LEPTIN:

EFFECT ON TRANSFORMING GROWTH FACTOR-β1 AND VASCULAR ENDOTHELIAL GROWTH FACTOR IN GLOMERULAR ENDOTHELIAL CELLS IN AN IN VITRO MODEL OF DIABETES

By

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To the faculty of Washington State University:

The members of the Committee appointed to examine the thesis of Matthew Halloran find it satisfactory and recommend that it be accepted.

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Chair

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ABSTRACT

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Diabetes and obesity are powerful contributors to glomerulosclerosis and end stage renal disease (ESRD). They are characterized by extracellular matrix (ECM) protein deposition (fibrosis), resulting in glomerulosclerosis, basement membrane thickening, mesangial expansion, glomerular and renal hypertrophy, progressive renal dysfunction and, eventually, ESRD. Recently, the adipokine leptin was described as a possible link between obesity and the increased risk for ESRD. Vascular endothelial growth factor (VEGF) is also important in progression of glomerulosclerosis. Leptin, high glucose, and VEGF can each increase markers of fibrosis, including transforming growth factor-beta (TGF-β), a known stimulator of fibrosis. Little is known about the effect of leptin on TGF-β1 or VEGF in glomerular endothelial cells.

HYPOTHESIS: Leptin will increase TGF-β1 and VEGF concentration in an in vitro model of diabetes using rat glomerular endothelial cells (RGEC).

METHODS: RGEC were exposed to increasing doses of leptin (0, 15, 25, 50,100 ng/mL) for 48h in either normal (5.55mM) or high (25mM) glucose. Media was sampled at 12, 24 and 48 hr and assayed for TGF-β1 and VEGF by sandwich ELISA. Data were analyzed by univariate ANOVA and t-tests.

RESULTS: Leptin, in combination with high glucose, caused a significant linear dose-related increase in TGF-β1 supernatant concentration at 12 hr and 48 hr ($p<0.05$), but significantly decreased TGF-β1 in supernatant from cells grown in normal glucose ($p<0.05$). High glucose increased VEGF protein concentration in supernatant at 48 hr ($p<0.05$). Leptin had no effect on VEGF in either normal or high glucose ($p>0.05$).

CONCLUSIONS: Leptin may contribute to the excessive glomerular ECM deposition seen in diabetes by increasing TGF-β1 production in the presence of a high glucose concentration. Glucose-induced increases in VEGF could enhance glomerulosclerosis due to the ability of VEGF to promote fibrosis

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Dedication

I would like to dedicate this thesis to my parents for their unwavering support and love throughout my life. Without you this would not have been possible. And to Anna, for her patience in allowing me to keep the light on a little longer while she tried to sleep.

Chapter I: Introduction

Obesity has become a first world epidemic over the last half century and the list of disease states to which it contributes, and in some cases causes, is growing.[1] Endothelial dysfunction, cardiovascular disease, insulin resistance, diabetes, hypertension, hypercholestemia, and hyperglucosemia, all components of the metabolic syndrome, are just a few of the comorbidities associated with excess adipose tissue, particularly when the adipose deposits are visceral.[1] Obesity also contributes to the progression of kidney dysfunction, as obesity in the presence of endothelial dysfunction is a major risk factor for chronic kidney disease (CKD) and congestive heart failure.[2, 3] Thus, it is thought that many of the same mechanisms that underlie coronary heart disease and atherosclerosis act on the kidney to induce glomerulosclerosis which can progress to CKD. The last ten years have produced a multitude of studies that directly connect vascular function, or dysfunction, with the endothelium.[1] This literature review will address the effects of obesity and diabetes in relation to renal glomerular endothelial cells with a special focus on the adipokine leptin.

In 2006, diabetes was the largest primary diagnosis contributor to end stage renal disease (ESRD) and CKD, contributing to more than half of new diagnoses. Of the total ESRD incidence, 30.6% are diagnosed with type 2 diabetes as the primary cause.[4] Obesity is a main driving force for the increase in incidence and prevalence of non-insulin dependent diabetes.[5] Currently the focus of much research is to understand the mechanisms and interactions that underlie these trends in obesity, diabetes, and ESRD.

One avenue of research focuses on the contribution of adipocyte-derived signaling molecules termed adipokines. Leptin is an adipokine that has received a good deal of attention since its discovery in 1994[6] and it may be associated with renal failure.[7] The interaction of leptin

with endothelial cells of the renal glomerulus is a likely way for leptin to contribute to renal disease.[8] In response to the milieu of signals present, endothelial cells in renal glomeruli significantly contribute to the progression of renal disease by altering the expression of pro-fibrotic proteins including transforming growth factor-β1 (TGF-β1), a known inducer of fibrosis and contributor to ESRD.[9, 10] Imbalance of cellular signals, including high glucose and leptin, can lead to thickening of the basement membrane via deposition of extracellular matrix proteins.[11, 12] The process of uncontrolled extracellular matrix deposition is known as fibrosis. Increased extracellular matrix deposition, in combination with mesangial proliferation and expansion, reduces glomerular filtration rate (GFR) and finally leads to CKD and ESRD.[13] Transforming growth factor-β1 is considered to be the final common mediator of fibrosis in diabetic nephropathy[14, 15] and will therefore be the main outcome measure as a pro-fibrogenic cytokine in this study.

Among the milieu of signals present, this research will focus on three important proteins known to interact with the renal endothelium: leptin, vascular endothelial growth factor (VEGF), and TGF-β1. There is strong support for the involvement of TGF-β1 in renal fibrosis, while the roles of leptin and VEGF are incompletely understood.[9] Both leptin and VEGF have been shown to induce increases in TGF-β1, a pro-fibrogenic cytokine and marker of fibrosis,[16, 17] and tissue inhibitor of metalloproteinase-1[18] production by glomerular endothelial cells. Leptin, in addition to increasing TGF-β1 protein expression, also induces an increase in VEGF protein expression in HECa10 endothelial cells of peripheral lymph node origin,[19] porcine retinal endothelial cells,[20] 4T1 mouse mammary epithelial cells,[21] and hepatic stellate cells.[22] These findings provide evidence that leptin may induce glomerular fibrosis through more than one pathway since leptin can upregulate both VEGF and TGF-β1.

Research Questions/Purpose

Determining whether leptin causes increases in VEGF and TGF-β1 in glomerular endothelial cells may provide insight into important interactions between leptin and two growth factors thought to stimulate fibrosis. We hypothesize that leptin will cause increased release of VEGF and TGF-β1 protein by RGEC in both normal and high glucose conditions in vitro. The effect of leptin on the concentration of VEGF protein and TGF-β1 protein is poorly defined in glomerular endothelial cells. Specifically, we sought to determine whether leptin plays a role in critical processes underlying the pathogenesis of glomerulosclerosis in a diabetic environment as it pertains to VEGF and the known inducer of fibrosis, TGF-β1.

The research questions we addressed in vitro were: 1) Does leptin increase VEGF protein production by rat glomerular endothelial cells (RGEC) in the presence and absence of high glucose? and 2) Does leptin increase TGF-β1 production by RGEC in the presence and absence of high glucose?

Chapter II: Literature Review

Methodology: This literature review was conducted using the online database PubMed. Additional supplemental information was gathered utilizing general internet searches. The following search terms were used alone or in combination and returned "hits" ranging in number from 0 to thousands: obesity; leptin; glomerulus; glomeruli; glomerular; extracellular matrix; ECM; vascular endothelial growth factor; VEGF; vascular endothelial growth factor receptor; VEGFR; endothelial cell; endothelial dysfunction; ED; high glucose; HG; nephropathy; diabetic; type 1; type 2; chronic kidney disease; CKD; kidney; renal; filtration rate; GFR; leptin receptor; obese receptor; Ob-R; Ob-Ra; Ob-Rb; fibronectin, collagen type IV; fibrosis and fibrosis markers. Articles were excluded based on: non-English language, non-peer reviewed journals/articles, reading of title and, for questionable titles, reading of abstract. Publication dates were not used as criteria for exclusion.

Obesity, Endothelial Dysfunction, and Chronic Kidney Disease

The mechanisms underlying obesity and associated diseases are being uncovered slowly. Adipose tissue is made up of adipocytes which are actually modified fibroblasts. These adipocytes are viable, functioning cells that store almost pure triglycerides in quantities that can reach 95% of the total cell volume. The discovery that adipocytes are not merely energy storage sites, but rather a collection of energy-storing cells with an endocrine-like function has greatly enhanced our understanding of obesity-related diseases. "Adipokine" and "adipocytokine" are blanket terms that refer to any bioactive molecule and/or protein that is secreted from adipocytes and adipose tissue. These adipokines can act either as autocrine, paracrine, or endocrine signals that affect different mechanisms according to their specific function.

A rapidly growing body of evidence supports the progression of chronic kidney disease (CKD) in the presence of obesity[2, 23, 24] where CKD is defined as a glomerular filtration rate (GFR) of < 60 mL/min/1.73m^2. Anthropomorphic measures such as a high waist circumference and body mass index (BMI) have been found to be predictive factors of CKD and both have also been shown to be inversely related to GFR.[25] Gelber et al.[26] showed that a higher baseline BMI increased an individual's risk for CKD. When compared to subjects with a BMI of < 22.7 kg/m^2, subjects with a BMI of > 26.2 kg/m^2 were 1.27 times more likely to develop CKD. While BMI and obesity are predictive of both endothelial dysfunction and CKD, there is no known mechanistic link between them. To help further our understanding it would be beneficial to know the mechanisms behind endothelial dysfunction and atherosclerosis as both have been associated with CKD.[25]

It is thought that many of the same mechanisms that underlie atherosclerosis act on the glomeruli as well. Recent research has provided a number of studies that directly connect vascular function, or dysfunction, with the endothelium.[27] The role of the endothelium is broad and multifaceted. This single layer of cells that lines the vascular system is responsible for interactions with smooth muscle cells of the vascular system, the transport and diffusion of metabolites to the surrounding parenchyma, response to hemodynamic mechanical stimuli, providing the first barrier between plasma and parenchyma, and mediating immune responses.[27] Additionally, endothelial cells express phenotypic heterogeneity depending upon which vascular bed they are contained in.[28, 29] So, for example, endothelial cells from an artery may not express all the same traits as endothelial cells from a capillary.[28]

The most common description of endothelial dysfunction (ED) is the inability to dilate a vessel via an endothelium-dependent mechanism, namely nitric oxide (NO), a critical

vasodilator. A common clinical test for this would be to measure the vasoactive response to occlusion (reactive hyperemia) or to a NO-dependent mediator such as acetylcholine. However, these measures have not been done in renal arteries. Evidence for NO as the main contributor to ED is supported by studies that show increased plasma concentrations of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO, to be highly correlated with ED.[30] Interestingly, ADMA has also been shown to be a determinant of CKD.[2]

Although a decrease in NO bioavailability is a main determinant of ED, a much more complete definition of ED is provided in a paper by Goligorsky[27(pF877)] who states:

> ED can be characterized by 1) decreased bioavailability and production of NO; 2) increased adhesiveness for monocytes and polymorphonuclear cells; 3) accumulation of cholesterol and oxidized LDL; 4) defective transmission of endothelium-derived hyperpolarizing factor; 5) enhanced expression of profibrotic genes; and 6) a tendency toward premature senescence and apoptosis.

All of the aforementioned are also prevalent risk factors in ESRD[27] and are common conditions associated with obesity. One condition that Goligarsky does not mention in his definition of endothelial dysfunction is an elevated production of reactive oxygen species (ROS) in combination with a decreased ability for removal. An increase in ROS causes severe ED and some consider it to be central to endothelial dysfunction-associated pathologies.[31] Leptin has also been shown to induce ROS within endothelial cells,[32-34] which may be another way that leptin could contribute to progressive renal dysfunction.

To complicate the matter further, metabolic syndrome (at least 3 of the following: hypertension, hypercholesterolemia, a skewed cholesterol profile favoring LDL, hyperinsulinemia and hyperglucosemia) has been shown to have negative effects on endothelial

function.[35] Metabolic syndrome can stimulate the immune system which has severe negative implications with regard to thrombosis, atherosclerosis, arteriosclerosis, and glomerulosclerosis.[35] Chronic activation of pro-inflammatory immune responses creates a state of inflammation within the endothelium and further enhances fibrosis, atherosclerosis, and glomerulosclerosis.

Finally, endothelial dysfunction leads to an increase in production and deposition of ECM proteins on the basement membrane that can lead diabetic nephropathy and ESRD.[12, 36] Basement membrane thickening is associated with many forms of diabetic vascular complications and has been studied extensively in diabetic retinopathy.[11, 37, 38] Thickening of the basement membrane in diabetic renal complications is observed with major contributions coming from collagen type IV and fibronectin.[39, 40] Mauer et al.[41] showed that the degree of basement membrane thickening is weakly associated with the amount of proteinuria and total mesangium is better correlated with the severity of proteinuria. The mesangium is the layer of cells and associated matrix that surrounds glomerular capillaries and therefore glomerular endothelial cells. Mesangial cell proliferation and expansion is central to glomerular hypertrophy and also increases in ECM protein.[42] Similar results were described by Fioretto et al.[43] with total mesangial area being a strong correlate to proteinuria.

Transforming Growth Factor-β and Fibrosis

Transforming growth factor-beta (TGF-β) is a cytokine that participates in a multitude of normal physiologic functions as well as several pathogenic processes including glomerular fibrosis. The TGF-β proteins belong to what is known as the TGF-β superfamily which contains over 100 proteins that are involved in a multitude of cellular functions.[9] Nearly every cell in the body expresses a receptor for a TGF-β family member including endothelial cells.[44] This brief

review will concentrate on three mammalian isoforms known as TGF-β1, β2 and β3. Under normal circumstances TGF-β participates in tissue repair or wound healing that results in a restored ECM and parenchymal architecture.[9] Adverse effects of TGF-β signaling begin when cells and tissue are chronically exposed to high concentrations of the activated protein. Briefly, when tissues or cells are injured or stretched, they release stimuli that activate latent TGF-β (LTGF-β). Activated TGF-β signaling then causes an increase in extracellular matrix protein production as well as a decrease in matrix protein degradation. This cycle, if unchecked, will eventually lead to excessive fibrosis.[9] It is therefore useful to understand the relationship between TGF-β1 and ECM deposition and degradation.

Transforming growth factor-β1 is generally considered the final common pathway for ECM deposition and mesangial expansion seen in diabetic nephropathy[14, 15] and glomerulosclerosis.[45] Type 2 diabetic patients express net renal production of TGF-β1 while their non-diabetic counterparts display net renal clearance of TGF-β1.[46] In cultured mesangial cells, inhibition of TGF-β1 by anti-TGF-β1 antibody can completely abolish the increase in soluble fibronectin seen in response to high glucose.[15] Additionally, Sharma et al.[47] showed that inhibition of TGF-β1 can significantly blunt (by 50%) the glomerular hypertrophy seen in streptozotocin-induced diabetic mice. Furthermore, transgenic mice overexpressing TGF-β1 develop progressive glomerulosclerosis at 3 weeks of age and by 15 weeks of age more than 25% die of uremic disease.[48, 49]

Transforming growth factor-β significantly increases a multitude of ECM proteins, including collagen types I, II, IV and V;[50-52] fibronectin,[50, 53] and thrombospondin,[54] in various cell types. In addition, TGF-β1 blunts ECM breakdown by inhibiting matrix-degrading enzymes. This is accomplished by increasing proteins such as plasminogen activator inhibitor-1 (PAI-1)[55]

and tissue inhibitor of metalloproteinase 1 (TIMP-1).[56] Increasing PAI-1 can have serious deleterious effects on renal function because plasminogen activator (PA) and matrix metalloproteinases (MMPs) are important enzymes in the continual breakdown of ECM and basement membranes.[57, 58] If MMPs and PA are chronically inhibited by TGF-β1signalling, build-up of ECM results and processes like glomerulosclerosis are exacerbated.

There are multiple isoforms of TGF-β but TGF-β1 is considered to be the predominant isoform involved in fibrosis[50] and is thus the one most commonly studied in regard to glomerulosclerosis. Isoforms TGF-β2 and TGF-β3 have less well established roles in fibrosis; however, it is thought that the fibrotic effects of TGF-β2 and 3 are at least partially mediated through increases in TGF-β1, although TGF-β2 and TGF-β3 have both been shown to be fibrotic independent of TGF-β1.[50] Transforming growth factor-β1 isoform was chosen for measure in this study due to its critical involvement in renal fibrosis, glomerulosclerosis and diabetic nephropathy.

TGF-β1 Secretion and Signaling

Transforming growth factor-β1 is secreted as an inactive form known as latent TGF-β1 (LTGF-β1). Latent TGF-β1 protein is unable to bind its receptor, contains a latency-associated peptide (LAP), and usually circulates in complex with latent TGF-β binding protein (LTBP).[59] This latent form of TGF-β1 is immature and requires activation which is an extracellular process resulting in proteolytic cleavage of the LTBP.[59] Latent TGF-β can be activated by a multitude of molecules including plasmin and thrombospondin-1 to result in the active 25kD protein.[60] A recent article by Kumpers et al.[61] demonstrates that leptin can act as a co-activator of LTGF-β1, suggesting that leptin, in addition to upregulating TGF-β1, can also increase its activity.

Transforming growth factor-β1 receptor-mediated signaling occurs through two transmembrane serine/threonine kinase receptors known as TGF-β type I (TβRI) and type II (TβRII) receptors.[59] The TβRI and TβRII act in concert in the TGF-β1 signaling pathway.[62] First, TGF-β1 binds TβRII which then recruits and phosphorylates TβRI in a glycine/serine rich residue. Phosphorylation of TβRI activates it and turns on its intracellular kinase activity. Intracellular signaling of TGF-β1 is thought to happen predominantly through activation of the Smad family of proteins by TβRI.[62] The Smad signaling cascade allows for nuclear transduction of TGF-β1 signaling to activate Smad-dependent genes. These genes code for almost all types of cellular matrix proteins, as well as inhibitors of matrix degradation such as PAI-1 and TIMP-1.[62]

Clearly, TGF-β1 plays a very important role in the glomerulosclerosis and renal hypertrophy associated with diabetes. A number of researchers have suggested that selective inhibition of all or part of the TGF-β1 signaling cascade could prove to be a valuable tool in the fight against renal disease.[14, 15, 45, 47]

Leptin

Leptin, Greek for "thin" and probably the most famous adipokine, is a protein almost exclusively released from adipocytes.[63] A 16-kDa protein, the first described function of leptin was to act on the hypothalamus to contribute to satiety.[64] One use for leptin is as a biomarker for adiposity because circulating leptin levels are proportional to adipose mass.[65] So, as adipose mass increases, so do leptin levels, a relationship that holds true for both humans and rodents.[65] Additionally leptin contributes to insulin sensitizing in skeletal muscle, liver, and the pancreas.[66] Leptin is involved in a wide variety of normal physiologic functions as well as pathogenic processes, including renal fibrosis,[8] and might be an independent risk factor for coronary heart

disease.[67] Additionally, leptin has been shown to promote angiogenesis both in vitro and in vivo[68-70] and is involved in immune cell maturation and responses to stimuli.[22, 71-74]

Research done in the field of leptin has primarily utilized two genetically altered species of mice. It is therefore important to briefly discuss the phenotypes of these mice. Two major classes of mice exist that have leptin abnormalities. They are: 1) leptin-deficient mice, also known as ob/ob mice; and 2) leptin receptor-mutated mice, also known as db/db or fa/fa mice. Leptin-deficient mice have an inability to produce a functioning leptin protein. Diabetic, or receptor-mutated (db/db) mice, lack the ability to phosphorylate the STAT3 residue at serine 1138 on the leptin receptor due to a truncated cytoplasmic domain.[75] The truncated receptor in db/db mice does not allow for normal leptin signaling, and thus their phenotype mimics a leptin-deficient ob/ob mouse, but there are differences. Some of the traits that ob/ob mice exhibit at adulthood are obesity, hyperglycemia, hyperinsulinemia, reduced skeletal muscle growth, insulin resistance,[76] infertility,[77] and blunted wound healing ability.[78] The traits that db/db express are similar[79] with hyperleptinemia being the main difference.[80]

To begin the discussion on leptin and renal fibrosis it will be helpful to look at in vivo models first and normal versus abnormal kidney function as it pertains to leptin in normal animals. Normally functioning kidneys are the site of clearance for leptin and contribute to almost 100% of circulating leptin clearance.[81, 82] In ESRD the filtration function of the kidney is no longer effective and thus serum leptin concentration is increased in patients with ESRD.[83]

Leptin and Fibrosis

In normal rats infused with leptin (at a rate of 250 ng/hr for 28 d), increased TGF-β1 was found specifically around the tubule cells.[84] Additionally, leptin-infused rats display increased diuresis[85] and naturesis along with an increase in proteinuria,[84] which are markers of decreased

renal function. Although not statistically significant, Gunduz et al.[84] also showed a trend for leptin infusion in rats to increase protein excretion in the urine. A study conducted by Wolf et al.[16] showed infusion of leptin (31.25 nmol/hr) in rats over a 3-week period to significantly increase both proteinuria and collagen IV. Additionally, Wolf et al. showed leptin to increase TGF-β1 mRNA by 50% and increase TGF-β1 protein in vitro and hypothesized the increases in TGF-β1 seen may be related to the degree of proteinurea seen in vivo.

As stated previously, endothelial dysfunction is predictive of both CKD and coronary heart disease,[25] so one could hypothesize that there is a causal relationship between endothelial dysfunction and vascular diseases including glomerulosclerosis. Several studies have shown increased leptin to alter the vasculature toward an endothelial dysfunction phenotype. Following wire-induced femoral arterial injury, leptin-deficient mice (ob/ob) showed significantly less neointimal formation than their wild-type controls.[86, 87] Additionally, db/db mice were protected from excessive neointimal formation following vascular injury[88] suggesting that intact leptin receptor signaling is required for leptin's endothelial specific proliferative and profibrotic effects.

Taken together, these studies demonstrate that leptin may have a significant role in renal fibrosis and ECM deposition in vivo. One final note is that the use of db/db mice in several studies has shown that these mice are not protected against the onset of diabetic nephropathy, but that does not exclude leptin from contributing to CKD. Because leptin-deficient mice are not protected against ESRD, the leptin and TGF-β1 relationship needs to be studied further. If leptin increases TGF-β1 in glomerular endothelial cells, there is a strong argument for leptin to contribute to glomerulosclerosis independent of diabetes.

Leptin Signaling

The leptin receptor belongs to the class I cytokine receptor superfamily, and is most closely related to the gp-130 signal transducing component of interleukin-6 (IL-6).[89] Currently, there are 5 known isoforms of the leptin receptor and they are designated Ob-R with isotype specificity portrayed via a subscript a-e (eg, Ob-Ra).[75] It is thought that most signaling happens via the Ob-Ra and Ob-Rb isoforms.[75] The kidneys express both Ob-Ra and Ob-Rb isoforms of the leptin receptor,[89, 90] with the highest concentration of receptors occurring in the renal medulla.[85] It is important to note that the kidney and adrenal glands both express the long form of the leptin receptor (Ob-Rb),[91] so designated because its intracellular domain is longer than all other isoforms. Glomerular endothelial cells also express the Ob-Rb isofom.[16] Expression of Ob-Rb within the multiple renal cell types suggests that, in addition to the hypothalamus, the kidney and adrenal glands are primary target organs for leptin.

A majority of leptin signaling involves coupling of the leptin receptor to the Janus kinase/signal transducer and activator of transcription pathway[64] which involves such mediators as insulin receptor substrate (IRS) and phosphatidylinositol 3 kinase (PI3-K). Leptin signaling has also been shown to activate the ras/raf - mitogen activated protein kinase (MAPK) pathway which can lead to a number of downstream effects.[64]

VEGF and Renal Function

A recent addition to pathogenic markers for renal fibrosis is vascular endothelial growth factor (VEGF).[92] Vascular endothelial growth factor is a strong mitogen for endothelial cells, increasing their proliferation and inducing migration.[93] This is of note because increased intimal-medial area of the vasculature via increased cell number and migration and increased ECM deposition is a marker for endothelial dysfunction which contributes to renal deficiency.

Classically, VEGF plays a major role in angio- and vasculogenesis. Developmentally, VEGF is extremely important as mice lacking a single VEGF allele undergo embryonic death between d 11 and 12.[94]

The role of VEGF and its receptors in diabetic nephropathy and renal fibrosis are slowly being defined. Diabetic nephropathy in humans is associated with an increased VEGF concentration in both the blood and urine.[95] For future research, it will be important to distinguish if increased VEGF is a cause or effect of decreased filtration in ESRD.

In support for the hypothesis that VEGF is a cause, Flyvbjerg et al.[96] showed that a VEGF neutralizing antibody given over 2 months was able to significantly attenuate the diabetes-associated increases in kidney weight, glomerular volume, and basement membrane thickness, suggesting that VEGF promotes renal fibrosis. Schrijvers et al.[97] also showed that inhibition of VEGF in Zucker diabetic fatty rats (fa/fa rats) significantly decreased glomerular hypertrophy associated with early diabetic nephropathy. Similarly, Vriese et al.[98] demonstrated that a neutralizing VEGF monoclonal antibody decreased hyperfiltration, albuminuria, and glomerular hypertrophy in diabetic rats. Taken together these studies provide strong evidence in favor of excessive VEGF playing a role in early stages of diabetic renal disease.

The contribution of VEGF to ECM protein production and fibrosis should also be considered. Li et al.[18] showed in vitro that mouse glomerular endothelial cells exposed to 10 ng/mL VEGF displayed a significant increase in TGF-β1 from hr 3 to 24. Additionally, the pro-fibrotic matrix metalloproteinase 2, 13 and tissue inhibitor of metalloproteinase (TIMP-1) showed similar increases over a 24-hr period.[18] An alternate study established exposure of human mesangial cells in vitro to VEGF (0-200 ng/mL) led to a dose-dependent increase in cell-

associated and secreted collagen.[99] In human smooth muscle cells in vitro, fibronectin is another ECM component that has been shown to be up-regulated in response to VEGF.[100]

When reviewing the many effects of VEGF, it is very possible that increased expression of VEGF during the early stages of renal disease could have deleterious effects on renal function. This is in part due to the ability of VEGF to increase TGF-β1, and thus increase deposition and accumulation of ECM components. As stated previously, there is no accepted effect of leptin and VEGF interactions as they pertain to glomerular endothelial cells. This is of importance because both act to increase proliferation of endothelial cells and contribute to ECM and pro-fibrotic signals.

VEGF Isoforms and Signaling

The VEGF protein has several identified isoforms named according to their amino acid length. In humans they are 206, 189, 165 and 121.[93] In rats the equivalent proteins all have one less amino acid per isoform and are so designated 205, 188, 164 and 120, respectively. Sometimes this family of VEGF is referred to as VEGF-A or simply VEGF with VEGF-B through E all expressed by different chromosomes.[101] Characteristics of the VEGF isoforms can be generalized by their amino acid length as well, with $VEGF_{206}$ and $VEGF_{189}$ having the highest affinity for heparin sulfate and thus being bound almost exclusively to cells and ECM. The $VEGF_{121}$ isoform is shorter, lacks the basic heparin sulfate binding region, is more acidic, and thus almost exclusively released into circulation as a free protein. Approximately 30-50% of intermediate $VEGF_{165}$ exists as free circulating protein with the rest remaining cell and ECM bound. Additionally, $VEGF_{165/164}$ is thought to be the most abundant and biologically active, although all isoforms have identified functions.[102] Several growth factors positively contribute to up-regulation of VEGF protein, including TGF-β.[103]

Receptors for VEGF belong to a family of receptor tyrosine kinases and include VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1) as the main mediators of VEGF signaling. Both receptors are required for fetal development, but it is thought that most angiogenic and mitogenic actions of VEGF in adulthood require VEGFR-2 signaling, while the vasculogenic effects are thought to require VEGFR-1.[103] Signaling through VEGFR-2 can activate mitogen activated protein kinase (MAP) and require phospholipase-C-γ (PLC- γ). After ligand binding of VEGF, it is thought that PLC- γ is tyrosine phoshorylated. Activated PLC- γ then stimulates hydrolysis of phosphatidyl inositol (PIP_2) which leads to protein kinase-C (PKC) activation and Ca^{2+} influx into the cytosol. Additionally, the Raf-MEK-MAP pathway is a key mediator of the proliferative effect of VEGF.[104]

Leptin and VEGF Interaction

In vitro studies have shown leptin to significantly increase VEGF protein expression in HECa10 endothelial cells or peripheral lymph node origin,[19] porcine retinal endothelial cells,[20] 4T1 mouse mammary cells,[21] and hepatic stellate cells.[22] From this, one could hypothesize that leptin signaling plays a role in increasing VEGF expression. Recently leptin has received attention as an angiogenic factor capable of inducing neovascularization in human endothelial cells to a similar extent as VEGF.[68] Proliferation of endothelial cells in response to leptin demonstrates that endothelial cells can readily respond to leptin signaling. Bouloumié et al.[69] demonstrated that leptin increases proliferation of human umbilical vein endothelial cells and increases the number of capillary-like structures in cultured endothelial cells at normal physiologic concentrations (10 ng/mL) of serum leptin. Interestingly the delineated signaling mechanism was different than “typical” leptin signaling which occurs through JAK/STAT.

Bouloumié et al.[69] found that the endothelial proliferative and angiogenic effects were a result of mitogen activated protein kinase (MAPK) and Erk1/2 activation.

Glucose Effects

As stated previously, diabetes is the number one primary diagnosis contributor to ESRD,[4] and obesity is the number one contributor to diabetes incidence.[5] Because plasma leptin concentrations are positively related to percent body fat, high leptin is often seen in combination with a high glucose concentration in diabetic patients as a result of poor metabolic control. This study will employ the use of an in vitro diabetic model to further analyze the interaction of leptin and glucose in glomerular endothelial cells. It is therefore useful to review the current knowledge of glucose effects on VEGF and TGF-β1.

Glucose effects on VEGF mRNA, VEGF protein, and VEGFR expression have been described in a multitude of cell types. Both low and high glucose concentrations in vitro can lead to up regulation of VEGF. Embryonic stem cells respond to low glucose by up-regulating VEGF mRNA.[105] This response was independent of hypoxia inducible factor-1α (HIF-1α), a powerful stimulus for VEGF production in response to hypoxia.[105] Similar results to glucose deprivation were also seen in various other cell types, including monocytes and hepatoblastoma cells.[106, 107]

High glucose will also induce VEGF production. Sone et al.[108] demonstrated that chronic exposure of retinal epithelial cells to high glucose (16.5 mM) over 10 d caused a significant increase in VEGF production. Also of interest in this study was the observation that reduction of the high glucose group to a normal glucose concentration of 5.5 mM after the 10-d trial period did not result in an increase in VEGF production, but rather a decrease. However, when glucose was again lowered from 5.5 mM to 2.75 mM, VEGF production increased. It appears that the interaction of VEGF and glucose is tightly regulated and that a euglycemic environment is

required for normal function. Cha et al.[95] also showed a high glucose concentration to stimulate an increase in VEGF mRNA. Rat mesangial cells were cultured with normal glucose (5.5 mM), medium glucose (15 mM), and high glucose (30 mM). It was found that VEGF mRNA was upregulated in a dose-dependent manner over a 24-hr period with the highest production occurring at hr 3. The observation that a high glucose concentration in cultured renal cells up-regulates VEGF with a peak at 2-3 hr is supported by others as well.[109, 110]

The effect of high glucose concentration on the expression of ECM proteins and pro-fibrotic markers has been studied extensively. Several in vitro studies utilizing endothelial cells showed that collagen IV production increased in response to high glucose.[111, 112] Glucose can also stimulate increases in TGF-β1, PAI-1, and collagen type I in mesangial cells in vitro.[113] The pro-fibrotic effect of high glucose has been shown to be dependent on TGF-β1 response to high glucose.[15, 113] Additionally, high glucose concentrations (25 mM or 30 mM) stimulate fibronectin production in endothelial cells.[11, 114, 115] In this study the effect of leptin will be measured in both a normal and high glucose in vitro model.

Until recently, high glucose was the only in vitro model of the diabetes effect on cells. In diabetes plasma amino acids are elevated similar to glucose, and thus are of concern for diabetics. The effects of high concentrations of amino acids in culture are less well defined than the effects of high glucose. However, in vivo studies have shown that high protein diets enhance the progression of chronic kidney disease, with a particular contribution in diabetes, as a result of the hypertensive effect amino acids have on the kidneys.[116] In vitro studies utilizing high amino acid concentrations are less prevalent than those utilizing high glucose concentrations, but show concentrations of high amino acids to increase markers of fibrosis, including collagen IV and fibronectin, independent of hypertension.[117] This increased ECM protein in response to high

amino acid concentrations is thought to be a result of increased availability of amino groups for glycation reactions which increases advanced glycation end products (AGE). These AGE then lead to reactive oxygen species formation and cellular damage.[118] It should be noted however that there is some debate as to the efficacy of low protein diets, and hence low plasma amino acid concentration, in the treatment and prevention of kidney disease.[119] This research will not elaborate on the effects of high amino acid concentrations, but it is important to note possible confounding variables as well as future areas of interest.

Summary

Combined, obesity and diabetes are powerful predictors of renal fibrosis and ESRD. Leptin, VEGF, and TGF-β1 could have additive or synergistic proliferative and fibrotic effects on endothelial cells and the associated ECM. Both leptin and high glucose concentrations increase pro-fibrotic markers and the ECM components collagen and fibronectin. To the best of the author's knowledge, the effect of leptin in high glucose versus the effect of leptin in normal glucose on TGF-β1 and VEGF has not been studied in an in vitro model using endothelial cells.

Chapter III: Methods and Materials

Culture of Rat Glomerular Endothelial Cells

Cells

RGEC have previously been isolated from glomeruli obtained by sieving the cortex of kidneys excised from 6-month-old Sprague-Dawley rats.[120] These cells had been previously characterized as endothelial cells using a panel of markers, including flk, flt and VE-cadherin. Cells between passages 17 and 25 were used for experiments.

Coating of Plates

Fibronectin (1 mg/mL, Roche Biochemicals, Indianapolis, IN) was diluted with phosphate buffered saline (PBS, KCl: 0.2 g/L; KH_2PO_4: 0.2 g/L; Na_2HPO_4: 2.16 g/L; NaCl: 8.0 g/L; deionized H_20) to a final concentration of 5 μg/μL and frozen at -20˚C in 1 mL aliquots. To coat culture plates (100 mm, Greiner, ISC Biologicals, Kaysville, UT), 1 mL of thawed fibronectin solution was added to a culture plate, evenly spread over the surface by repeated manual tapping and then placed on a rocker for > 30 min and evenly distributed over culture plate by repeated tapping throughout the incubation period. The solution that remained was aspirated prior to addition of cells.

Thawing, Plating and Culture

Frozen RGEC were removed from liquid nitrogen and thawed briefly (~20 sec) in a warm (37ºC) water bath. Contents of the Nunc® cryotube vial (Nunc, Rocksilde Denmark) were transferred into an empty 15 mL centrifuge tube and 14 mL of cold (5ºC) complete Dulbecco's Modified Eagle Medium (DMEM, Gibco Life Technologies, Rockford, MD) containing 10% FBS (Atlas Biologicals, Ft. Collins, CO), 2 mM sodium pyruvate, 90 μg/mL heparin, 100 IU/mL

penicillin, 100 μg/mL streptomycin, and a total concentration of 4 mM $MgSO_4$ (complete DMEM) was slowly added. Cells were centrifuged (4°C, 800 x g, 5 min) and the media aspirated. Warm complete DMEM was added to the cell pellet and the solution aspirated gently and repeatedly to break up the cell clump. The resulting cell suspension was added to tissue culture plates pre-coated with fibronectin as described above. Dibutyryl cyclic adenosinemonophosphate (cAMP) (Sigma-Alrdich, St. Louis, MO) was added to the media to achieve a 500 μM concentration and the cells incubated at 37ºC in 5% CO_2 and humidified atmosphere. Culture plates were washed with 5 mL and complete DMEM (without cAMP added) was changed 18-24 hr after the initial plating and every 48 hr thereafter. Prior to changing media, all solutions (PBS, complete DMEM) were warmed to 37ºC in a water bath. Culture plates were washed with 2 mL PBS, aspirated, and 9 mL complete DMEM containing 10% FBS was added.

Passaging

Cells were passaged at 75-80% confluence. For each plate to be passaged, 3 new plates were coated with fibronectin as described above. All solutions were warmed to 37ºC in a water bath. The media was aspirated and cells were exposed to 2 mL of a 0.25% trypsin/EDTA (Gibco BRL, Rockford, MD) solution for 5 minutes. A cell scraper helped to loosen any cells that did not detach from the plate and 5 mL warm complete DMEM was added to neutralize the trypsin. This trypsin, cell and DMEM solution was then transferred to a 15 mL centrifuge tube and centrifuged (800 x g) for 5 min at room temperature. Supernatant was aspirated, complete DMEM (3 mL) was added, and the cell pellet was gently re-suspended by repeated aspiration. The cell solution (1 mL/new plate) was then placed in a previously coated 100-mm plate. At confluence, RGEC were washed and exposed to fresh complete DMEM with 2% FBS for 24 hr prior to experimental conditions.

Experimental Conditions

All experiments were carried out using 10 mL complete DMEM supplemented with 2% FBS added at time 0 hr in 100-mm cell culture plates. RGEC were exposed to the conditions described below for a total of 48 hr with 800 µL media samples taken at 12, 24 and 48 hr. Leptin (Affinity Bio-Reagents, Golden, CO) concentrations used were chosen based on literature review of both in vitro research and in vivo measures. Briefly, RGEC (n = 3-6 100-mm plates for each condition) were exposed to leptin at a concentration of 0, 15, 25, 50 or 100 ng/mL. Soluble TGF-β1 and VEGF in supernatant were then assayed as described below. Experimental conditions were as follows: **A)** Control: complete DMEM with 2% FBS and normal glucose (5.55 mM); **B)** High glucose (25.5 mM); **C)** Leptin (15, 25, 50 or 100 ng/mL) in normal glucose (5.55 mM); and **D)** Leptin (15, 25, 50 or 100 ng/mL) in high glucose (25.5 mM).

Supernatant Collection

After exposure to experimental conditions outlined above, 800 µL supernatant samples were collected and EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) was added to achieve a ratio of 10:1 (media:protease inhibitor). Samples were centrifuged (2000x, 5 min) and assayed immediately or frozen at -70ºC until tested. Prior to assay, frozen samples were immediately placed in 37°C water bath until liquefied (~ 1 min). All samples were measured in triplicate.

Measuring Proteins in the Supernatant

TGF-β1 Protein Measurement

Soluble TGF-β1 was measured using a human TGF-β1 Duoset sandwich enzyme linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to manufacturer's directions. All incubation periods were at room temperature. The kit includes: Capture antibody (360 µg/mL in PBS); detection antibody (54 µg/mL in reagent diluent; TGF-β1

standard (145 ng/mL in reagent diluent), and streptavadin-horseradish peroxidase (HRP, 1:200 dilution in reagent diluent). To determine TGF-β1 concentration, 96-well plates (Corning Costar, Lowell, MA) were coated with capture antibody (working concentration: 2.0 µg/mL in PBS) and allowed to incubate overnight. The plate was washed 3 times with wash buffer (0.05% Tween 20 (Bio-Rad, Hercules, CA) in PBS (300 µL/well), aspirated, and incubated with block buffer (5% Tween 20 in PBS) for 1.5 hr. Standards were created using a serial dilution of TGF-β1 standard in reagent diluent (standard range: 0 – 2000 pg/mL). See Table 3.2. The standard curve was then created using a computer-generated 4-parameter logistic curve.

Vial	*Volume Reagent Diluent (µL)*	*Volume Source TGF-β1*	*Final [TGF-β1] (pg/mL)*
A	715	10 µL stock TGF-β1 std.	2,000
B	362.5	362.5 µL of A	1,000
C	362.5	362.5 µL of B	500
D	362.5	362.5 µL of C	250
E	362.5	362.5 µL of D	125
F	362.5	362.5 µL of E	62.5
G	362.5	362.5 µL of H	31.25
H	362.5	0	0

Table 3.1: Volumes and solutions utilized in preparation of TGF-β1 standard curve.

Latent TGF-β1 in media samples were acid activated using the following method: 25 µL 1 N HCl was added to 100 µL media samples and allowed to incubate for 10 min. Immediately 25 µL of 1.2 N NaOH/0.5 M hepes (Sigma-Aldrich, St. Louis, MO) was added to neutralize the samples. Activated samples were then transferred to a 96-well plate pre-coated with capture antibody and allowed to incubate on plate for 2 hr. Plate wells were washed as described above and 100 µL of detection antibody solution (300 ng/mL in reagent diluent) was added and allowed to incubate for 2 hr. Plates were washed and 100 µL of streptavidin-horseradish peroxidase (HRP) solution (1:200 dilution) was added to each well and allowed to incubate (20 min). Wells were washed and 100 µL of substrate solution (1:1 mixture of Reagent A and Reagent B) (R&D Systems, Minneapolis, MN) was added to each well and the plate incubated (20 min). Stop

solution (50 µL, 2 N H_2SO_4) was added and plates were read immediately on a spectrophotometer (SpectraMax Pro384, Software: Softmax Pro v. 3.1.2, Molecular Devices, Sunnyvale, CA). Absorbances were measured according to directions and measurements at 540 nm were subtracted from those at 450 nm.

VEGF Protein Measurement

VEGF protein in the media was measured using a rat VEGF Duoset ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. All incubation times were at room temperature. The materials provided in the kit include: Capture antibody (180 µg/mL in PBS); detection antibody (18 µg/mL in reagent diluent); VEGF standard (70 ng/mL in reagent diluent); and streptavidin-horseradish peroxidase (HRP) (1:200 dilution in reagent diluent). Standards were created using an eight-step serial dilution with the maximum concentration equal to 1000 pg/mL. See Table 3.3. A standard curve was created using a computer-generated 4-parameter logistic curve.

Vial	*Volume Reagent Diluent (µL)*	*Volume Source VEGF*	*Final [VEGF] (pg/mL)*
A	690	10 µL stock VEGF std.	1,000
B	350	350 µL of A	500
C	350	350 µL of B	250
D	350	350 µL of C	125
E	350	350 µL of D	62.5
F	350	350 µL of E	31.25
G	350	350 µL of H	15.75
H	350	0	0

Table 3.2: Volumes and solutions utilized in preparation of VEGF standard curve

To determine VEGF protein concentration in unknowns, a 96-well plate (Costar, Lowell, MA) was coated with capture antibody (100 µL/well), sealed and allowed to incubate overnight. The plates were washed 3 times with wash buffer (0.05% tween 20, Bio-Rad, Hercules, CA, in PBS, 300 µL/well), aspirated and incubated with block buffer (reagent diluent, 300 µL/well) for 1 hr. The plates were again washed and aspirated. 100 µL of media samples or standards were

then added to each well and allowed the plate to incubate on a rocker for 2 hr followed by a repeat of the wash/aspiration step. Detection antibody (100 µL/well) was then added, allowed to incubate (2 hr) and washed as described previously. Streptavidin-HRP (100 µL/well) was then incubated (20 min) and the plate was again washed. Substrate solution, a 1:1 mix of 30% H_20_2 (Reagent A, R&D Systems, Minneapolis, MN) and tetramethylbenzidine (Reagent B, R&D Systems, Minneapolis, MN) was added (100 µL/well) and the plate incubated for 20 min. Stop solution (2 N H_2SO_4, 50 µL/well) was added and absorbance was read immediately on a spectrophotometer. Absorbance was measured according to directions and measurements at 540 nm were subtracted from those at 450 nm.

Determination of Cell Injury and Cell Viability

Viability of cells was assessed with an in vitro cytotoxicicity detection kit for lactate dehydrogenase (LDH, Roche Applied Science, Indianapolis, IN). Briefly, standards for LDH assay were created for both a low control (complete DMEM with 2% FBS only) and a high control. High control was prepared by adding a 1:20 dilution (Triton X-100:supernatant) of cell lysis solution to the confluent culture plates in order to lyse all cells. The plate was shaken and allowed to incubate for 15 min at room temperature. High control supernatant samples were then centrifuged (800 x g) for 3 min. Triplicate 100 µL samples (high control, low control, and experimental conditions) were added to a 96-well plate. Immediately, 100 µL of supplied reaction mixture (250 µL of catalyst with 11.25 µL of dye solution) was added to each well and allowed to incubate at room temperature for 20 min. Catalyst contains tris, diaphorase, and nicotinamide-adenine dinucleotide dissolved in deionized water. Dye solution contains Triton X-100, sodium lactate, and tris. Stop solution (50 µL of 1 N HCl) was added after incubation and absorbance read immediately at 490 nm on a spectrophotometer [SpectraMax Pro384 (Software:

Softmax Pro v. 3.1.2); Molecular Devices, Sunnyvale, CA]. Percent cytotoxicity was determined using the following equation. $Cytotoxicity(\%) = \left(\frac{ExpermentalValue - LowControl}{HighControl - LowControl} \right) * 100$.

Statistics

Statistical analyses were done utilizing SPSS version 16. Data for TGF-β1 were analyzed utilizing univariate ANOVA at each time point and each leptin and glucose concentration. This was a test for linear change in response to leptin dose. VEGF results were analyzed utilizing T-Tests comparing control group with leptin group at the same time point. Cytotoxicity was analyzed utilizing a repeated measures test for linearity. Data are expressed as mean +/- standard error [SD÷(√n)]. Statistical significance was accepted at a probability value of 0.05 or less.

Chapter IV: Alternate Format

ABSTRACT

Diabetes and obesity stimulate renal fibrosis, often resulting in glomerulosclerosis and eventually end stage renal disease (ESRD). Leptin and vascular endothelial growth factor (VEGF) increase markers of fibrosis, including transforming growth factor-beta1 (TGF-β1). Little is known about the effect of leptin on TGF-β1 or VEGF in glomerular endothelial cells.

HYPOTHESIS: Leptin will increase TGF-β1 and VEGF concentration in an in vitro model of diabetes using rat glomerular endothelial cells (RGEC).

METHODS: RGEC were exposed to leptin for 48 hr in either normal or high glucose. Supernatant was assayed for TGF-β1 and VEGF by ELISA.

RESULTS: Leptin combined with high glucose caused a dose-related increase in TGF-β1 at 12 and 48 hr ($p<0.05$). In normal glucose, leptin caused a dose-related decrease in TGF-β1 at 48 hr ($p<0.05$). High glucose, but not leptin, increased VEGF at 48 hr ($p<0.05$).

CONCLUSIONS: Leptin and glucose-induced VEGF could contribute to the fibrosis seen in diabetes by increasing TGF-β1 production.

Introduction

In 2006, diabetes was the largest primary diagnosis contributor to end stage renal disease (ESRD) and chronic kidney disease (CKD), contributing to more than half of new diagnoses. Of the total ESRD incidence, 30.6% are diagnosed with Type 2 diabetes as the primary cause.[4] Obesity is a main driving force for the increase in incidence and prevalence of Type 2 diabetes.[5] Multiple autocrine and paracrine factors, termed "adipokines", are released from adipose tissue and many are inflammatory.[121] The adipokine leptin, which increases in plasma in direct

proportion to percent body fat,[65] appears to have a role in the progressive renal dysfunction seen in diabetes and obesity as leptin can alter the expression of pro-fibrotic proteins,[8, 16] and has been shown to increase fibrosis in vivo.[8] Normally functioning kidneys are responsible for almost 100% of circulating leptin clearance.[81, 82] In ESRD the filtration function of the kidney is no longer effective and thus serum leptin concentration is increased in patients with ESRD, leading to higher than normal plasma leptin concentrations.[83]

Fibrosis of the renal glomerulus is the main contributing factor to renal insufficiency.[39] Endothelial cells in renal glomeruli significantly contribute to the progression of renal disease in diabetes by increasing the expression of pro-fibrotic proteins such as transforming growth factor-β1 (TGF-β1) and fibrotic proteins themselves, including fibronectin and collagen[114, 115, 122] which contribute to basement membrane thickening.[9] In diabetes, elevated leptin concentration as a result of increased adiposity[65] is often seen in plasma along with high glucose concentrations.[4, 5] Consequently, in diabetic subjects, endothelial cells lining blood vessels are often exposed to concomitant high levels of leptin and glucose. Endothelial cells of the glomerulus express the long form (Ob-Rb) of the leptin receptor,[16] suggesting that the interaction of leptin with endothelial cells of the renal glomerulus is a likely way for obesity to contribute to renal disease.[8]

Transforming growth factor-β1 is generally considered the final common pathway for extracellular matrix (ECM) protein deposition and fibrosis that accompanies diabetic nephropathy.[14, 15] Thickening of the basement membrane via ECM deposition in diabetic renal complications is observed with major contributions coming from collagen type IV and fibronectin,[39, 40] both of which are increased by TGF-β1.[50, 52, 53]

The role of leptin in renal fibrosis and dysfunction is just being recognized. In normal rats infused with leptin at a rate of 250 ng/hr for 28 d, increased TGF-β1 was found specifically around the tubule cells.[84] Wolf et al.[16] also demonstrated that leptin infusion in rats (31 nmol/hr) increased TGF-β1 mRNA and protein. Additionally, leptin-infused rats display increased diuresis[85] and natiuresis, along with increases in proteinuria and collagen IV,[84] all measures of decreased renal function. Taken together, these studies provide evidence that leptin may play a role in glomerulosclerosis by altering expression of pro-fibrotic proteins such as TGF-β1.

A recent addition to the pathogenic inducers of renal fibrosis is vascular endothelial growth factor (VEGF).[92] Vascular endothelial growth factor is a strong mitogen for endothelial cells, increasing proliferation and migration of these cells.[93] In humans diabetic nephropathy is associated with an increased VEGF concentration in both the blood and urine.[95] In db/db rats, Flyvbjerg et al.[96] showed that a VEGF-neutralizing antibody given over two months was able to significantly attenuate (14%) the diabetes-associated increases in basement membrane thickness, suggesting that VEGF can promote fibrosis.

VEGF may promote fibrosis and basement membrane thickening by increasing TGF-B1 and other pro-fibrotic proteins. Li et al.[18] showed in vitro that mouse glomerular endothelial cells exposed to 10 ng/mL of VEGF displayed a significant increase in TGF-β1 from hr 3 to 24. Additionally, the pro-fibrotic matrix metalloproteinase 2, 13 and tissue inhibitor of metalloproteinase (TIMP-1) were measured, with significant increases seen over a 24-hr period.[18] Finally, Misztal-Dethloff et al.[19] demonstrated in vitro that leptin could increase VEGF concentrations in endothelial cells of peripheral lymph-node origin.

Determining whether leptin causes increases in VEGF and TGF-β1 in glomerular endothelial cells may provide insight into important interactions between leptin and two growth

factors thought to stimulate fibrosis. The effect of leptin on the concentration of VEGF protein and TGF-β1 protein is poorly defined in glomerular endothelial cells. Specifically, we sought to determine whether leptin plays a role in critical processes underlying the pathogenesis of glomerulosclerosis in a diabetic environment as it pertains to VEGF and the known inducer of fibrosis, TGF-β1. The research questions we addressed were: 1) Does leptin increase VEGF protein production by rat glomerular endothelial cells (RGEC) in the presence and absence of high glucose? and 2) Does leptin increase TGF-β1 production by RGEC in the presence and absence of high glucose?

We hypothesize that leptin will cause increased release of VEGF and TGF-β1 protein by RGEC in both normal and high glucose conditions.

Methods and Materials

Cells

RGEC have previously been isolated from glomeruli obtained by sieving the cortex of kidneys excised from 6-month-old Sprague-Dawley rats.[120] Cells between passages 17 and 25 were used for experiments.

Coating of Plates

Fibronectin (1 mg/mL, Roche Biochemicals, Indianapolis, IN) was diluted with phosphate buffered saline (PBS, KCl: 0.2 g/L; KH_2PO_4: 0.2 g/L; Na_2HPO_4: 2.16 g/L; NaCl: 8.0 g/L; deionized H_20) to a final concentration of 5 μg/μL and frozen at -20˚C in 1 mL aliquots. To coat culture plates (100 mm, Greiner, ISC Biologicals, Kaysville, UT), 1 mL of thawed fibronectin solution was added to a culture plate and evenly spread over the surface by repeated manual tapping, then placed on a rocker for > 30 min. Even coating of the plate was maintained

by repeated tapping and rotation of the plate at regular intervals. Remaining fibronectin solution was aspirated prior to addition of cells.

Thawing, Plating and Culture

Frozen RGEC were removed from liquid nitrogen and thawed briefly (~20 sec) in a warm (37ºC) water bath. Contents of the Nunc® cryotube vial (Nunc, Roskilde, Denmark) were transferred into an empty 15 mL centrifuge tube and 14 mL of cold (5ºC) Dulbecco's Modified Eagle Medium (DMEM, Gibco Life Technologies, Rockford, MD) containing 10% fetal bovine serum (FBS, Atlas Biologicals, Ft. Collins, CO), 2 mM sodium pyruvate, 90 μg/mL heparin, 100 IU/mL penicillin, 100 μg/mL streptomycin, and a total concentration of 4 mM $MgSO_4$ (complete DMEM) was slowly added. Cells were centrifuged (4°C, 800 x g, 5 min) and the media aspirated. Warm complete DMEM was added to the cell pellet and the solution aspirated gently and repeatedly to break up the cell clump. The resulting cell suspension was added to tissue culture plates pre-coated with fibronectin as described above. Dibutyryl cyclic adenosinemonophosphate (cAMP) (Sigma-Aldrich, St. Louis, MO) was added to the media to achieve a 500 μM concentration and the cells incubated at 37ºC in 5% CO_2 and humidified atmosphere. Culture plates were washed with 2 mL PBS and 9 mL complete DMEM (without cAMP) added. Cells were washed and media changed 18-24 hr after the initial plating and every 48 hr thereafter. Prior to changing media, all solutions (PBS, complete DMEM) were warmed to 37ºC in a water bath.

Passaging

Cells were passaged at 75-80% confluence. All solutions were warmed to 37ºC in a water bath before use. The media was aspirated and cells were exposed to 2 mL of a 0.25% trypsin/EDTA (Gibco BRL, Rockford, MD) solution for 5 minutes. A cell scraper helped to

loosen any cells that did not detach from the plate and 5 mL warm complete DMEM was added to neutralize the trypsin. This trypsin, cell and DMEM solution was then transferred to a 15 mL centrifuge tube and centrifuged (800 x g) for 5 min at room temperature. Supernatant was aspirated and complete DMEM (3 mL) was added and the cell pellet was gently re-suspended by repeated aspiration. The cell solution (1 mL/new plate) was then placed in a previously coated 100-mm plate. At confluence, RGEC were washed and exposed to fresh complete DMEM with either high glucose for high glucose groups or normal glucose for normal glucose groups and 2% FBS for 24 hr prior to experimental conditions.

Experimental Conditions

All experiments were carried out using 10 mL complete DMEM supplemented with 2% FBS added at time 0 hr in 100-mm cell culture plates. Briefly, RGEC were exposed to leptin (Affinity Bio-Reagents, Golden, CO) at a concentration of 0, 15, 25, 50 and 100 ng/mL, respectively, for 48 hr. Experimental conditions were as follows: **A)** Control: complete DMEM with 2% FBS and normal glucose (5.55 mM); **B)** High glucose (25.5 mM); **C)** Leptin (15, 25, 50 or 100 ng/mL) in normal glucose (5.55 mM); and **D)** Leptin (15, 25, 50 or 100 ng/mL) in high glucose (25.5 mM). Four independent dose response experiments were conducted. The final n after the experiments was n of 4-6 culture plates per bar on fig. 1-3.

Supernatant Collection

After exposure to experimental conditions outlined above, 800 µL media samples were collected and EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) was added to achieve a ratio of 10:1 (media:protease inhibitor). Samples were centrifuged (2000x, 5 min) and assayed immediately or frozen at -70ºC until tested. Prior to assay, frozen samples were immediately placed in 37°C water bath until liquefied (~ 1 min).

TGF-β1 Protein Measurement

Soluble TGF-β1 was measured in supernatant using a rat TGF-β1 Duoset sandwich enzyme linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to manufacturer's directions. The materials provided are TGF-β1 specific and include: capture antibody; detection antibody; TGF-β1 standard, and streptavadin-horseradish peroxidase (HRP). Standards were created using a serial dilution of TGF-β1 standard in reagent diluent (R&D Systems, Minneapolis, MN), with a maximum concentration of 2000 pg/mL. Measurements made on a spectrophotometer (SpectraMax Pro384, Software: Softmax Pro v. 3.1.2, Molecular Devices, Sunnyvale, CA) at 540 nm were subtracted from those made at 450 nm. Four independent experiments for TGF-β1 were conducted.

VEGF Protein Measurement

Soluble VEGF protein in the supernatant was measured using a rat VEGF Duoset ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. The materials provided are rat $VEGF_{164}$ specific and include: capture antibody; detection antibody; VEGF standard; and streptavidin-horseradish peroxidase (HRP). Standards were created using an eight-step serial dilution with the maximum concentration equal to 1000 pg/mL. Absorbances were measured according to manufacturer's directions on a spectrophotometer (SpectraMax Pro384, Software: Softmax Pro v. 3.1.2, Molecular Devices, Sunnyvale, CA). Absorbance measurements at 540 nm were subtracted from those made at 450 nm. A standard curve was created using a computer-generated 4-parameter logistic curve. Four independent experiments for VEGF were conducted.

Determination of Cell Injury and Cell Viability

Viability of cells was assessed with an in vitro cytotoxicity detection kit for lactate dehydrogenase (LDH, Roche Applied Science, Indianapolis, IN) following manufacturer's

directions. Briefly, standards for LDH assay were created for both a low control (complete DMEM with 2% FBS only) and a high control. High control was prepared by adding a 1:20 dilution (Triton X-100:supernatant) of cell lysis solution to a confluent culture plate to lyse all cells. The resulting media contained only lysed, unviable cells. Cytotoxicity of all samples is represented as percent of the high control.

Statistics

Statistical analyses were done utilizing SPSS version 16. Data for TGF-β1 were analyzed utilizing univariate ANOVA at each time point and each leptin and glucose concentration as a test for linear change in response to leptin dose. VEGF results were analyzed utilizing T-Tests comparing control group with leptin group at the same time point. Cytotoxicity was analyzed utilizing a repeated measures test for linearity. Data are expressed as mean +/- standard error [SD÷(√n)]. Statistical significance was accepted at a probability (p) value of 0.05 or less.

Results

Transforming Growth Factor-β1

Incubation of RGEC with increasing doses of leptin (0, 15, 25, 50 or 100 ng/mL) in the presence of high glucose (25.5 mM) caused a linear dose-related increase in TGF-β1 at 12 hr and 48 hr (both $p < 0.05$), but not at 24 hr (n = 4-6 per bar, Fig. 1). Alternately, leptin in the presence of normal glucose concentration (5.55mM) lead to a linear decrease in TGF-β1 at 48 hr ($p < 0.05$). No significant linear change was found at either 12 or 24 hr for leptin in normal glucose conditions (Fig. 2).

Vascular Endothelial Growth Factor

Leptin (100 ng/mL) did not have a significant effect on VEGF protein concentration at any time point in either normal or high glucose conditions. High glucose concentration increased VEGF protein at 48 hr ($p<0.05$) as compared to the normal glucose group (n = 4-6 per bar, Fig. 3).

Cytotoxicity

Cytotoxicity significantly increased over time from 12 to 24 hr ($p< 0.01$) and 24 to 48 hr ($p<0.01$) in all conditions. However, leptin had no significant effect on cytotoxicity at any time point or concentration in either normal or high glucose concentrations. (Data not shown.)

Discussion

Increases in obesity and diabetes are the two major contributors to the increase in incidence of CKD and ESRD.[4] It is crucial to understand the underlying mechanisms that stimulate fibrosis and progressive renal dysfunction with a specific focus on the connections with obesity and diabetes. In the present study, we sought to determine whether leptin plays a role in critical processes underlying the pathogenesis of glomerulosclerosis in a diabetic environment as it pertains to VEGF and the known inducer of fibrosis, TGF-β1.

The finding that leptin stimulates a dose-related linear increase in TGF-β1 by RGEC in a high, but not normal, glucose concentration (Fig. 1, Fig 2) does not support our original hypothesis. We expected leptin to increase TGF-β1 protein in both high glucose and normal glucose conditions. In high glucose, a significant linear change at 24 hr was not seen (Fig 1), although the trend at 24 hr was towards significance ($p = 0.11$) and would likely have become significant with more statistical power. Using rat glomerular endothelial cells, Wolf et al.[16] showed in vitro that leptin (100 – 10,000 ng/mL) significantly increased TGF-β1 protein

expression in high glucose (25 mM), which is in agreement with our observations on high glucose concentration. A literature search did not identify any research utilizing normal glucose concentrations when exposing endothelial cells to leptin in vitro. Therefore, this study appears to be the first to show in RGEC that leptin decreases TGF-β1 expression in vitro in the presence of a normal glucose concentration.

In vivo, Gunduz et al[84] showed that leptin infusion into normal rats (without diabetic complications such as high blood glucose concentrations) caused a significant increase in TGF-β1, specifically around the tubule cells and not endothelial cells. However, the infusion rate for leptin that Gunduz et al.[84] utilized was very high at 250 ng/hr over 28 d. Serum concentrations of leptin in normal rats range from 1 – 10 ng/mL[123] while normal values for serum leptin in humans range from 2-7 ng/mL in lean individuals and up to 45 ng/mL in obese persons.[124, 125] Infusion of 250 ng of leptin every hr over 28 d will lead to supraphysiologic leptin concentrations and possibly irrelevant results.

Our results suggest that the effects of leptin on TGF-β1 protein in RGEC are modulated by glucose concentration. Serum leptin concentrations undergo diurnal variation,[126] decrease with fasting, and increase with ingestion of food.[127, 128] In normal rats, increased plasma leptin results in decreased food intake, increased energy expenditure, and decreased body weight.[129-131] Based on the effect that leptin has on energy expenditure and satiety, it seems reasonable that leptin may have different effects in vivo in varying energy availability states such as those represented by high or normal plasma glucose concentrations. However, further research regarding leptin and differential effects based on glucose concentrations in vitro are needed to determine whether this is the case.

Leptin (100 ng/mL) was not observed to have a significant effect on VEGF protein concentration at any time point in either normal or high glucose conditions (Fig. 3). These results differ to those of Misztal-Dethloff et al.[19] Using HECa10 endothelial cells of peripheral lymph-node origin, these researchers showed that leptin at concentrations of 0.5 – 25 ng/mL stimulated increases in VEGF protein at 24 hr. However, in this same study, 250 ng/mL of leptin did not cause a significant difference in VEGF when compared to control at any time point. Interestingly, the leptin concentration that induced the highest mean VEGF protein was 5 ng/mL, suggesting that there may be an inverse relationship between concentrations of leptin and VEGF protein. Consequently, the leptin concentration of 100 ng/mL used in the present study, may have been too high to stimulate increases in VEGF protein from endothelial cells. Further research would be needed to delineate these observations.

Our finding that high glucose increased VEGF protein, specifically at 48 hr ($p= 0.016$), as compared to the normal glucose group (Fig. 3), is supported by findings of others. In human umbilical vein endothelial cells, Li et al.[132] observed increases in both VEGF mRNA and protein after 48 hr incubation in response to high glucose. After a 3-d incubation period, Sone et al.[108] showed that high glucose (16.6 mM) significantly increased VEGF protein concentration in retinal epithelial cells. Additionally, using rat mesangial cells, Cha et al.[95] observed high glucose to increase VEGF mRNA (at 3 hr) in a dose-dependent manner with a peak VEGF mRNA at 30 mM of glucose.

In vitro, both concentrations of high glucose[111-114] and VEGF[18, 99, 100] have been shown to increase TGF-β1, fibronectin, and collagen production independently in various cell types. Therefore, both high glucose alone, and high glucose-induced increases in VEGF protein from

glomerular endothelial cells, could significantly contribute to the progression of basement membrane thickening and fibrosis in ESRD.

Although cytoxicity in our cells significantly increased with time, our cytoxicity values of 6% at 24 hr and 14% at 48 hr are comparable to, or even lower than, cytoxicity values for endothelial cells reported by others. With cells grown in normal glucose, Bresgen et al.[133] had similar cytotoxicity results with a mean mouse capillary endothelial cell cytotoxicity of 6 % at 24 hr as determined by morphology of DAPI-stained cells under a microscope. When LDH activity was measured after just 6 hr, thoracic aortic endothelial cells grown in normal glucose conditions showed even higher cytotoxicity with a mean value of 23.8%.[134]

Our results suggest that leptin, in the presence of high glucose, may contribute to the basement membrane thickening seen in diabetes and obesity. Conversely, when in culture with normal glucose, leptin may have protective effects against fibrosis by decreasing TGF-β1 protein production in RGEC. Results of this pilot work, as well as those of others, provide intriguing evidence that leptin may play an important role in excess renal ECM deposition in diabetes and ESRD by increasing VEGF and TGF-B1.

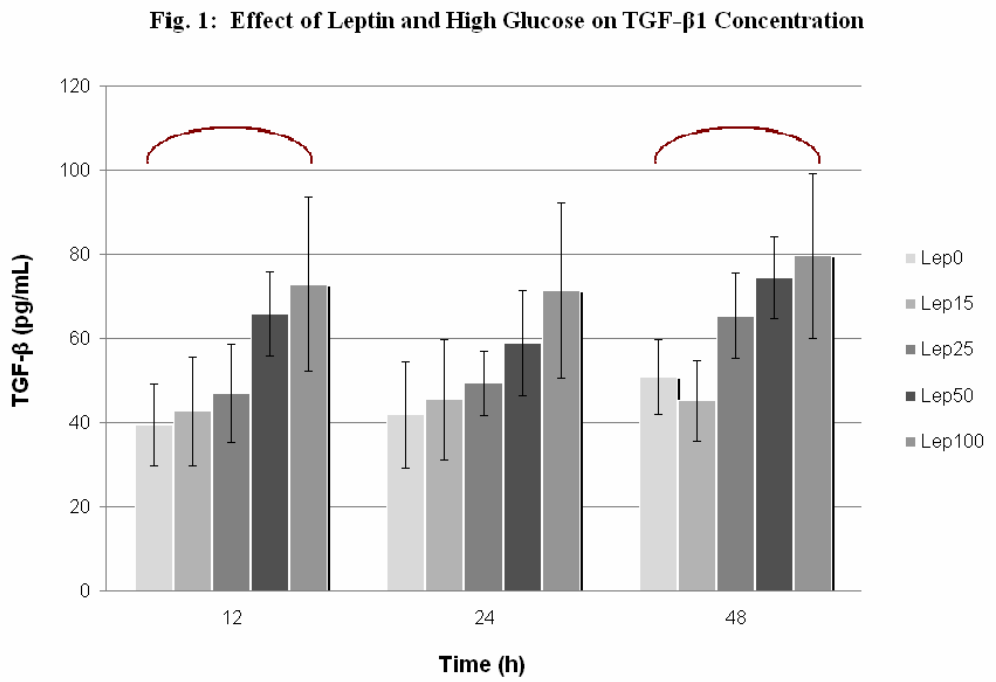

Figure 1: Effect of leptin and high glucose on TGF-β1 protein concentration in rat glomerular endothelial cell (RGEC) culture supernatant. RGEC were exposed to increasing doses of leptin (0, 15, 25, 50 or 100 ng/mL) in high glucose (25.5 mM) media. Supernatant samples (n = 4-6 per bar) were collected at 12, 24 and 48 hr and analyzed in triplicate by sandwich ELISA. A linear dose-related increase in TGF-β1 was seen at 12 and 48 hr (both $p<0.05$). Significance was not found at 24 hr although the trend was toward significance ($p=0.11$). Data are represented as the mean +/- the SEM.

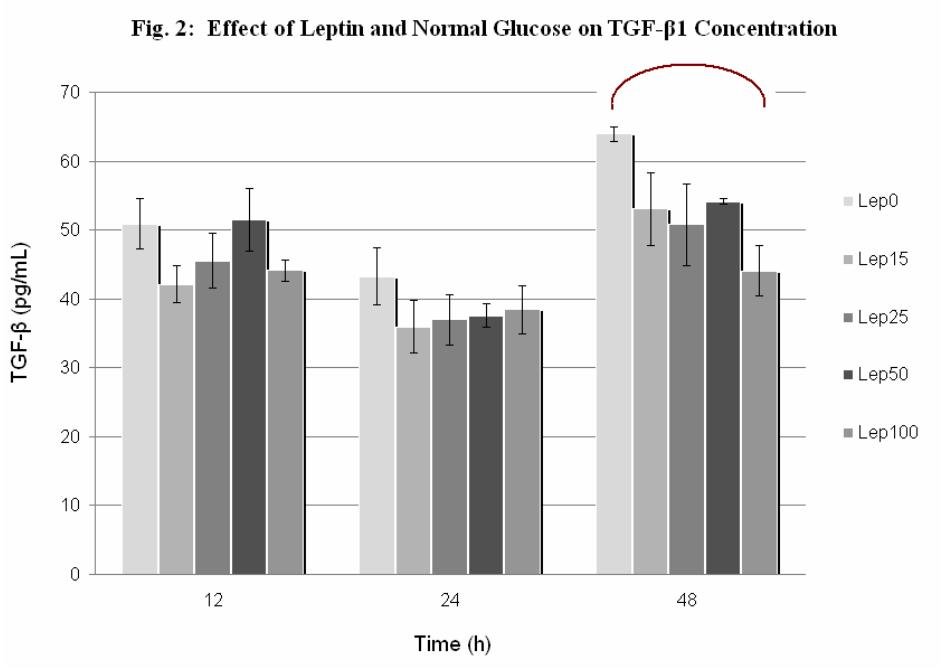

Figure 2: Effect of leptin and normal glucose on TGF-β1 protein concentration in rat glomerular endothelial cell culture supernatant. RGEC were exposed to increasing doses of leptin (0, 15, 25, 50 or 100 ng/mL) in normal glucose (5.55 mM) media. Supernatant samples (n = 4-6 per bar) were collected at 12, 24 and 48 hr and analyzed by sandwich ELISA. A linear dose-related decrease in TGF-β1 was seen at 48 hr ($p<0.05$). Data represented as the mean +/- the SEM.

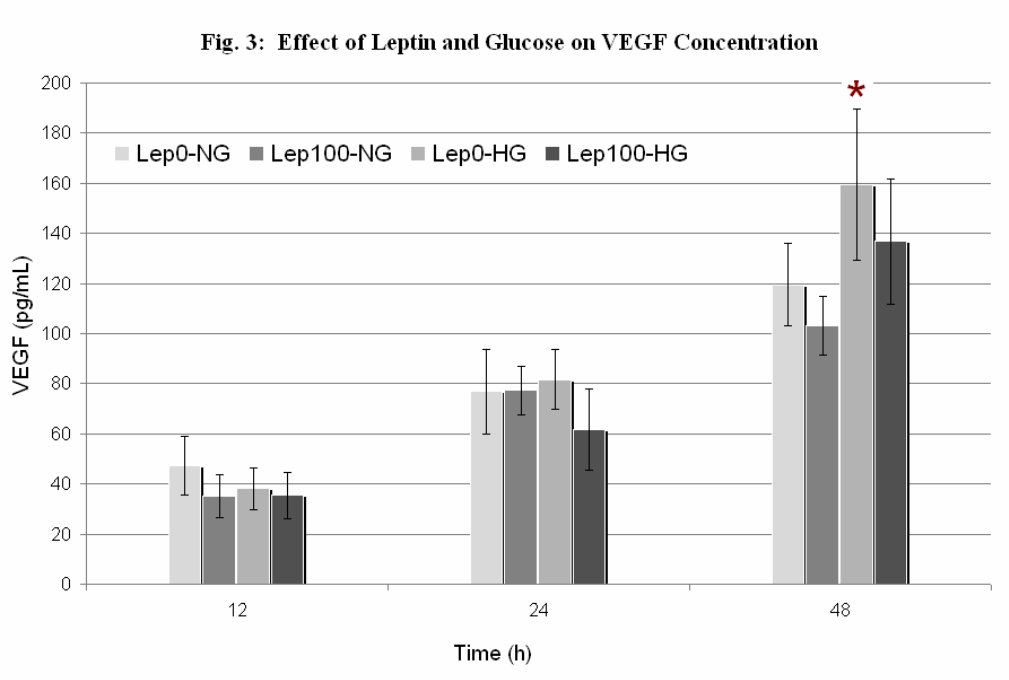

Figure 3: Effect of leptin and glucose concentration on VEGF protein content in rat glomerular endothelial cell culture supernatant. RGEC were exposed to leptin (100 ng/mL) in normal glucose (NG, 5.55 mM) or high glucose (HG, 25.5 mM) media. Supernatant samples (n = 4-6 per bar) were collected at 12, 24 and 48 hr and analyzed in triplicate by sandwich ELISA. * At 48 hr, high glucose, but not leptin, stimulated a significant increase in VEGF protein concentration ($p<0.05$). Data are represented as the mean +/- the SEM.

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