsinized, labeled with Annexin V and propidium iodide, and analyzed by flow cytometry.

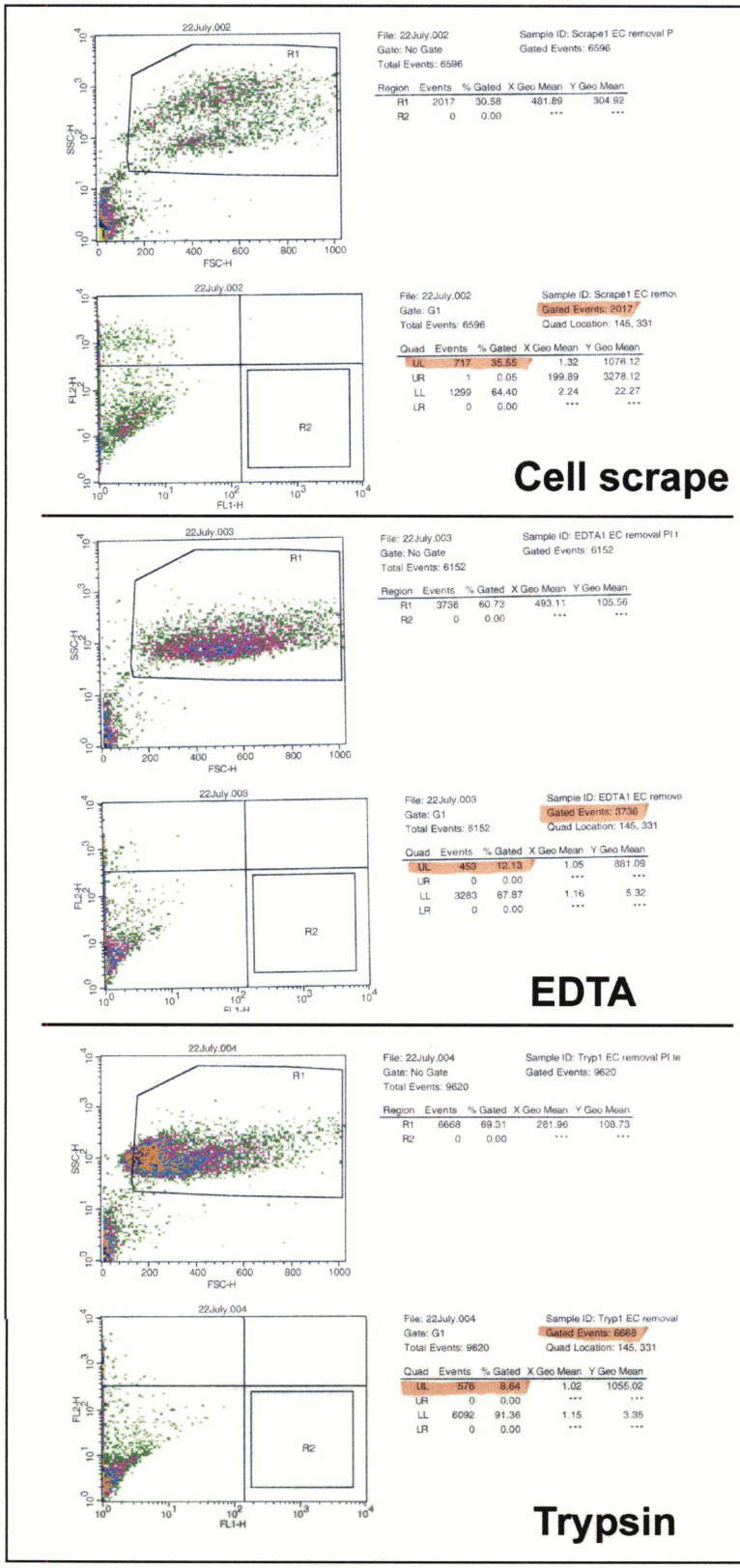


FIGURE 4.3: FLOW CYTOMETRY DATA FOR CELL REMOVAL METHODS: CELL SCRAPING, EDTA, AND TRYPSIN. Trypsinization produced the tightest cell size distribution (FSC-H) and the lowest percentage of cell fragments (population in lower left corner).

The latter two techniques were further evaluated in the annexin V – propidium iodide assay. Cells harvested with trypsin were less likely to be propidium iodide positive (damaged, dead) than those harvested with EDTA, indicating less potentially confounding damage from cell removal (FIGURE 4.4). Therefore, PAEC were prepared for the annexin V – propidium iodide assay by removing attached cells with trypsin and combining them with floating cells from the media. Samples were centrifuged to pellet cells, washed thoroughly in PBS, resuspended in annexin binding buffer, and labeled with annexin V-FITC and propidium iodide as per kit instructions (BD Pharmingen). Samples were analyzed immediately by flow cytometry.

Caspase 3, a cysteine protease activated during early apoptosis, is considered the point of "no return" for apoptotic cells. Activated caspase 3 was measured in PAEC by a spectrofluorometric assay and flow cytometry (BD Pharmingen). For spectrofluorimetry, PAEC were lysed per kit instructions, and 50 μl of cell lysate was

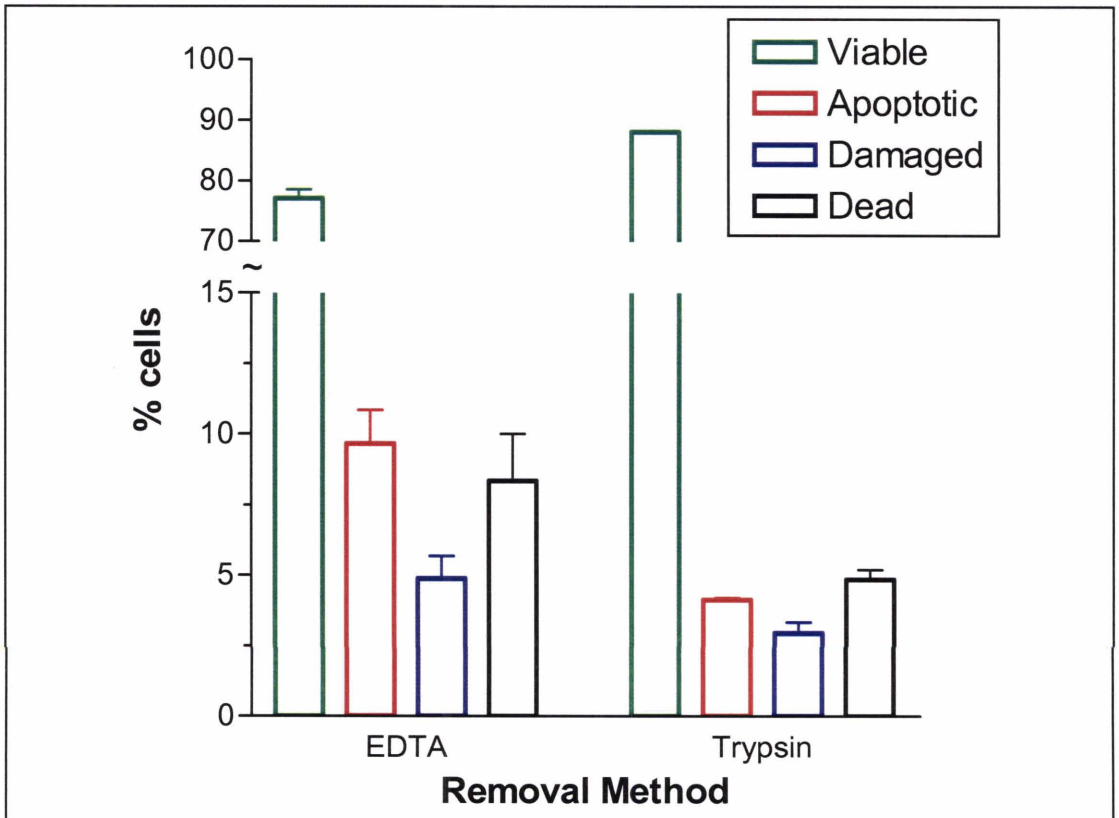


FIGURE 4.4: TRYPSIN PRODUCES AN OPTIMAL CELL DISTRIBUTION IN THE ANNEXIN V – PROPIDIUM IODIDE ASSAY. Trypsinization of cells as opposed to EDTA produces more viable cells and fewer damaged or dead cells, as analyzed by flow cytometry.

incubated with 5 μl Ac-DEVD-AMC in a multiwell plate for one hour at 37 °C. Ac-DEVD-CHO, a caspase inhibitor, was used as negative control. AMC liberated from Ac-DEVD-AMC by activated caspase 3 was measured using a Fluoroskan plate reader at excitation/emission wavelengths of 380/450 nm. For flow cytometry, PAEC were collected as for the annexin V assay. Cells were fixed and permeabilized, then labeled with FITC-conjugated monoclonal rabbit anti-active caspase 3 antibody as per kit instructions. Samples were analyzed immediately by flow cytometry.

The TUNEL assay employed an APO-BrdU kit (BD Pharmingen) in which TdT catalyzes 5-bromo-2-deoxyuridine (BrdU) addition to 3'-OH ends of double and single stranded DNA fragments in late apoptosis. PAEC were collected in the same way as for the annexin V assay, after which cells were fixed in paraformaldehyde, washed, resuspended in 70% v/v ethanol in PBS, and stored at -20 °C until use. For each experiment, samples were thawed, labeled as per kit instructions, and analyzed immediately by flow cytometry.

4.2.4 Endothelial cell permeability

Cell permeability assays were performed on PAEC cultured for four days on 0.4 μm Costar Transwell inserts (Corning). After washing cells with PBS, serum-free media with 1 mg/ml 10 kDa tetramethylrhodamine-labeled dextran (Molecular Probes) was added to the insert. At this size dextran has a molecular radius near that of the FGF-2 monomer (3 nM) (172). 50 μl samples were collected from media outside the insert well from 30 to 480 minutes and measured at 544/590 nm in a Fluoroskan (ThermoLabsystems). Cell permeability to FGF-2 and subsequent binding to basement membrane was measured by adding 1 μg/ml FGF-2 in serum-free media to PAEC cultured for four days in tissue culture plates. Short and long term glucose exposure effects were tested by growing cells in either 5 or 30 mM glucose media (long term), and at the time of experiment, adding the FGF-2 load in either 5 or 30 mM glucose serum-free media (short term). Following a two hour incubation at 37 °C, basement membrane was isolated and FGF-2 extracted as described previously.

4.2.5 Fluorescence microscopy

PAEC cultured for four days on coverslips were washed with PBS and fixed in 4% w/v paraformaldehyde (pH 7.4). Fixed cells were thoroughly washed in PBS and labeled with a mouse monoclonal antibody to Pecam (1:100, MCA1746, Serotec), Ve-cadherin (1:50, MCA1748, Serotec), and collagen IV (1:100, C1926, Sigma). After thorough washing, cells were labeled with Alexa Fluor 488 goat anti-mouse secondary antibody (1:100, A11017, Molecular Probes) with Hoescht nuclear stain (1:1000). Coverslips were mounted on microscope slides with 1:1 glycerol-PBS and stored at 4 °C.

A similar process was used for imaging PKC and reactive oxygen species, however instead of antibodies, fluorescently labeled moieties targeted intracellular agents. Fim-1 diacetate (Molecular Probes), a fluorescein labeled bisindolylmaleimide derivative, imaged and quantified intracellular PKC. After fixation, cells were permeabilized in ice cold methanol for 10 minutes, washed, and incubated with 200 nM Fim-1 for 30 minutes at room temperature. Cells were then washed again and mounted as described previously. A chemically reduced and acetylated form of dichlorofluorescein (DCF), which is not fluorescent until acetate groups are removed by intracellular esterases and oxidation occurs within cells, was used to measure reactive oxygen species. Attached or trypsinized live cells were incubated with 2.5 μM carboxy-H_2DCFDA (Molecular Probes) for 30 minutes at 37 °C, after which cells were washed with PBS and imaged using either flow cytometry or fluorescent microscopy.

4.2.6 Statistics

All statistical analyses were performed with Prism software (Graphpad). Data were normally distributed and expressed as mean ± standard deviation. Comparisons between two groups were analyzed by Student's t test, and comparisons between more than two groups were analyzed by ANOVA. A value of $p < 0.05$ was considered statistically significant and is indicated in the text as such or in figures with a pound sign (#). A value of $p < .01$ is indicated with an asterisk (*). If no statistical significance is reported, none was observed.

4.3 Results

Basement membrane FGF-2 increases with media glucose concentration without any change in binding kinetics or amount of basement membrane produced (chapter 3). Binding kinetics further showed that basement membrane bound FGF-2 increased linearly with available soluble FGF-2. We therefore sought to determine whether the increase in basement membrane FGF-2 is related to an alteration in endothelial cell release of or permeability to FGF-2.

4.3.1 FGF-2 release and apoptosis

FGF-2 is produced by and released from endothelial cells. FGF-2 inside endothelial cells increased slightly with glucose, although the difference was not statistically significant ($.01 < p < .05$) (FIGURE 4.5). In response to an environmental stress such as glucose or TNFα, endothelial cells released FGF-2 into conditioned media. Released FGF-2 increased with stress and time, yet released FGF-2 was consistently higher in 30 mM glucose rather than 5 mM independent of other stressors (FIGURE 4.6, FIGURE 4.7, FIGURE 4.8). We are the first to show that with glucose alone, endothelial cells released twice as much FGF-2 over 48 hours when exposed to 30 mM instead of 5 mM glucose media (FIGURE 4.9).

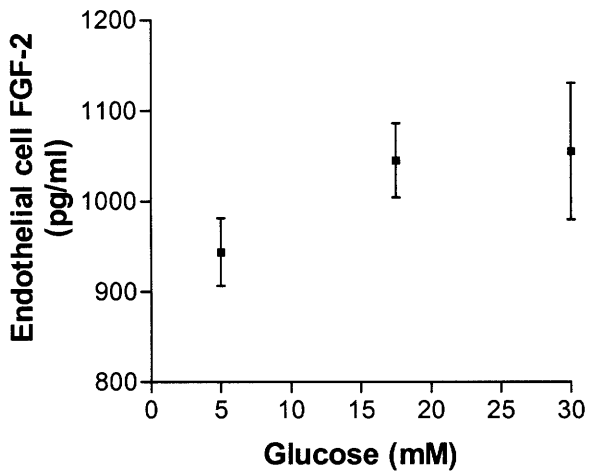


FIGURE 4.5: ENDOTHELIAL CELL INTRACELLULAR FGF-2 INCREASES SLIGHTLY WITH GLUCOSE. Endothelial cells were cultured for four days in 5, 17.5, or 30 mM glucose growth media, after which they were removed and homogenized. FGF-2 was determined by ELISA. ($.01 < p < .05$) Cell extract total protein was unchanged with glucose.

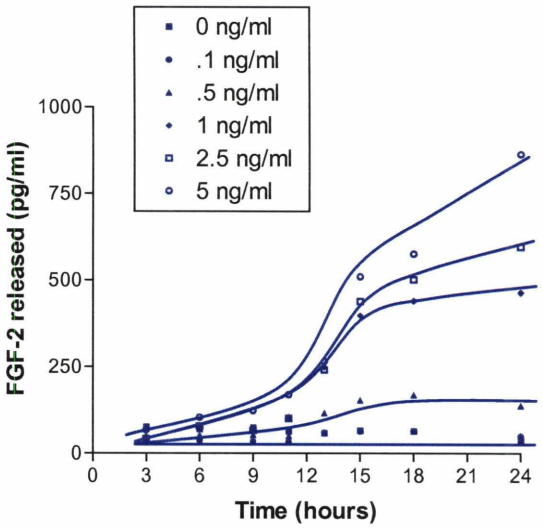


FIGURE 4.6: ENDOTHELIAL CELL FGF-2 RELEASE INCREASES WITH TNFα ADDITION OVER A 24 HOUR PERIOD (5 MM GLUCOSE). Endothelial cells were grown in 5 mM growth medium for four days, after which cells were exposed to TNFα. FGF-2 in media was quantified via ELISA.

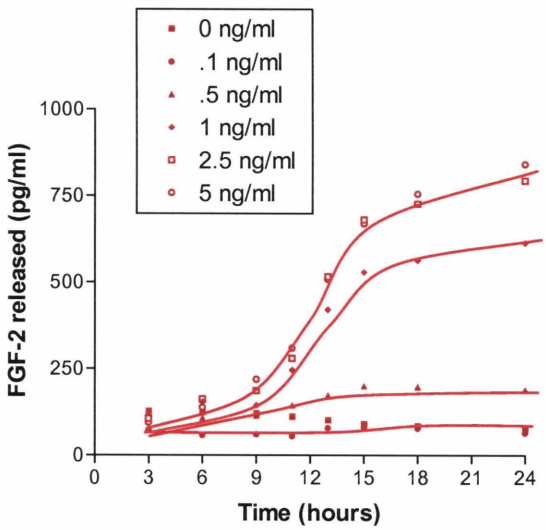


FIGURE 4.7: ENDOTHELIAL CELL FGF-2 RELEASE INCREASES WITH TNFα ADDITION OVER A 24 HOUR PERIOD (30 MM GLUCOSE). Endothelial cells were grown in 30 mM growth medium for four days, after which cells were exposed to TNFα. FGF-2 in media was quantified via ELISA.

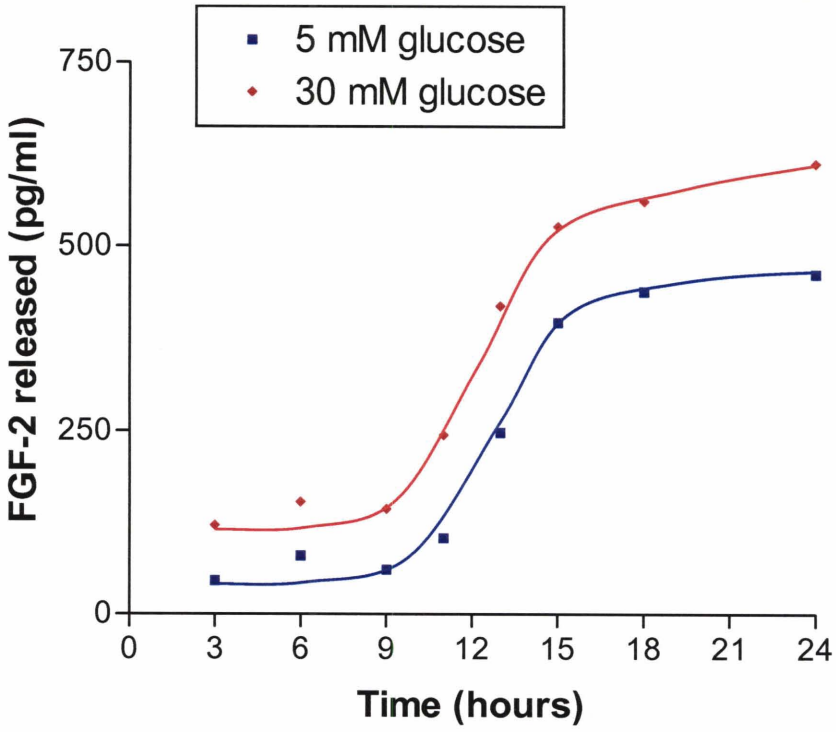


FIGURE 4.8: ENDOTHELIAL CELL FGF-2 RELEASE WITH TNFα IS HIGHER IN 30 MM GLUCOSE MEDIA. The amount of FGF-2 released by endothelial cells across all TNFα exposures and time points is higher for cells cultured in 30 mM glucose media. Data shown here is for 1 ng/ml TNFα. ($p < .01$)

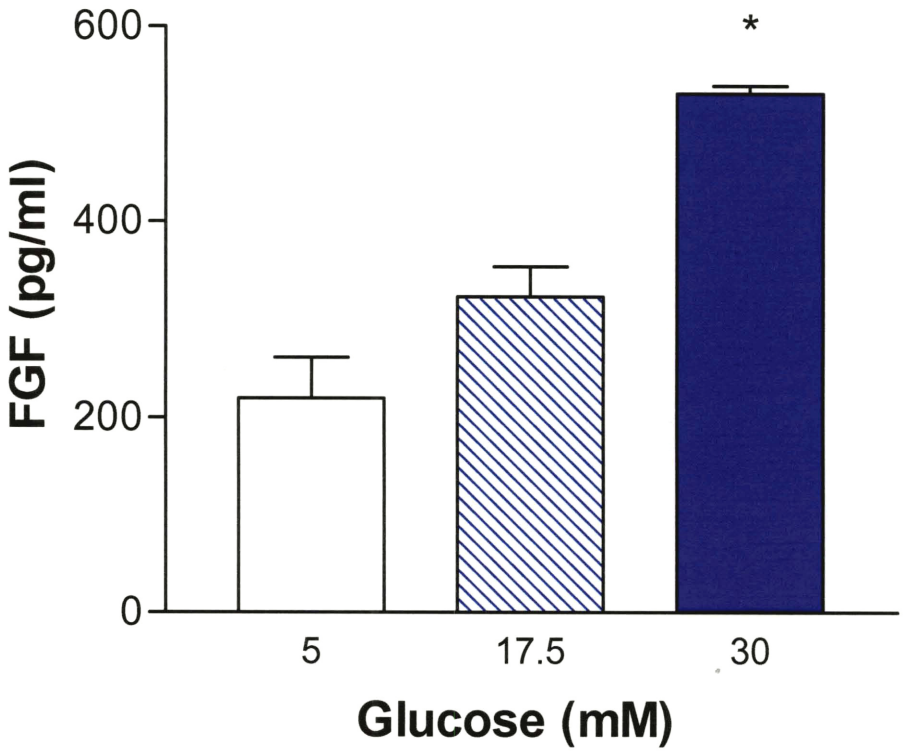


FIGURE 4.9: ENDOTHELIAL CELL FGF-2 RELEASE INCREASES WITH CULTURE GLUCOSE CONCENTRATION. Endothelial cells were cultured in 5, 17.5, or 30 mM glucose growth media for four days. On day four, media samples were collected, pelleted to remove floating cells, and FGF-2 were quantified via ELISA. (*) $p < .01$

Since FGF-2 has no signal sequence for secretion, we hypothesized that FGF-2 release in high glucose might occur through cell membrane damage or cell death by apoptosis. Cell counts could not be used to assess differences in viable cell numbers with glucose, as high glucose decreases overall cell number by increasing cell size (Chapter 2). However, apoptosis increased with culture glucose concentration in a dose dependent fashion as confirmed by three independent assays: *annexin V – propidium* iodide, caspase 3 activation and TUNEL flow cytometry (FIGURE 4.10, FIGURE 4.11, FIGURE 4.12).

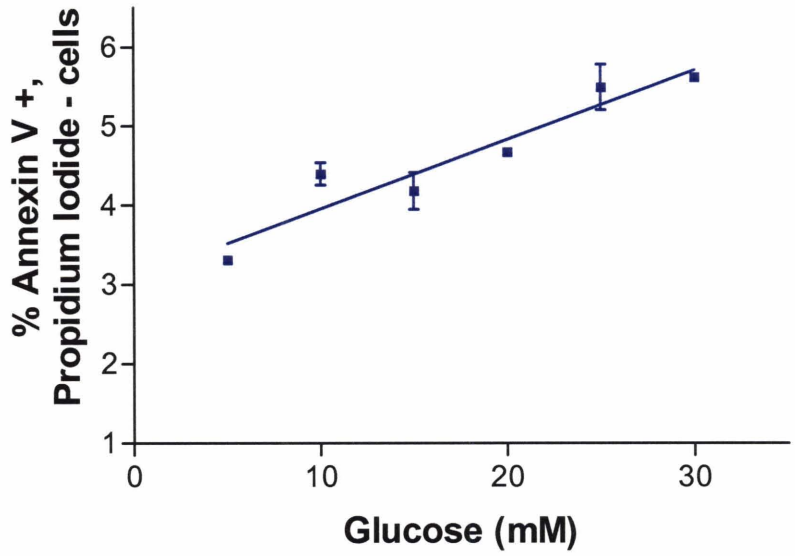


FIGURE 4.10: ENDOTHELIAL CELL APOPTOSIS INCREASES WITH CULTURE GLUCOSE CONCENTRATION (ANNEXIN V – PROPIDIUM IODIDE). Endothelial cells were cultured for four days in graded glucose media, after which cells were trypsinized, labeled with annexin V and propidium iodide, and analyzed by flow cytometry. Correlation $p < .0001$ (ANOVA); $R^2 = .88$

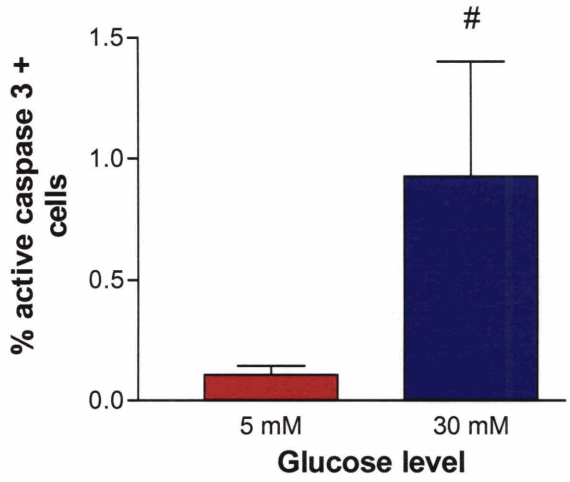


FIGURE 4.11: ENDOTHELIAL CELL APOPTOSIS, MEASURED BY ACTIVE CASPASE 3, INCREASES WITH CULTURE GLUCOSE CONCENTRATION. Endothelial cells were cultured for four days in 5 or 30 mM glucose growth medium, after which cells were trypsinized, labeled with a specific antibody to active caspase 3, and analyzed by flow cytometry. (#) $p < .05$

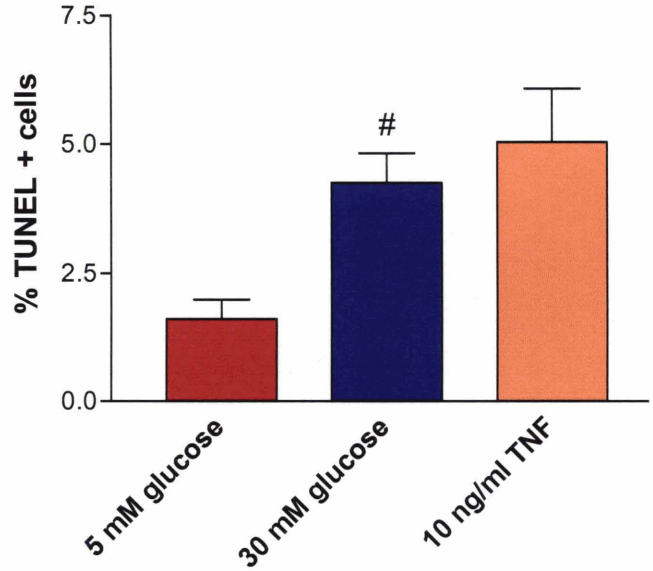


FIGURE 4.12: ENDOTHELIAL CELL APOPTOSIS INCREASES WITH GLUCOSE MEASURED BY TUNEL. Endothelial cells cultured for four days in 5 or 30 mM glucose growth medium were trypsinized, fixed in paraformaldehyde, suspended in 70% ethanol, and frozen overnight. An APO-BrdU kit was used to label apoptotic DNA fragments, which were quantified via flow cytometry. (#) $p < .05$. 24 hour exposure to 10 ng/ml TNFα was the positive control.

4.3.2 Endothelial cell permeability

Increased FGF-2 release from endothelial cells raises FGF-2 available to bind to basement membrane, but FGF-2 must still access basement membrane through the endothelial cell layer. We therefore examined changes in endothelial cell permeability with glucose. Cell permeability to 10 kDa dextran increased in a dose-dependent manner with glucose (FIGURE 4.13), peaking at ~90 minutes and returning to baseline conditions by 180 minutes for 17.5 mM glucose and 360 minutes for 30 mM glucose. Specific endothelial cell permeability to FGF-2, measured by extracting FGF-2 in basement membrane after applying a two hour FGF-2 load, rose over three fold with exposure to glucose but not with exposure to osmotic control mannitol (FIGURE 4.14, FIGURE 4.15).

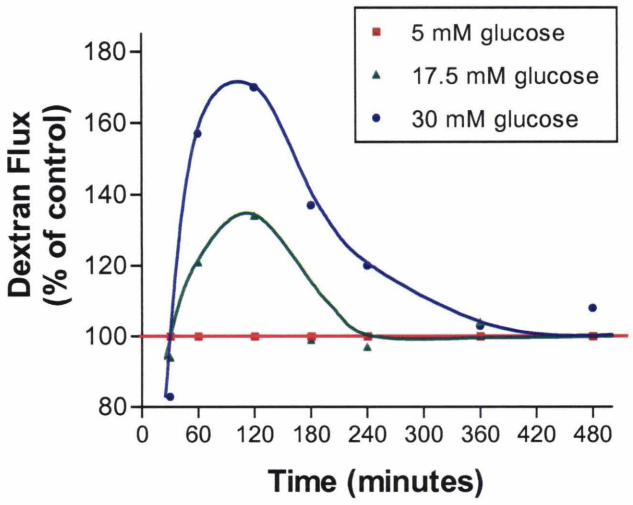


FIGURE 4.13: ENDOTHELIAL CELL PERMEABILITY TO 10 KDA TMR LABELED DEXTRAN INCREASES WITH GLUCOSE. Endothelial cells were cultured for four days in 5, 17.5, or 30 mM glucose on 0.4 μm cell culture inserts. 1 mg/ml of TMR-dextran was added inside the culture well, and fluorescent samples from outside the well were quantified with a Fluoroskan. Data are a representative experiment of three, and samples are normalized to 5 mM glucose ($p < .01$).

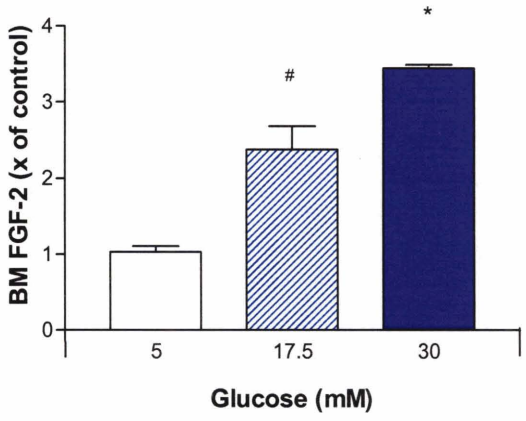


FIGURE 4.14: ENDOTHELIAL CELL PERMEABILITY TO FGF-2 INCREASES WITH GLUCOSE. Endothelial cells were cultured in 5, 17.5, or 30 mM glucose for four days. After a two hour 1 μg/ml FGF-2 load, cells were removed, FGF-2 extracted, and measured via FGF ELISA. (#) $p < .05$ (*) $p < .01$

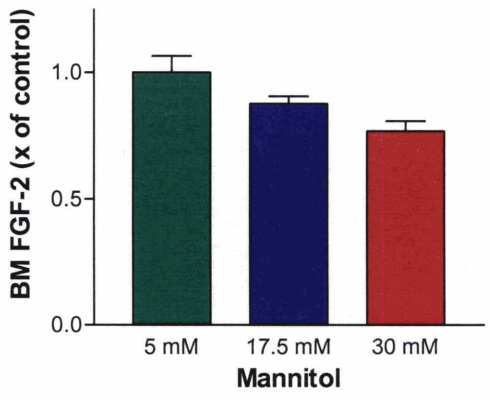


FIGURE 4.15: ENDOTHELIAL CELL FGF-2 PERMEABILITY DOES NOT INCREASE WITH MANNITOL OSMOTIC CONTROL. Endothelial cells were cultured for four days in mannitol, after which cells were exposed to a two hour 1 μg/ml FGF-2 load. Cells were removed, FGF-2 extracted, and measured via FGF ELISA ($p > .05$).

Increased endothelial cell permeability to FGF-2 has both short and long term exposure components (FIGURE 4.16). When cells cultured in 5 mM glucose are exposed to 30 mM glucose concurrent with FGF-2 load, they are more permeable (+33%) than cells maintained in 5 mM glucose ($p < .05$). Similarly, when cells cultured in 30 mM glucose are exposed to 5 mM glucose concurrent with FGF-2 load, they are less permeable than cells maintained in 30 mM glucose (-20%). However, their permeability does not return to the 5 mM level but is still 33% higher than cells maintained at 5 mM glucose ($p < .05$).

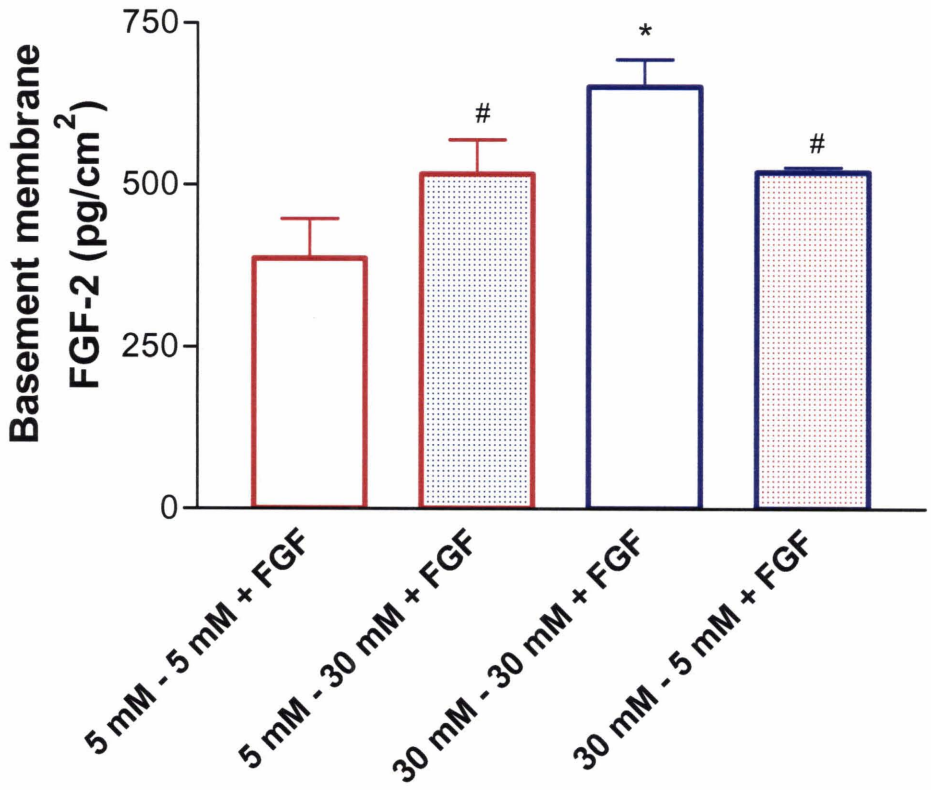


FIGURE 4.16: HIGH GLUCOSE-INDUCED INCREASE IN CELL PERMEABILITY TO FGF-2 HAS SIGNIFICANT SHORT ($P < .05$) AND LONG ($P < .01$) TERM COMPONENTS. Endothelial cells were cultured in 5 or 30 mM glucose growth media for four days (long term). An FGF-2 load was then added in either 5 or 30 mM glucose serum-free media for two hours (short term). Cell were lysed, FGF-2 extracted from basement membrane with 2M NaCl, and quantified via ELISA. (*) $p < .01$; (#) $p < .05$

This increase in endothelial cell permeability occurred without significant changes in cell—cell junctions, as indicated by continuous Pecam and Ve-cadherin along cell borders independent of glucose concentration (FIGURE 4.17). The osmotic increase in cytoplasmic area was accompanied by an increase in intercellular area with glucose concentration. 30 mM glucose cells additionally showed 15 fold greater luminosity of fluorescently labeled collagen IV in basement membrane, perhaps visually demonstrating increased small molecule access to basement membrane in hyperglycemia.

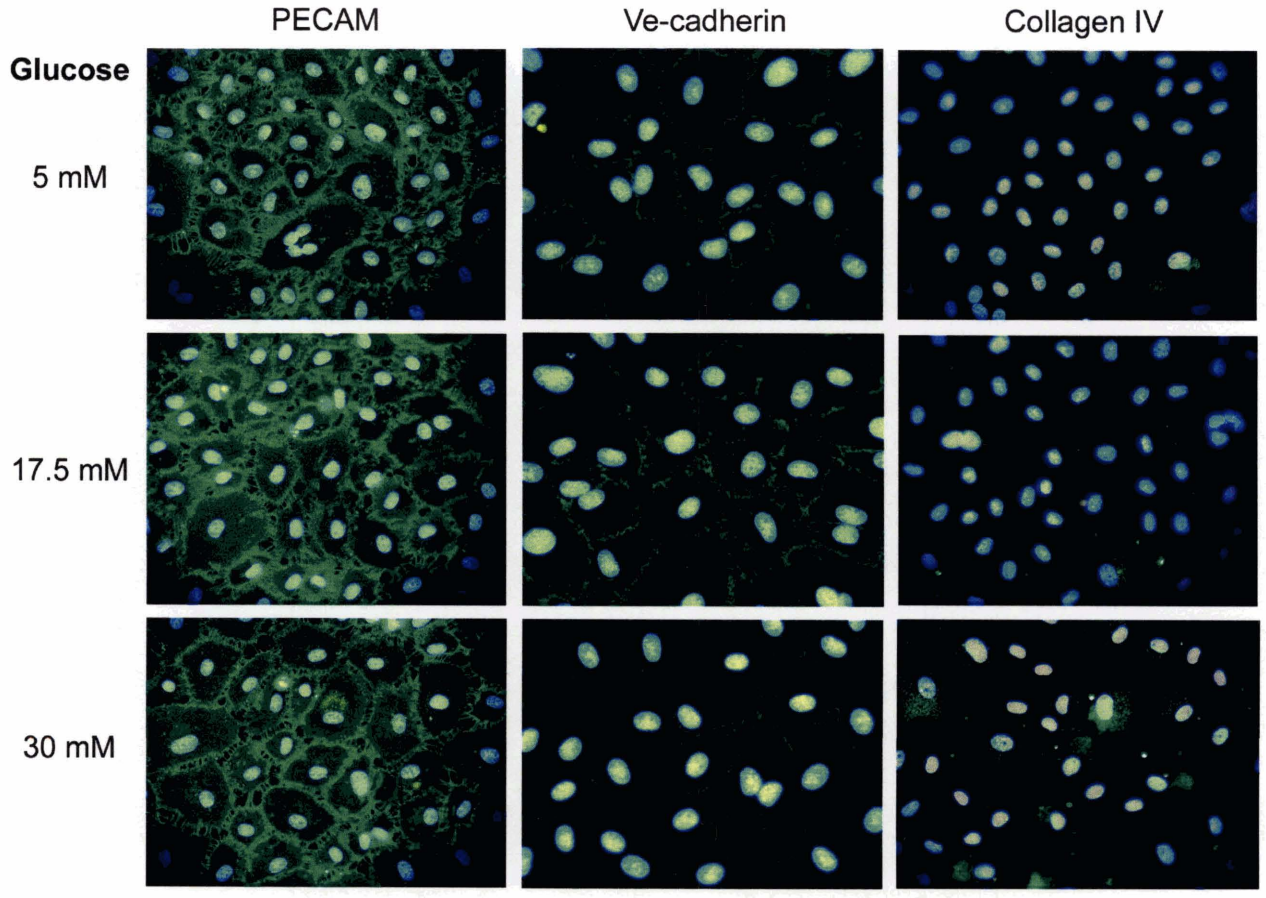


FIGURE 4.17: GLUCOSE INCREASES CELL SIZE, BUT INTERCELLULAR JUNCTIONS REMAIN INTACT. Fluorescent micrographs of PAEC cultured for 4 days in 5, 17.5, or 30 mM glucose. Cells were labeled for PECAM (CD31), Ve-cadherin, or collagen IV (FITC, green) in addition to a Hoescht nuclear stain (UV, blue).

4.3.3 Protein kinase C and VEGF

Protein kinase C (PKC) has been reported as a mediator of glucose-induced endothelial cell dysfunction. We investigated whether PKC blockade with the specific inhibitor bisindolylmaleimide would abrogate glucose effects on basement membrane FGF-2 storage, apoptosis, and endothelial cell permeability specifically to FGF-2. Endothelial cells labeled with a fluorescein tagged bisindolylmaleimide derivative show elevated PKC in high glucose cells (FIGURE 4.18). However, when PKC was inhibited with bisindolylmaleimide, cells appeared unhealthy with jagged cellular edges and altered shape. We further showed that when PKC was inhibited in endothelial cells exposed to high or low glucose, no decrease in basement membrane FGF-2 storage

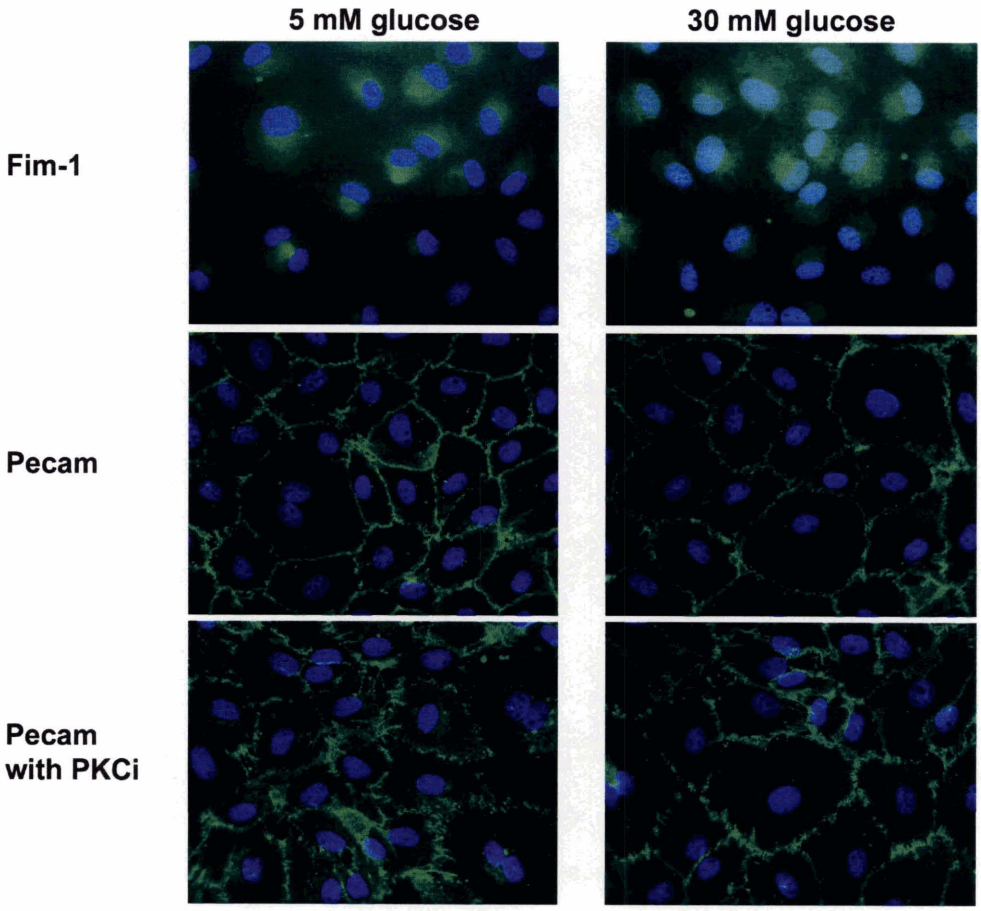


FIGURE 4.18: ENDOTHELIAL CELLS CULTURED IN HIGH GLUCOSE HAVE HIGHER PKC, BUT LONG TERM PKC INHIBITION DAMAGES CELLS. Endothelial cells were cultured in 5 or 30 mM glucose growth media for four days. PKC was inhibited by adding 1 nM bisindolylmaleimide to culture media on day 2. Cells were then labeled with either Fim-1 for PKC or a Pecam antibody (green) as well as Hoescht nuclear stain (blue).

occurred (FIGURE 4.19). Interestingly, apoptosis assays showed no change in annexin V positive – propidium iodide negative cells, but a significant ($p < .05$) decrease in caspase 3 positive cells (FIGURE 4.20,FIGURE 4.21). As opposed to reported accounts of PKC inhibition blocking endothelial cell permeability with glucose, in our experiments PKC inhibition only seemed to increase endothelial cell FGF-2 permeability in high glucose conditions ($p < .01$) (FIGURE 4.22).

VEGF is similarly thought to be a factor in hyperglycemic endothelial cell dysfunction. Despite VEGF's function as an important permeability enhancer, FGF-2 extracted from basement membrane in high glucose did not decrease significantly ($p > .05$) with VEGF inhibition (FIGURE 4.23).

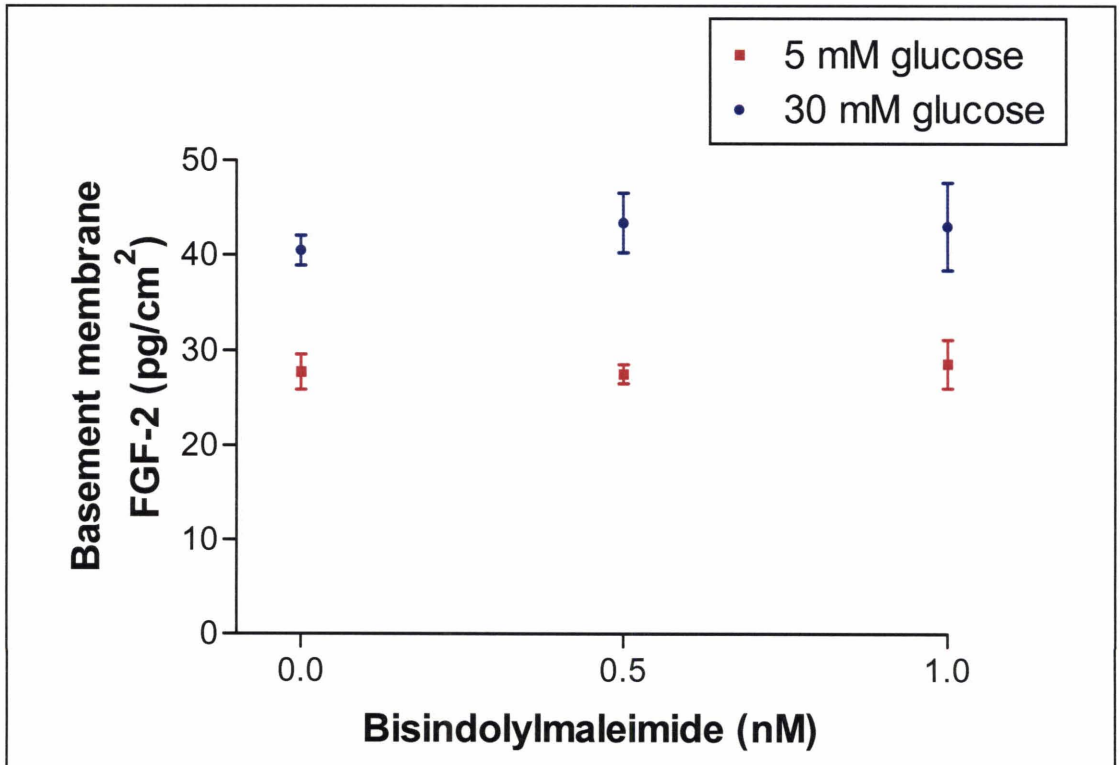


FIGURE 4.19: PKC INHIBITION DOES NOT DECREASE BASEMENT MEMBRANE FGF-2 IN HIGH GLUCOSE. Endothelial cells were cultured in 5 or 30 mM glucose media, and increasing bisindolylmaleimide was added on day two to inhibit PKC. At four days, cells were lysed, after which FGF-2 was extracted from the basement membrane and quantified via FGF ELISA.

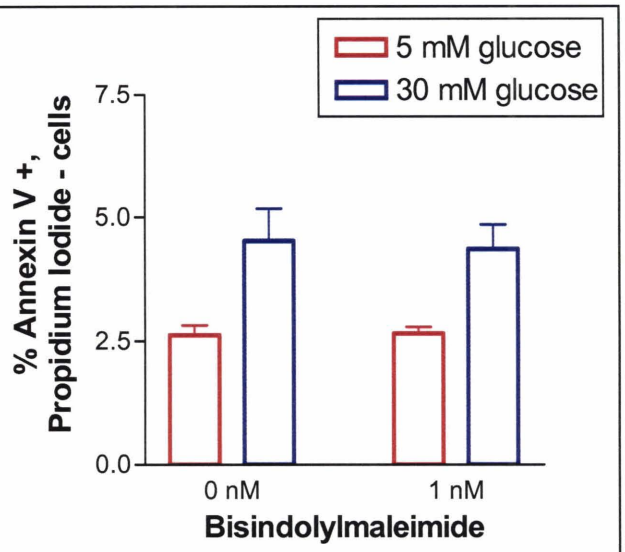


FIGURE 4.20: ENDOTHELIAL CELL APOPTOSIS DOES NOT CHANGE WITH PKC INHIBITION (ANNEXIN V – PROPIDIUM IODIDE). Endothelial cells were cultured for four days in 5 or 30 mM glucose growth medium, and 1 nm bisindolylmaleimide was added for the final two days to inhibit PKC. Cells were then trypsinized, labeled with annexin V and propidium iodide, and analyzed by flow cytometry. ($p > .05$).

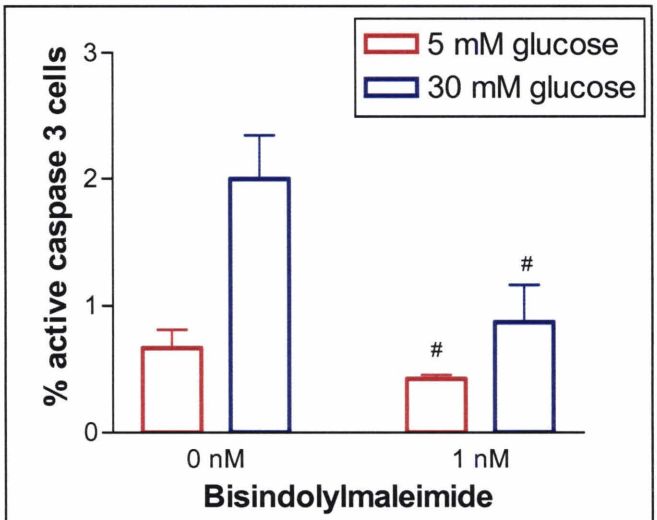


FIGURE 4.21: PKC INHIBITION DECREASES ACTIVE CASPASE 3+ ENDOTHELIAL CELLS. Endothelial cells cultured for four days in 5 or 30 mM glucose media with or without bisindolylmaleimide for PKC inhibition were trypsinized and labeled with a specific antibody to active caspase 3. Cell percentages were quantified with flow cytometry. (#) $p < .05$ compared to no PKCi

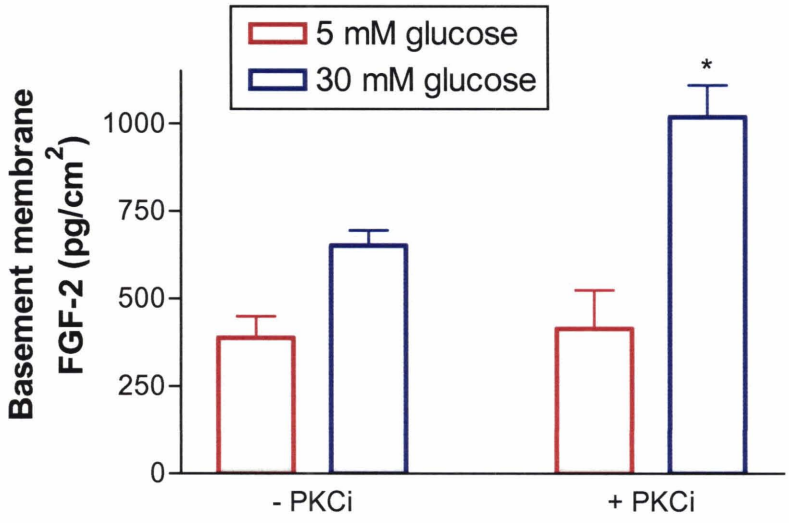


FIGURE 4.22: ENDOTHELIAL CELL PERMEABILITY TO FGF-2 IN HIGH GLUCOSE INCREASES WITH PKC INHIBITION. Endothelial cells cultured for four days in 5 or 30 mM glucose media with or without bisindolylmaleimide as a PKC inhibitor were exposed to 1 μg/ml FGF-2 for two hours. Cells were lysed, FGF-2 extracted from basement membrane with 2 M NaCl, and quantified via FGF ELISA (*) $p < .01$

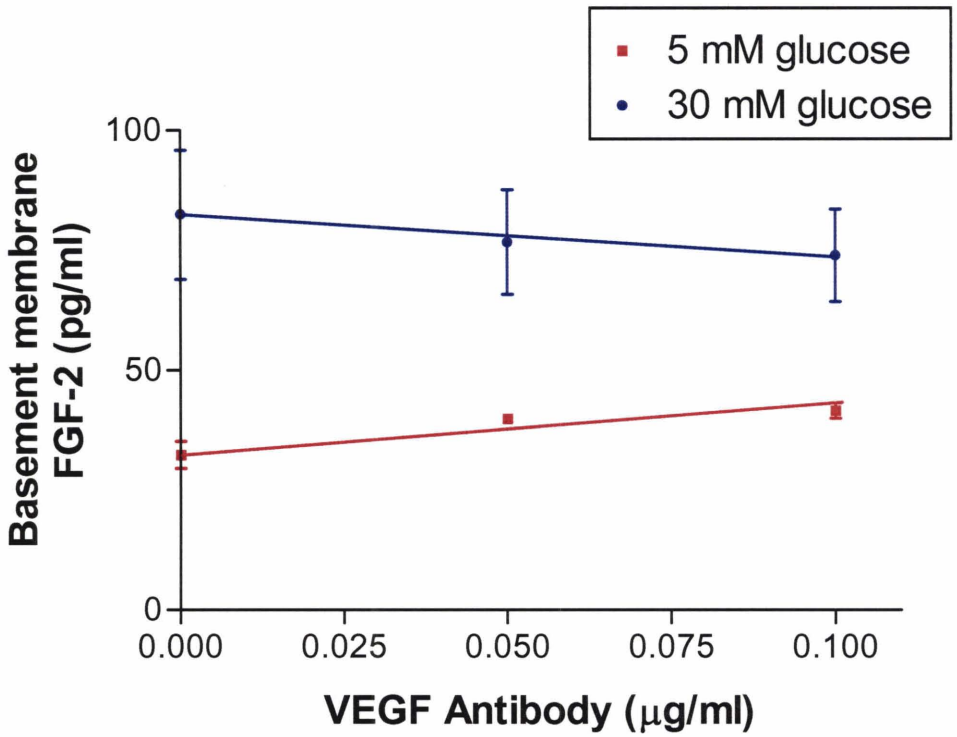


FIGURE 4.23: A NEUTRALIZING VEGF ANTIBODY DOES NOT ABROGATE INCREASED BASEMENT MEMBRANE FGF-2 WITH GLUCOSE. Endothelial cells were cultured for four days in 5 or 30 mM glucose media. Increasing neutralizing VEGF antibody was added to remove permeability effects from cell-released VEGF. Cells were then lysed, basement membrane FGF-2 extracted with 2 M NaCl, and quantified via FGF ELISA. ($p > .05$)

4.3.4 Caspases

Caspases are considered the "point of no return" in apoptotic cell death. To determine if apoptosis plays a critical role in increased basement membrane FGF-2, we blocked caspase activation with either a general caspase inhibitor (Z-VAD-FMK) or a specific caspase 3 inhibitor (Ac-DEVD-CHO). Caspase inhibition with either caspase inhibitor did not abrogate the increase in basement membrane FGF-2 storage with glucose ($p > .05$) (FIGURE 4.24). Similarly, FGF-2 release from endothelial cells exposed to TNFα decreased only slightly with caspase inhibition for low TNFα concentrations (1 ng/ml) but actually increased at high TNFα concentrations (5 ng/ml) (FIGURE 4.25). When cell viability was assayed, we were surprised to find that attached viable cell number among endothelial cells exposed to TNFα did not increase with caspase inhibition (FIGURE 4.26). Similarly, the percentage of annexin V positive – propidium

iodide negative cells did not decrease (FIGURE 4.27). The only apoptotic indicator that decreased was the percentage of active caspase 3 positive cells, indicating effective caspase inhibition (FIGURE 4.28). However, loss of caspases did not alter other apoptotic measures.

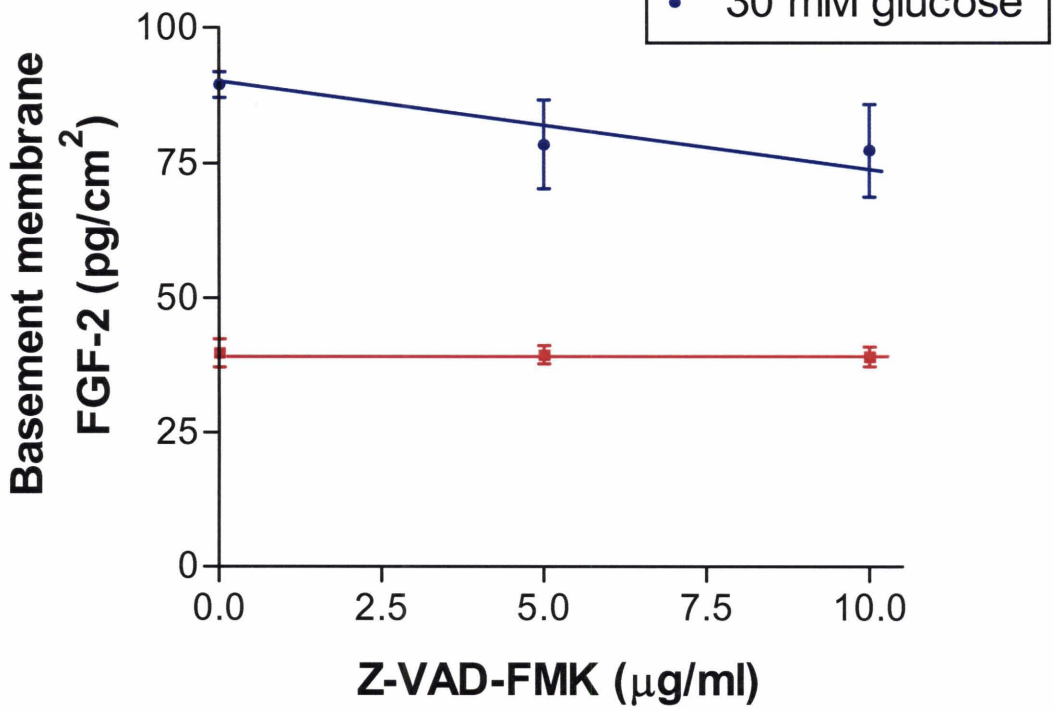


FIGURE 4.24: A GENERAL CASPASE INHIBITOR DOES NOT ABROGATE INCREASED BASEMENT MEMBRANE FGF-2 WITH GLUCOSE. Endothelial cells were cultured for four days in 5 or 30 mM glucose media. Increasing Z-VAD-FMK was added to block endothelial cell apoptosis by limiting caspase activation. Cells were then lysed, basement membrane FGF-2 extracted with 2 M NaCl, and quantified via FGF ELISA. ($p > .05$)

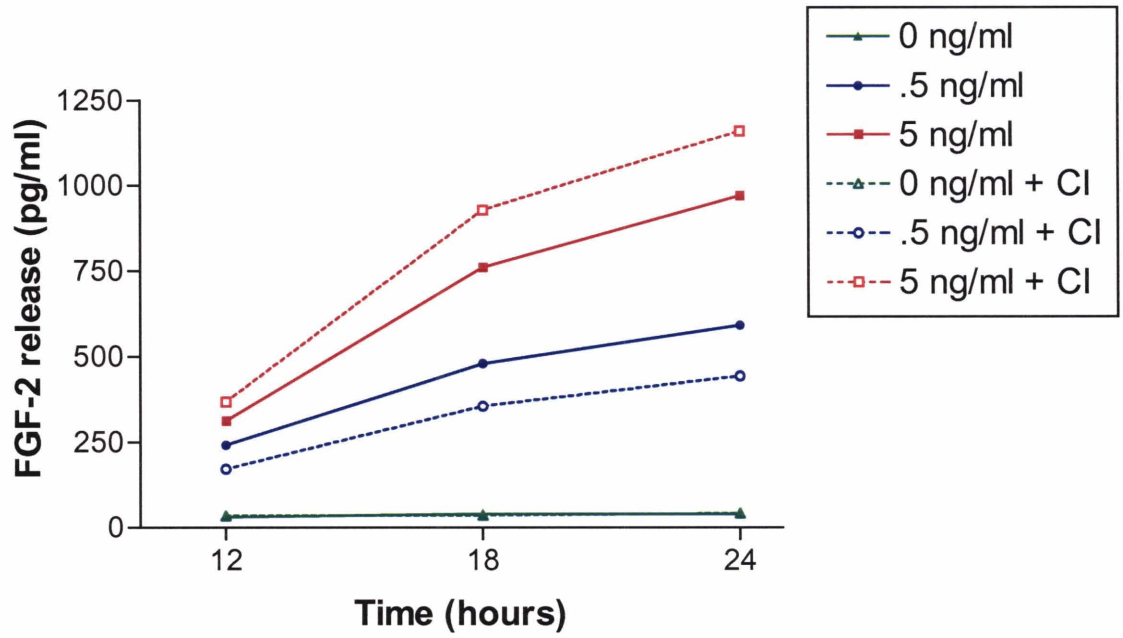


FIGURE 4.25: TNFα INDUCED ENDOTHELIAL CELL FGF-2 RELEASE DOES NOT DECREASE WITH CASPASE INHIBITION. Confluent endothelial cells were exposed to TNFα for 24 hours. Some cells were pre-incubated with a caspase inhibitor. FGF-2 was quantified in media samples via FGF ELISA.

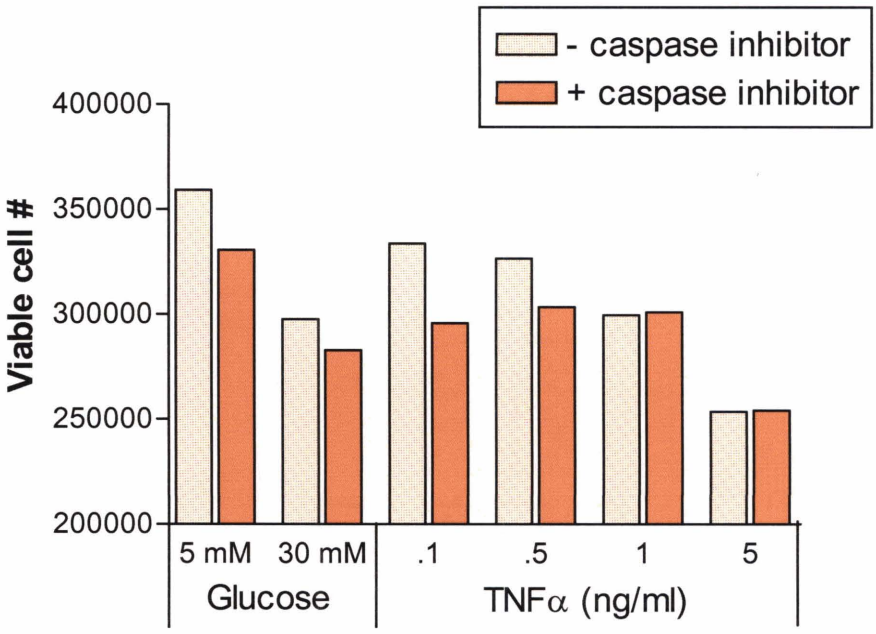


FIGURE 4.26: VIABLE ENDOTHELIAL CELL NUMBER DOES NOT INCREASE WITH CASPASE INHIBITION. Endothelial cells were cultured for four days in 5 or 30 mM glucose, with TNFα added to 5 mM samples for 24 hours. Prior to exposure to glucose or TNFα, 100 μM Ac-DEVD-Cho was added to block caspase 3 activation. Cells were trypsinized and counted with a Coulter counter.

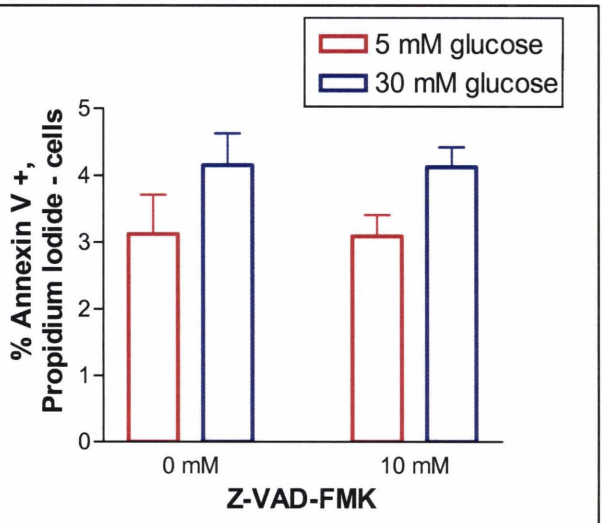


FIGURE 4.27: CASPASE INHIBITION DOES NOT DECREASE APOPTOSIS MEASURED BY THE ANNEXIN V - PROPIDIUM IODIDE ASSAY. Endothelial cells cultured in 5 or 30 mM glucose were incubated with general caspase inhibitor Z-VAD-FMK for the final two days of culture. Cells were then trypsinized, labeled with annexin V and propidium iodide, and analyzed by flow cytometry. ($p > .05$)

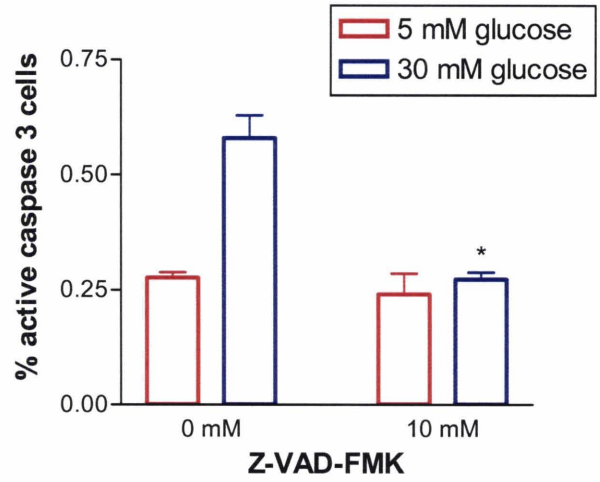


FIGURE 4.28: CASPASE INHIBITION DECREASES APOPTOSIS MEASURED BY THE CASPASE 3 ASSAY. Endothelial cells cultured in 5 or 30 mM glucose were incubated with general caspase inhibitor Z-VAD-FMK for the final two days of culture. Cells were then trypsinized, labeled with a specific antibody to active caspase 3, and analyzed by flow cytometry. (*) $p < .01$

4.3.5 Reactive oxygen species

Nishikawa et al identified reactive oxygen species production as a common pathway for glucose-induced endothelial cell dysfunction. We measured higher reactive oxygen species in endothelial cells cultured in high glucose both by fluorescent microscopy (FIGURE 4.29) and flow cytometry (FIGURE 4.30). The flow cytometry studies quantitatively show that with either long or short term exposure to high glucose, intracellular reactive oxygen species increase. However, reactive oxygen species reduction with superoxide dismutase did not abrogate the increase in basement membrane FGF-2 seen with glucose (FIGURE 4.31). In fact, reactive oxygen species blockade was unable to alter endothelial cell apoptotic markers, either by annexin V – propidium iodide or caspase 3 activation (FIGURE 4.32, FIGURE 4.33).

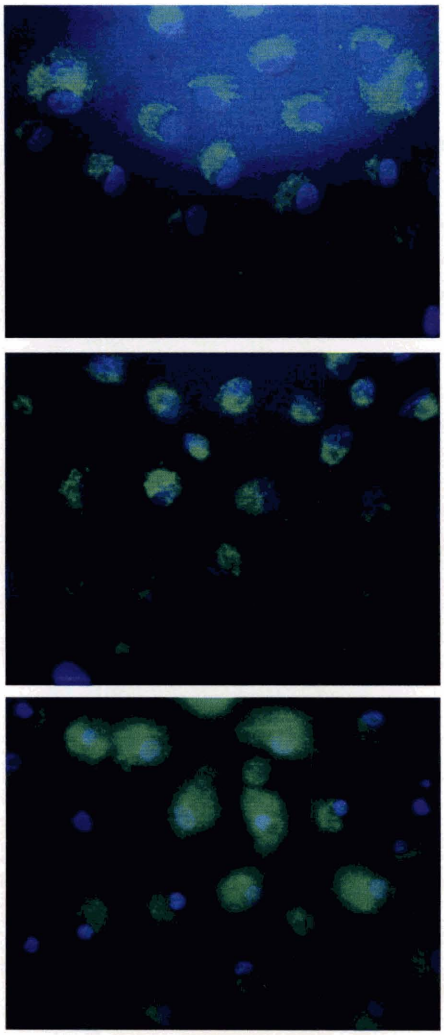
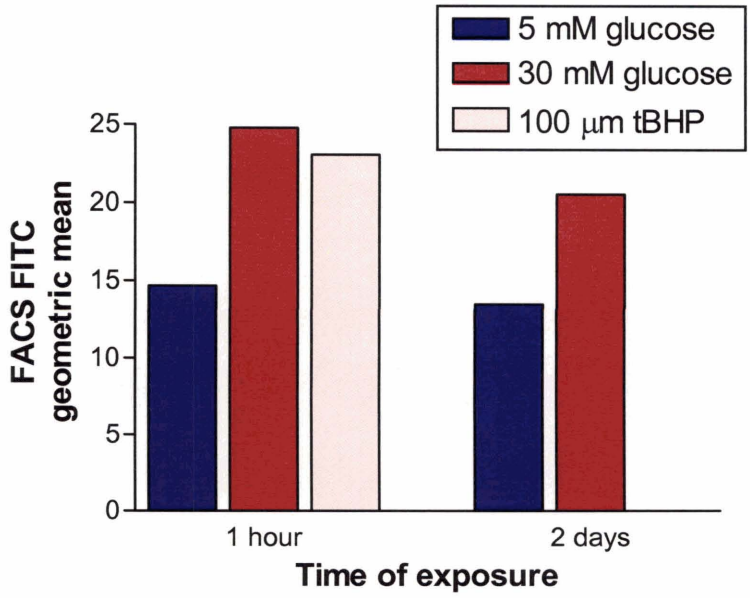


FIGURE 4.29: CELLS CULTURED IN 30 MM GLUCOSE HAVE GREATER REACTIVE OXYGEN SPECIES. Endothelial cells were grown in 5 or 30 mM glucose growth media for four days. Carboxy-H_2DCFDA, which is broken down by intracellular reactive oxygen species to release a fluorophore, was added to the cells for thirty minutes. Cells were then fixed in paraformaldelyde and imaged in a fluorescent microscope. 100 μM tBHP exposure for one hour was the positive control.

FIGURE 4.30: ENDOTHELIAL CELLS EXPOSED TO HIGH GLUCOSE HAVE GREATER REACTIVE OXYGEN SPECIES WITH EITHER SHORT OR LONG TERM EXPOSURE. Endothelial cells were grown in 5 mM glucose media. After one hour or two days of exposure of 5 mM glucose cells to 30 mM glucose media, carboxy-H_2DCFDA was added to cells for thirty minutes to measure reactive oxygen species. Cells were trypsinized and reactive oxygen species quantified by flow cytometry geometric mean. 100 μM tBHP exposure for one hour was the positive control.



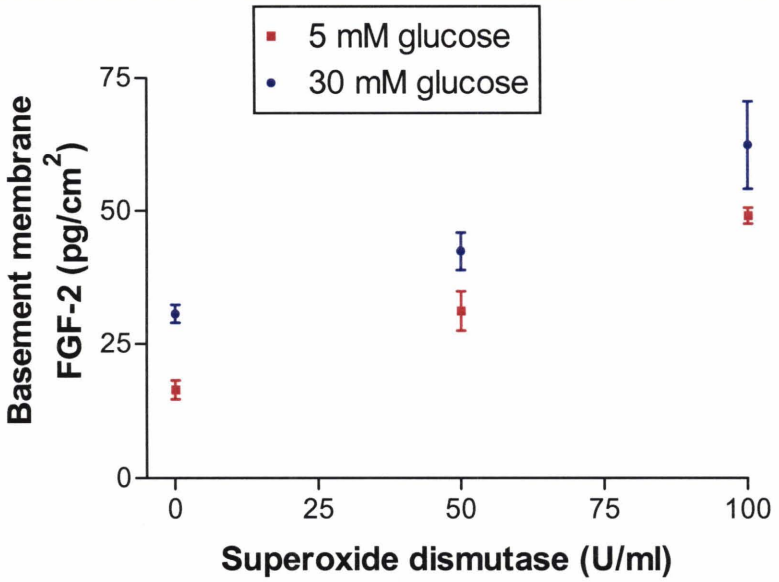


FIGURE 4.31: BASEMENT MEMBRANE FGF-2 INCREASES WITH ADDITION OF SUPEROXIDE DISMUTASE. Endothelial cells were cultured for four days in 5 or 30 mM glucose, with increasing cell permeable superoxide dismutase added at day two. Cells were lysed, basement membrane FGF-2 extracted with 2 M NaCl, and quantified via FGF ELISA ($p < .01$)

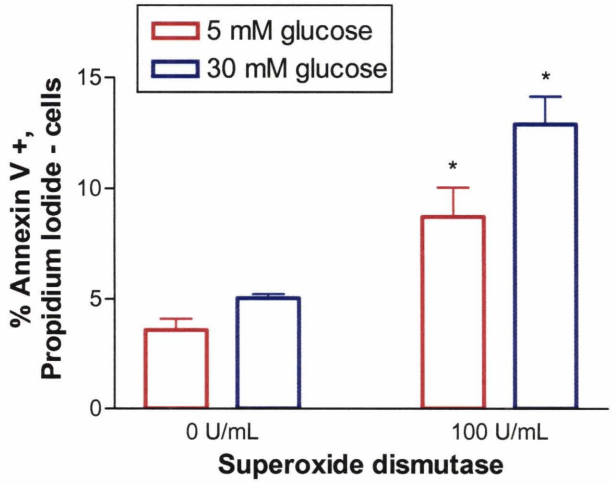


FIGURE 4.32: SUPEROXIDE DISMUTASE INCREASES ENDOTHELIAL CELL APOPTOSIS (ANNEXIN V – PROPIDIUM IODIDE ASSAY). Endothelial cells were cultured for four days in 5 or 30 mM glucose growth media, with 100 U/ml cell-permeable superoxide dismutase added for the final two days. Cells were then trypsinized, labeled with annexin V and propidium iodide, and analyzed by flow cytometry. ($p < .01$)

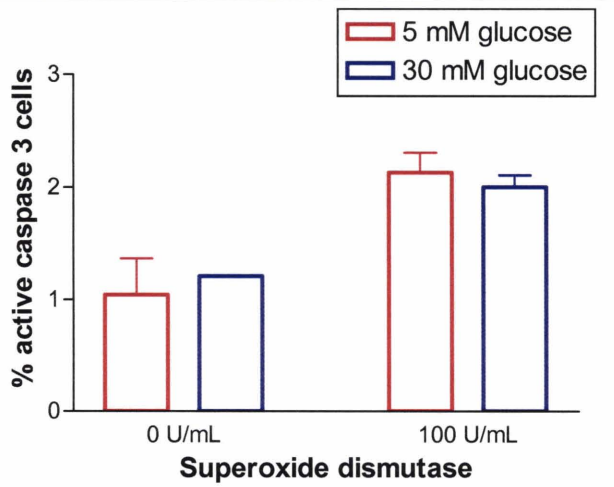


FIGURE 4.33: SUPEROXIDE DISMUTASE DOES NOT CHANGE ENDOTHELIAL CELL CASPASE 3 ACTIVATION. Endothelial cells were cultured for four days in 5 or 30 mM glucose growth media, with 100 U/ml cell-permeable superoxide dismutase added for the final two days. Cells were then trypsinized, labeled with a specific antibody for active caspase 3, and analyzed by flow cytometry. ($p > .05$)

4.3.6 Alternative environmental stressors

Since we were unable to block the increase in basement membrane FGF-2 with glucose, we applied alternative forms of environmental stress to determine if the effect was reproducible. Indeed, either TNFα or tBHP at low concentrations led to increased FGF-2 release from cells ($p < .01$) (FIGURE 4.34, FIGURE 4.35). At high TNFα and tBHP concentration, extensive cell death occurred, marked by few viable attached cells, resulting in very high media FGF-2. This FGF-2 release was associated with increased apoptosis ($p < .01$), as indicated by the annexin V – propidium iodide assay (FIGURE 4.36, FIGURE 4.37). TNFα and tBHP also both enhanced endothelial cell permeability specifically to FGF-2 six fold over cells not exposed to environmental stress (FIGURE 4.38, FIGURE 4.39). Therefore it was not surprising that at low doses, both TNFα and tBHP resulted in increased basement membrane FGF-2 storage (Table 4.).

Environmental Stress	Glucose (mM)	TNFα (ng/ml)	tBHP (μM)
Low	15.6 ± 1.2	17.9 ± 2.6	13.6 ± 0.7
Medium	23.6 ± 4.3#	25.4 ± 2.4#	20.3 ± 2.5#
High	30.0 ± 3.6*	41.3 ± 6.4*	59.3 ± 4.6*

TABLE 4.1: BASEMENT MEMBRANE FGF-2 INCREASES WITH ENVIRONMENTAL STRESS. Porcine aortic endothelial cells were exposed to graded glucose (4 days), TNFα (24 hours), or tBHP (48 hours). After the environmental stress exposure, cells were removed, FGF-2 extracted from basement membrane, and measured via FGF ELISA. Levels of each environmental stress are: glucose (low = 5 mM, medium = 17.5 mM, high = 30 mM), TNFα (low = 0 ng/ml, medium = 0.1 ng/ml, high = 0.5 ng/ml), and tBHP (low = 0 μM, medium = 5 μM, high = 10 μM). (#) $p < .05$, (*) $p < .01$.

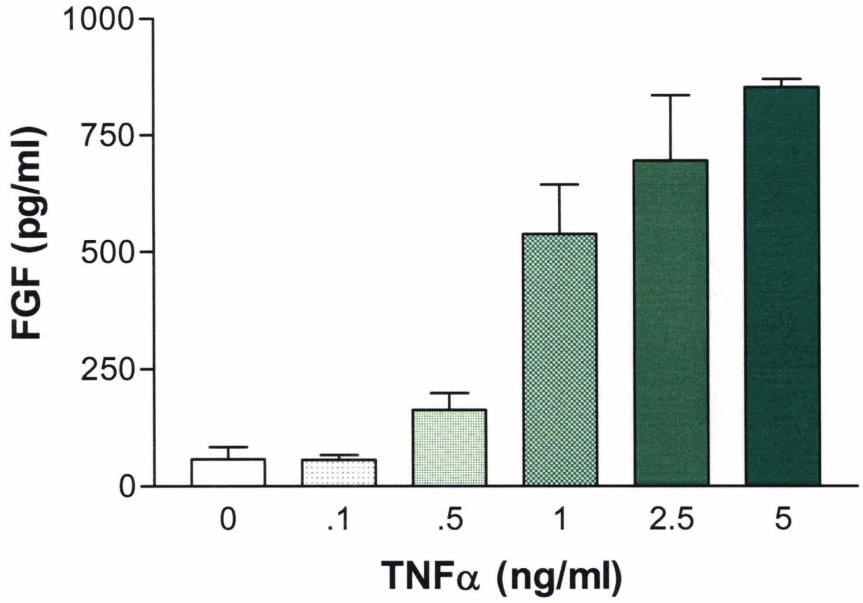


FIGURE 4.34: ENDOTHELIAL CELL FGF-2 RELEASE INCREASES WITH TNFα EXPOSURE. Porcine aortic endothelial cells were incubated in graded concentrations of TNFα for 24 hours. 100 μl of culture media was collected and FGF-2 quantified via FGF ELISA. ($p < .01$)

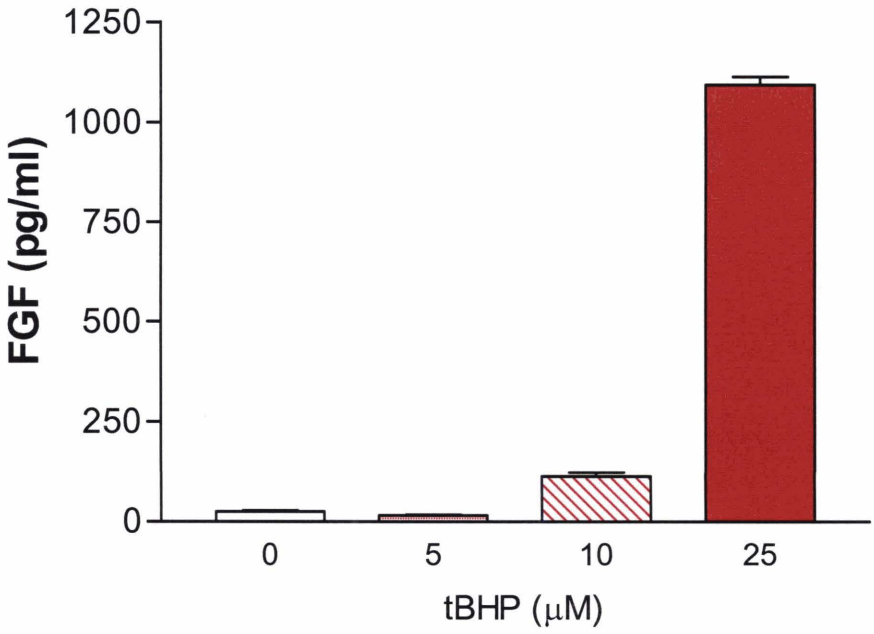


FIGURE 4.35: ENDOTHELIAL CELL FGF-2 RELEASE INCREASES WITH REACTIVE OXYGEN SPECIES. Porcine aortic endothelial cells were incubated in graded concentrations of tBHP for 24 hours. 100 μl of culture media was collected and FGF-2 quantified via FGF ELISA. ($p < .01$).

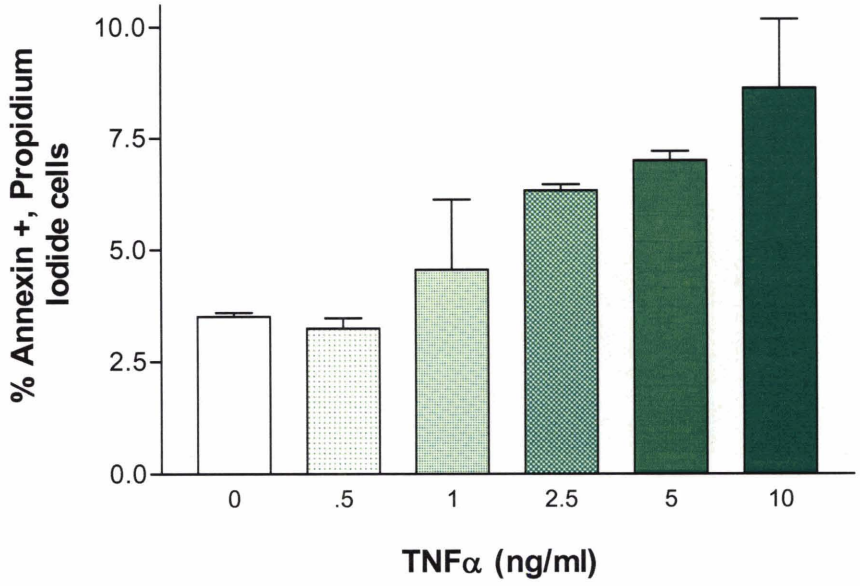


FIGURE 4.36: ENDOTHELIAL CELL APOPTOSIS INCREASES IN A DOSE DEPENDENT MANNER WITH TNFα. Porcine aortic endothelial cells were cultured for four days in 5 mM glucose and exposed to TNFα for 24 hours. Floating and attached cells were harvested, labeled with annexin V and propidium iodide, and analyzed by flow cytometry. ($p < .01$)

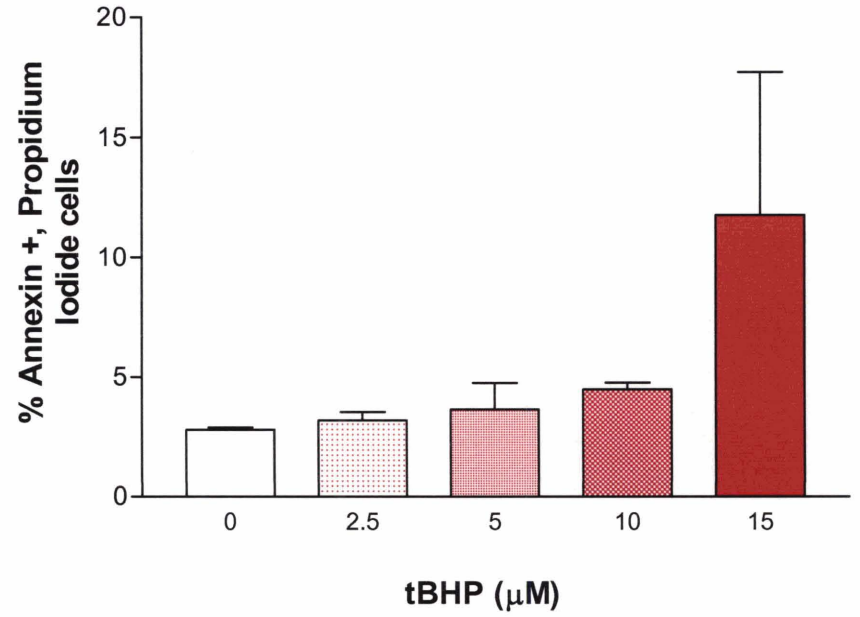


FIGURE 4.37: ENDOTHELIAL CELL APOPTOSIS INCREASES WITH REACTIVE OXYGEN SPECIES. Porcine aortic endothelial cells were cultured for four days in 5 mM glucose and exposed to tBHP for 48 hours. Floating and attached cells were harvested, labeled with annexin V and propidium iodide, and analyzed by flow cytometry. ($p < .01$)

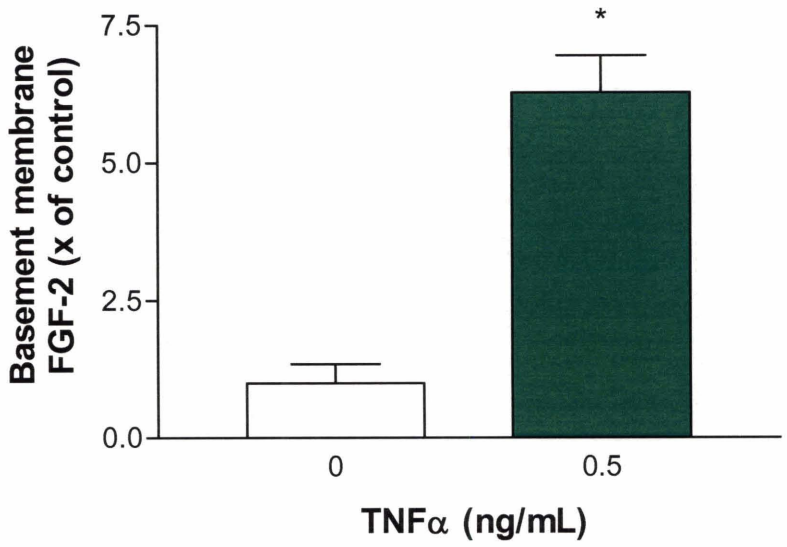


FIGURE 4.38: ENDOTHELIAL CELL PERMEABILITY TO FGF-2 INCREASES WITH TNFα EXPOSURE. Porcine aortic endothelial cells were cultured for four days at 5 mM glucose and incubated with TNFα for 24 hours. Cells were exposed to a 1 μg/ml FGF-2 load for 2 hours, after which cells were removed, basement membrane FGF-2 extracted with 2M NaCl and measured via FGF ELISA. (*) $p < .01$

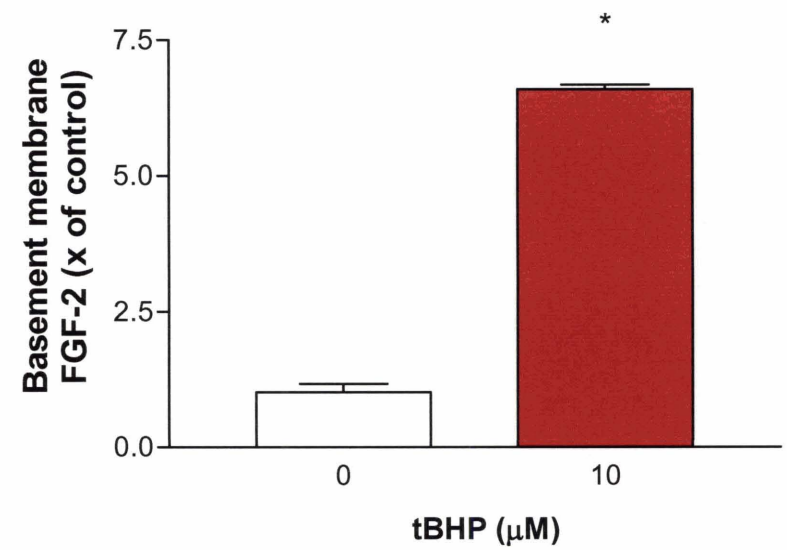


FIGURE 4.39: ENDOTHELIAL CELL PERMEABILITY TO FGF-2 INCREASES WITH REACTIVE OXYGEN SPECIES. Endothelial cells were cultured for four days at 5 mM glucose and incubated with tBHP for 48 hours. Cells were exposed to a 1 μg/ml FGF-2 load for 2 hours, after which cells were removed, basement membrane FGF-2 extracted with 2M NaCl and measured via FGF ELISA. (*) $p < .01$

4.4 Discussion

Investigation of endothelial cell regulation of basement membrane composition brings us closer to understanding the cooperative loop between cells and surfaces on which they grow. Though FGF-2 storage in basement membrane is well documented and well defined, it remains an incompletely understood observation of unclear regulatory significance (72). We now show that endothelial cells control FGF-2 storage by altering vascular basement membrane exposure to FGF-2 through modulation of cellular FGF-2 release and permeability. Environmental stress in disease, such as hyperglycemia in diabetes, adjusts the basement membrane protein balance via endothelial cell function.

4.4.1 Endothelial cell FGF-2 release and apoptosis

With no change in the amount of basement membrane produced or FGF-2 basement membrane binding kinetics (Chapter 3), the increase in basement membrane FGF-2 could be related to increased basement membrane FGF-2 exposure. The two components of increased exposure, which could occur individually or in combination, are increased available soluble endothelial cell-derived FGF-2 and/or increased FGF-2 access to basement membrane. Each component is controlled by an inherent endothelial cell function: FGF-2 release and permeability. Endothelial cell released FGF-2 increases with culture glucose without a significant increase in intracellular FGF-2 (FIGURE 4.9). Since FGF-2 has no signal sequence for secretion, FGF-2 release from endothelial cells is postulated to occur only at cell injury or death (98). In fact, for cells exposed to any of the environmental stresses applied in these experiments, the increase in FGF-2 release with dose and time correlates well with an increase in cellular apoptosis with dose and time (FIGURE 4.10). When environmental stressors TNFα and tBHP are added to cells in low or high glucose, high glucose cells release more FGF-2 and undergo apoptosis at a higher rate than low glucose cells. Thus glucose accentuates effects of both inflammatory and reactive oxygen species induced stress.

Despite association between FGF-2 release and apoptosis, caspase blockade by either a specific caspase 3 inhibitor or a general caspase inhibitor does not alter FGF-2 release, viable cell number, the percentage of annexin V positive – propidium iodide negative cells, or the increase in basement membrane FGF-2 (FIGURE 4.24). This is true for both high glucose and alternative chemical stressors. The only distinct effect of caspase inhibitors is that they effectively inhibit caspase activation, as shown by the decrease in caspase 3 positive cell percentage (FIGURE 4.28). Several possible conclusions arise from these data. In each case, FGF-2 release is associated with annexin V labeling. Annexin V binds to phosphatidylserine after it translocates from inner to outer cell membrane early in the apoptotic process likely prior to caspase activation (173). FGF-2 release from endothelial cells could occur concomitant with early membrane integrity loss, and therefore would not be prevented with caspase inhibitors that block apoptosis at a later stage. An appealing teleologic explanation could be that an injured cell releases a survival factor to save itself from death.

Alternatively, it has recently been postulated that a form of cell death similar to apoptosis can occur without caspase activation or despite caspase inhibition. Caspase-independent cell death has been defined as "the loss of cell viability that is induced by pro-apoptotic conditions, and which proceeds despite the inhibition or disruption of caspase function" (174). While caspase 3 activation increases with endothelial cell exposure to high glucose, when caspases are blocked high glucose cells continue to show higher annexin V labeling and death by cell counts. These endothelial cells exposed to high glucose may continue to die through caspase independent pathways, resulting in cellular FGF-2 release.

FGF-2 release concurrent with endothelial cell apoptosis is in itself interesting. Apoptosis has classically been thought of as a clean cell death, with little to no cellular content release. This is in direct contrast to necrosis, where cell death causes cellular contents spillage which affects surrounding cells. However, this apoptotic paradigm is also coming into question. Rabinovitch et al. recently showed that caspases are released during apoptosis, and that these released caspases affect the surrounding environment by degrading elastase (175). We hypothesize that FGF-2 is similarly released from endothelial cells in sub-lethal membrane injury, apoptosis, and caspase

independent cell death. Since FGF-2 promotes endothelial cell survival and proliferation, FGF-2 release could be critical in limiting surrounding cell death and promoting regrowth both during initial injury and over time through FGF-2 basement membrane storage.

4.4.2 Endothelial cell permeability to FGF-2

Increased cellular FGF-2 release provides only part of the formula for increased basement membrane FGF-2 storage. Soluble FGF-2 must access basement membrane binding sites through the endothelial cell barrier, which requires an increase in endothelial cell permeability. High glucose increases endothelial cell permeability to inert proteins such as albumin *in vitro* (64). Increased permeability in atherosclerosis prone areas in hyperglycemic states *in vivo* has been linked to increased albumin, fibrinogen, and LDL cholesterol deposition in the arterial wall (22, 64). We similarly show that endothelial cell permeability to 10 kDa dextran, an inert molecule chosen for its similarity in size to FGF-2, increases transiently with glucose concentration (FIGURE 4.13). The permeability increase peaks at around 90 minutes, indicating that permeability changes occur with short term exposure to glucose. However, we did not observe the permeability change when cells cultured in 5 mM glucose were suddenly exposed to 30 mM glucose, suggesting that a component of the effect takes days to develop.

Our data extend these observations on dextran to vasoactive compounds by definitively demonstrating an increase in endothelial cell permeability to FGF-2 (FIGURE 4.14). This permeability increase results in more FGF-2 bound to basement membrane. Similar to dextran, we show both short and long term glucose exposure effects on endothelial cell permeability (FIGURE 4.16). Permeability of cells cultured in 5 mM glucose and changed to 30 mM glucose at time of FGF-2 load is enhanced in contrast to cells maintained in 5 mM glucose for the entire experiment. Similarly, cells cultured in 30 mM glucose and changed to 5 mM glucose for the FGF-2 load show decreased permeability to cells maintained in 30 mM glucose. However, in neither case does switching the glucose level at the time of FGF-2 load bring permeability entirely to the long term level. Both short and long term components of endothelial cell permeability

changes with glucose occur on the time scale of hours to days of stress exposure, which is similar to the time scale of endothelial cell FGF-2 release, endothelial cell apoptosis, increased basement membrane FGF-2, and physiologic glucose fluctuations *in vivo*.

Short and long term glucose exposure permeability effects differ whether the test molecule is dextran or FGF-2. For dextran, four days of high glucose culture was required to see a permeability change. When dextran was added in high glucose media to low glucose cells, no permeability increase was observed. With FGF-2, however, cells cultured in 5 mM glucose and given FGF-2 in 30 mM glucose do show an increased permeability. Since FGF-2 does induce changes in cytoskeleton and cell-cell junctions, there is undoubtedly some interaction between glucose, FGF-2, and endothelial cell permeability regulation.

High glucose induced permeability likely occurs through loss of intercellular junction integrity. Pecam endothelial cell labeling indicates a larger intracellular area in high glucose, as well as an increase in intercellular area. The intracellular area change had been attributed to increased cellular metabolic function in high glucose (176). Since we observed similar changes in cell area for cells cultured in 30 mM mannitol, we believe increased cell area is an osmotic effect nonspecific to glucose. The intercellular area increase, which has also been noted in transmission electron microscopy studies, could potentially cause an increase in permeability, perhaps through cell-cell junction loss (177). Although AGE has been shown to decrease Ve-cadherin in endothelial cells, Ve-cadherin was unchanged with glucose as measured either qualitatively by fluorescence microscopy or quantitatively by cellular ELISA, implying that adherens junctions remain intact in hyperglycemia (17, 178). The intercellular area and permeability alterations could be related to tight junctions instead.

4.4.3 Inhibition of glucose effects

Though both PKC and VEGF are specifically implicated in endothelial cell permeability control, neither appear to control the increase in basement membrane FGF-2. While VEGF does increase endothelial cell permeability, a VEGF antibody which neutralized any cell-released VEGF only led to a minor decrease in basement

membrane FGF-2 (FIGURE 4.23). Permeability changes in high glucose have also been linked to PKC, as others were able to block glucose-induced permeability increases using staurosporine as a PKC inhibitor (64). PKC inhibition is complicated by the critical role of PKC in cellular signal transduction. PKC is increased in high glucose cells, but when a PKC inhibitor is applied in culture for longer than several hours, cell physical structure is altered. Cells remain viable for several days but undoubtedly have altered cellular function beyond permeability (FIGURE 4.18). Contrary to the literature on cellular permeability to inert proteins, endothelial cell permeability specifically to FGF-2 actually increases with PKC inhibition, possibly due to interaction between PKC and FGF-2 in cytoskeletal reorganization (FIGURE 4.22). PKC inhibition does decrease caspase 3 activation but not annexin V labeling, supporting a role for PKC in preventing cell apoptosis but not initial membrane damage. In keeping with these findings, no PKC inhibition level decreases basement membrane FGF-2 in 30 mM glucose.

The failure of VEGF and PKC inhibition to alter high glucose basement membrane FGF-2 led us to reactive oxygen species, which are thought to be a common mediator among different pathways of glucose-induced endothelial cell dysfunction. Reactive oxygen species increase in cells cultured in 30 mM glucose, whether culture is short or long term. However, study of reactive oxygen species is complicated by the lack of effective inhibition. Superoxide dismutases decrease intracellular reactive oxygen species but so inhibit vital cell functions that long term experiments are not possible. Endothelial cells carefully balance reactive oxygen species levels, with small quantities critical for intracellular signaling but large quantities severely impairing cell viability. It is therefore not surprising that long term culture with superoxide dismutase actually increases endothelial cell apoptosis.

4.4.4 Reactive oxygen species induction

As high glucose effects were difficult to counteract *in vitro*, we instead used alternative methods of stressing endothelial cells to recreate the glucose effect. Glucose, TNFα, and tBHP all increase FGF-2 release from endothelial cells, increase endothelial cell permeability to FGF-2, and thereby increase basement membrane FGF-

2. Each factor functions using intracellular reactive oxygen species (ROS), perhaps indicating that ROS are common mediators (179).

The TNFα–induced endothelial cell permeability increase is critical to inflammation. TNFα causes FGF-2 release from endothelial cells, which in concert with the permeability increase, results in increased basement membrane FGF-2 deposition. This dual process is of interest in inflammatory processes, especially chronic inflammatory states. Several cytokines also bind basement membrane, including interleukins and interferon-γ (73). Recent data have shown that basement membrane binding of these factors drastically increases their half life and effect. High TNFα concentration in inflammation could cause increased endothelial cell permeability, perhaps resulting in interleukin and interferon-γ deposition into the vascular wall where they may contribute to inflammatory process extension.

4.4.5 Limitations

While our data support the concept that endothelial cells control vascular basement membrane FGF-2 storage by modulating cellular FGF-2 release and permeability, there are limitations to these data. A primary limitation is the difficulty in determining the role of apoptosis. Since cells were trypsinized and apoptosis measured by flow cytometry, increased apoptosis measured in high glucose could be due to increased cell damage in the removal process. We were able to validate high glucose apoptosis by measuring the process at different phases, however alternative methods to the annexin assay were challenging. The active caspase 3 antibody had a low affinity for endothelial cells, and in TUNEL, it was difficult to maintain adequate cell number to achieve statistical significance. Despite using these varied assays, we could not correlate FGF-2 release with any particular apoptotic stage. More specific means of measuring membrane changes and blocking apoptosis at particular stages would be necessary.

To measure endothelial cell permeability to FGF-2, creative measures had to be employed. We were unable to extend cell culture insert experiments from dextran to FGF-2 because FGF-2 bound to proteins and the cell culture insert itself, and therefore was not detectable outside the well. To further complicate matters, FGF-2 can alter cell

permeability, in particular in the setting of pharmacological PKC inhibition. All permeability experiments were extremely sensitive to monolayer quality, and loss of monolayer integrity could and did often lead to false results.

It is also difficult to determine the permeability change mechanism. We believe that FGF-2 moves between cells, but our intercellular junction measurements have limitations. Increase in intercellular space could be a fixation artifact, and while Ve-cadherin was constant by fluorescence microscopy or cellular ELISA, Ve-cadherin changes could be transient or occur in a different plane than the one we were able to measure. Again, more detailed techniques to closely analyze intercellular junction integrity would be needed. The fluorescent collagen IV images are interesting as the labeling pattern appears diffuse rather than as an ordered network. We cannot, however, be sure if increased labeling was caused by increased cell permeability or an increase in total basement membrane collagen IV.

The true challenge in these experiments came in inhibiting increased basement membrane FGF-2. Neither PKC, reactive oxygen species, nor caspase inhibition was able to block the glucose effect. We believe that blockade of the first two factors compromises cellular health and thereby monolayer integrity. Cells are still damaged and die despite caspase inhibition, so perhaps a genetic modification could be programmatically introduced to counter this phenomenon. However, since we do not know at what apoptotic stage FGF-2 release occurs, we might still see FGF-2 release if we blocked apoptosis at too late a phase. As we were unable to block basement membrane FGF-2 increase, we alternatively relied on inducing the increase through alternative means to show that endothelial cells control basement membrane FGF-2 through modulation of FGF-2 release and cell permeability.

4.5 Conclusions

Endothelial cells actively control FGF-2 storage in basement membrane through a combination of FGF-2 release and cell permeability. We are the first to demonstrate that the environmental stress of glucose causes increased FGF-2 release from endothelial cells, perhaps mediated by early membrane changes in apoptosis. Increased cell permeability with glucose has been linked to a variety of factors, including VEGF and PKC. We were unable to block endothelial cell permeability to FGF-2 using any of these methods, but we were able to induce permeability increases by creating intracellular reactive oxygen species. Cell functional alterations occur on a timescale of hours to days, which correlates well with the rapid increase in basement membrane FGF-2.

4.6 Chapter References

1. Bikfalvi, A., Klein, S., Pintucci, G., and Rifkin, D.B. 1997. Biological roles of fibroblast growth factor-2. *Endocrine Reviews* 18:26-45.
2. McNeil, P.L., Muthukrishnan, L., Warder, E., and Damore, P.A. 1989. Growth-Factors Are Released By Mechanically Wounded Endothelial-Cells. *Journal Of Cell Biology* 109:811-822.
3. Haimovitz-Friedman, A., Vlodavsky, I., Chaudhuri, A., Witte, L., and Fuks, Z. 1991. Autocrine Effects Of Fibroblast Growth-Factor In Repair Of Radiation-Damage In Endothelial-Cells. *Cancer Research* 51:2552-2558.
4. Gloe, T., Sohn, H.Y., Meininger, G.A., and Pohl, U. 2002. Shear stress-induced release of basic fibroblast growth factor from endothelial cells is mediated by matrix interaction via integrin alpha(V)beta(3). *Journal Of Biological Chemistry* 277:23453-23458.
5. Kerr, J.F., Wyllie, A.H., and Currie, A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26:239-257.
6. Hengartner, M.O. 2000. The biochemistry of apoptosis. *Nature* 407:770-776.
7. Baumgartnerparzer, S.M., Wagner, L., Pettermann, M., Grillari, J., Gessl, A., and Waldhausl, W. 1995. High-Glucose-Triggered Apoptosis In Cultured Endothelial-Cells. *Diabetes* 44:1323-1327.
8. Risso, A., Mercuri, F., Quagliaro, L., Damante, G., and Ceriello, A. 2001. Intermittent high glucose enhances apoptosis in human umbilical vein endothelial cells in culture. *American Journal Of Physiology-Endocrinology And Metabolism* 281:E924-E930.
9. Du, X.L., Sui, G.Z., Stockklauser-Farber, K., Weiss, J., Zink, S., Schwippert, B., Wu, Q.X., Tschope, D., and Rosen, P. 1998. Induction of apoptosis by high proinsulin and glucose in cultured human umbilical vein endothelial cells is mediated by reactive oxygen species. *Diabetologia* 41:249-256.
10. Sheu, M.L., Ho, F.M., Yang, R.S., Chao, K.F., Lin, W.W., Lin-Shiau, S.Y., and Liu, S.H. 2005. High glucose induces human endothelial cell apoptosis through a phosphoinositide 3-kinase-regulated cyclooxygenase-2 pathway. *Arteriosclerosis Thrombosis And Vascular Biology* 25:539-545.
11. Ho, F.M., Liu, S.H., Liau, C.S., Huang, P.J., and Lin-Shiau, S.Y. 2000. High glucose-induced apoptosis in human endothelial cells is mediated by sequential activations of c-Jun NH2-terminal kinase and caspase-3. *Circulation* 101:2618-2624.
12. Quagliaro, L., Piconi, L., Assaloni, R., Martinelli, L., Motz, E., and Ceriello, A. 2003. Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cell - The role of protein kinase C and NAD(P)H-oxidase activation. *Diabetes* 52:2795-2804.
13. Chavakis, E., and Dimmeler, S. 2002. Regulation of endothelial cell survival and apoptosis during angiogenesis. *Arteriosclerosis Thrombosis And Vascular Biology* 22:887-893.
14. Choy, J.C., Granville, D.J., Hunt, D.W.C., and McManus, B.M. 2001. Endothelial cell apoptosis: Biochemical characteristics and potential implications for atherosclerosis. *Journal Of Molecular And Cellular Cardiology* 33:1673-1690.

15. Yamashita, T., Mimura, K., Umeda, F., Kobayashi, K., Hashimoto, T., and Nawata, H. 1995. Increased Transendothelial Permeation Of Albumin By High Glucose-Concentration. *Metabolism-Clinical And Experimental* 44:739-744.

16. Hempel, A., Maasch, C., Heintze, U., Lindschau, C., Dietz, R., Luft, F.C., and Haller, H. 1997. High glucose concentrations increase endothelial cell permeability via activation of protein kinase C alpha. *Circulation Research* 81:363-371.

17. Sander, B., Larsen, M., Engler, C., Lundandersen, H., and Parving, H.H. 1994. Early Changes In Diabetic-Retinopathy - Capillary Loss And Blood-Retina Barrier Permeability In Relation To Metabolic Control. *Acta Ophthalmologica* 72:553-559.

18. Nannipieri, M., Pilo, A., Rizzo, L., Penno, G., Rapuano, A., and Navalesi, R. 1995. Increased Transcapillary Escape Rate Of Albumin In Microalbuminuric Type-Ii Diabetic-Patients. *Diabetes Care* 18:1-9.

19. Bell, F.P., Adamson, I.L., and Schwartz, C.J. 1974. Aortic Endothelial Permeability To Albumin - Focal And Regional Patterns Of Uptake And Transmural Distribution Of I-131 Albumin In Young Pig. *Experimental And Molecular Pathology* 20:57-68.

20. Park, L., Raman, K.G., Lee, K.J., Lu, Y., Ferran, L.J., Chow, W.S., Stern, D., and Schmidt, A.M. 1998. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nature Medicine* 4:1025-1031.

21. Ishii, H., Jirousek, M.R., Koya, D., Takagi, C., Xia, P., Clermont, A., Bursell, S.E., Kern, T.S., Ballas, L.M., Heath, W.F., et al. 1996. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science* 272:728-731.

22. Williamson, J.R., Ostrow, E., Eades, D., Chang, K., Allison, W., Kilo, C., and Sherman, W.R. 1990. Glucose-Induced Microvascular Functional-Changes In Nondiabetic Rats Are Stereospecific And Are Prevented By An Aldose Reductase Inhibitor. *Journal Of Clinical Investigation* 85:1167-1172.

23. Nishikawa, T., Edelstein, D., Du, X.L., Yamagishi, S., Matsumura, T., Kaneda, Y., Yorek, M.A., Beebe, D., Oates, P.J., Hammes, H.P., et al. 2000. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787-790.

24. Astafieva, I.V., Eberlein, G.A., and Wang, Y.J. 1996. Absolute on-line molecular mass analysis of basic fibroblast growth factor and its multimers by reversed-phase liquid chromatography with multi-angle laser light scattering detection. *Journal Of Chromatography A* 740:215-229.

25. Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., Klagsbrun, M. 1987. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *PNAS* 84:2292-2296.

26. Nugent, M.A., and Iozzo, R.V. 2000. Fibroblast growth factor-2. *International Journal Of Biochemistry & Cell Biology* 32:115-120.

27. Martin, S.J., Reutelingsperger, C.P.M., McGahon, A.J., Rader, J.A., Vanschie, R., Laface, D.M., and Green, D.R. 1995. Early Redistribution Of Plasma-Membrane Phosphatidylserine Is A General Feature Of Apoptosis Regardless Of The Initiating Stimulus - Inhibition By Overexpression Of Bcl-2 And Abl. *Journal Of Experimental Medicine* 182:1545-1556.

28. Chipuk, J.E., and Green, D.R. 2005. Do inducers of apoptosis trigger caspase-independent cell death? *Nature Reviews Molecular Cell Biology* 6:268-275.

29. Cowan, K.N., Leung, W.C.Y., Mar, C., Bhattacharjee, R., Zhu, Y.H., and Rabinovitch, M. 2005. Caspases from apoptotic myocytes degrade extracellular matrix: a novel remodeling paradigm. *Faseb Journal* 19.

30. Salameh, A., Zinn, M., and Dhein, S. 1997. High D-glucose induces alterations of endothelial cell structure in a cell-culture model. *Journal Of Cardiovascular Pharmacology* 30:182-190.

31. Mandal, A.K., Puchalski, J.T., Lemley-Gillespie, S., Taylor, C.A., and Kohno, M. 2000. Effect of insulin and heparin on glucose-induced vascular damage in cell culture. *Kidney International* 57:2492-2501.

32. Dejana, E. 2004. Endothelial cell-cell junctions: Happy together. *Nature Reviews Molecular Cell Biology* 5:261-270.

33. Otero, K., Martinez, F., Beltran, A., Gonzalez, D., Herrera, B., Quintero, G., Delgado, R., and Rojas, A. 2001. Albumin-derived advanced glycation end-products trigger the disruption of the vascular endothelial cadherin complex in cultured human and murine endothelial cells. *Biochemical Journal* 359:567-574.

34. Simon, H.U., Haj-Yehia, A., and Levi-Schaffer, F. 2000. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* 5:415-418.

35. Gibbs, R.V. 2003. Cytokines and glycosaminoglycans (GAGs). In *Glycobiology And Medicine*. 125-143.

CHAPTER 5

FGF-2 EFFECTS ON ENDOTHELIAL CELLS IN HYPERGLYCEMIA

Abstract

Endothelial cell basement membrane FGF-2 increases with culture glucose, as mediated by changes in endothelial cell FGF-2 release and permeability. This effect becomes more intriguing in light of the FGF-2 effect on endothelial cell proliferation and survival in varied glucose conditions. In this chapter, we show that glucose decreases endothelial cell proliferation and increases apoptosis, resulting in an overall decreased viable cell number. Increased basement membrane FGF-2 partially mitigates this effect by enhancing proliferation, but the anti-apoptotic FGF-2 effect is decreased in high glucose conditions. Since endothelial cell FGF-2 binding is independent of glucose, the functional change is likely related to intracellular alterations in Erk and Akt signaling pathways.

5.1 Introduction

Endothelial cells and basement membrane form a co-regulatory unit for FGF-2. In previous chapters, we demonstrated that FGF-2 basement membrane storage is modulated by glucose-induced endothelial cell dysfunction. In this chapter we investigate how the FGF-2 effect on endothelial cells is in turn modulated by glucose. FGF-2 induces varied endothelial cell functions, including proliferation, migration, survival, and phenotype (109, 111, 114). However, we will focus on the role of FGF-2 in viable endothelium maintenance through balance of cell proliferation and death.

FGF-2 binds to endothelial cells through a cooperative process between two cell surface molecules: heparan sulfate proteoglycans and FGF receptors. Heparan sulfate proteoglycans, considered low-affinity binding sites, facilitate dimerization of FGF receptors, which are high-affinity binding sites, and hence intracellular FGF-2 signaling (119). Normal levels, structure, and function of both cell surface molecules are critical for FGF-2 to influence endothelial cells.

FGF-2 induces varied endothelial cell functions via different intracellular signaling pathways. Upon FGF-2 binding and receptor dimerization, intrinsic receptor tyrosine kinase is activated leading to multiple receptor tyrosine residue phosphorylation. These residues provide docking sites for SH2 or signaling enzyme PTB domains, which allows signaling complex assembly. Depending on which signaling complex attaches to the activated FGF-2 receptor, signaling can occur down the RAS-MAP kinase pathway (proliferation), PI-3-kinase/Akt pathway (survival), or PKC pathway (cytoskeletal organization) (123-125). The particular mechanism by which one pathway is selected over another is an active research area, but it appears to depend on nature of the ligand, the particular receptor, the signal transduction pathways utilized, or the transcriptional regulation of specific genes (180).

The glucose effect on FGF-2, as well as the FGF-2 effect on cells in high glucose, has not been extensively studied. Patients with diabetic retinopathy have elevated FGF-2, and in type 2 diabetics, high plasma FGF-2 correlated with poor glycemic control, diabetic retinopathy, and albuminuria (134, 135). In contrast, gastric wounds in diabetic rats showed impaired healing which was ameliorated by exogenous

FGF-2 addition (136). *In vitro* documentation of glucose effects on FGF-2 show that intracellular FGF-2 stores are glycated, and therefore rendered less potent, in high glucose culture (150). However, FGF-2 that is bound to heparin and heparan sulfate is protected from glycation and hence inactivation (103).

A critical function of the endothelial cell-basement membrane unit is regulation of cell proliferation and death. We investigated whether glucose modifies the FGF-2 effect on endothelial cells. We did not study glycated FGF-2, specifically because FGF-2 bound to basement membrane seems protected from glycation both by binding heparan sulfate and by short exposure to an extracellular high glucose environment. As we had already measured an increase in FGF-2 concomitant with an increase in endothelial cell apoptosis, we focused on the role of FGF-2 in the balance between endothelial cell proliferation and apoptosis.

5.2 Materials and Methods

5.2.1 Endothelial cell culture

Porcine aortic endothelial cells were cultured in 5, 17.5, or 30 mM glucose growth DMEM with 5% FBS, 2% glutamine, and 1% penicillin-streptomycin as described previously. For basement membrane growth, cells were seeded in 6, 12, or 24 well tissue culture plates (Corning) near confluence and cultured for four days with a change to supplemented DMEM with 4% w/v dextran on day three. Basement membrane was isolated by lysing cells using a Triton-NH_4OH cell lysis buffer followed by thorough washing in PBS. Prior to addition of new endothelial cells, basement membrane was incubated with serum free DMEM for thirty minutes. Basement membrane FGF-2 was extracted using a 2 M NaCl salt buffer followed by thorough washing. Additional FGF-2 (Peprotech) was added to basement membrane by incubating it for three hours at room temperature in binding buffer with the appropriate FGF-2 concentration, after which the FGF-2 solution was removed and basement membrane washed with PBS followed by serum free DMEM to remove any unbound FGF-2.

5.2.2 Endothelial cell FGF-2 binding kinetics

Endothelial cell FGF-2 binding kinetics were investigated using similar protocols to those used for basement membrane binding kinetics, except all experiments were performed on ice to decrease cellular metabolism and minimize FGF-2 internalization (119). As FGF-2 binds both to cell surface heparan sulfate proteoglycans as well as cell surface receptors, two extraction methods were used. FGF-2 bound to heparan sulfate proteoglycans was released by 10 second incubation with high salt buffer (2 M NaCl, 20 mM Hepes, pH 7.4). FGF-2 bound to cell surface receptors was released by 5 minute incubation with acidic salt buffer (2 M NaCl, 20 mM sodium acetate, pH 4.0).

To determine FGF-2 equilibrium binding capacity, endothelial cells were plated in a 96 well plate and grown to confluence. Prior to each experiment, cells were washed in ice cold binding buffer (25 mM Hepes, 0.05% w/v gelatin, pH 7.4) and incubated on ice for 10 minutes. ^{125}I-FGF-2 (Perkin Elmer) in binding buffer was added to endothelial cells at concentrations from 0 to 25 ng/ml. Equilibrium, defined as the time at which association and dissociation occur at equal rates resulting in no change in bound FGF-2, occurred between two and three hours after growth factor addition. The ^{125}I-FGF-2 solution was aspirated and cells were washed quickly in cold binding buffer to remove unbound ^{125}I-FGF-2. Bound ^{125}I-FGF-2 was extracted from both heparan sulfate proteoglycans and cell surface receptors as previously described and quantified in a gamma counter (Packard).

Basement membrane FGF-2 association was measured on confluent endothelial cells in 24 well plates. 5 ng/ml FGF-2 or ^{125}I-FGF-2 in binding buffer was added to cells on ice for 0 to 360 minutes. This concentration (5 ng/ml) is well within the linear binding range and results in physiologically relevant levels of bound FGF-2. After the incubation period, FGF-2 was aspirated, cells washed quickly in cold binding buffer, and bound FGF-2 extracted and measured as previously described.

To determine FGF-2 basement membrane dissociation kinetics, confluent endothelial cells in 24 well plates were incubated on ice with 5 ng/ml ^{125}I-FGF-2 in binding buffer to equilibrium (3 hours). The ^{125}I-FGF-2 solution was removed, followed by three quick washes in cold binding buffer. Binding buffer containing unlabeled FGF-2 (1 μg/ml) was added to each well for 0 to 360 minutes. Unlabeled FGF-2 was included in dissociation buffer to decrease rebinding of released ^{125}I-FGF-2 to cells. After the

dissociation period, dissociation buffer was removed and cell bound ^{125}I-FGF-2 was extracted from heparan sulfate proteoglycans and cell surface receptors. ^{125}I-FGF-2 in dissociation buffer, salt extraction buffer, and acid extraction buffer was quantified in a gamma counter.

5.2.3 Cell counts and proliferation

Viable endothelial cell number was determined by removing media with floating (nonviable) cells, washing the cell monolayer with PBS, and adding trypsin-EDTA (Gibco) to cover the bottom of the dish. After a five minute incubation at 37 ºC, DMEM with 5% FBS was added and the trypsin-media-cell solution was counted with a Coulter counter (Beckman Coulter).

Propidium iodide intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centered around 600 nm (181). Cells can be identified as being in G1, S, or G2/M phase of the cell replication cycle based on their cellular fluorescence, which is directly proportional to DNA content. Since propidium iodide also binds double-stranded RNA, cells must be treated with RNAse for maximum resolution. For cell cycle analysis, endothelial cells were seeded in 6 well tissue culture plates at 80,000 cells per well, and 1 ng/ml FGF-2 was added at the appropriate time point. At time of analysis, endothelial cells were harvested by trypsinization and washed by repeated centrifugation and resuspension in PBS. After the final wash, cells were incubated for 20 minutes at 37 ºC in propidium iodide staining solution consisting of 3% w/v polyethylene glycol 6000 (Sigma), 50 μg/ml propidium iodide (Molecular Probes), 180 U/ml RNAse (Worthington Biochemical), and 0.1% v/v Triton X-100 in 4 mM citrate buffer (pH 7.2). Following incubation, an equal salt solution volume was added (3% w/v polyethylene glycol 6000, 50 μg/ml propidium iodide, and 0.1% v/v Triton X-100 in 0.4 M NaCl, pH 7.2). Cells were stored overnight at 4 ºC and analyzed the following morning by flow cytometry.

Tritiated (^{3}H) thymidine provided a second method to assess DNA replication, and hence cellular proliferation, in response to FGF-2. Radioactive thymidine is a nucleoside that is incorporated into newly formed DNA during cell proliferation, therefore proliferation is directly proportional to sample radioactivity by liquid scintillation counting.

Cells were seeded near confluence in 24 well tissue culture plates at either 5 or 30 mM glucose. At two days, cells were given fresh media, and at four days, cells were given fresh media with the appropriate FGF-2 amount. 21 hours after FGF-2 addition, 1 μCi ^{3}H-thymidine was added to each well for three hours. Cells were thoroughly washed and then lysed with 1 mL 1 M NaOH. Cell lysate was added to UltimaGold liquid scintillation fluid (PerkinElmer) and measured in a liquid scintillation counter (Packard).

5.2.4 Apoptosis

Endothelial cells were analyzed for apoptosis using the annexin V – propidium iodide assay described in Chapter 4. Briefly, annexin V binds phosphatidylserine translocated from inner to outer cell membrane in programmed, rather than catastrophic, cell death. Cells in early apoptosis are identified as annexin V positive while negative for the vital dye propidium iodide. PAEC were prepared for the annexin V – propidium iodide assay by combining floating and attached cells, which were removed by trypsinization. Samples were centrifuged to pellet cells, washed thoroughly in PBS, resuspended in annexin binding buffer, and labeled with annexin V-FITC and propidium iodide as per kit instructions (BD Pharmingen). Samples were analyzed immediately by flow cytometry.

For experiments testing the combined effect of FGF-2 and environmental stimuli on apoptosis, cells cultured in 5 or 30 mM glucose media were given either a saturating dose of additional exogenous FGF-2 (10 ng/ml) or neutralizing FGF-2 antibody (1 μg/ml; Upstate Biotechnology) for the final two days of culture. Cells were then exposed either to TNFα (5 ng/ml; R&D systems) for 24 hours or tBHP (10 μM; Sigma) for 48 hours.

5.2.5 Western blot

Intracellular signaling pathway activation was analyzed by Western blot. Confluent endothelial cells in 10 cm tissue culture dishes were washed with PBS and then lysed with ice cold lysis buffer (20mM Tris, 150mM NaCl, 1% Triton-X-100, 1% sodium deoxycholate, 0.1% SDS, 2mM EDTA, 2mM Na_3VO_4, 2mM PMSF, 50mM NaF, Complete protease inhibitor pellet and 10% glycerol) for 15 minutes at 4 °C. Cells were scraped using a rubber policeman and spun at 10,000 g for 10 minutes to pellet

insoluble material. Supernatant protein concentration was measured by bicinchoninic acid assay (Pierce), after which samples were aliquoted and stored at -20 ºC.

For Western blot, samples were boiled for 5 minutes with Laemmli sample buffer, and equal protein amounts were run on a 10% SDS-polyacrylamide gel, transferred to PVDF membrane and blocked for 1 hour with 5% nonfat dry milk. Membranes were incubated overnight at 4 ºC with anti-Akt, anti-ERK (p44/p42 MAP kinase), anti-phospho-Akt (Ser473) and anti phospho-ERK (p44/p42 MAP kinase; Thr202/Tyr204) (1:1000, Cell Signaling). Horseradish peroxidase-labeled IgG secondary antibodies (1:2000, Santa Cruz) with an enhanced chemiluminescence kit (Perkin Elmer) were used to visualize protein bands.

5.2.6 Statistics

All statistical analyses were performed with Prism software (Graphpad). Data were normally distributed and expressed as mean ± standard deviation. Comparisons between two groups were analyzed by Student's t test, and comparisons between more than two groups were analyzed by ANOVA. A value of $p < 0.05$ was considered statistically significant and is indicated in the text as such or in figures with a pound sign (#). A value of $p < .01$ is indicated with an asterisk (*). When no statistical significance was observed none are reported.

5.3 Results

As a co-regulatory unit for FGF-2, endothelial cells and basement membrane cooperate in FGF-2 storage and release to ensure effective delivery. We showed previously that basement membrane FGF-2 increases with culture glucose, and this increase is mediated through changes in endothelial cell FGF-2 release and permeability rather than inherent changes in basement membrane FGF-2 binding kinetics. We now show that higher FGF-2, whether added in soluble form or released from basement membrane, enhances endothelial cell proliferation to a similar degree in both low and high glucose culture. For apoptosis, however, the ability of FGF-2 to increase cell survival is dependent on secondary mediators.

5.3.1 Endothelial cell response to FGF-2

Viable endothelial cell number depends on basement membrane FGF-2. Endothelial cells were seeded on isolated basement membrane, basement membrane with FGF-2 extracted, basement membrane with exogenous FGF-2 added, or basement membrane with added soluble tissue inhibitors of matrix metalloproteinases. Cells were grown in 5 or 30 mM glucose media and counted on the seventh day. Basement membrane with its FGF-2 extracted surprisingly did not lower cell number from the native basement membrane level. However, endothelial cells showed 40% higher cell number on basement membrane with added FGF-2 compared to native basement membrane, whether media glucose was 5 or 30 mM (FIGURE 5.1). Cell number was lowered by tissue inhibitors of matrix metalloproteinases (20% lower for 5 mM glucose, 46% lower for 30 mM glucose; $p < .01$ for glucose effect). This suggests an important role for basement membrane breakdown in viable endothelial cell maintenance, in particular in high glucose.

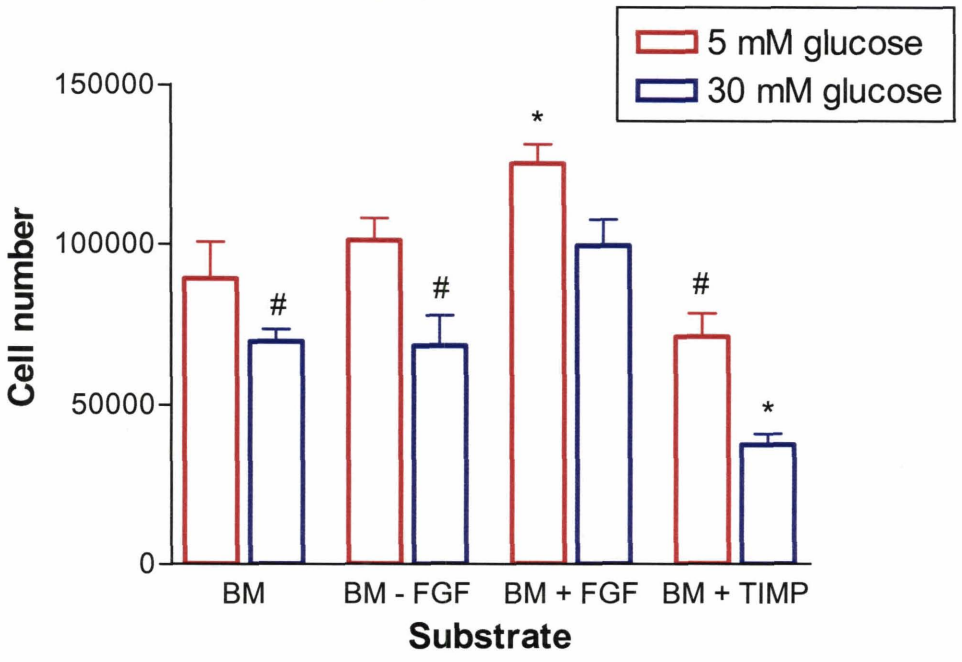


FIGURE 5.1: BASEMENT MEMBRANE FGF-2 INCREASES ENDOTHELIAL CELL PROLIFERATION. Endothelial cells were seeded on 5 mM glucose basement membrane (BM), 5 mM basement membrane with FGF-2 extracted with 2 M NaCl (BM – FGF), 5 mM basement membrane with added FGF-2 (BM + FGF), or 5 mM basement membrane with soluble tissue inhibitors of matrix metalloproteinases in the media (BM + TIMP). Cells were cultured for seven days in either 5 or 30 mM glucose media, after which they were trypsinized and counted in a Coulter counter. All samples are compared to 5 mM baseline. 30 mM samples of BM + FGF and BM + TIMP are also significantly different from 30 mM baseline ($p < .01$). (*) $p < .01$; (#) $p < .05$

Exogenous FGF-2 addition to low glucose basement membrane equalized high glucose basement membrane effects on endothelial cells. (FIGURE 5.2, FIGURE 5.3). At seven days of culture in growth media, basement membrane samples showed twice the viable cell number as tissue culture polystyrene controls. Furthermore, endothelial cells on 30 mM glucose basement membrane had 30-35% higher viable cell number than cells on 5 mM glucose basement membrane ($p < .01$), a difference that was negated fully by FGF-2 addition. Basement membrane glucose exposure predominated over media glucose exposure as this basement membrane effect was observed across media glucose conditions (FIGURE 5.1 - FIGURE 5.3).

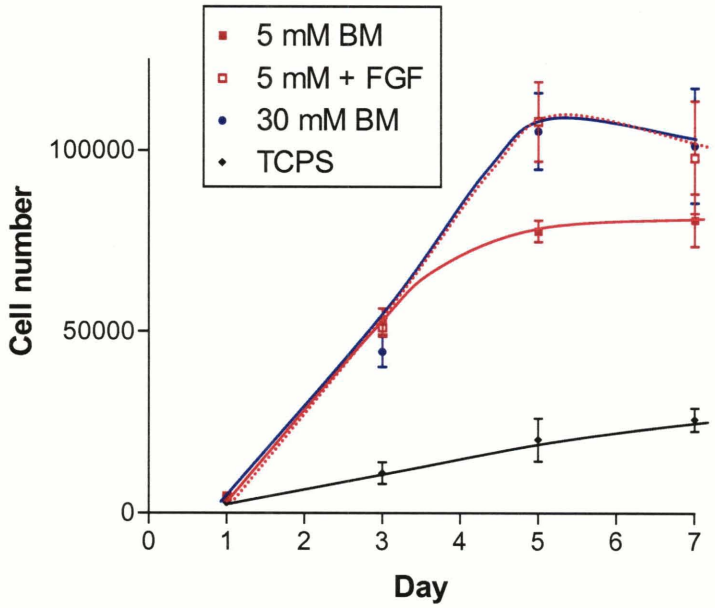


FIGURE 5.2: ENDOTHELIAL CELL NUMBER ON 5 MM GLUCOSE BASEMENT MEMBRANE WITH ADDED FGF-2 IS EQUIVALENT TO 30 MM GLUCOSE BASEMENT MEMBRANE (5 MM GLUCOSE MEDIA). Porcine aortic endothelial cells were seeded in 5 mM glucose media on 5 mM glucose basement membrane (5 mM BM), 5 mM glucose basement membrane pre-incubated with FGF-2 (5 mM + FGF), 30 mM glucose basement membrane (30 mM BM), or tissue culture polystyrene (TCPS). At each time point, cells were trypsinized and counted in a Coulter counter.

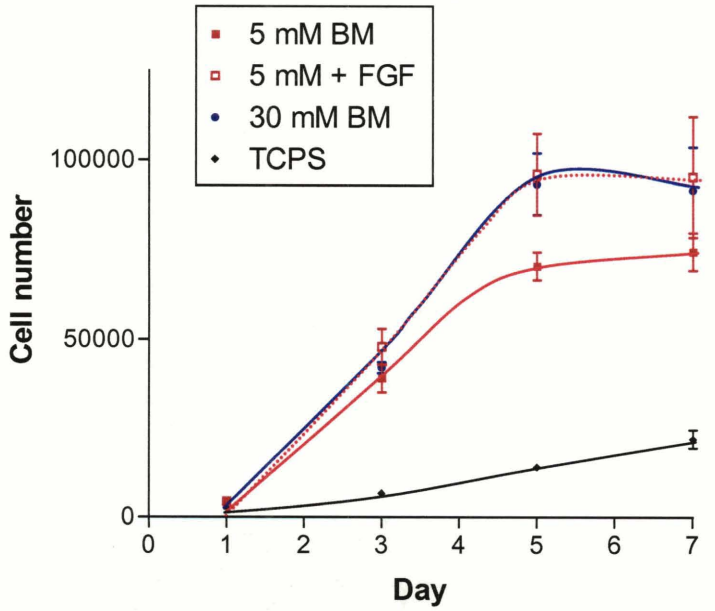


FIGURE 5.3: ENDOTHELIAL CELL NUMBER ON 5 MM GLUCOSE BASEMENT MEMBRANE WITH ADDED FGF-2 IS EQUIVALENT TO 30 MM GLUCOSE BASEMENT MEMBRANE (30 MM GLUCOSE MEDIA). Porcine aortic endothelial cells were seeded in 30 mM glucose media on 5 mM glucose basement membrane (5 mM BM), 5 mM glucose basement membrane pre-incubated with FGF-2 (5 mM + FGF), 30 mM glucose basement membrane (30 mM BM), or tissue culture polystyrene (TCPS). At each time point, cells were trypsinized and counted in a Coulter counter.

Basement membrane preserved endothelial cell health in the most stressful conditions. Viable cell number is a balance of proliferation and survival. When proliferation was decreased by starving cells in serum free media for seven days, endothelial cell overall survival increased 9% when seeded on 30 rather than 5 mM glucose basement membrane ($p < .05$), and both were greater still than cells seeded onto a serum-coated tissue culture polystyrene control ($p < .05$) (FIGURE 5.4).

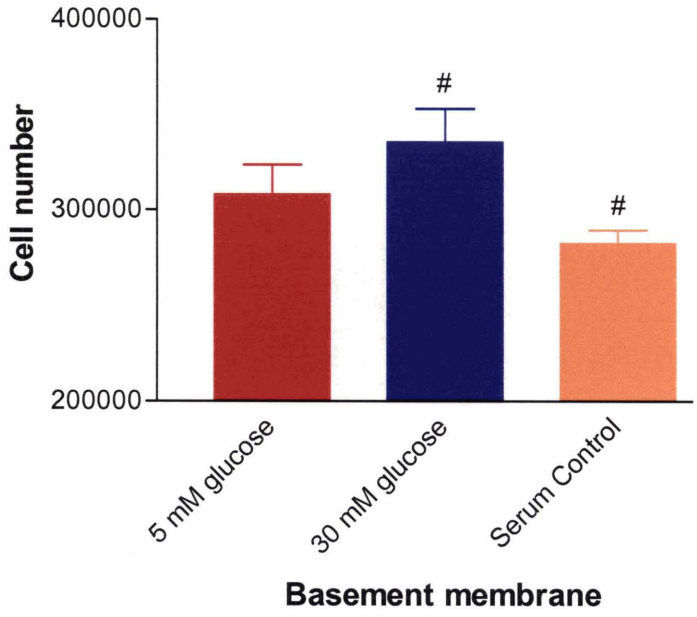


FIGURE 5.4: ENDOTHELIAL CELL SURVIVAL IS HIGHER ON 30 MM GLUCOSE BASEMENT MEMBRANE. Endothelial cells were seeded in 5 mM serum-free media on 5 mM glucose basement membrane, 30 mM glucose basement membrane, or serum coated tissue culture polystyrene. After seven starvation days, cells were trypsinized and counted. (#) $p < .05$

Viable endothelial cell count depends on FGF-2 dose, whether FGF-2 is soluble or bound to basement membrane. Endothelial cell number increased in a dose dependent manner with both time and soluble FGF-2 ($p < .01$), whether cells were cultured in 5 or 30 mM glucose media (FIGURE 5.5, FIGURE 5.6). After five days, cultures with 5 ng/ml FGF-2 had 93% greater cell number in 5 mM glucose and 63% greater cell number in 30 mM glucose compared to control. For all FGF-2 concentrations, endothelial cells cultured in 5 mM glucose consistently showed 15 to 20% higher cell numbers than endothelial cells cultured in 30 mM glucose ($p < .01$) (FIGURE 5.7).

FGF-2 affects endothelial cells in a similar manner whether it is added in soluble form or released from basement membrane. Cells seeded on basement membrane with added FGF-2 demonstrated increasing cell number with time ($p < .01$) and basement membrane bound FGF-2 ($p < .01$) (FIGURE 5.8). At five days, the maximum response to basement membrane FGF-2 was a 40% increase in viable cells.

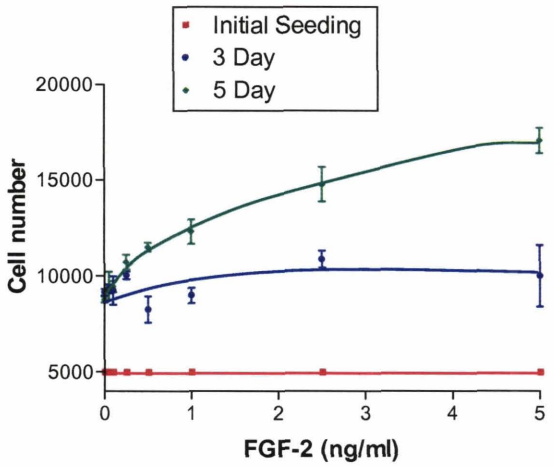


FIGURE 5.5: VIABLE ENDOTHELIAL CELL NUMBER INCREASES IN A DOSE-DEPENDENT MANNER WITH TIME ($P < .0001$) AND ADDED SOLUBLE FGF-2 ($P < .0001$) (5 MM GLUCOSE MEDIA). Endothelial cells were cultured in 5 mM glucose growth media with increasing exogenous FGF-2. At each time point, cells were trypsinized and counted.

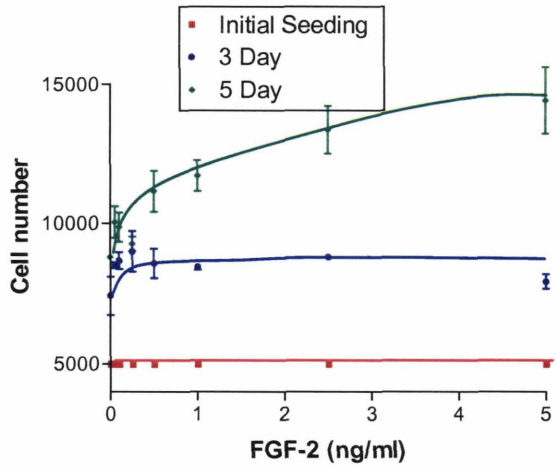


FIGURE 5.6: VIABLE ENDOTHELIAL CELL NUMBER INCREASES IN A DOSE-DEPENDENT MANNER WITH TIME ($P < .0001$) AND ADDED SOLUBLE FGF-2 ($P < .0001$) (30 MM GLUCOSE MEDIA). Endothelial cells were cultured in 30 mM glucose growth media with increasing exogenous FGF-2. At each time point, cells were trypsinized and counted.

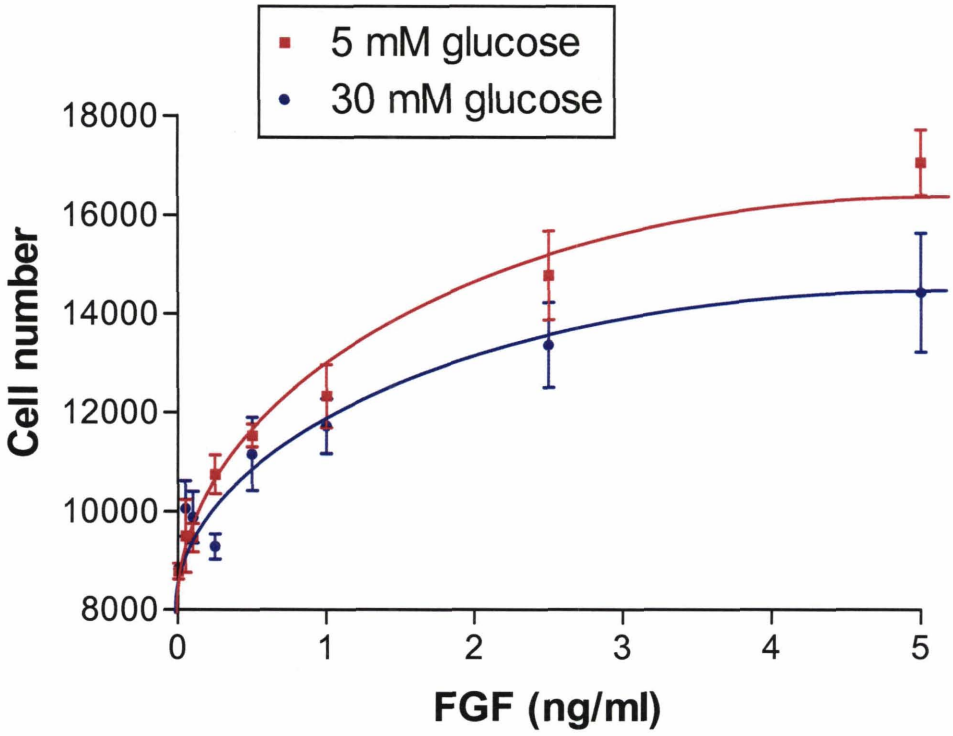


FIGURE 5.7: ENDOTHELIAL CELL NUMBER IS CONSISTENTLY HIGHER IN 5 MM GLUCOSE THAN 30 MM GLUCOSE IN RESPONSE TO FGF-2. Endothelial cell count at five days is compared for 5 and 30 mM glucose media with various exogenous FGF-2 levels.

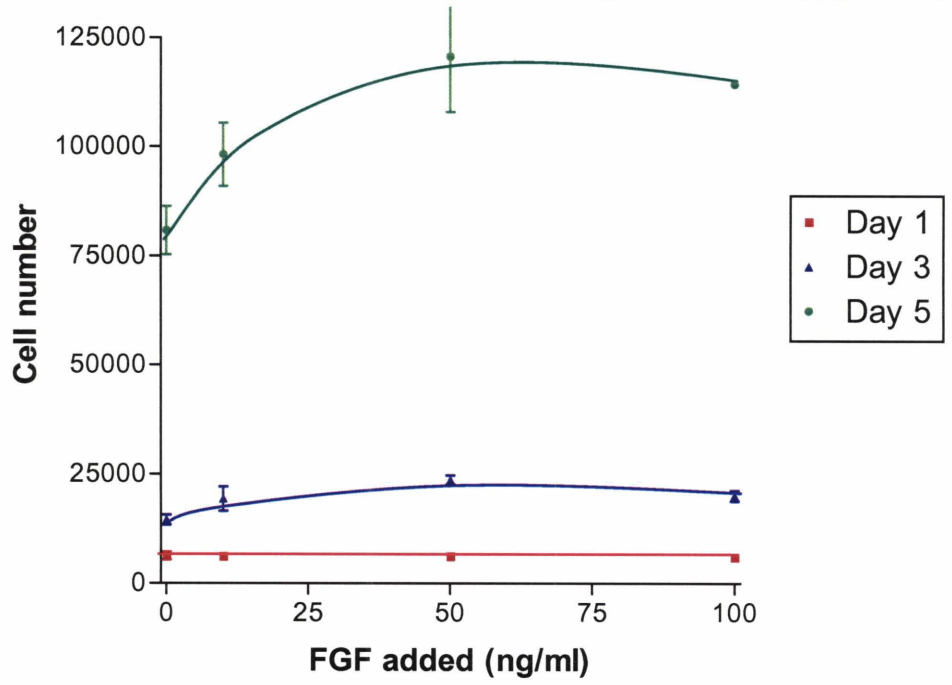


FIGURE 5.8: VIABLE ENDOTHELIAL CELL NUMBER INCREASES WITH BOTH TIME ($P < .01$) AND BASEMENT MEMBRANE BOUND FGF-2 ($P < .01$) (5 MM GLUCOSE MEDIA). Endothelial cells were seeded on 5 mM basement membrane that had been incubated with various amounts of FGF-2 prior to cell seeding. Cells were cultured in 5 mM glucose growth media, and at each time point, cells were trypsinized and counted.

5.3.2 FGF-2 endothelial cell binding kinetics

Endothelial cell FGF-2 binding kinetics were investigated to determine if FGF-2 bound to endothelial cells in a similar manner whether cells were grown in 5 or 30 mM glucose or whether there was 5 or 30 mM glucose in binding buffer. As was the case for heparan sulfate proteoglycans in basement membrane, endothelial cell surface heparan sulfate proteoglycan equilibrium capacity for FGF-2 was unchanged with glucose throughout the linear, physiologic range ($p = 0.96$) (FIGURE 5.9). Cell surface receptor FGF-2 binding was also similar for all glucose conditions ($p = 0.93$) (FIGURE 5.10).

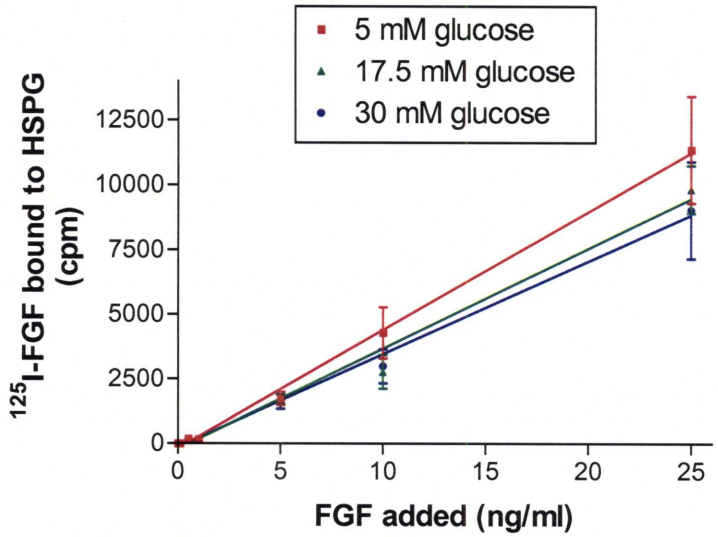


FIGURE 5.9: ENDOTHELIAL CELL SURFACE HEPARAN SULFATE PROTEOGLYCAN EQUILIBRIUM FGF-2 CAPACITY DOES NOT CHANGE WITH CULTURE GLUCOSE. Porcine aortic endothelial cells were cultured for four days in 5, 17.5, or 30 mM glucose growth media. On day four, cells were incubated on ice with increasing FGF-2 in binding buffer to equilibrium (three hours). FGF-2 was extracted from cell surface heparan sulfate proteoglycans with a ten second 2 M NaCl wash and quantified via FGF ELISA. ($p > .05$)

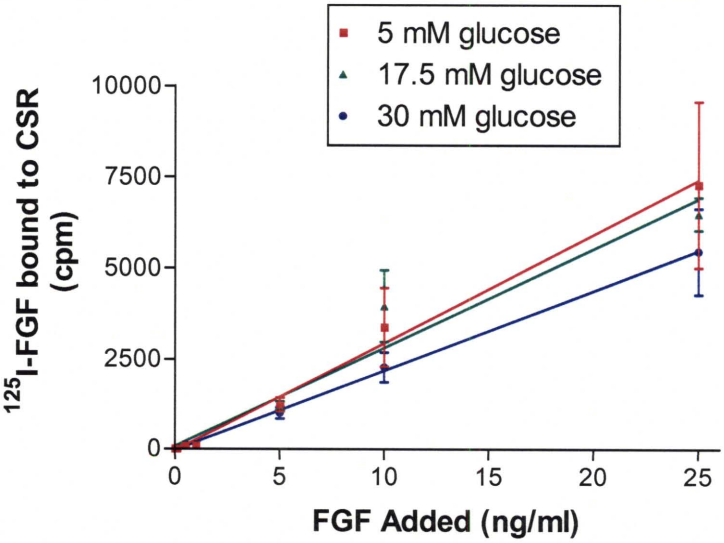


FIGURE 5.10: ENDOTHELIAL CELL SURFACE RECEPTOR EQUILIBRIUM FGF-2 CAPACITY DOES NOT CHANGE WITH CULTURE GLUCOSE. Porcine aortic endothelial cells were cultured for four days in 5, 17.5, or 30 mM glucose growth media. On day four, cells were incubated on ice with increasing FGF-2 in binding buffer to equilibrium (three hours). FGF-2 was extracted from cell surface receptors with a five minute acidic salt wash and quantified via FGF ELISA. ($p > .05$)

FGF-2 association with endothelial cell heparan sulfate proteoglycans and cell surface receptors was unchanged with glucose culture ($p = .73$) or glucose in binding buffer ($p = .71$). For cell surface heparan sulfate proteoglycans, bound FGF-2 increased rapidly within the first hour of exposure and reached equilibrium by two hours at approximately 1 ng/cm^2 FGF-2 bound : 40 ng/ml added (FIGURE 5.11). This ratio of bound : soluble FGF-2 was the same for basement membrane and cell surface heparan sulfate proteoglycans (Chapter 3). Association with cell surface receptors followed the same time course and did not vary with glucose ($p = .90$) but reached equilibrium at a slightly lower level of 1 ng/cm^2 FGF-2 bound : 66 ng/ml added (FIGURE 5.12). A second set of experiments using radiolabeled FGF-2 were performed to avoid confounding by native endothelial cell produced growth factor. These experiments confirmed that culture glucose concentration did not affect endothelial cell FGF-2 association kinetics for either heparan sulfate proteoglycans ($p = .75$) or cell surface receptors ($p = .91$) (FIGURE 5.13, FIGURE 5.14). Finally, FGF-2 association with endothelial cells did not change with glucose in binding buffer (FIGURE 5.15, FIGURE 5.16).

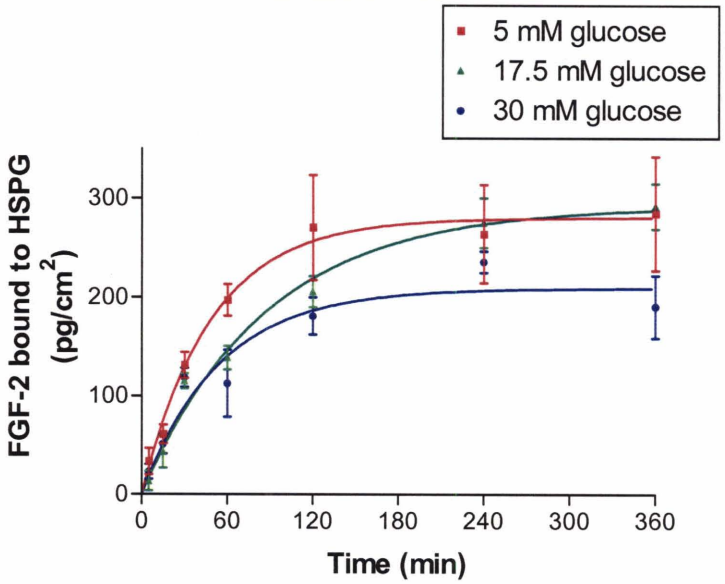


FIGURE 5.11: FGF-2 ASSOCIATION WITH ENDOTHELIAL CELL SURFACE HEPARAN SULFATE PROTEOGLYCANS DOES NOT CHANGE WITH CULTURE GLUCOSE. Endothelial cells were cultured for four days in 5, 17.5, or 30 mM glucose media. On day four, cells were incubated on ice with 5 ng/ml FGF-2 in binding buffer. At each time point, FGF-2 was extracted from cell surface heparan sulfate proteoglycans with a ten second 2 M NaCl wash and quantified via FGF ELISA. ($p > .05$)

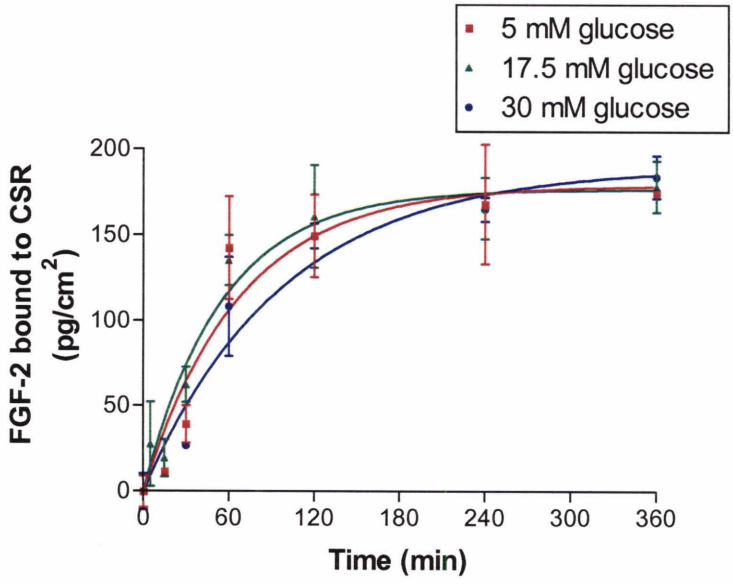


FIGURE 5.12: FGF-2 ASSOCIATION WITH ENDOTHELIAL CELL SURFACE RECEPTORS DOES NOT CHANGE WITH CULTURE GLUCOSE. Endothelial cells were cultured for four days in 5, 17.5, or 30 mM glucose growth media. On day four, cells were incubated on ice with 5 ng/ml FGF-2 in binding buffer. At each time point, FGF-2 was extracted from cell surface receptors with a five minute acidic salt wash and quantified via FGF ELISA ($p > .05$)

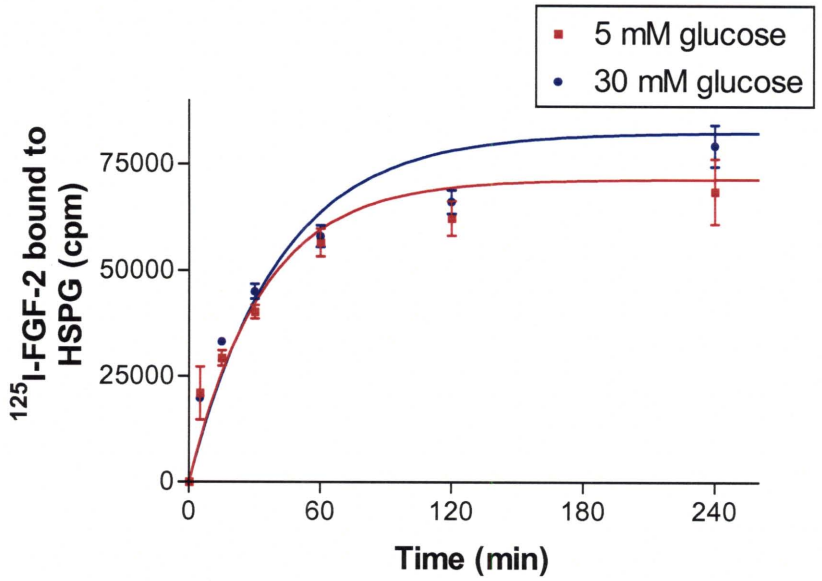


FIGURE 5.13: ASSOCIATION OF ^{125}I-FGF-2 WITH ENDOTHELIAL CELL HEPARAN SULFATE PROTEOGLYCANS SHOWED NO CHANGE WITH CULTURE GLUCOSE. Association experiments were repeated as described previously for ^{125}I-FGF-2 to ensure that data was not confounded by endothelial cell produced FGF-2. ($p > .05$)

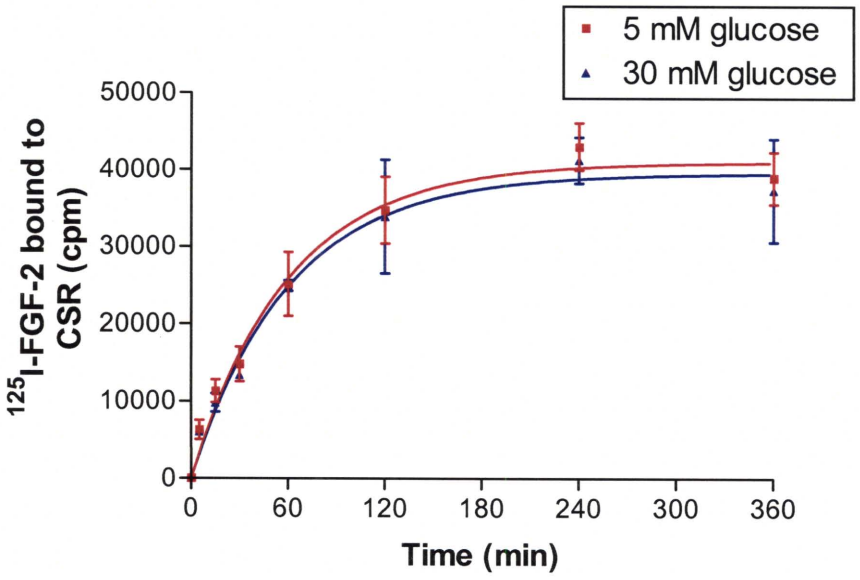


FIGURE 5.14: ASSOCIATION OF ^{125}I-FGF-2 WITH ENDOTHELIAL CELL SURFACE RECEPTOR SHOWED NO CHANGE WITH CULTURE GLUCOSE. Association experiments were repeated as described previously for ^{125}I-FGF-2 to ensure that data was not confounded by endothelial cell produced FGF-2. ($p > .05$)

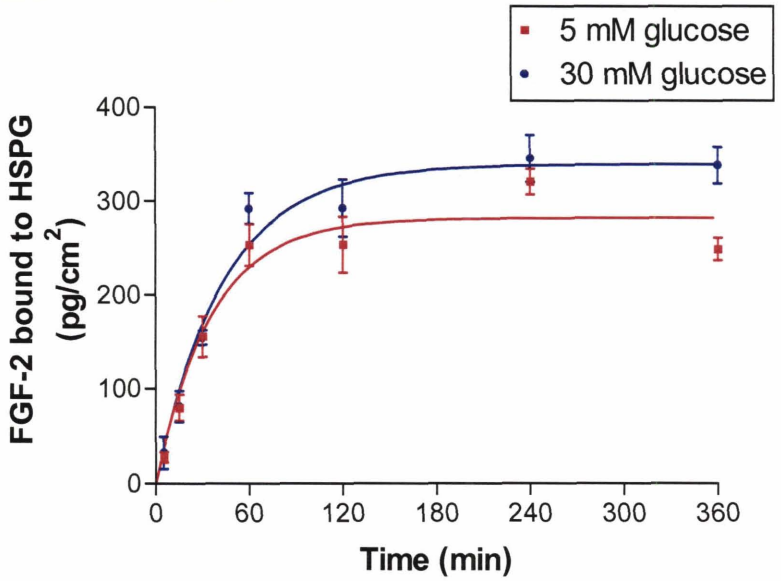


FIGURE 5.15: FGF-2 ASSOCIATION TO CELL SURFACE HEPARAN SULFATE PROTEOGLYCANS IS UNCHANGED WITH BUFFER GLUCOSE. Endothelial cells were cultured for four days in 5 mM glucose growth media. On day four, cells were incubated on ice with 5 ng/ml FGF-2 in binding buffer of 5 or 30 mM glucose. At each time point, FGF-2 was extracted from cell surface heparan sulfate proteoglycans with a ten second 2 M NaCl wash and quantified via FGF ELISA. ($p > .05$)

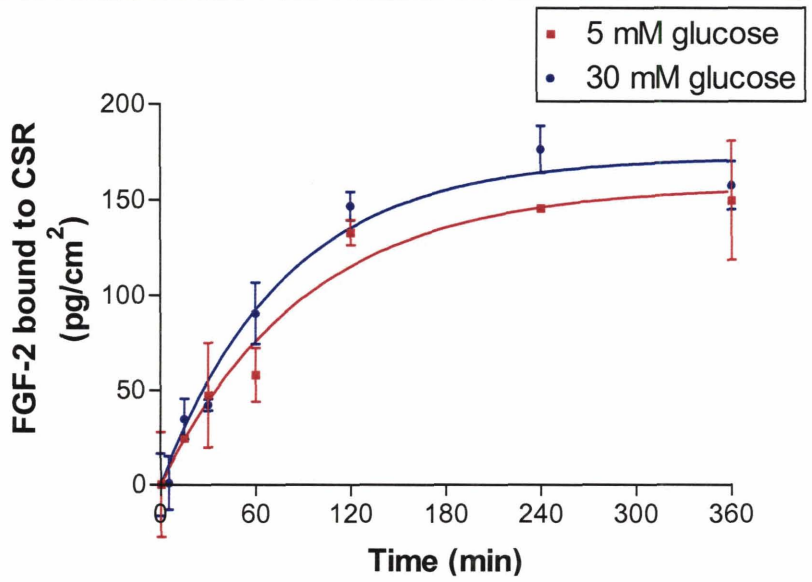


FIGURE 5.16: FGF-2 ASSOCIATION WITH CELL SURFACE RECEPTORS IS UNCHANGED WITH BUFFER GLUCOSE. Endothelial cells were cultured for four days in 5 mM glucose growth media. On day four, cells were incubated on ice with 5 ng/ml FGF-2 in binding buffer with 5 or 30 mM glucose. At each time point, FGF-2 was extracted from cell surface receptors with a five minute acidic salt wash and quantified via FGF ELISA ($p > .05$)

FGF-2 dissociated from endothelial cells with time was unchanged for either culture glucose or glucose in dissociation buffer. For both cases, dissociated FGF-2 rose rapidly until one hour and reached equilibrium at around two hours ($p = .71$ for culture glucose, $p = .54$ for glucose in buffer) (FIGURE 5.17, FIGURE 5.18). FGF-2 that remained bound to cell surface heparan sulfate proteoglycans or receptors decreased similarly with time independent of culture or buffer glucose (FIGURE 5.19, FIGURE 5.20, FIGURE 5.21, FIGURE 5.22). Similarly, FGF-2 that remained bound to either heparan sulfate proteoglycans ($p = .96$ for culture glucose, $p = .98$ for glucose in buffer) or cell surface receptors ($p = .66$ for culture glucose, $p = .19$ for glucose in buffer) did not change significantly with cell culture or binding buffer glucose.

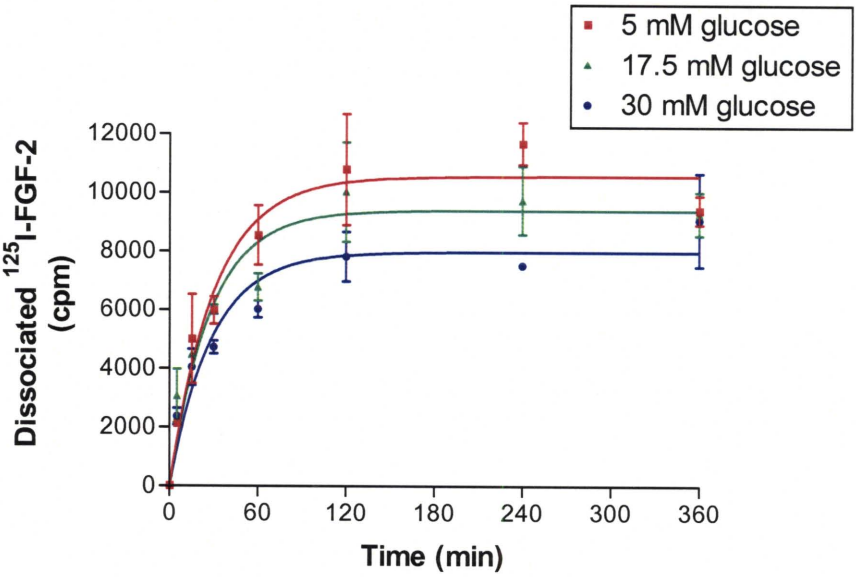


FIGURE 5.17: FGF-2 DISSOCIATION FROM ENDOTHELIAL CELLS INCREASES WITH TIME AND IS UNCHANGED WITH CULTURE GLUCOSE. Endothelial cells were cultured for four days in 5, 17.5, or 30 mM glucose growth media. On day four, cells were incubated on ice with 5 ng/ml ^{125}I-FGF-2 in binding buffer to equilibrium. Fresh binding buffer was added, and at each time point, dissociated ^{125}I-FGF-2 was measured in the buffer with a gamma counter. ($p > .05$)

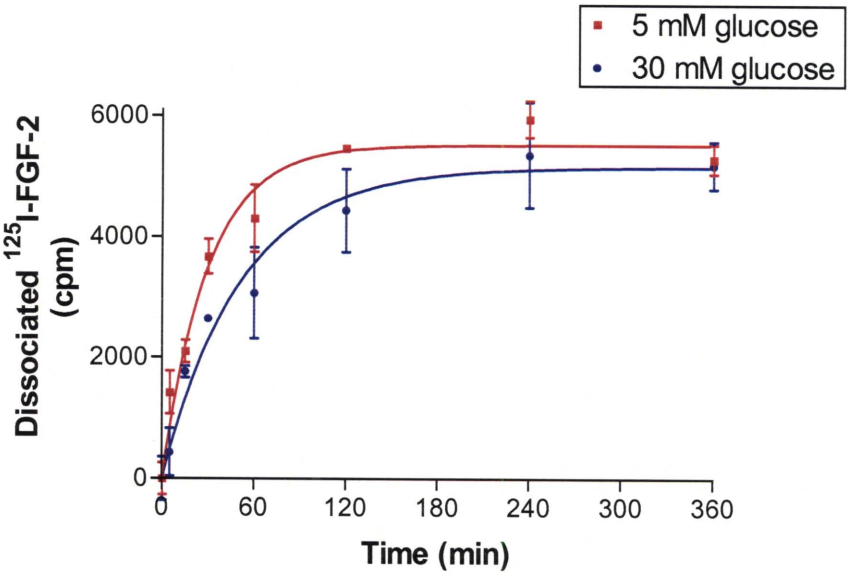


FIGURE 5.18: FGF-2 DISSOCIATED FROM ENDOTHELIAL CELLS IS UNCHANGED WITH BUFFER GLUCOSE. Endothelial cells were cultured for four days in 5 mM glucose growth media. On day four, cells were incubated on ice with 5 ng/ml ^{125}I-FGF-2 in binding buffer to equilibrium. Fresh binding buffer with 5 or 30 mM glucose was added, and at each time point, dissociated ^{125}I-FGF-2 was measured in the buffer with a gamma counter. ($p > .05$)

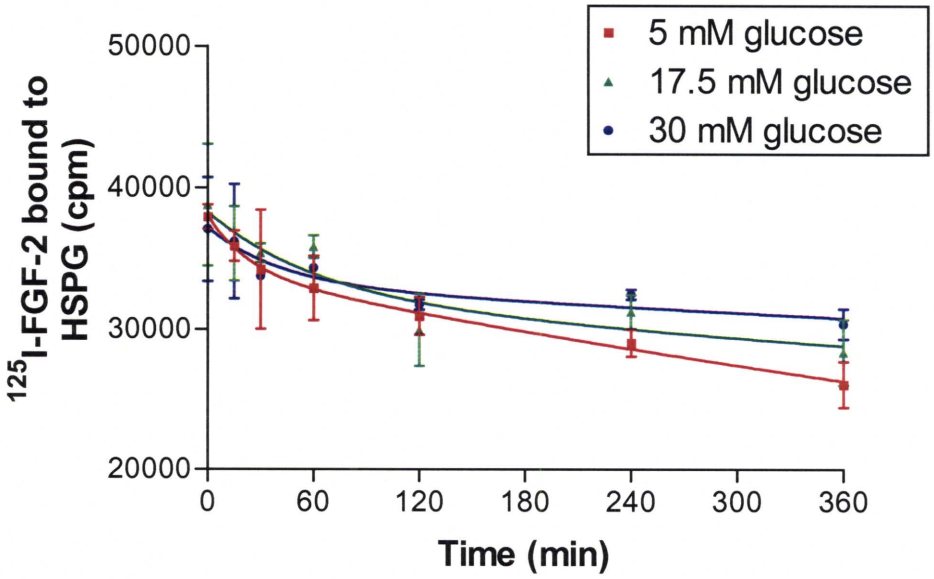


FIGURE 5.19: FGF-2 BOUND TO ENDOTHELIAL CELL HEPARAN SULFATE PROTEOGLYCANS DECREASES WITH TIME AND IS UNCHANGED WITH CULTURE GLUCOSE. Endothelial cells were cultured for four days in 5, 17.5, or 30 mM glucose growth media. On day four, cells were incubated on ice with 5 ng/ml ^{125}I-FGF-2 in binding buffer to equilibrium. Fresh binding buffer was added, and at each time point, ^{125}I-FGF-2 was extracted from cell surface heparan sulfate proteoglycans with a ten second 2 M NaCl wash and quantified in a gamma counter. ($p > .05$)

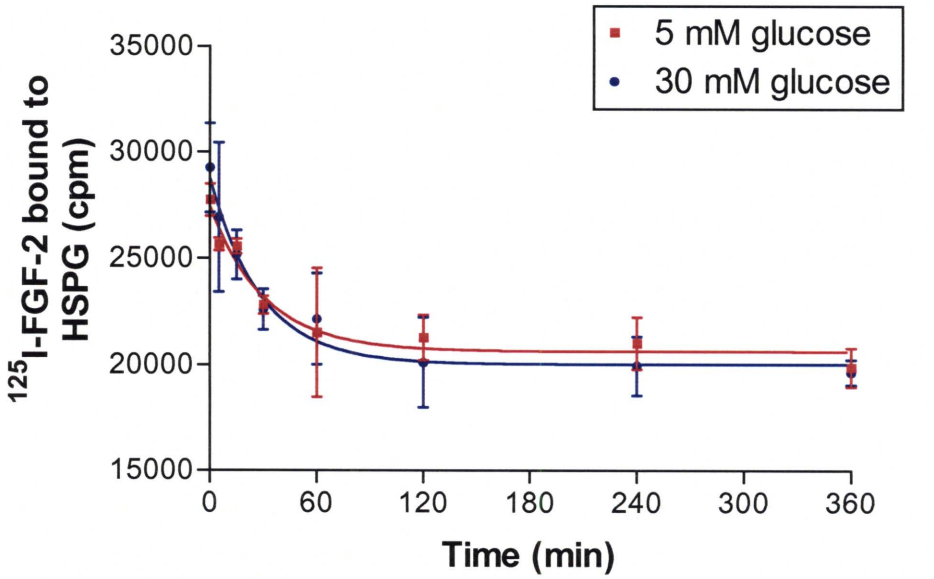


FIGURE 5.20: FGF-2 BOUND TO ENDOTHELIAL CELL HEPARAN SULFATE PROTEOGLYCANS IS UNCHANGED WITH BUFFER GLUCOSE. Endothelial cells were cultured for four days in 5 mM glucose growth media. On day four, cells were incubated on ice with 5 ng/ml ^{125}I-FGF-2 in binding buffer to equilibrium. Fresh binding buffer with either 5 or 30 mM glucose was added, and at each time point, ^{125}I-FGF-2 was extracted from cell surface heparan sulfate proteoglycans with a ten second 2 M NaCl wash and quantified in a gamma counter. ($p > .05$)

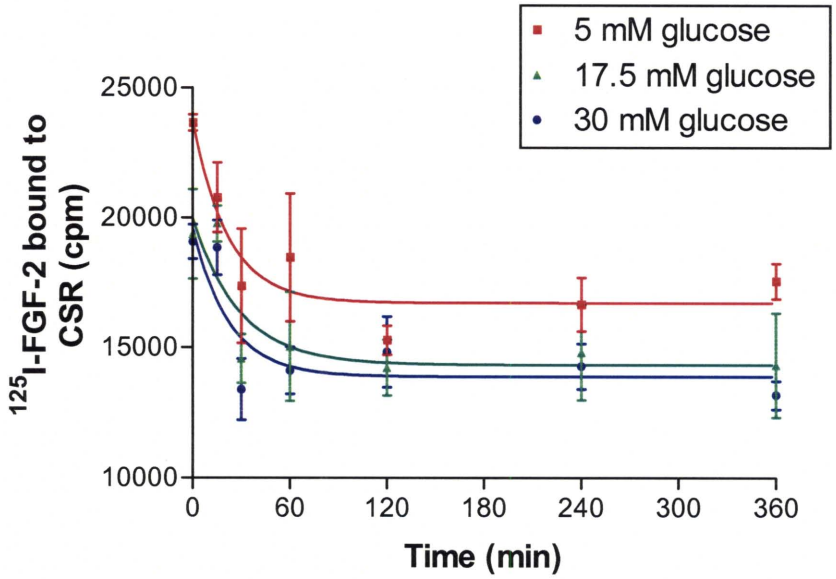


FIGURE 5.21: FGF-2 BOUND TO ENDOTHELIAL CELL SURFACE RECEPTORS DECREASES WITH TIME BUT NOT WITH CULTURE GLUCOSE. Endothelial cells were cultured for four days in 5, 17.5, or 30 mM glucose growth media. On day four, cells were incubated on ice with 5 ng/ml ^{125}I-FGF-2 in binding buffer to equilibrium. Fresh binding buffer was added and at each time point, ^{125}I-FGF-2 was extracted with a five minute acidic salt wash and quantified in a gamma counter. ($p > .05$)

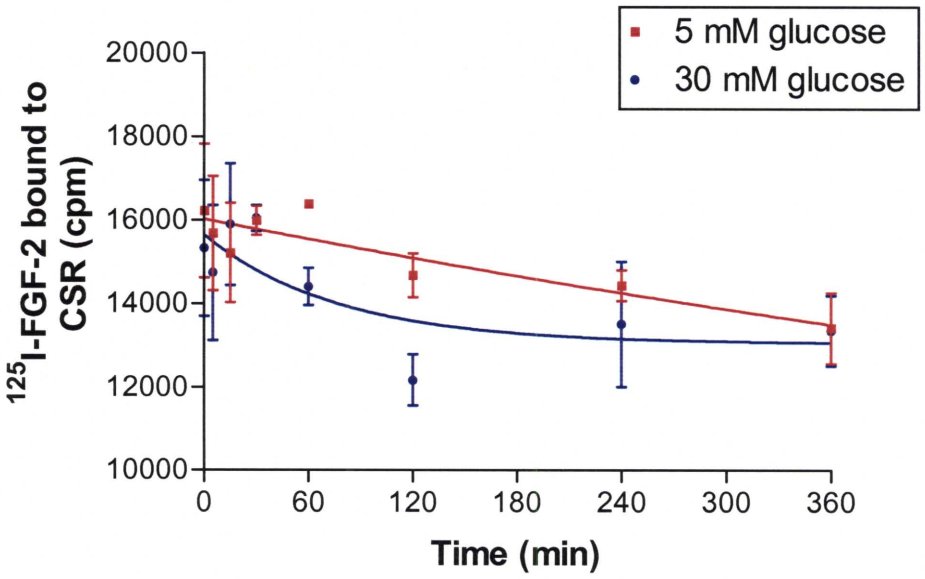


FIGURE 5.22: FGF-2 BOUND TO ENDOTHELIAL CELL SURFACE RECEPTORS IS UNCHANGED WITH BUFFER GLUCOSE. Endothelial cells were cultured for four days in 5 mM glucose growth media. On day four, cells were incubated on ice with 5 ng/ml ^{125}I-FGF-2 in binding buffer to equilibrium. Fresh binding buffer with 5 or 30 mM glucose was added, and at each time point, ^{125}I-FGF-2 was extracted with a five minute acidic salt wash and quantified in a gamma counter. ($p > .05$)

5.3.3 Endothelial cell proliferation with FGF-2

Endothelial cell proliferation was measured through cell cycle phase and DNA production in response to FGF-2. Timing studies for both cell cycle analysis and DNA production showed a peak response approximately 24 hours after growth factor exposure and remained elevated up to 72 hours ($p < .01$) (FIGURE 5.23). Cells cultured in 30 mM glucose showed the same response timing as those in 5 mM glucose but the percentage of S phase cells was ~10 percent lower throughout all time points ($p < .05$). The percentage of S-phase cells increased four fold as FGF-2 was added up to a concentration of 0.5 ng/ml and reached equilibrium afterwards with ~30% of cells actively entering the mitotic cycle (FIGURE 5.24). The percentage of S-phase cells remained consistently 10% lower ($p < .01$) for cells cultured in 30 mM glucose as opposed to 5 mM glucose (FIGURE 5.24, FIGURE 5.25). However, when these data were normalized to percentage of S-phase cells without any FGF-2 added, normalized values for 30 mM glucose were no different from 5 mM glucose ($p = .19$) (FIGURE 5.26).

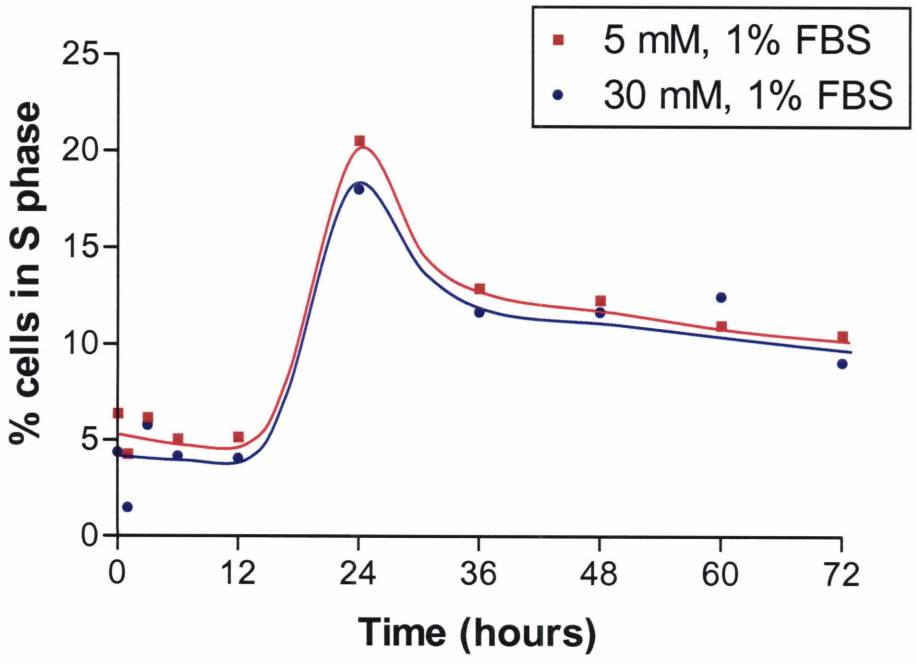


FIGURE 5.23: ENDOTHELIAL CELL S-PHASE RESPONSE TO FGF-2 PEAKS AT 24 HOURS BUT IS LOWER IN 30 MM GLUCOSE. Subconfluent endothelial cells were incubated with 1 ng/ml FGF-2. At each time point, cells were trypsinized, labeled with propidium iodide, stored overnight at 4 °C, and analyzed by flow cytometry the following morning. ($p < .05$)

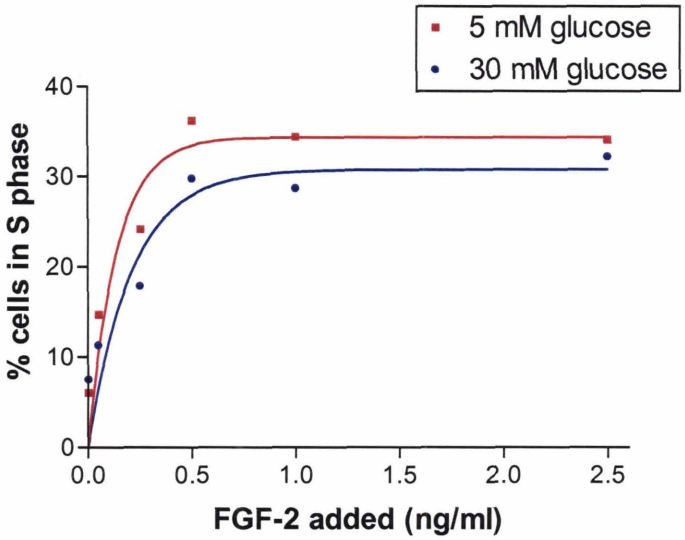


FIGURE 5.24: ENDOTHELIAL CELLS ENTER S PHASE IN A DOSE DEPENDENT MANNER IN RESPONSE TO FGF-2, BUT 30 MM GLUCOSE LOWERS THE RESPONSE. Subconfluent endothelial cells were incubated with increasing levels of FGF-2 for 24 hours. Cells were trypsinized and labeled with propidium iodide, stored over night at 4 °C and analyzed by flow cytometry the following morning. ($p < .0001$)

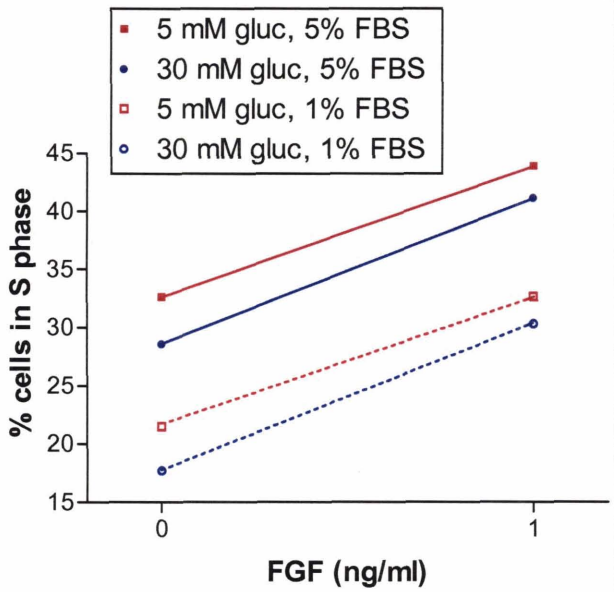


FIGURE 5.25: ENDOTHELIAL CELLS IN 30 MM GLUCOSE ENTER S-PHASE AT A LOWER RATE THAN CELLS IN 5 MM GLUCOSE 24 HOURS AFTER EXPOSURE TO FGF-2. Endothelial cells cultured in 5 or 30 mM growth medium, with either 1% or 5% fetal bovine serum (FBS), show consistently decreased 30 mM cells in S-phase after 24 hour exposure to FGF-2. ($p < .01$) .

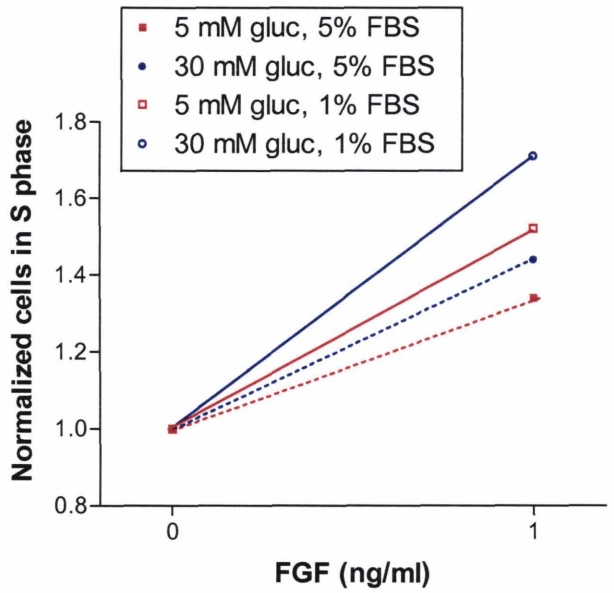


FIGURE 5.26: DECREASED HIGH GLUCOSE ENTRY INTO S-PHASE IS NOT MEDIATED BY FGF-2. Though high glucose media appeared to inhibit cell entry into S phase in response to FGF-2, when data are normalized to the case without added FGF-2, there was no statistical difference between 5 and 30 mM glucose cells.

Endothelial cell DNA production measurement via ^{3}H-thymidine incorporation confirmed cell cycle results. Endothelial cells increased DNA production in a dose dependent manner to FGF-2, with ^{3}H-thymidine incorporation increasing nearly four fold at 5 ng/ml added FGF-2 (FIGURE 5.27). DNA production by cells in 30 mM as opposed to 5 mM glucose media decreased by an average of 15% ($p < .01$) (FIGURE 5.28). However, cell number was lower in 30 mM glucose due to increased cell size. When ^{3}H-thymidine incorporation was normalized to cell number, there was no difference in DNA production with glucose.

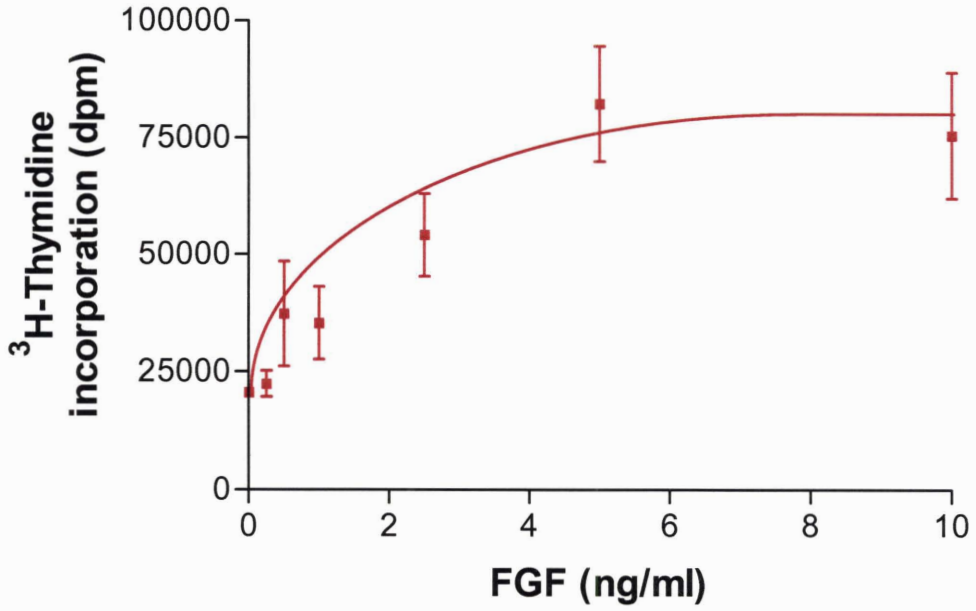


FIGURE 5.27: ENDOTHELIAL CELL DNA PRODUCTION IN RESPONSE TO SOLUBLE FGF-2 IS DOSE DEPENDENT. Confluent porcine aortic endothelial cells were incubated with increasing levels of FGF-2. After 21 hours, 1 μCi ^{3}H-thymidine was added for three hours. Cells were then lysed with 1 M NaOH, and the cell lysate was measured in a liquid scintillation counter. ($p < .0001$)

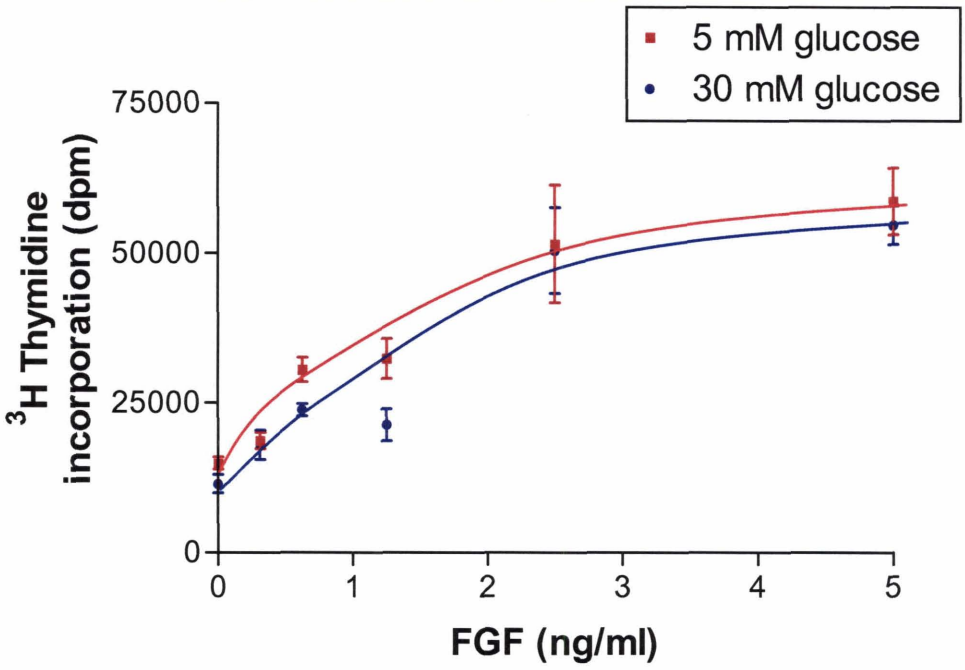


FIGURE 5.28: GLUCOSE DECREASES ENDOTHELIAL CELL DNA PRODUCTION IN RESPONSE TO FGF-2, BUT THE DIFFERENCE DISAPPEARS WHEN DATA IS NORMALIZED TO THE CASE WITHOUT ADDED FGF-2. Confluent porcine aortic endothelial cells cultured in 5 or 30 mM glucose were incubated with increasing levels of FGF-2. After 21 hours, 1 μCi ^{3}H-thymidine was added for three hours. Cells were lysed with 1 M NaOH, and cell lysate was measured in a liquid scintillation counter. ($p < .01$)

5.3.4 Endothelial cell apoptosis with FGF-2

The FGF-2 effect on endothelial cell survival was elucidated in the annexin V – propidium iodide assay using both added FGF-2 and a neutralizing FGF-2 antibody. We demonstrated in Chapter 4 that endothelial cells release FGF-2 concomitant with apoptotic membrane damage, therefore neutralizing antibody removed native FGF-2 released from cells themselves. We first determined that the FGF-2 antibody effectively neutralized FGF-2 added in media FBS, added exogenously, or released from endothelial cells. For all FGF-2 types, the neutralizing antibody reduced active FGF-2 by two to three fold (FIGURE 5.).

Endothelial cell apoptosis increased by 20% in 30 mM glucose conditions, as described in Chapter 4. Addition of a saturating FGF-2 dose did not significantly decrease apoptosis for either 5 or 30 mM glucose cells ($p > .05$), although standard deviation did decrease (FIGURE 5.). However, neutralizing antibody addition increased apoptosis by 50% for 30 mM glucose cells compared to those with added FGF-2 ($p <$

.05). Similarly, when reactive oxygen species production was induced in endothelial cells using tBHP, presence of tBHP increased apoptosis for all conditions ($p < .05$), and FGF-2 neutralizing antibody addition increased apoptosis for cells with high reactive oxygen species ($p < .05$) (FIGURE 5.).

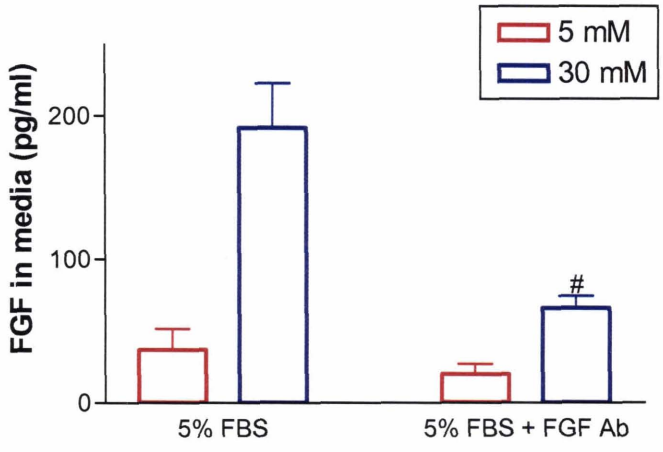


FIGURE 5.29: A NEUTRALIZING FGF-2 ANTIBODY DECREASES AVAILABLE CELL RELEASED FGF-2. Endothelial cells were cultured for four days in 5 or 30 mM glucose media. 1 μg/ml of a neutralizing FGF-2 antibody was added on day three, and on day four, media FGF-2 was quantified via FGF ELISA. ($p < .01$); (#) $p < .05$ when compared to 30 mM without antibody.

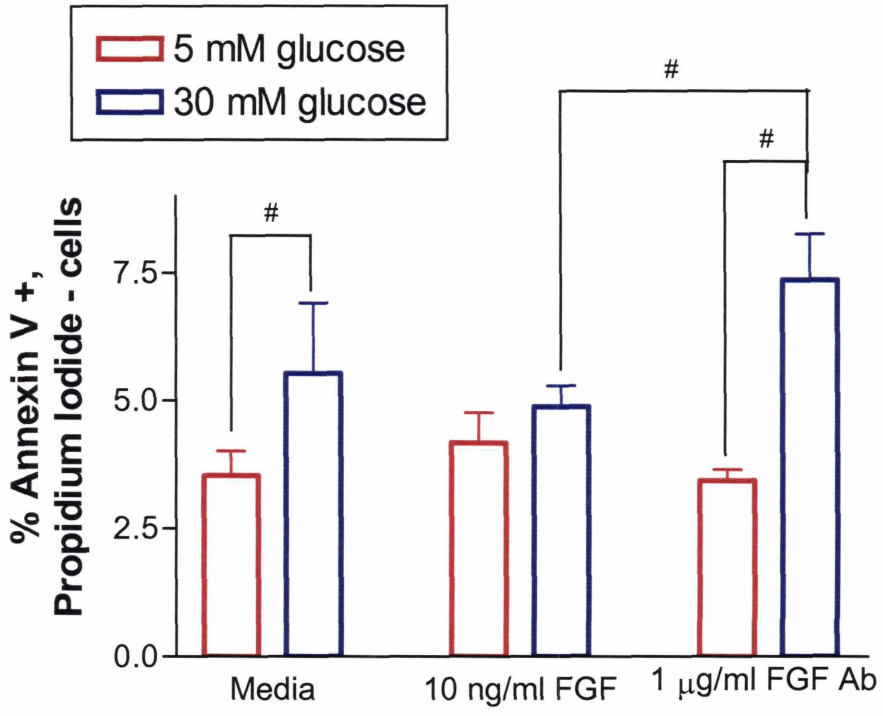


FIGURE 5.30: ENDOTHELIAL CELL APOPTOSIS IS HIGHER IN 30 MM GLUCOSE CULTURE, WHICH IS PARTIALLY INHIBITED BY FGF-2. Endothelial cells were cultured for four days in 5 or 30 mM glucose media. For the final two days, exogenous FGF-2 or a neutralizing FGF-2 antibody was added to media. Cells were trypsinized, labeled with annexin V and propidium iodide, and analyzed by flow cytometry. (#) $p < .05$

Studies of endothelial cell exposure to TNFα showed the complex role of FGF-2 in the apoptotic process. At 12 hours, apoptosis for cells exposed to TNFα was almost twice that of unexposed cells (FIGURE 5.32). FGF-2 neutralizing antibody eliminated the difference between cells exposed and not exposed to TNFα ($p > .05$). After 24 hours TNFα exposure, apoptosis was significantly decreased by FGF-2 antibody addition ($p < .05$) but was still higher than that of cells not exposed to TNFα ($p < .01$) (FIGURE 5.33). Antibody addition shows that part of the TNFα apoptotic effect was increased through FGF-2 release.

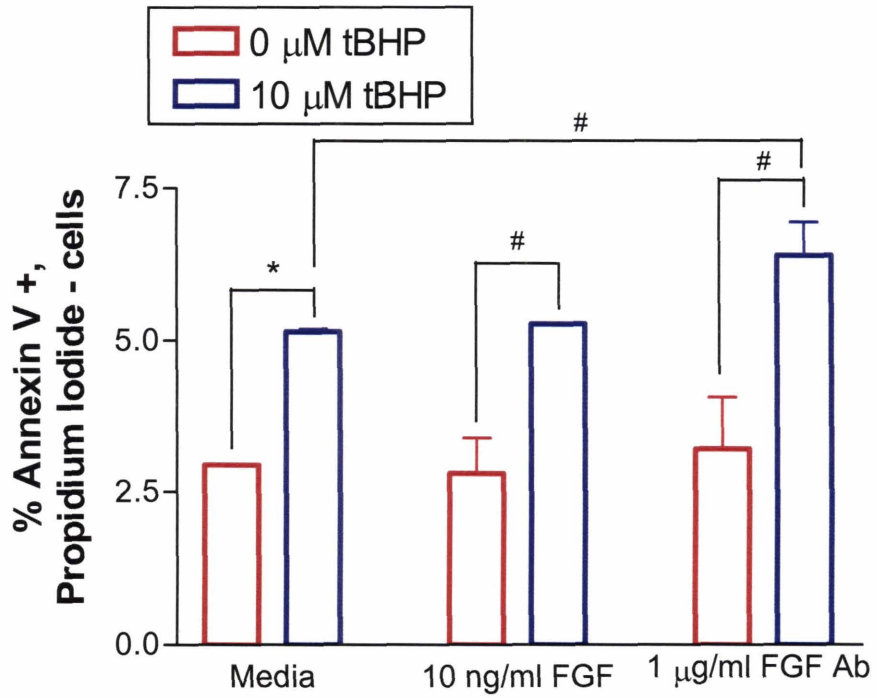


FIGURE 5.31: ENDOTHELIAL CELL APOPTOSIS DUE TO REACTIVE OXYGEN SPECIES IS PARTIALLY ABROGATED BY ENDOTHELIAL CELL FGF-2 RELEASE. Endothelial cells cultured for four days in 5 mM glucose media were exposed to tBHP for the final two days, along with either exogenous FGF-2 or a neutralizing FGF-2 antibody. Cells were trypsinized, labeled with annexin V and propidium iodide, and analyzed by flow cytometry. (*) $p < .01$; (#) $p < .05$

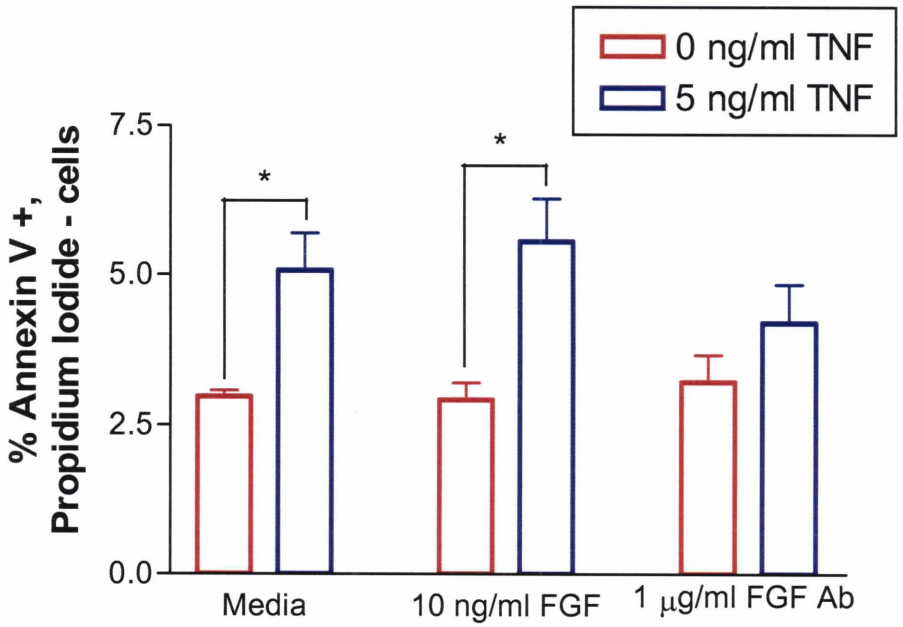


FIGURE 5.32: FGF-2 INCREASES ENDOTHELIAL CELL APOPTOSIS DUE TO TNFα (12 HOURS). Porcine aortic endothelial cells were exposed to TNFα for 12 hours in the presence of exogenous FGF-2 or a neutralizing FGF-2 antibody. Cells were then harvested, labeled with annexin V and propidium iodide, and analyzed by flow cytometry. (*) $p < .01$

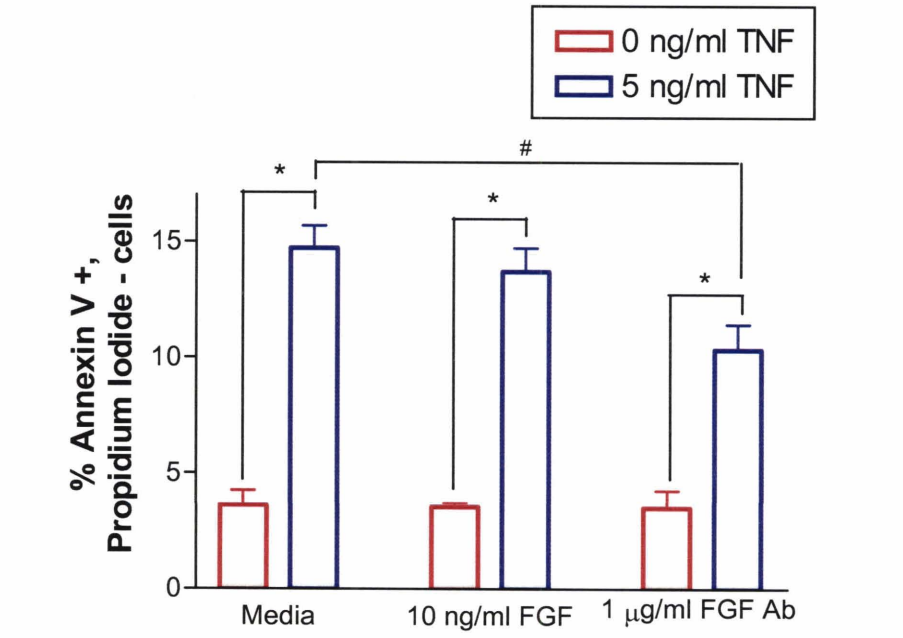


FIGURE 5.33: FGF-2 INCREASES ENDOTHELIAL CELL APOPTOSIS DUE TO TNFα (24 HOURS). Endothelial cells were exposed to TNFα for 24 hours in the presence of exogenous FGF-2 or a neutralizing FGF-2 antibody. Cells were then harvested, labeled with annexin V and propidium iodide, and analyzed by flow cytometry. (*) $p < .01$; (#) $p < .05$

5.3.5 Signaling pathway alterations

FGF-2 intracellular signaling pathways were investigated by Western blot. Endothelial cells cultured in either 5 or 30 mM glucose showed similar 1.7 fold increases in phosphorylated-Erk with constant Erk after FGF-2 exposure, indicating normal progression along the proliferative pathway (TABLE 5.1, FIGURE 5.34). However, active phosphorylated-Akt increased 50% in 5 mM cells with FGF-2 exposure but did not increase at all in high glucose cells exposed to FGF-2. Signaling down the anti-apoptotic survival pathway appears to be disrupted in high glucose cells.

	5 mM glucose		30 mM glucose	
	- FGF-2	+ FGF-2	- FGF-2	+ FGF-2
Erk	5607	6347	5393	5371
p-Erk	3808	10246	4899	12756
Akt	5977	4814	5519	4534
p-Akt	719	1111	401	393

TABLE 5.1: FGF-2 INDUCES A 50% INCREASE IN P-AKT IN LOW BUT NOT HIGH GLUCOSE CULTURE. Endothelial cells were cultured for four days in 5 or 30 mM glucose media, after which an FGF-2 stimulus was applied. Densitometry of the Western blot (FIGURE 5.34) shows that p-Erk increases by 1.7 fold for both 5 and 30 mM glucose cells, whereas p-Akt increases 50% for 5 mM glucose cells only.

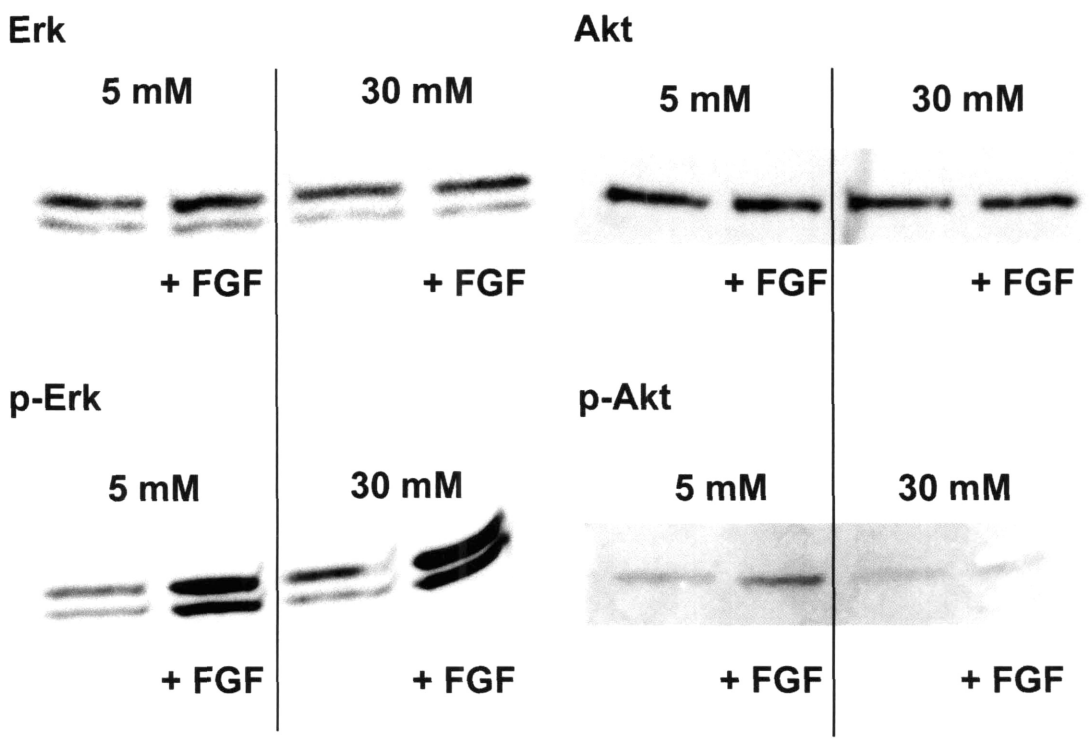


FIGURE 5.34: HIGH GLUCOSE CULTURE DECREASES ENDOTHELIAL CELL FGF-2 AKT SIGNALING WITHOUT CHANGING ERK SIGNALING. Endothelial cells cultured in 5 or 30 mM glucose were serum starved overnight and then incubated with FGF-2 for 10 minutes. Cells were lysed and cell extracts run on a gel. After transfer, specific antibodies to Erk, phospho-Erk, Akt, and phospho-Akt were used to identify specific signaling proteins. Decreased intensity of the phospho-Akt band indicates lower FGF-2 signaling down the anti-apoptotic Akt pathway in high glucose culture.

5.4 Discussion

The increase in basement membrane FGF-2 in high glucose is interesting in itself, in that it shows how endothelial cells actively control basement membrane protein content. However, the data become far more intriguing when we consider how basement membrane FGF-2 in turn affects endothelial cell function. In this chapter, we propose a new model for the role of FGF-2 in the basement membrane-endothelial cell co-regulatory loop under high glucose stress conditions.

5.4.1 Viable cell count and FGF-2

The population of endothelial cells that retain their viability is determined by a balance between proliferation and death. Since FGF-2 promotes both proliferation and survival, cell number provides a starting point for determination of FGF-2 effects on endothelial cells. Cell number increases in a dose dependent manner with FGF-2 addition independent of delivery method and culture glucose, indicating a favorable balance of proliferation and apoptosis. Cell number consistently peaks at 5 days and maximal response is at approximately 5 ng/ml added FGF-2 (FIGURE 5.5).

We now show that the increase in basement membrane FGF-2 with glucose has a measurable effect on endothelial cell number. Cells seeded on basement membrane grown in 30 mM glucose have higher cell numbers than cells seeded on basement membrane grown in 5 mM cell glucose, whether put in growth media to measure proliferation and survival or in starvation media to examine survival in particular (FIGURE 5.2 - FIGURE 5.4). Incubation of 5 mM basement membrane with FGF-2 prior to cell seeding abrogated the difference in cell number between 5 and 30 mM basement membrane, pointing to FGF-2 as the critical factor (FIGURE 5.2, FIGURE 5.3).

However, basement membrane post-FGF-2 extraction does not show decreased endothelial cell number when compared to basement membrane with its native FGF-2 (FIGURE 5.1). This could indicate that salt extraction removes only the surface FGF-2 fraction, and as cells break down the basement membrane, they are still able to release adequate FGF-2 from deeper in the basement membrane to support cell proliferation and survival. This concept is supported by the lower cell number when tissue inhibitor of

matrix metalloproteinases (TIMP) is added to media to inhibit basement membrane breakdown. For all conditions, TIMP addition decreases cell number.

Exposing endothelial cells to serum-free media begins to dissect out survival versus proliferation FGF-2 effects, as cell proliferation is severely impeded in starvation media. After extended starvation, cells seeded on 5 mM glucose basement membrane have decreased survival over cells on 30 mM glucose basement membrane, but both are still improved over serum-coated tissue culture polystyrene (FIGURE 5.4). The increased survival with high glucose basement membrane is only ~10%, which accounts for only part of the total change in growth media (30-35%). Basement membrane FGF-2 appears to have additive survival and proliferation effects on endothelial cells.

Throughout all cell count experiments, endothelial cell number is lower in high glucose media even for the same FGF-2 level (FIGURE 5.1, FIGURE 5.7). Since it is unclear whether this effect is related to decreased proliferation or increased apoptosis, more experiments are needed to dissect out the particular mechanism.

5.4.2 Endothelial cell FGF-2 binding kinetics

Similar to basement membrane FGF-2 binding kinetics, equilibrium capacity and association of FGF-2 to endothelial cell surface heparan sulfate proteoglycans are unchanged with either culture glucose or glucose in the environment. The ratio of bound to soluble FGF-2 (1:40) is the same for both basement membrane and cell surface heparan sulfate proteoglycans, indicating similar binding conditions in both locations (FIGURE 5.11). FGF-2 capacity and association to cell surface receptors are also unchanged with glucose, but the bound to soluble FGF-2 ratio is about a third lower (1:66). At first, this seems counterintuitive since the cell surface receptor is a high affinity binding site. However, there are 10 to 10000 times more cell surface heparan sulfate proteoglycans than cell surface receptors (146). The increased binding ratio of bound to soluble FGF-2 for heparan sulfate proteoglycans is likely an effect of quantity overriding affinity.

FGF-2 dissociation from both cell surface heparan sulfate proteoglycans and receptors similarly does not change with glucose. The FGF-2 off rate from heparan

sulfate proteoglycans is reported to be an order of magnitude higher than that for cell surface receptors (119). Especially for samples with glucose in the buffer, we show a higher off rate for heparan sulfate proteoglycans, indicating good agreement with the literature (FIGURE 5.20, FIGURE 5.22). These data show that FGF-2 is able to bind normally to endothelial cells independent of glucose, which points to intracellular signaling changes as the cause of altered endothelial cell response to FGF-2.

5.4.3 FGF-2 effects on proliferation and apoptosis

Individual analyses of endothelial cell proliferation and apoptosis are necessary to determine the cause of decreased cell number in 30 mM glucose after exposure to FGF-2. We show that endothelial cell entry into S-phase is highly dependent on FGF-2. The level peaks at 24 hours and remains elevated for at least 72 hours, indicating a prolonged proliferative effect without additional FGF-2 stimulation (FIGURE 5.23). The extended FGF-2 effect could be related to continued elevation of FGF-2 in culture media, FGF-2 binding and slow release from basement membrane, slow internalization of bound FGF-2, or lasting effects of internalized FGF-2.

Both cell cycle and DNA production show dose dependent proliferative responses to FGF-2. Both also show less proliferation for cells cultured in 30 mM glucose. However, when the percentage of S-phase cells with 1 ng/ml FGF-2 added is normalized to the percentage of S-phase cells with no FGF-2 added, the difference between high and low glucose cells becomes insignificant. Similarly, when the difference in ^{3}H-thymidine incorporation is normalized, the difference between high and low glucose becomes insignificant. Thus glucose clearly has an anti-proliferative effect on endothelial cells, but the effect does not seem to be mediated through FGF-2.

Apoptosis is an altogether different situation. Apoptosis increases with culture glucose, as seen in Chapter 4, and FGF-2 is released concomitant with apoptotic membrane damage. When endothelial cells are cultured with a high FGF-2 level, the apoptotic difference between low and high glucose cells becomes statistically insignificant (FIGURE 5.29). These data imply a protective survival role of FGF-2 in high glucose apoptosis, but question the role of cell-released FGF-2 with apoptotic cell membrane damage. The cell-released FGF-2 does have some protective effect,

however, as when an FGF-2 neutralizing antibody is used to block cell- released FGF-2, apoptosis does increase.

These data imply that FGF-2 release with apoptosis is protective, but constant high FGF-2 levels are more protective. Apoptotic FGF-2 release occurs slowly over time, so perhaps high FGF-2 levels are needed before or early in the process of environmental stress to be protective. In fact, when we examined short term intracellular signaling in response to FGF-2 in low and high glucose, survival signaling was diminished. A detailed analysis of FGF-2 intracellular survival signaling over time with glucose would clarify the difference in added versus apoptosis-released FGF-2.

5.4.4 Importance of secondary mediators

Glucose effects on FGF-2 survival signaling begin to elucidate the importance of secondary mediators. In particular, we show here that reactive oxygen species and TNFα have opposite effects on FGF-2 survival signaling. Similar to the case for glucose, reactive oxygen species cause increased apoptosis, which only increases when FGF-2 is removed with a neutralizing antibody (FIGURE 5.). Apoptosis due to TNFα, on the other hand, actually decreases when an FGF-2 neutralizing antibody is added (FIGURE 5.32, FIGURE 5.33). Removal of FGF-2 completely removes any significant increased in apoptosis with TNFα at 12 hours. At 24 hours, it is likely that high endothelial cell-released FGF-2 does not allow the antibody to fully neutralize FGF-2, so some effect is still seen.

The increase in TNFα-induced apoptosis with FGF-2 has been previously presented in the literature in glomerular endothelial cells, but the signaling remains unclear (182). It has been proposed that FGF-2 increases cytochrome C release from mitochondria. However, as both TNFα and FGF-2 signal through NFκB and JNK, dysregulation of these pathways may contribute to the pro-apoptotic FGF-2 effect with TNFα (183). The implications of this finding are far-reaching in terms of diabetic wound healing. If a wound becomes infected, and a high TNFα level is present, then increased FGF-2 released by basement membrane would only further inhibit healing.

5.4.5 A mechanistic model for the endothelial cell – basement membrane unit

We now propose a mechanistic model for interaction between endothelial cells and basement membrane in cooperative FGF-2 regulation (FIGURE 5.35). Glucose increases intracellular reactive oxygen species formation, which decreases signaling down the Akt pathway and leads to increased apoptosis and FGF-2 release. By an unknown mechanism, but possibly also reactive oxygen species-mediated, glucose increases endothelial cell permeability. High FGF-2 release and cell permeability lead to increased basement membrane FGF-2, which likely builds with time. Basement membrane FGF-2 then helps to modulate decreased cell proliferation due to glucose stress (FIGURE 5.36). However, FGF-2 cannot completely abrogate glucose-induced apoptosis due to loss of Akt activity.

5.4.6 Limitations

While these data point to a critical role for basement membrane FGF-2 in cell proliferation and survival, they are not without limitations. We demonstrated similar effects of soluble and basement membrane bound FGF-2 on viable endothelial cell number, but subsequent experiments were performed entirely with soluble FGF-2 due to ease of controlling FGF-2 levels. However, it is possible basement membrane bound FGF-2 differentially affects specific endothelial cell processes such as proliferation and apoptosis.

The endothelial cell FGF-2 binding kinetics data is of lower quality than basement membrane FGF-2 binding kinetics data. This makes it challenging to determine if alterations in FGF-2 binding could lead to some functional cell changes because in some cases it appears as though slightly more FGF-2 binds endothelial cells in high glucose, and in some cases it appears as though less binds. Even more important is internalization, since FGF-2 must not only bind to cells but be brought inside the cell to begin signaling cascades. Our preliminary data show no significant change in FGF-2 internalization with glucose, but the issue was not fully explored. Similar to limitations of basement membrane binding kinetics experiments, each cell binding kinetics experiments was performed on a macro time and quantity scale. Smaller transient

changes at the cell surface level could cause larger cell signaling alterations, so more detailed measurements of micro- and nano-binding kinetics would be needed.

Limitations of endothelial cell apoptosis measurements have been described in Chapter 4. Endothelial cell proliferation measurements have their own limitations. ^{3}H thymidine has been shown to affect cell function, therefore the assay cannot independently measure cell proliferation. Determination of cell entry into S-phase can be confounded by low data quality, but our data was of a relatively high quality and showed clear cell differentiation between cells with normal, intermediate, and twice normal levels of DNA.

FGF-2 effect on endothelial cell survival with different secondary mediators is complicated and likely dependent on FGF-2 signal timing. Our experiments only investigated FGF-2 addition or neutralization at one time point. In addition, the ability of the FGF-2 antibody to completely remove any FGF-2 signaling over time is limited, and it is likely that some FGF-2 could still bind to cells. Since FGF-2 is potent at extremely low levels, any neutralizing antibody failure could have profound effects.

Finally, we used Western blot to detect intracellular signaling pathways in response to FGF-2. The sample amount added to each lane was normalized to total sample protein to reduce any inconsistencies, however total Erk and Akt varied slightly among samples. Our data also could be confounded by protein and signaling molecule glycation, or reduced ability of antibodies to bind to altered Akt and Erk forms. The phospho-Akt signal was difficulty to detect, and it is therefore possible that the decreased level in high glucose cells is an artifact.

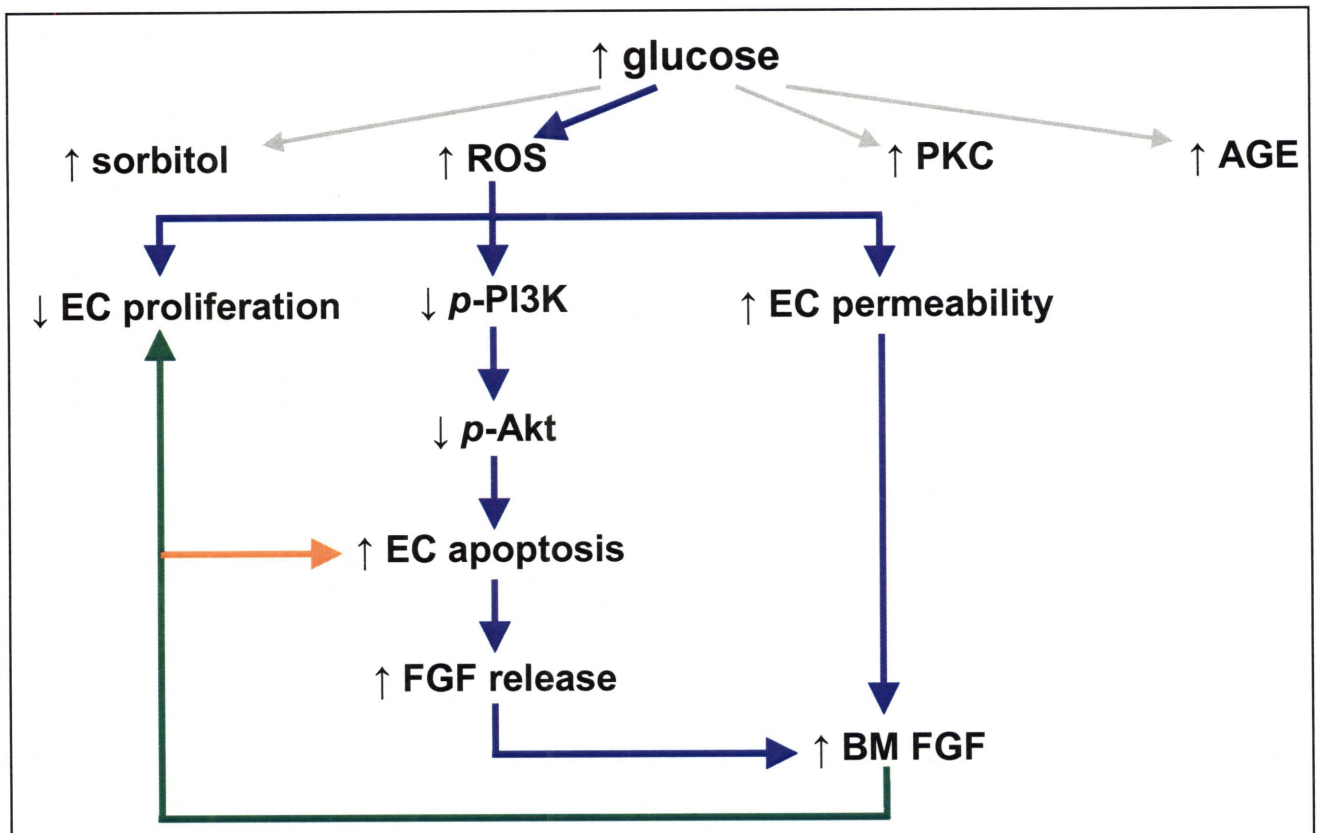


FIGURE 5.35: A MECHANISTIC MODEL FOR FGF-2 IN THE ENDOTHELIAL CELL – BASEMENT MEMBRANE CO-REGULATORY SYSTEM UNDER HIGH GLUCOSE STRESS CONDITIONS. Glucose increases reactive oxygen species, which decreases signaling down the Akt pathway, leading to increased apoptosis and FGF-2 release. By an unknown mechanism, glucose and reactive oxygen species also increase endothelial cell permeability. High levels of FGF-2 release and cell permeability lead to increased basement membrane FGF-2. Basement membrane FGF-2 then increases cell proliferation, which is decreased in glucose stress. However, FGF-2 cannot abrogate glucose-induced apoptosis due to loss of Akt activity.

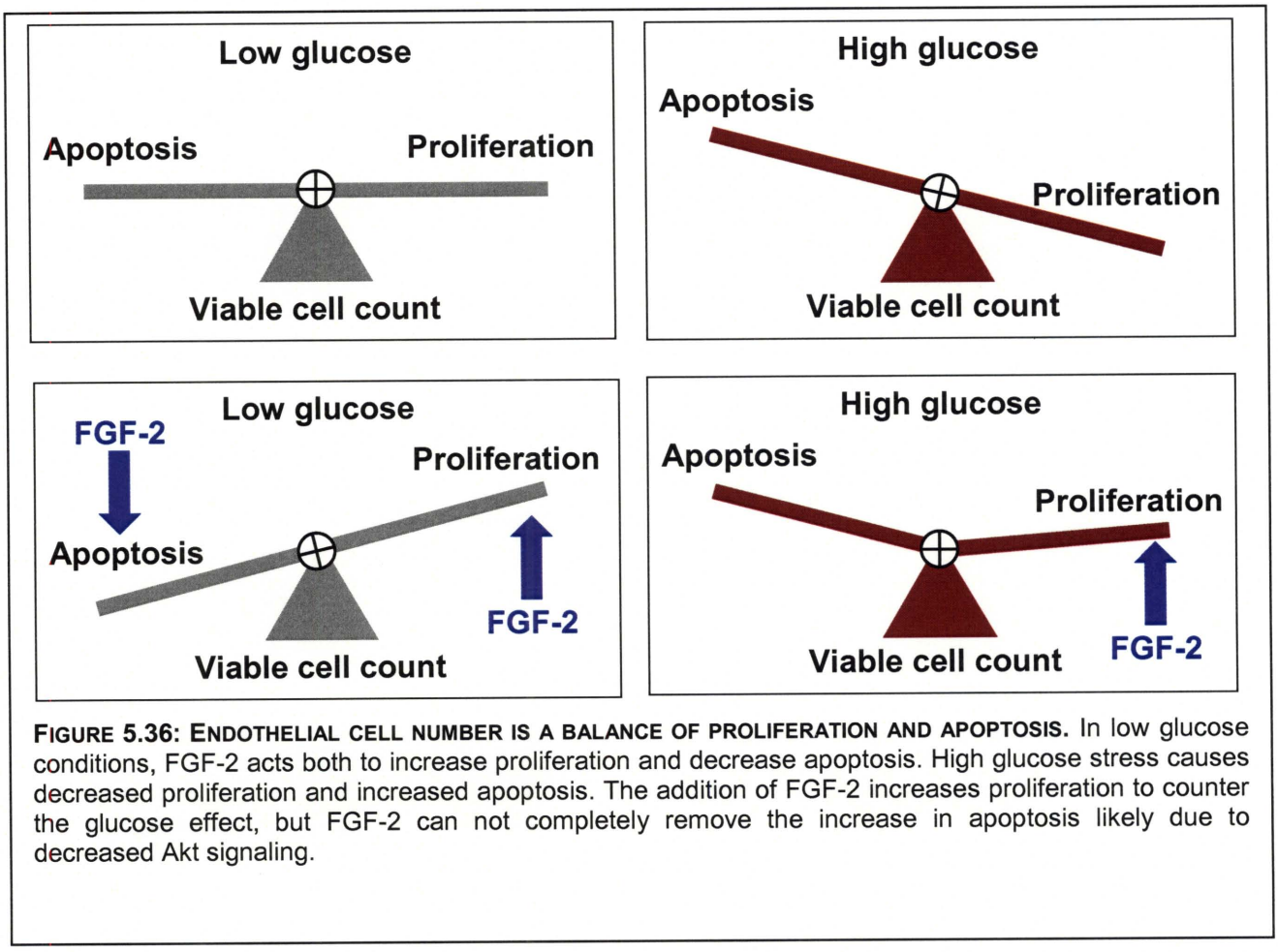


FIGURE 5.36: ENDOTHELIAL CELL NUMBER IS A BALANCE OF PROLIFERATION AND APOPTOSIS. In low glucose conditions, FGF-2 acts both to increase proliferation and decrease apoptosis. High glucose stress causes decreased proliferation and increased apoptosis. The addition of FGF-2 increases proliferation to counter the glucose effect, but FGF-2 can not completely remove the increase in apoptosis likely due to decreased Akt signaling.

5.5 Conclusions

Viable endothelial cell number is decreased in high glucose, in particular because proliferation decreases and apoptosis increases. Increased basement membrane FGF-2 in high glucose helps mitigate these effects by increasing proliferation. However, the anti-apoptotic FGF-2 effect is lost in high glucose likely due to decreased signaling down the intracellular Akt pathway. These data illustrate the complexities of a new mechanistic model for endothelial cell – basement membrane FGF-2 co-regulation. Furthermore, FGF-2 has the opposite effect on apoptosis in the presence of TNFα, which demonstrates that glucose is not the only factor to modulate growth factor effects on endothelial cells.

5.6 Chapter References

1. Cross, M.J., and Claesson-Welsh, L. 2001. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends In Pharmacological Sciences* 22:201-207.
2. Terranova, V.P., Diflorio, R., Lyall, R.M., Hic, S., Friesel, R., and Maciag, T. 1985. Human-Endothelial Cells Are Chemotactic To Endothelial-Cell Growth-Factor And Heparin. *Journal Of Cell Biology* 101:2330-2334.
3. Klein, S., Giancotti, F.G., Presta, M., Albelda, S.M., Buck, C.A., and Rifkin, D.B. 1993. Basic Fibroblast Growth-Factor Modulates Integrin Expression In Microvascular Endothelial-Cells. *Molecular Biology Of The Cell* 4:973-982.
4. Nugent, M.A., and Edelman, E.R. 1992. Kinetics Of Basic Fibroblast Growth-Factor Binding To Its Receptor And Heparan-Sulfate Proteoglycan - A Mechanism For Cooperativity. *Biochemistry* 31:8876-8883.
5. Kouhara, H., Hadari, Y.R., SpivakKroizman, T., Schilling, J., BarSagi, D., Lax, I., and Schlessinger, J. 1997. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell* 89:693-702.
6. Johnson, G. 2002. Signal transduction - Scaffolding proteins - More than meets the eye. *Science* 295:1249-1250.
7. Schlessinger, J. 2000. Cell signaling by receptor tyrosine kinases. *Cell* 103:211-225.
8. Boilly, B., Vercoutter-Edouart, A.S., Hondermarck, H., Nurcombe, V., and Le Bourhis, X. 2000. FGF signals for cell proliferation and migration through different pathways. *Cytokine & Growth Factor Reviews* 11:295-302.
9. Zimering, M.B., and Eng, J. 1996. Increased basic fibroblast growth factor-like substance in plasma from a subset of middle-aged or elderly male diabetic patients with microalbuminuria or proteinuria. *Journal Of Clinical Endocrinology And Metabolism* 81:4446-4452.
10. Schultz, G.S., and Grant, M.B. 1991. Neovascular growth factors. *Eye* 5 (Pt 2):170-180.
11. Takeuchi, K., Takehara, K., Tajima, K., Kato, S., and Hirata, T. 1997. Impaired healing of gastric lesions in streptozotocin-induced diabetic rats: Effect of basic fibroblast growth factor. *Journal Of Pharmacology And Experimental Therapeutics* 281:200-207.
12. Giardino, I., Edelstein, D., and Brownlee, M. 1994. Nonenzymatic Glycosylation In-Vitro And In Bovine Endothelial-Cells Alters Basic Fibroblast Growth-Factor Activity - A Model For Intracellular Glycosylation In Diabetes. *Journal Of Clinical Investigation* 94:110-117.
13. Nissen, N.N., Shankar, R., Gamelli, R.L., Singh, A., and DiPietro, L.A. 1999. Heparin and heparan sulphate protect basic fibroblast growth factor from non-enzymic glycosylation. *Biochemical Journal* 338:637-642.
14. Krishan, A. 1975. Rapid Flow Cytofluorometric Analysis Of Mammalian-Cell Cycle By Propidium Iodide Staining. *Journal Of Cell Biology* 66:188-193.
15. Moscatelli, D. 1987. High And Low Affinity Binding-Sites For Basic Fibroblast Growth-Factor On Cultured-Cells - Absence Of A Role For Low Affinity Binding In The

Stimulation Of Plasminogen-Activator Production By Bovine Capillary Endothelial-Cells. *Journal Of Cellular Physiology* 131:123-130.

16. Messmer, U.K., Briner, V.A., and Pfeilschifter, J. 2000. Basic fibroblast growth factor selectively enhances TNF-alpha-induced apoptotic cell death in glomerular endothelial cells: Effects on apoptotic signaling pathways. *Journal Of The American Society Of Nephrology* 11:2199-2211.

17. Varfolomeev, E.E., and Ashkenazi, A. 2004. Tumor necrosis factor: An apoptosis JuNKie? *Cell* 116:491-497.

CHAPTER 6

CONCLUSIONS

Thesis Summary

With the rising worldwide diabetes epidemic and its associated vascular morbidity and mortality comes the need to understand diabetic vascular dysfunction. Diabetes is a complex disease that causes a wide array of molecular, cellular, and tissue effects over a range of time. We investigated one diabetic change (hyperglycemia) in one system (FGF-2 regulation in the endothelial cell – basement membrane unit) over one time range (hours to days).

We have shown that over the course of days, basement membrane FGF-2 storage increases linearly with glucose. This change is not caused by alterations in basement membrane protein composition and structure, which seem to occur on a longer time scale. Rather, endothelial cells actively control FGF-2 basement membrane storage through FGF-2 release and cellular permeability. These endothelial cell dysfunctions with glucose are perhaps mediated through early membrane changes in apoptosis and intracellular reactive oxygen species. Glucose in turn mediates basement membrane FGF-2 effect on endothelial cells by inhibiting signaling down the anti-apoptotic Akt pathway without altering the proliferative Erk pathway. Thus glucose disturbs FGF-2 storage, release, and signaling within the endothelial cell – basement membrane unit.

This thesis helps elucidate the complex interaction between endothelial cells and basement membrane, as well as the role of FGF-2 in diabetes. Perhaps most important clinically, we have shown that physiologic glucose fluctuations on the order of hours to days can have long term detrimental effects in the vasculature. This may help explain why tight glucose control does not correlate with decreased vascular morbidity and mortality in diabetes.

6.1 Specific Findings

This thesis investigated the interaction between endothelial cells and basement membrane as a co-regulatory unit, focusing in particular on biochemical cooperation. We hypothesized that glucose could perturb this system, altering interaction at a level with biochemical impact that then translates to functional impact. Our specific model tested how a physiologic glucose range mediated altered handling of vasoactive compounds such as FGF-2 within the endothelial cell-basement membrane unit. This dysregulation between cells above and basement membrane below could contribute to disease initiation and progression.

In support of these concepts, this thesis consisted of a series of studies designed to elucidate the role of FGF-2 in glucose-induced vascular dysfunction, in particular as FGF-2 is cooperatively stored, released, and metabolized by endothelial cells and basement membrane.

In Chapter 2, we developed novel methods to examine FGF-2 binding kinetics with isolated basement membrane *in vitro*. In addition to demonstrating techniques to measure capacity, association, and dissociation of FGF-2 with isolated basement membrane, we showed that FGF-2 binds to tissue culture polystyrene and developed a correction scheme to account for this binding in the basement membrane model.

In Chapter 3, we used an *in vitro* hyperglycemic cell culture model to show that basement membrane FGF-2 increases with culture glucose. Basement membrane binding kinetics methods clearly demonstrated that this change was not related to alterations in basement membrane FGF-2 binding kinetics.

In Chapter 4, we redefined endothelial cell control of basement membrane FGF-2. We discovered that endothelial cells released more FGF-2 in high glucose and were more permeable to FGF-2, which combined to allow more FGF-2 to bind into basement membrane. Our data further suggest that FGF-2 release is associated with reactive oxygen species mediated apoptosis, in particular with early apoptotic cell membrane changes.

In Chapter 5, we examined how FGF-2 released from basement membrane affected endothelial cell proliferation and survival. After confirmation of consistent

endothelial cell FGF-2 binding kinetics with physiologic glucose concentrations, we demonstrated similar proliferation but increased apoptosis in response to FGF-2 in high glucose. This change appeared to be mediated through decreased signaling down the Akt pathway with glucose.

This thesis has made significant progress towards understanding the cooperative activity of endothelial cells and basement membrane. We have shown that endothelial cells actively control basement membrane content, which is particularly important in terms of vasoactive factors which can in turn affect cells. By elucidating interaction between cells and natural materials, we can move closer to smart biomimetic material design and therapies that target natural basement membrane as a therapeutic device.

6.1.1 FGF-2 release and storage as protection

The endothelium functions as an endocrine organ composed of a single cell monolayer along all blood contacting surfaces in the body. It is logical that injury to or death of one cell induces protective mechanisms to rescue not only itself but cells around it. Thus when an endothelial cell is injured by environmental stress, it releases FGF-2 in an attempt to save its own life and protect its neighbors' lives. In addition to the protective effect, the FGF-2 signal induces neighboring cells to migrate and proliferate in case the injured cell does die.

The system seems more ingenious when basement membrane is factored in. Upon cell injury, FGF-2 is released not only into the temporary soluble environment but into the lasting bound basement membrane storage reservoir. Thus a previous injury can actually help protect cells against a future injury. In the case of repeated glucose loads, this system may become dysfunctional as FGF-2 builds up in basement membrane and contributes to diabetic vascular disease. As with many physiologic adaptations, what starts out protective turns destructive.

6.1.2 Time scale of glucose effects

While hyperglycemia has been linked to diabetic vascular dysfunction, tight blood glucose control does not correlate with decreased morbidity and mortality. We therefore

asked the fundamental question: do short term glucose fluctuations within the range defined as normal lead to vascular dysfunction?

Certainly short term deleterious effects of glucose extremes, whether osmotic or ketoacidotic, are well recognized clinically. Long term protein-based advanced glycation end products have been extensively studied in their role in diabetic complications. We now show through this thesis that physiologic fluctuations in glucose on the order of hours and days have important effects on vascular function.

Clinical definitions of dangerous hyperglycemia may need to be reconsidered. Recent studies have shown that our strict cut-offs for normal blood lipoproteins or blood pressure oversimplify biological processes. In fact, clinical evidence has shown that a reduction in blood lipoproteins or blood pressure—even from high normal range to low normal range —is beneficial. We may need to consider glycemia in the same manner. Patients with glucose in the high normal range, in particular those with other risk factors leading to endothelial cell dysfunction, should possibly be treated to reduce their vascular disease risk.

6.1.3 Hyperglycemic memory

The vascular effects of hyperglycemia do not disappear once the glucose load has been removed. Restoration of euglycemia in both animals and humans does not reverse or even prevent diabetic vascular disease progression (1-5). This suggests some means of hyperglycemic memory, in which glucose causes an effect that lasts long after the stimulus has been removed.

Others have suggested AGE, or even mitochondrial DNA damage, as potential mechanisms for hyperglycemic memory (6). However, this thesis shows that basement membrane serves as a memory device. FGF-2 is released from endothelial cells and stored in basement membrane during hyperglycemic episodes, only to be released later. If FGF-2 is released in a euglycemic period, it may in fact have a completely different effect on endothelial cells given the alteration in intracellular signaling pathways with glucose.

For example, streptozotocin-induced diabetic dogs showed no proliferative diabetic retinopathy until after they were returned to euglycemia (2). It is possible that

the prolonged hyperglycemic period led to FGF-2 buildup in retinal capillary basement membranes, but FGF-2 did not have a net proliferative effect on endothelial cells because of the high glucose-induced apoptosis rate. However, once the glucose load was removed, cells were stimulated both to proliferate and survive by stored FGF-2 release. Also, in diabetic wounds, the combination of high stored FGF-2 in concert with inflammatory TNFα could block the normal angiogenic process by inducing endothelial cell apoptosis.

The basement membrane provides a means for vascular memory. In understanding endothelial cell – basement membrane co-regulation, we need to consider long term effects of short term hyperglycemia even when glucose is not beyond what we define as normal. And when we target growth factors for a drug therapy, we need to consider both soluble and bound growth factors.

6.2 Next Steps

6.2.1 A quantitative basement membrane model

This thesis examined how the endothelial cell – basement membrane unit regulates one particular growth factor, with a specific release mechanism, binding site, and cellular signaling scheme. To truly understand interaction between endothelial cells and basement membrane, we must examine other growth factors and cytokines with different release, binding, and signaling properties to determine their binding and release kinetics. In this way, we can develop a quantitative model of the endothelial cell – basement membrane co-regulatory unit which will be useful for biomaterials design as well as predicting functional changes in disease.

Vascular endothelial growth factor (VEGF) binds and releases basement membrane in a similar way to FGF-2, yet it has an alternative release mechanism and cellular signaling (7). Cytokines, such as interferon-γ and interleukins, similarly bind basement membrane heparan sulfate proteoglycans, but again have different release and signaling (8). Using molecules with varied release, transport, binding, and signaling mechanisms, we can probe kinetics of the endothelial cell – basement membrane system and thereby develop a quantitative understanding of these critical parameters.

Growth factor and cytokine basement membrane binding kinetics form an important basis for mechanistic insight but have limited applicability to real systems. We must move forward to study basement membrane binding kinetics in the presence of cells. We could begin with cell-produced matrix metalloproteinases (MMPs), and their antagonists, tissue inhibitors of matrix metalloproteinases (TIMPs). These are perhaps the soluble cellular factors that most influence growth factor and cytokine binding and release from basement membrane. Cells can then be added into the system in co-culture, and finally in direct contact with basement membrane to slowly elucidate how the endothelial cell – basement membrane unit functions as an ensemble.

The experiments in this thesis examine endothelial cells and basement membrane in bulk rather than on the micro- or nanoscale. While we have assumed that basement membrane acts as a reservoir, we do not fully understand growth factor release from cell, storage in basement membrane, release from basement membrane, and growth factor binding to cell at the protein level. Detailed study of the microscale endothelial cell – basement membrane unit will require novel application of emerging techniques, including micropatterning, atomic force microscopy, and fluorescence resonance energy transfer (FRET). While these techniques present intrinsic challenges, they are critical to developing a complete quantitative model.

6.2.2 Endothelial cell – basement membrane mechanics

The endothelial cell – basement membrane unit is a structural and biochemical system. It is naïve to consider either in isolation. For this thesis, experiments were performed in static cell culture for the sake of isolating and clearly studying biochemical relationships. Now these relationships must be tested in the face of concomitant mechanical influences.

The cell response to a soluble stimulus may be regulated by the local mechanical environment (9). Basement membrane connects to the endothelial cell cytoskeleton through integrins, which are critical cell signaling features. In particular for endothelial cells, which are in direct contact with shear and normal stresses from blood flow, mechanical interaction between cell and basement membrane influences cell signaling. In fact, mechanical forces alone may alter basement membrane binding kinetics.

With new techniques, we can not only strain endothelial cells and basement membrane on a large scale, but we can probe mechanical interactions protein by protein. We can learn if alterations in basement membrane proteins, through quantity or glycation or crosslinking, alter cell focal adhesion complex strength and thus alter cellular response to a growth factor stimulus. We can learn if stretching basement membrane, or the endothelial cell – basement membrane unit, increases FGF-2 binding by opening up new binding sites, or alternatively decreases binding by altering heparan sulfate proteoglycan conformation. And how does strain affect endothelial cell FGF-2 release and permeability? Endothelial cells at altered flow sites are known to be more permeable. Are glucose and flow effects additive?

6.2.3 Toward biomimetic materials and basement membrane therapies

Biomaterials investigation is confounded by the sheer quantity of literature in the field. Yet while it is relatively simple to find an investigation of a particular cell type on a particular material, it is nearly impossible to find a discussion of governing properties of cell-material interactions. A new approach is clearly needed. If we seek instead to understand how natural materials interact with cells, perhaps we can design materials that meet these specifications rather than continue the trial and error process.

The first step is developing a quantitative model of the basement membrane – endothelial cell unit, including both biochemical and mechanical interactions. We can then build these features into a material and test the novel system. Current technology already allows us to incorporate basement membrane topography, protein components, and mechanical properties into polymers that support delivery of factors for drug therapy or cellular adhesion for tissue engineered structures (10).

However, even though we can carefully control design of these systems *in vitro*, a major challenge remains designing materials that can sense and react to biochemical signals in the body (11). For example, FGF-2 enhances endothelial cell survival in the presence of glucose but would be more effective if glucose were not present. FGF-2 actually decreases endothelial cell survival if TNFα is present. Could we create an FGF-2 delivery system that would absorb or neutralize glucose and TNFα so FGF-2 could fully stimulate endothelial cell survival and proliferation? Since nearly all therapies are

most effective in the presence or absence of specific secondary signals, polymeric systems should be designed in which release of a given therapeutic substance only occurs in the optimal biochemical environment. These advanced materials would deliver therapy when it would provide benefit rather than harm.

Many current biomaterials must stand up to harsh mechanical conditions, which are often an impediment to biomaterial function. However, mechanical forces could be used to deliver a therapy when it is most needed, whether that therapy is drug release or simply an alteration in mechanical material properties. Can we design materials that respond and reorganize in response to mechanical loads, so that cells can also respond and reorganize?

Alternatively, instead of trying to create materials that mimic basement membrane, we can create drugs that target basement membrane. If we want to neutralize growth factors in the retina or kidney, we need to consider not just soluble growth factors but those bound to basement membrane as well. Perhaps a drug therapy could decrease the FGF-2 dissociation rate from basement membrane, thereby decreasing the FGF-2 stimulus for cells. Or perhaps we could use increased endothelial cell permeability in high glucose or abnormal blood flow to target a therapy to basement membrane? It is possible that something as simple as giving a drug during a hyperglycemic episode could increase basement membrane deposition and therefore drug half life in the body. This would allow basement membrane to serve as a local drug delivery device, creating slow therapeutic release with time.

We can better design smart materials and drug delivery only when we understand how nature has ordered the system in which we work. An understanding of basic biological mechanisms should be used to develop materials that will bind and release vasoactive factors in a manner similar to natural basement membrane, as well as target drugs and treatments to basement membrane as a natural drug delivery device.

Far too often, materials used in medicine are selected because they are inert or minimally damaging in the body. In fact, most biomaterials used today were not designed for their specific purpose—rather, they were tested *in vivo*, determined to be safe, and then used repeatedly. With our greater understanding of biology, our

improved experimental methods in both biology and materials science, and our increased ability to create new materials, the next generation of biomaterials will be designed for specific tasks. This thesis demonstrates how engineering techniques can be used to study natural basement membrane properties. As this work continues, I hope these discoveries will be translated into medical therapies to ameliorate morbidity and mortality in cardiovascular disease.

6.3 Chapter References

1. Kowluru, R.A. 2003. Effect of reinstitution of good glycemic control on retinal oxidative stress and nitrative stress in diabetic rats. *Diabetes* 52:818-823.
2. Engerman, R.L., and Kern, T.S. 1987. Progression Of Incipient Diabetic-Retinopathy During Good Glycemic Control. *Diabetes* 36:808-812.
3. Kador, P.F., Takahashi, Y., Akagi, Y., Neuenschwander, H., Greentree, W., Lackner, P., Blessing, K., and Wyman, M. 2002. Effect of galactose diet removal on the progression of retinal vessel changes in galactose-fed dogs. *Investigative Ophthalmology & Visual Science* 43:1916-1921.
4. Shannon, H., Duffy, H., Dahms, W., Mayer, L., Brillion, D., Lackaye, M., Whitehouse, F., Kruger, D., Bergenstal, R., Johnson, M., et al. 2000. Retinopathy and nephropathy in patients with type 1 diabetes four years after a trial of intensive therapy. *New England Journal Of Medicine* 342:381-389.
5. Steffes, M.W., Chavers, B.M., Molitch, M.E., Cleary, P.A., Lachin, J.M., Genuth, S., Nathan, D.M., Genuth, S., Nathan, D., Engel, S., et al. 2003. Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy - The Epidemiology of Diabetes Interventions and Complications (EDIC) study. *Jama-Journal Of The American Medical Association* 290:2159-2167.
6. Brownlee, M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820.
7. Ferrara, N. 2004. Vascular endothelial growth factor: Basic science and clinical progress. *Endocrine Reviews* 25:581-611.
8. Gibbs, R.V. 2003. Cytokines and glycosaminoglycans (GAGs). In *Glycobiology And Medicine*. 125-143.
9. Ingber, D.E. 2002. Mechanical signalling and the cellular response to extracellular matrix in angiogenesis and cardiovascular physiology. *Circulation Research* 91:877-887.
10. Ito, Y. 1999. Surface micropatterning to regulate cell functions. *Biomaterials* 20:2333-2342.
11. Langer, R., and Tirrell, D.A. 2004. Designing materials for biology and medicine. *Nature* 428:487-492.