ROLE OF STROMAL CELL-DERIVED FACTOR 1 IN PROLIFERATIVE RETINOPATHY

By

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To my beautiful wife Diana and wonderful son Tyson. Your love and support have made all of this possible.

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Diabetic retinopathy is the leading cause of blindness in working-age adults. It is caused by oxygen starvation in the retina, inducing aberrant formation of blood vessels that destroy retinal architecture. In humans, vitreal stromal cell-derived factor 1 (SDF-1) concentration increases as proliferative diabetic retinopathy progresses. Treating patients with triamcinolone decreases SDF-1 levels in the vitreous and leads to marked visual improvement. SDF-1 induces human retinal endothelial cells to increase expression of vascular cell adhesion molecule-1 (VCAM-1), a receptor for very late antigen-4 (VLA-4) found on many hematopoietic progenitors, and reduces tight cellular junctions by reducing occludin expression. Both changes would serve to recruit both hematopoietic and endothelial progenitor cells along an SDF-1 gradient. We have shown, using a murine model of proliferative adult retinopathy, that the majority of new vessels formed in response to oxygen starvation are of hematopoietic stem cell-derived endothelial progenitor cell origin. We now show that the levels of SDF-1 found in human patients

with proliferative retinopathy induce retinopathy in our murine model. Intravitreal injection of blocking antibodies to SDF-1 prevents retinal neovascularization in our murine model, even in the presence of exogenous VEGF.

We further set out to elucidate how SDF-1 could be working mechanistically to promote neovascularization by analyzing our model at various time points. Using immunohistochemistry and ELISA, we have shown that SDF-1 is upregulated in the bone marrow and the retina following ischemic insult. The percentage of bone marrow-derived CD133+ cells increased in the circulating peripheral blood in response to the ischemic insult. These bone marrow-derived CD133+ cells were positive for CXCR4 (the only known receptor for SDF-1) and possessed chemotatic activity toward a SDF-1 gradient. Intravenous injection of bone marrow-derived CD133+/GFP+ cells following ischemic insult results in the recruitment and incorporation of these cells into the repairing vasculature of the retina. These results demonstrate that SDF-1 can form a gradient sufficient to promote neovascularization and that the source of endothelial progenitor cells (EPC) that are recruited to the site of preretinal neovascularization could be distinguished by the cell surface marker CD133. Together these data show that SDF-1 plays a major role in proliferative retinopathy and may be an ideal target to prevent proliferative retinopathy.

CHAPTER 1
INTRODUCTION AND BACKGROUND

For many years researchers have been trying to understand the body's ability to repair and replace cells and tissues of organs. Work in elucidating into the cellular mechanisms of repair has led researchers to begin focusing their efforts on the potential of adult stem cells to facilitate the process of repair. Adult stem cells, like all stem cells, share two pivotal characteristics. First, they can give rise to all mature lineages that have the morphologies and specialized functions of the tissue they are harvested from. This characteristic allows the adult stem cell to continuously generate all cell types necessary to maintain the existence of their native organ. Second, they have the ability to undergo asymmetrical division. This allows the adult stem cell to make an identical copy of itself (termed self-renewal) and allows for lineage-committed progeny for the life of the animal [1].

Adult stem cells are extremely rare, with their primary function to maintain homeostasis. Most adult stem cells have no definitive means of characterization, and no one truly knows the origin of adult stem cells in any mature tissue. Current methods for characterizing adult stem cells are dependent on determining cell surface markers and observations about their differentiation patterns in culture dishes. Most of the information on the adult stem cells comes from work done in the mouse. Using the adult mouse, the list of organs that contain cells with "stem cell-like" properties has been growing. Adult stem cells have been reported to exist in the bone marrow [2], skeletal muscle [3], liver [4], pancreas [5], intestinal lining [6], skin [7], and brain [8-10].

Some adult stem cells possess the capability to differentiate into tissues other than the one from which they originated; this phenomenon is known as stem cell plasticity. To be able to claim that an adult stem cell is plastic, the cell population that is isolated from the tissue of interest has to have the identifying features of stem cells; it is important to note that most adult stem cell populations fail to meet the requirements to be considered a true stem cell. Most plasticity experiments reported to date involve adult stem cells differentiating into cells that have developed from the same primary germ layer. The hematopoietic stem cell (HSC), which is derived from the mesoderm layer, has been shown to differentiate into other mesodermally derived tissues such as skeletal muscle [11, 12], cardiac muscle [13, 14], or liver [15-19].

Our unifying goal of this body of work was to begin to further describe the characteristics of the HSC in relation to its plastic ability to produce the endothelial tissue lining the blood vessel wall. In particular, this study will focus on the capability of the HSC to participate in preretinal neovascularization during the progression of diabetic retinopathy. This body of work will first introduce you to the HSC and its close developmental relationship to the endothelial cell, and how the HSC can give rise to a subset of cells with endothelial potential known as endothelial progenitor cells (EPC). Next, this study will show how preretinal neovascularization, caused by the HSC and local endothelial cells, can be blocked by modulating the effects of stromal cell-derived factor 1 (SDF-1). Finally, this body of work will begin to elucidate into the mechanisms that SDF-1 may be working on to drive such a deleterious condition.

Hemangioblast: The Link between Blood and Blood Vessels

The first visible sign of hematopoietic activity in the mouse embryo is the appearance of blood islands in the developing extraembryonic yolk sac around day 7.5 of

gestation [20]. The progression of hematopoiesis within the yolk sac blood islands primarily produces erythrocytes and goes on to establish intraembryonic sites of blood production [21-23]. The primary site of intra-embryonic hematopoiesis is in the fetal liver, but recent evidence has shown that there are sites present prior to establishment of hematopoiesis in the fetal liver in the mouse [24,25]. These regions are known as the paraaortic splanchopleura (P-sp) in day 8.5 to 9.5 embryos and the aorta gonad mesonephros (AGM) in older (day 10.5 to 11.5) embryos. The P-sp/AGM is not an area of hematopoietic maturation, but rather serves as a source of definitive hematopoietic progenitor cells [25-32]. Initially these hematopoietic progenitor cells are a very rare population, but they rapidly increase in number and stem cell activities, seen by the ability of this cell population to reconstitute the bone marrow in a lethally irradiated adult mouse [32].

Yolk sac blood islands also contain a subset of cells known as angioblasts, precursors to endothelial cells. At day 7.5 of gestation angioblasts begin to form vascular lumens and organize into vascular networks in a process known as vasculogenesis [33-35]. Studies over the past 100 years have shown that blood cells develop in close proximity to the vascular system during embryogenesis. Endothelial cells can be found on the ventral surface of the aorta derived from the P-sp/AGM regions, and HSC are found nestled in the endothelial floor of the aorta. This tight relationship has sparked an idea of a common progenitor that gives rise to both blood and blood vessels. In 1932, Murray began detailed work on chick embryos, where he dissected out and cultured cells capable of producing both blood and blood vessels. Murray called these cells “hemangioblast” [36].

Blood Vessel Formation in the Adult

Vasculogenesis and angiogenesis are two distinct processes that lead to the formation of blood vessels. Vasculogenesis is thought to be the de novo differentiation of primitive endothelial progenitor cells that aggregate to form a primary capillary plexus. This process was only thought to occur during vascular development of embryogenesis [37]. Angiogenesis, on the other hand, is defined as the formation of new blood vessels by a process of sprouting from preexisting vessels. This process occurs both during embryogenesis and in postnatal life [37-39]. In the adult, angiogenesis is a tightly control process. It occurs in the healthy body for healing wounds and for restoring blood flow to tissues after injury or insult. Angiogenesis is regulated through a series of "on" and "off" switches within the body. These switches are known as angiogenesis-stimulating growth factors and angiogenesis inhibitors. When angiogenenic growth factors are produced in excess or angiogenenic inhibitors, signals are given for new blood vessel growth, and vice versa. The normal healthy body normally maintains and excess of angiogenenic inhibitors, which helps maintain homeostasis of angiogenesis modulators [40].

Until recently, angiogenesis was thought to be the only process in which new blood vessels were formed in postnatal life. In 1991, Dr. Sampol's group from Marseille, France isolated human endothelial cells from whole blood. They used a pan-endothelial cell surface marker, S-Endo1 [41]. They found that there was an increase in the circulating endothelial cells following endothelial injury after angioplasty. This study's discovery of a circulating endothelial cell sparked a general curiosity to determine the origin and characterization of this cell population.

Endothelial Progenitor Cells

Origin and Characterization of Endothelial Progenitor Cells

The dogma that the differentiation of mesodermal cells to angioblast to form vascular networks only occurs during embryonic development was overturned in 1997, when Asahara et al. showed that hematopoietic progenitor cells could be expanded *ex vivo* and could differentiate into cells that have an endothelial phenotype. In this study, CD34+ cells were isolated and showed expression of various endothelial markers, and incorporated into the sites of ischemic neovascularization [42]. These cells were named "endothelial progenitor cells (EPCs)." In 1998 the existence of a bone marrow-derived circulating EPC was confirmed by Rafii and colleagues [43]. Once again, purifed CD34+ hematopoietic progenitor cells were shown to express endothelial markers and differentiate into cells of the endothelial lineage. Most interestingly, Rafii et al. [43] showed that implanted Dacron grafts were covered with genetically tagged bone marrow cells that had been transplanted.

Those landmark studies were among the first to show evidence of a circulating hemangioblast-like cell. These cells were initially characterized and defined by being positive both for CD34 and an endothelial protein marker vascular endothelial growth factor receptor 2 (VEGFR2). Further studies excluded CD34 as a defining marker due to CD34 not being exclusively expressed on the HSC. It is also expressed, albeit at low levels, on mature endothelial cells. Studies began using the more immature HSC marker, CD133. Purified CD133+ cells were shown to differentiate into endothelial cells *in vitro* [44]. CD133, also known as AC133 and prominin-1, is a highly conserved antigen and its biological function is not known. Most importantly it is expressed on immature HSC

and is not expressed on mature endothelial cells, making CD133+VEGFR2+ cells more likely to represent a bone marrow-derived EPC [45].

Controversy still exists in regards to the characterization and origin of the EPC. When isolating EPC from peripheral blood mononuclear cells there are many possible sources for the EPC, which includes a rare population of HSC [42,43], CD14+/CD34- myeloid cells which coexpress endothelial cell markers and form tube-like structures *ex vivo* [46] and incorporate into newly formed blood vessels *in vivo* [47], and circulating mature endothelial cells, which shed off the blood vessel wall [48]. In general, it is believed that there are multiple sources other than the HSC that give rise to EPC. In 2002, Dr. Rozing's group showed that resident blood vessel endothelial cells play a pivotal role in repairing the vasculature of rats that underwent transplant arteriosclerosis, with limited contribution (1-3%) from bone marrow-derived EPC [49]. In addition, small subset population of cells derived from the bone marrow, such as side population cells and multipotent adult progenitor cells that are distinct from the HSC, have been shown to differentiate into cells of the endothelial lineage [50,51]. These data support more than the fact that there are many possible sources of EPC; they support the realization that it will be difficult to characterize a "true" EPC. One can only hope that better profiling and fate mapping studies will be able to discover the cell surface marker codes that will help us distinguish between bone marrow-derived EPC and the non-bone marrow-derived EPC.

Role of EPC in Neovascularization

Improving the rate of neovascularization is becoming a primary therapeutic option to rescue critically injured tissue from ischemia [52]. The discovery that bone marrow-derived EPC can incorporate into sites of ischemic injury has led to the proposal that EPC

for therapeutic vasculogenesis. In recent animal studies using a model of myocardial infarction, the injection of various cell populations isolated from the bone marrow or through *ex vivo* expansion was shown to augment capillary density and neovascularization of the ischemic tissue, and was also shown to increase blood flow and cardiac output [53,54]. Isolation of peripheral blood mononuclear cells has shown similar results in augmenting neovascularization [47,55].

There has been overwhelming evidence showing that EPC can improve neovascularization, but the question of how still remains. Several groups have used genetically modified bone marrow cells for transplantation to assess the incorporation of bone marrow-derived EPC. These studies have had conflicting results, with incorporation percentages ranging from 0% to 90% [56-60]. A reasonable explanation for this discrepancy could be that the model of ischemia could dramatically influence the incorporation rates. At any rate, the general consensus is that the incorporation of bone marrow-derived EPC is quite low. So how could such a low number of cells lead to increase neovascularization? Many believe that EPC may act like monocytes/macrophages, in that they may serve as a source of proangiogenenic growth factors. So the rate of neovascularization may not be solely dependent on the incorporation of bone marrow-derived EPC, but may be influenced by the secretion of growth factors in a paracrine manner. It has been shown that EPC cultivation results in an increase of expression in growth factors such as vascular endothelial growth factor (VEGF) [61]. The release of these growth factors may support local endothelial cells to participate in classical angiogenesis, particularly in the steps of proliferation, migration, and survival of the mature endothelial cells.

Stromal Cell-Derived Factor 1: Role in Embryogenesis and HSC Maintenance

The chemokine stromal cell-derived factor 1 (SDF-1) and its only known receptor CXCR4 are required for normal development of the nervous, hematopoietic, and cardiovascular systems. Targeted deletion of either the SDF-1 or CXCR4 genes in the mouse causes death in utero, with primary defects in the generation of large vessels supplying the gastrointestinal tract and in B-cell lymphopoiesis and myelopoiesis [62-65]. Most importantly, fetal liver hematopoiesis is not affected, suggesting that the SDF-1/CXCR4 axis plays a pivotal role in transposition of definitive hematopoiesis from the fetal liver to the bone marrow [62]. In the adult mouse, SDF-1 is constitutively expressed by stromal cells of various tissues [66,67], dendritic cells, endothelial cells and pericytes [68], osteoblasts and endothelial cells from the bone marrow [69], and astrocytes and neurons from the brain [70]. SDF-1 is the primary chemokine responsible for chemotaxis of cells that express CXCR4, such as CD34+ HSC, monocytes, lymphocytes, and endothelial cells, and can promote transendothelial migration of CD34+ HSC and other cell types [71-75]. Through complex interactions with adhesion molecules, SDF-1 can promote attachment of CD34+ HSC to the vascular endothelium [76-78]. The SDF-1/CXCR4 axis can also regulate the retention of HSC to the bone marrow and promote HSC engraftment and survival [79-82].

Role of the SDF-1/CXCR4 Axis in Angiogenesis

Chemokines are multifunctional regulators that can promote immune responses, stem-cell survival, development, and homeostasis. Chemokines have also been shown to trigger chemotaxis and angiogenesis [72,83-85]. Chemokines are divided into four subfamilies, based on structural properties and primary amino acid sequence: CXC, CC, C or CX3C [83]. Recent evidence has shown that CXC chemokines play a pivotal role in

the control of angiogenesis, with the SDF-1/CXCR4 axis being the most important [86]. First evidence of the importance of the SDF-1/CXCR4 interaction for angiogenesis was seen in targeted gene deletion of CXCR4, where the large vessels of the gastrointestinal tract failed to grow [64]. These data led to CXCR4 being the first angiogenic chemokine receptor identified. The existence of a regulatory loop between VEGF-A and SDF-1/CXCR4 further supports the crucial role of the SDF-1/CXCR4 axis in the regulation of angiogenesis. Indeed, SDF-1 upregulates VEGF-A production and VEGF-A upregulates CXCR4 expression, thus generating an amplification circuit influenced directly by hypoxia [87,88].

Effect of Angiogenic Cytokines on CXCR4 Expression

Endothelial cells express CXCR4 at low constitutive levels. This low level can be increased 4-fold by VEGF and basic fibroblast growth factor (bFGF), rendering endothelial cells more responsive to SDF-1 [88,89]. The ability of VEGF and bFGF to increase expression is solely restricted to CXCR4, because they do not elicit a response in other CXC receptors at both the protein and mRNA levels [87]. The facts that VEGF, bFGF, and SDF-1 are widely expressed throughout the body of mice and humans, and that their respective receptors are expressed on vascular cells, suggest that these interactions contribute to the maintenance of the endothelium [35,90-94].

Angiogenesis is a highly regulated process in which quiescent endothelial cells can react either to an increase of angiogenic mediators such as tumor necrosis factor α (TNF-α) or to a decrease in angiostatic factors such as interferon γ (INF-γ). TNF-α has a biphasic effect on CXCR4 expression, eliciting an early down-regulation [95], and then a late induction [87]. This late effect is in part due to TNF-α induction of VEGF and

bFGF. In contrast to the positive effect of the classic angiogenic factors of CXCR4 expression, INF-γ acts as a negative regulator on CXCR4. This was shown during INF-γ treatment where basal levels of CXCR4 expression were down regulated. This resulted in the inhibition of SDF-1-mediated chemotaxis [87,95]. Thus, the angiostatic ability of mediators such as INF-γ might be in part dependent upon the down-regulation of CXCR4. These data suggest that angiogenesis can be modulated by the upregulation of CXCR4 by mediators such as TNF-α or by angiogenic factors such as VEGF and bFGF.

Diabetes

Diabetes is a syndrome of abnormal carbohydrate metabolism that is characterized by hyperglycemia. It is associated with a relative or absolute impairment in insulin secretion, along with varying degrees of peripheral resistance to the action of insulin [96]. There two main forms of diabetes, type 1 diabetes and type 2 diabetes.

Type 1 diabetes results from autoimmune destruction of the insulin-producing β-cells in the islets Langerhans. This process, which occurs in genetically susceptible subjects, is probably triggered by one or more environmental agents, and usually progresses over many months or years [97]. The pathogenesis of this disorder is quite different from that of type 2 diabetes, in which both decreased insulin release and insulin resistance plays a role.

Type 2 diabetes, or adult onset diabetes, is characterized by hyperglycemia, insulin resistance, and relative impairment in insulin secretion. It is by far the most common type of diabetes, with over 80% of the world's cases of diabetes being classified as type 2 diabetes [98]. Insulin resistance is the best predictor of type 2 diabetes. The causes of insulin resistance are still unclear, but insulin resistance becomes more severe with

increasing age and weight, thereby unmasking a concurrent defect in insulin secretion in susceptible subjects to cause impaired glucose tolerance and eventually hyperglycemia [99-101]. Obesity also causes impairment in insulin processing. Insulin production in healthy subjects involves the cleavage of proinsulin into insulin. In the normal, healthy body, approximately 10-15 percent of secreted insulin is proinsulin and its byproducts. In subjects with type 2 diabetes, the amount of proinsulin increases dramatically to over 40 percent. The increase in proinsulin secretion persists after matching for degree of obesity, suggesting that it represents β-cell dysfunction, and not merely the response to the increased secretory demand imposed by the insulin resistance of obesity [102,103].

There is currently no cure for diabetes. Patients with type 1 or type 2 must take insulin several times a day and test their blood glucose levels three to four times a day for the rest of their lives [104]. Maintaining glucose levels near a healthy range is very important because in can significantly decrease many complications of diabetes, such as diabetic retinopathy (discussed below).

Diabetic Retinopathy

Introduction and Pathogenesis

Diabetic retinopathy is the leading cause of blindness in the working class of developed countries. It accounts for nearly 12 percent of all new cases of blindness per year in the United States alone. It is a major cause of morbidity in patients with both type 1 diabetes and type 2 diabetes. For instance, the incidence of blindness in patients with diabetes is 25 times higher than the general public [104].

Chronic hyperglycemia is thought to be the primary cause of diabetic retinopathy, but why hyperglycemia is a direct cause of diabetic retinopathy still remains a mystery [105]. What is known is that there is probably an interaction of hemodynamic,

biochemical, and hormonal mechanism involved [106]. There are currently three main hypotheses that may explain the cause of diabetic retinopathy by hyperglycemia. The first is retinal blood flow. Retinal blood flow remains at a constant until the mean arterial pressure is raised above 40 percent. This autoregulatory mechanisms is negatively affected by hyperglycemia. The increase in retinal blood flow causes an undue amount of shear stress on the blood vessels of the retina, which produces proangiogenenic factors such as VEGF [106]. The second hypothesis is an accumulation of sorbitol within retinal cells. Sorbitol plays a major role in the metabolism of glucose within the cells via the enzyme aldose reductase. Sorbitol accumulation within the cell causes an increase in osmolality (an increase of water in the cell causing swelling), which causes an interference with glucose metabolism. The role of sorbitol in the progression of diabetic retinopathy remains unclear, but it is known that a gene defect in aldose reductase is associated with the early onset of retinopathy in some patients [107,108]. The last hypothesis is the accumulation of advanced glycosylation end products (AGE) in the extracellular fluid. When a patient has chronic hyperglycemia, some of the excess glucose has the tendency to bind to free amino acids, serum, or tissues. This process produces reversible early glycosylation products and later irreversible AGE. In diabetic patients, there is an accumulation of AGE in the retina. The AGE may cross link with collagen, initiating vascular complications [109].

Development of Retinopathy

The retina is one of the most sensitive organs in the body. It has a high rate of aerobic energy metabolism and is particularly sensitive to imbalances and ischemia [105]. In the very early stages of diabetes, a loss of retinal pericytes and microvascular endothelial cells is seen. Apoptosis of retinal pericytes and microvascular endothelial

cells results and thickening of the retinal basement membrane leads to the formation of retinal capillary microaneurysms (hypercellular outpouchings of weakened retinal capillaries). Microaneurysms cause an excessive increase in vascular permeability and increase the activity of vasoproliferative substances such as VEGF. The initial stage of cell death and increased vascular permeability may be followed by cycles of renewal and further cell death. These cycles lead to progressive destruction of the microvascularature, ischemic injury, and unregulated angiogenesis [110]. Microaneurysms and the leakage of lipid and proteinacieous material, referred to as "hard" exudates, are the intial clinical signs of diabetic retinopathy. These symptoms are difficult to notice during normal health exams, and are usually noticed when significant damage has already occurred and complications have developed [111]. These first clinical signs are closely associated with the following pathological and clinical changes: hemorrhaging of the microvascular network of the retina, proliferation of the endothelial cells of retinal vein that form tortuous loops, and severe ischemia that leads to new vessel formation [105].

There are two stages of diabetic retinopathy: nonproliferative and proliferative. Nonproliferative retinopathy is the early stage in which hyperglycemia weakens the microvascularature of the retina. The vessels develop microaneursyms (as mentioned above) that may rupture into the vitreous humor. Proliferative retinopathy is the later and more severe stage of diabetic retinopathy. The main feature of proliferative retinopathy is the formation of new blood vessels. These new vessels can arise from arteries or veins and can spread out within the retinal layers or push forward into the vitreous. The new vessels are extremely fragile and are prone to rupture. As the vessels mature, the fibrous

component becomes more prominent, leading to constriction. This causes distortion of the retina and a potential retinal detachment [105].

HSC Role in Diabetic Retinopathy

In early 2001, our laboratory began a collaborative effort with the laboratory of Dr. Maria Grant. Our goal was to show definitive evidence that the hemangioblast existed within the adult bone marrow compartment, and that the HSC itself could provide hemangioblast activity. Using a unique murine model for retinal neovascularization that closely mimics the pathology seen in human diabetic retinopathy, we have shown that the HSC could indeed be plastic enough to participate in new blood vessel formation in an ischemia challenged retina. This study was of great importance because it was the first to demonstrate that retinal neovascularization results not only from the local endothelial cells participating in the normal process of angiogenesis, but also relies on the participation of bone marrow-derived progenitor cells to aid in vasculogenic means. It also defined our unique murine model as one of the best models to study diabetic retinopathy [112].

Using this unique murine model, we have begun to try to understand the angiogenenic factors that regulate the recruitment and incorporation of HSC and their circulating progenitor cells to new vessel formation. This effort may provide additional ways to influence the process of neovascularization. Furthermore, our experiments may lead to the development of new therapeutic regimens used to intervene in HSC participation to block unwanted vessel formation as in diabetic retinopathy.

CHAPTER 2
GENERAL METHODS

This chapter discusses the generation of an adult mouse model of proliferative retinopathy. The approach of combining VEGF-A administration (via an intravitreal protein injection or delivery of an rAAV2-VEGFA expression vector) with laser occlusion of retinal vessels to induce ischemia produces significant levels of retinal neovascularization. This adult model of retinal neovascularization failed if VEGF-A administration or laser-induced ischemia was used alone. The combination of both stimuli produced the formation of new blood vessels throughout the area of ischemia including new vessels intruding into the vitreous of the eye (preretinal neovascularization) thereby generating a phenotype that is highly reminiscent of the proliferative stage of diabetic retinopathy. The adeno-associated virus serotype 2 expression system chosen was based on evidence that serotype 2 preferentially infects Muller and retinal ganglion cells, which are thought to be the source of VEGF that initiates diabetic retinopathy in humans.

The methods described in this section have been used extensively throughout my entire graduate school career. Many of the general methods here have been used to help develop our unique animal model for proliferative retinopathy. This chapter will discuss all methods in details

Generating the GFP/BL6 Chimera

Isolation of Whole Bone Marrow

The generation of the GFP/BL6 chimera animals requires extensive animal use and cell manipulation. The donor strain is on a BL6 background which carries a green fluorescent protein (GFP) driven by chicken beta-actin promoter and CMV intermediate early enhancer and is ubiquitously expressed. All donor animals are male. Recipient animals are BL6 females that were obtained from Jackson Laboratories (Bar Harbor, Maine) and were at least 5 weeks old at the time of bone marrow transplantation. Recent controversy concerning the events during stem cell transdifferentiation for repair has led to the possibility that this may not be due to an inherent ability of the stem cells, but rather a fusion event occurring between the stem cell and target tissue. The transplantation of male HSC into female recipients directly addresses this issue by allowing for fluorescent *in situ* hybridization of tissue samples looking for the Y chromosome and determination if a fusion event has occurred. After fully-grown (> 6 weeks of age) GFP males were euthanized and sacrificed, the long bones in the legs were immediately removed. All muscle, tendon, and ligature were dissected from the bones and were immediately placed in ice-cold PBS. Each bone end was then pruned back about 1-2 millimeters to expose the hollow core of the marrow space. The bone marrow was flushed out into a tissue culture treated plate by inserting a 26-gauge needle into one end of the bone and washing 1-2 mL of Dulbecco's Modified Eagle's Medium (Gibco) through the hollow bone core. The cells were kept on ice at all times. The marrow was then manipulated into a single cell suspension with a 26-gauge needle. The marrow was then allowed to adhere to a tissue culture treated plate (Gibco) for 120 minutes. This step allows for an initial enrichment of HSC from other adherent progenitor cells such as

mesenchymal stem cells (MSC) since hematopoietic progenitor and stromal cells adhere to the tissue culture treated plastic, while HSC will remain suspended in the media. The complete volume of media containing the nonadherent HSC was then gently drawn up, washed in >10mL volume of cold media, and pelleted by centrifugation at 1000 x g performed at 4 degrees Celsius.

Purification of HSC for Whole Bone Marrow

Initial HSC purification was done through the sorting of the cells by magnetic beads using the Milteny Magnetic Activated Cell Sorting (MACS) system. Briefly, cells were stained with an antibody conjugated to a magnetic bead. The antibody, and subsequently the bead, is bound to the cell. When these cells are then run over a column in the presence of a magnetic field, those cells that have the specific surface antigens, and thus the antibody-bead bound to them, will adhere to the column (termed positive fraction). Cells that do not present that surface marker (negative fraction) will pass directly through the magnetic field and be removed from the positive fraction of cells. The magnetic field can then be removed and the positive fraction collected from the column.

To begin the MACS enrichment, cell number and viability were determined from the total marrow flushed from the long bones to ensure that the correct amount of antibody, beads, and staining volume will be used. To determine the cell number and viablility, washed cells were resuspended in trypan blue and bright cells were counted using a hemacytometer under a phase-contrast microscope. The enumerated cells were then washed in >10mL cold PBS and stained with a 100µl of lineage cocktail (B220, CD3, CD4, CD8, CD11B, GR-1, and TER-119) microbeads (supplied by Dr. Bill

Slayton). The cells were run over 3 separate columns to insure enrichment, and the flow-through was retained and at this time a >90% lineage negative purity typically was achieved. After lineage depletion, cells were immediately pelleted and placed back on ice for fluorescent antibody staining for FACS sorting.

For further HSC purification I used two different fluorochromes: C-KIT conjugated to APC and Sca-1 conjugated to PE (Pharmingen). All antibody concentrations and incubation times were followed according to the parameters described by the manufacturer guidelines. The FACSvantage SE is able to isolate single cells based on the surface antigen bound by antibodies and hence the spectrum of absorbance and fluorescence emitted by that cell. The flow rate was set at 10,000 events per second with no greater than a 10% abort proportion. These cells were then collected in media immediately after completion of the sort and pelleted.

During the sorting step described above, recipient animals (female BL6) were lethally irradiated with 950 RADS of gamma radiation. Gamma radiation nicks the DNA of cycling cells within the bone marrow. These cells then undergo apoptosis clearing the bone marrow compartment of host cells. This allows donor HSCs to take up resisdence within the recipients' bone marrow and establish hematopoiesis. Finally, all lethally irradiated BL6 animals were anaesthetized, and were injected with 100 highly enriched SKL (Sca-1+, c-kit+, Lin -) cells in the retro-orbital sinus cavity. The animals were monitored until they overcame the effects of the anesthetic and then were placed on a regime of antibiotics for the one month.

Verification of Multilineage Reconstitution

The recipient animals were given six months for the HSC to home to the bone marrow niche and begin to divide to produce progenitor cells which will contribute to the

various hematopoietic cell lineages. Determination of engraftment was resolved by peripheral blood sampling and FACS analysis to determine whether the marrow was repopulated or if the animal's native marrow recovered. Each animal had a peripheral blood sample drawn through a tail vein bleed and the blood was collected in a tube containing PBS and 5mM EDTA to act as an anticoagulant. The erythrocytes were removed with a FICOLL PLAQUE (Amersham Biosciences) purification. Briefly, the blood/PBS sample was layered on top of two times greater volume of FICOLL. The emulsion was centrifuged and the "buffy" layer containing the nucleated cells at the interface was removed. The lymphocyte layer containing the nucleated cells was washed in 5X volumes of PBS and stained with the various lineage marker antibodies conjugated to PE (CD3, CD11b, and B220). Samples were analyzed by FACS caliber, and animals exhibiting GFP positive cells of the various lineages were scored positive for engraftment. The controls used were C57/BL6 and Gfp mice that either had the "buffy" layer stained with various lineage markers or did not have the "buffy" layer stained. The Gfp "buffy" layer stained with various lineage markers helped determine what an engrafted animal "should" resemble. The positive animals were then monitored at the end of all experimental models, where multi-lineage reconstitution was reconfirmed to demonstrate long-term engraftment by HSC.

Induction of Retinal Ischemia

Induction of retinal ischemia involves the administration of an endogenous cytokine and vessel damage in order to promote blood vessel growth in the retina. GFP/BL6 chimeric animals were selected and anaesthetized. SDF-1 (75ng/μl) or (2 x 10^8 particles) AAV-murine VEGFA 188 (VectorCore, UF), where CMV promoter drives

expression of VEGF in an Adeno Associated Vector, was administered directly into the vitreous using a 36-gauge needle and Hamilton syringe. VEGF is an endothelial cell-specific mitogen which is transcriptionally regulated by the cytomegalovirus promoter/enhancer when packaged in AAV. AAV mediates long-term expression in nondividing cells, which allows for stable expression and constant amounts of VEGF to reach the area of ischemia to promote neovascularization.

Peak expression of VEGF by AAV has been determined to be at 3-6 weeks, therefore the physical disruption of the blood vessels was done during this time (unpublished data). First, mice were anaesthetized normally with a general anesthetic, and concurrently a 10% sodium fluorescein (Akorn) solution was administered intraperitineally. This dye labels blood vessels facilitating visualization during photocoagulation. The eyes were dilated with 1% atropine (Akorn) for 5 minutes, washed with PBS (Gibco), and subsequently dilated with 2.5% phenylephrin (Akorn) for 5 minutes. Immediately after the two 5 minute treatments the mice underwent laser treatment. An Argon Green laser system (HGM Corporation) was used for retinal vessel photocoagulation with the aid of a 78-diopter lens. The blue-green argon laser (wavelength 488-514 nm) was applied to various venous sites juxtaposed the optic nerve. The venous occlusion was accomplished with >60 burns of 1-sec duration, 50 mM spot size, and 50-100 mW intensity. The animals were allowed to recover for 30 days while the transplanted HSC, directed by the ischemia and induced by the VEGF, contributed to the neovascularization in order to relieve the hypoxia produced by the cauterizing of the existing vessels.

One month after ischemic injury the eyes were ready to be enucleated and neovascularization imaged by confocal microscopy or by hemotoxylin and eosin (HnE) staining. Mice were first anesthetized and then perfused while sedated. Peripheral blood and bone marrow were collected to confirm donor contribution analysis by FACS with lineage specific antibodies conjugated to PE (BD BioSciences) similarly to the procedure outlined above. First, the chest cavity was opened and the ribs cut away to expose the heart completely. The left atrium was punctured with a 26-gauge needle and injected with >3 mL of 50 mg/mL rhodamine isothiocyanate (RITC)-conjugated dextran (160,000 avg. MW, Sigma Chemical) in phosphate-buffered formaldehyde, pH 7.4. The perfusion was performed slowly into the left ventricle and is integral for the functional assay. Immediately afterwards the eyes were removed by sliding a curved forceps underneath the eyeball and pulling the globe out. For confocal imaging, the eyes were punctured with a 26-gauge needle to allow complete perfusion. The eyes were placed in fresh 4% PFA and shaken at room temperature for 30 minutes. The globes were then transferred to 1X PBS and washed by shaking at room temperature for 30 minutes to overnight. After washing with PBS the eyes were dissected. The eyes were placed under a surgical microscope and an initial incision was made in the cornea. The opening was enlarged until it could accommodate the lens of the eye. The lens was gently pushed forward until it exited through the hole cut in the cornea. The remaining cornea was then trimmed to where the sclera and cornea meet. The retina was dissected away from the retina pigment epithelial (RPE). The retina then detached and was readily mounted. The thickness of the retina (>200um) prevents adequate perfusion of antibody, therefore the retina was placed on a glass slide and 5-6 cuts were made around the periphery so that the retina lies

flat when mounted. The tissue was placed in Vectashield mounting medium (Vector Laboratories) to inhibit photo-bleaching. The retinas were immediately imaged. An Olympus IX-70, with inverted stage, attached to the Bio-Rad Confocal 1024 ES system for fluorescence microscopy was used for analysis. A Krypton-Argon laser with emission detector wavelengths of 598nm and 522nm differentiated the red and green fluorescence. The lenses used in our system were the (Olympus) 10X/0.4 Uplan Apo, 20X/0.4 LC Plan Apo, 40X/0.85 Uplan Apo, 60X/1.40 oil Plan Apo and 100X/1.35 oil Uplan Apo. The software was OS/2 Laser Sharp.

HnE was performed on eyes that were not imaged by confocal microscopy. Enucleated eyes were placed in 4% buffered PFA overnight. The next day the eyes were transferred to 30% sucrose until they sank. The eyes were then embedded in O.C.T. embedding medium (Sakura Finetechnical Co) and flash frozen in dry ice with methylbutane and placed in -80^{o}C for 24 hours. Once completely frozen, the eyes were sectioned and stained following the standard HnE protocol. Sections were imaged by light microscopy (Leica TCS SP2) and images were taken using an Optronics camera system and the software Magnafire.

Administration of SDF-1 Antibody

Immediately following laser photocoagulation, as described above, cohorts (n=10) of GFP/BL6 chimeric mice underwent intravitreal injections into the right eye, or lasered eye. Mice were anesthetized, and a SDF-1 neutralizing antibody (MAB 310, R&D Systems) or PBS plus isotype control was injected intravitreally (2μl total volume) to achieve a final concentration of 1μg/μl or 0.1μg/μl of antibody in the vitreous. A 36-

gauge needle and a Hamilton syringe were used for the administration of the antibodies. Some additional cohorts were given weekly booster injections for four weeks.

Triamcinolone Treatment

Vitreous samples were obtained at the time of vitreous aspiration for treatment with Triamcinolone in 46 patients with diffuse macular edema. Vitreous samples from nondiabetic patients having vitrectomy surgery for macular pucker and epiretinal membrane were used as controls. All patients received the standard treatment for DME with removal of 0.2 cc of liquid vitreous and injection of 4mg (0.1-0.2 cc) of Triamcinolone. Triamcinolone was injected through the par plana with the remaining volume replaced with a balanced salt solution. Vitreous aspirates that were collected were frozen at -20^{o} C until analysis. This experimental protocol was performed by the laboratory of Dr. Maria B. Grant.

Measurement of Intravitreal SDF-1 Levels in Patients

We obtained vitreous samples at the time of vitreous aspiration prior to and during treatment for DME with triamcinolone in diabetic patients. Patients were classified with respect to the status of their diabetic retinopathy, gender and duration of diabetes. All patients' protocols and consents were fully IRB reviewed and approved.

Levels of SDF-1 were measured using a commercially available ELISA assay (R&D Systems). Each sample (0.05cc) was run in triplicate and compared with a standard curve. All samples were assigned a random number and run without knowledge of disease or treatment status. Once the data were compiled, the sample classifications were revealed. The mean concentration was determined per sample and per group classification. Data received both a chi-square and rank statistical analysis to determine

significance. This experimental protocol was performed by the laboratory of Dr. Maria B. Grant.

Isolation of Protein from SDF Treated Cells

Human retinal endothelial cells (HRECs) were grown in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum. Cells were grown in a 37^{o}C incubator with 5% CO_2. The HRECs cultures were washed twice with ice cold PBS (Biowhittaker, Walkersville, MD) and scraped in lysis buffer (20mM Tris-HCl [Biorad Laboratories Inc.], 1mM EDTA [Sigma Aldrich, ST. Louis, MO], 255mM sucrose [Fisher Scientific, Atlanta, GA], 1% Igepal CA-630 [Sigma Aldrich, St. Louis MO], 1% protease inhibitor cocktail [Sigma Aldrich, St. Louis, MO]). The lysed cells were sonicated (Sonic dismembrator, model 100; Fisher Scientific) for 2 seconds and centrifuged (5415D eppendorf; Fisher Scientific) at 13,200 rpm for 5 minutes at 4°C. The pellet was discarded and the amount of protein was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). This experimental protocol was performed by the laboratory of Dr. Maria B. Grant.

ELISA for VCAM-1

HRECs were grown in Dulbecco's Modified Eagle's Medium (Gibco) and supplemented with 10% fetal bovine serum. Cells were cultured beyond confluence for three weeks to establish tight cellular junctions. Cells were grown in a 37^{o}C incubator with 5% CO_2. Triplicate HREC cultures were then treated for 48 hours with varying concentrations of SDF-1 and total protein extracts were prepared as indicated above. Each triplicate assay was repeated a total of three times. Equal amounts of protein were used for vascular cell adhesion molecule 1 (VCAM-1). ELISA assay for VCAM-1 was

performed according to the manufacturer's instructions (R&D Systems). This experimental protocol was performed by the laboratory of Dr. Maria B. Grant.

Determination Occludin Levels in SDF Treated Cells

HRECs were obtained from two independent donors and were cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum. Cells were grown in 37^{o}C incubator with 5% CO_2. Cells were grown to confluency and protein was isolated as mentioned above. A total of 50μg of total protein was blotted to a nitrocellulose membrane (Millipore Corp., Bedford, MA) and a western blot performed according to the manufacturers instructions. For Occludin detection the membrane was incubated with a 1:125 dilution of a rabbit polyclonal anti-occludin antibody (Zymed Laboratories incorporated, San Francisco, CA). Following occludin detection, the membrane was also used to detect β-actin protein levels using a 1:5000 dilution of mouse monoclonal anti-β-actin antibody (Sigma-Aldrich) and an HRP conjugated anti-mouse IgG secondary antibody (Sigma-Aldrich). The protein bands were visualized with an enhanced chemiluminescence (ECL) Western Blot Detection Kit (Amersham Biosciences Ltd., Amersham, UK). Standard molecular weight markers (Bio-Rad Laboratories Inc) served to verify the molecular size of occludin at 65 kDa and of β-actin at 42 kDa. Analysis of occludin and β-actin protein levels was performed using "Image" analysis software (Scion Corp., Frederick, MD). This experimental protocol was performed by the laboratory of Dr. Maria B. Grant.

Isolation of Tissues for SDF-1 Elisa

The tissues that were collected for the detection of SDF-1 by Elisa included bone marrow, serum, and vitreous fluid. The isolation of whole bone marrow was performed

as previously mentioned. The erythrocytes were removed with a FICOLL PLAQUE (Amersham Biosciences) purification. Briefly, the bone marrow/PBS sample was layered on top of two times greater volume of FICOLL. The emulsion was centrifuged and the "buffy" layer containing the nucleated cells at the interface was removed. The lymphocyte layer containing the nucleated cells was washed in 5X volumes of PBS. The nucleated cells were then counted using a hemacytometer. 2.5×10^5 cells were collected from each animal. These cells were pelleted at 1,100 rpm at 4^oC for 5 minutes. The supernatant was discarded and the cells were resuspended in 500 μl of a protease cocktail inhibitor (BD Biosciences)/PBS solution. Cells were sonicated using a Sonifier 450 (Branson) for 2 seconds (20% duty cycle at level 4 output control). Samples were immediately placed at -80^oC until time of analysis. Serum was collected by isolating peripheral blood from the retro-orbital sinus cavity. This method was easily accomplished by anaesthetizing the mice and slightly breaking the vascular bed of the retro-orbital sinus cavity using a Natelson blood collecting tube (Fisherbrand). Blood flowed freely by capillary action and is collected in 4ml Falcon tubes (BD Falcon). The collection tubes were pre-coated with heparin so that the blood would not clot during the collection process. The collected blood is placed in a 4^oC refrigerator over night to allow the red blood cells to clot. The next day the samples were centrifuged at 1,500 rpm at 4^oC for 20 minutes. Serum was collected using a pipetman, serum was the clear top layer. Samples were immediately placed at -80^oC until time of analysis. Vitreous fluid was collected by anaesthetizing the mice and using a 36-gauge needle and Hamilton syringe. The needle was placed directly into the vitreous and 5 μl of vitreal fluid was removed. The fluid was placed in a 1.5 mL collection tube. 45 μl of PBS were added to

the tubes for a final volume of 50 μl. Samples were immediately placed at –80^{o}C until time of analysis. Once all the samples were collected (bone marrow, vitreous, serum) and placed at –80^{o}C, the samples were placed on ice and allowed to thaw. All samples were analyzed for SDF-1 using ELISA (R&D Systems). ELISA assay for SDF-1 was performed according to the manufacturers instructions (R&D Systems).

Isolation and Injection of CD133+/GFP Bone Marrow Cells

The isolation of whole bone marrow was performed as previously mentioned. The erythrocytes were removed with a FICOLL PLAQUE (Amersham Biosciences) purification. Briefly, the bone marrow/PBS sample was layered on top of two times greater volume of FICOLL. The emulsion was centrifuged and the "buffy" layer containing the nucleated cells at the interface was removed. The lymphocyte layer containing the nucleated cells was washed in 5X volumes of PBS. The lymphocyte layer was then resuspended in 100 microliters of PBS and stained with an antibody to CD133 directly conjugated to PE (Pharmingen) according to the manufacturer's guidelines. Briefly, 5 microliters of CD133-PE was added to every 10^{7} cells counted by hemacytometer. Samples were then place at 4^{o}C and protected from light for 20 minutes. The samples were centrifuged to pellet the cells at 1,100 rpm at 4^{o}C for 5 minutes and then washed in five volumes of PBS. The cells were FACS sorted using the FACSvantage SE as previously described.

The CD133+/GFP were sorted the day after mice had undergone the laser photocoagulation phase of the induction of retinal ischemia, as previously mentioned. These mice were unmanipulated BL6 mice and did not undergo a bone marrow

transplant. The mice were anaesthetized and 2,000 CD133+/GFP were injected into the tail vein of the mice. The eyes were analyzed as previously described.

Isolation and Preparation of Bone and Eye for Immunohistochemistry

BL6 mice that were not manipulated but underwent every phase of induction of retinal ischemia were anesthetized and then perfused while sedated. First, the chest cavity was opened and the ribs cut away to expose the heart completely. The left atrium was punctured with a 26-gauge needle and injected with >3 mL of phosphate-buffered formaldehyde, pH 7.4. The perfusion was performed slowly into the left ventricle. Immediately following the perfusion, the long bones in the legs were removed and the eyes were removed by sliding a curved forceps underneath the eyeball and pulling the globe out. All muscle, tendon, and ligature were dissected from the bones. Both bones and eyes were immediately placed in 3mL of 4% PFA and placed in 4^{o}C refrigerator over night. Bones and eyes were transferred to 3mL of 70% ethanol and placed in 4^{o}C refrigerator over night. The following day, the bones and eyes were given to the Pathology Core for paraffin embedding.

Sectioning and Preparation of Paraffin Embedded Tissues

Both bones and eyes were sectioned and prepared in the same fashion. A cold plate (Tissue Tek II) was removed from -20^{o}C freezer and wet paper towels were placed on top of the cold plate. Once paper towels were cold to the touch, paraffin embedded samples were placed on the paper towels for 15 minutes. This allowed for the sectioning blade to pass smoothly through the paraffin wax. Samples were sectioned using a Microm sectioning apparatus (Heidelberg) at a thickness of 5 microns. Once the tissues were exposed, the samples were once again placed on the cold plate with wet paper towels for 15 minutes. This once again allowed for the sectioning blade to pass smoothly through

the paraffin wax, but also made sectioning of the tissue more smooth. Samples are then sectioned further, until desired number of sections was acquired. Paraffin sections were placed in a 42^{o}C water bath (Triangle Biomedical Sciences) and were guided onto glass slides. The glass slides with sections were placed in vertical slide holders and allowed to dry over night at room temperature.

Immunohistochemistry for SDF-1 and HIF-1α on Whole Eye Sections

Slides that were allowed to air dry over night are pretreated to deparaffanize and for retrieval of the antigens of interest (SDF-1 and HIF-1a). To deparaffanize, we simply ran our slides through a series of dips in different solutions:

Xylene 2X for 5 minutes
100 % Ethanol 2X for 2 minutes
95 % Ethanol for 2 minutes
70% Ethanol for 1 minute
H_2O twice for 1 minute (Keep in H_2O until you are ready for the retrieval step)

We found that the best retrieval method for both SDF-1 and HIF-1a was to submerge the deparaffanized slides in a container filled with citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0). We next placed this container in another container filled with water. This setup was then placed in a GE microwave oven and set to 50% power for seven minutes. Once the seven minutes was up, we kept the slides in the microwave for an additional 18 minutes, for a total of 25 minutes. The slides were then removed from the citrate buffer and rinsed twice with a Tris/Saline buffer. (Note: The slides are never allowed to dry. If the tissue on the slides dries, there is an increased potential for unspecific binding of your antibodies.) We removed excess buffer and blocked the slides with horse serum (15 μl/mL) for 20 minutes. This step decreases the potential of unspecific binding of the secondary antibodies. After the 20 minutes, excess

serum was blotted from slides, and slides were placed with SDF-1 primary antibody (Santa Cruz) and HIF-1a primary antibody (Novus) at a dilution of 1:40 for each. Since the SDF-1 antibody was made in goat and the HIF-1a antibody was made in rabbit, set up goat and rabbit IgG controls (Pharmagin) at dilutions of 1:500. Primary and IgG control antibodies were diluted using Tris/Saline buffer. Incubation time for all the antibodies are overnight and temperature was at 4^{o}C. The following day, slides were placed at room temperature and washed 3X for 5 minutes with Tris/Saline buffer. Blott excess buffer and stain slides with fluorescent anti-primary species (Donkey anti-Goat 594{red} for SDF-1 and Donkey anti-Rabbit 488{green} for HIF-1a). Fluorescent anti-primary antibodies were diluted 1:200 using Zymed diluent. The incubation period for this stain is 60 minutes at room temperature in a staining box that protects from the light. Once 60 minutes is up, wash slides 3X for 3 minutes using Tris/Saline buffer at room temperature (Remember to protect from light). Remove all excess buffer and place one drop of Vectashield with Dapi (counterstain) mounting media and cover with glass cover slip. Place slides in slide folder and put in 4^{o}C until ready for use. Fluorescent is good for approximately two weeks.

Immunohistochemistry for SDF-1 on Bone Sections

Slides that were allowed to air dry over night were pretreated to deparaffanize and retrieval of the antigens of interest (SDF-1). To deparaffanize, we simply ran our slides through a series of dips in different solutions:

Xylene 2X for 5 minutes
100 % Ethanol 2X for 2 minutes
95 % Ethanol for 2 minutes
70% Ethanol for 1 minute
H_2O twice for 1 minute (Keep in H_2O until you are ready for retrieval step)

We found that the best retrieval method for SDF-1 in the bone was to place the slides in Target Retrieval Solution, High pH (pH 9.9, Dako). These slides were then placed in a 37^{o}C water bath for over night. The slides were then removed from the Target Retrieval Solution and rinsed 2X with a Tris/Saline buffer. (Note: The slides are never allowed to dry. If the tissue on the slides dries, you have an increase potential for unspecific binding of your antibodies.) We remove excess buffer and block the slides with horse serum (15 l/mL) for 20 minutes. This step decreases the potential of unspecific binding of the secondary antibodies. After the 20 minutes, excess serum was blotted from slides and placed SDF-1 primary antibody (Santa Cruz) at a dilution of 1:40. Since the SDF-1 antibody was made in goat, set up Goat and IgG controls (Pharmagin) at dilutions of 1:500. Primary and IgG control antibodies were diluted using Tris/Saline buffer. Incubation time for all the antibodies were overnight and the temperature was at 4^{o}C. The following day, slides were placed at room temperature and washed 3X for 5 minutes with Tris/Saline buffer. Blott excess buffer and stain slides with fluorescent anti-primary species (Donkey anti-Goat 594{red} for SDF-1). Fluorescent anti-primary antibody were diluted 1:200 using Zymed diluent. The incubation period for this stain is 60 minutes at room temperature in a staining box that protects from the light. Once 60 minutes is up, wash slides 3X for 3 minutes using Tris/Saline buffer at room temperature (Remember to protect from light). Remove all excess buffer and place one drop of Vectashield with Dapi (counterstain) mounting media and cover with glass cover slip. Place slides in slide folder and put in 4^{o}C until ready for use. Fluorescent was good for approximately two weeks.

CHAPTER 3
SDF-1 IS BOTH NECESSARY AND SUFFICIENT TO PROMOTE PROLIFERATIVE RETINOPATHY

Introduction

Diabetic retinopathy is a major cause of blindness among Americans under the age of 65. There are approximately 16 million diabetics in the United States, with nearly 8 million having some form of diabetic retinopathy. Diabetes is caused when the body can no longer produce enough insulin or is not able to utilize the insulin produced. Without insulin, blood sugar levels cannot be regulated and an increase of blood glucose levels occurs. These prolonged high levels of blood glucose in diabetic patients destroy the small blood vessels in the eye. As the vessels are damaged, vascular permeability increases, resulting in fluid leakage into the surrounding tissue, often resulting in a swelling. When swelling occurs in the macula of the eye (the area of the retina responsible for sharp central vision), vision can often become distorted. This condition is called macular edema. Further vessel deterioration results in poor blood flow and the onset of ischemia or oxygen starvation. Ischemia promotes new blood vessel proliferation in an attempt to restore blood flow. Vision loss during this proliferative stage of diabetic retinopathy is caused by aberrant neovascularization resulting in newly formed blood vessels intruding into the vitreous of the eye (referred to as preretinal neovasculatization). These new vessels destroy the normal retinal architecture and may hemmorrage, easily causing bleeding into the eye, ultimately impairing vision [113].

The mechanisms governing this aberrant neovascularization during diabetic retinopathy are still being elucidated. We have recently demonstrated in two murine models of ocular neovascularization that adult HSC function as hemangioblasts producing both blood cells and the circulating EPC that give rise to new blood vessels in the eye [112,114]. $CD34^+$ cells, which are highly enriched for human HSC, from umbilical cord blood also produce new blood vessels in a murine xenograft adaptation of our model [115]. In this study we use a unique murine model that induces adult onset retinal neovascularization that closely mimics the pathology of neovascularization observed in diabetic humans. Retinal neovascularization in the adult mouse requires the administration of exogenous VEGF in addition to ischemic injury to promote new vessel formation. We have also shown that chronic vascular injury alone can be sufficient to induce EPC production from adult HSC [116]. The cytokine VEGF is a major inducer of angiogenesis and the resultant migration of endothelial progenitor cells [117]. Within the retina, VEGF expression is increased in response to ischemia to promote vascular repair. VEGF induces vascular permeability, protease production, and promotes endothelial cell migration and proliferation– key steps in angiogenesis. VEGF is widely recognized as a potential therapeutic target for regulating angiogenesis [118, 119]. We were interested in investigating other cytokines/chemokines that may work in conjunction with VEGF to promote the recruitment of endothelial progenitors from remote locations such as the bone marrow into the ischemic retina. We examined the role SDF-1 in the process of retinal neovascularization. SDF-1 is the predominant chemokine that mobilizes HSC/Progeny and EPC [120-122]. SDF-1 has been shown to be upregulated in many damaged tissues as part of the injury response and is thought to call stem/progenitor cells

to promote repair [123]. We have shown that SDF-1 levels increase in diabetics with proliferative diabetic retinopathy (PDR) and that SDF-1 may play an important role in the migration of HSC-derived EPCs to the site of vascular injury by regulating molecules important in the injury/repair response. SDF-1 can also replace VEGF to drive retinal neovascularization in our murine model. Futhermore, blocking SDF-1 function can prevent neovascularization and may serve as an important advancement in the treatment of ocular disease such as diabetic retinopathy.

Results

Measurement of SDF-1 in Patients with Varying Severity of Diabetic Retinopathy

Previously we demonstrated that HSC can be a major source of endothelial progenitor cells [112]. We now postulate that SDF-1 plays a key role in the recruitment of these progenitors to sites of vascular injury to produce new blood vessels. We further hypothesize that retinal ischemia results in increased SDF-1 expression. Our data suggest that vascular permeability may be increased by angiogenic factors, such as SDF-1 and VEGF produced in response to ischemia. The increased permeability will allow for a portion of the SDF-1 produced by the damaged retina to leak into the vitreous of the eye. SDF-1 leaking into the vitreous may create an artificially high SDF-1 concentration gradient due to the relative lack of proteases within the vitreous [124]. New vessel growth would be directed into the vitreous by the SDF-1 gradient. If our hypothesis is correct, we postulate that the addition of SDF-1 protein in the eye should augment preretinal neovascularization within the vitreous. Conversely, blocking SDF-1 activity in the eye should abrogate preretinal neovascularization within the vitreous.

To test our first hypothesis we obtained vitreous samples from 46 patients undergoing treatment for diabetic macular edema (DME) with and without proliferative diabetic retinopathy (PDR). Forty-four of the 46 patients were type II diabetics. Vitreous samples from nondiabetic patients having vitrectomy for non-PDR related conditions were used as controls. ELISA were performed in a masked fashion to measure SDF-1 levels in the vitreous samples. Once the ELISA data was compiled the samples were matched with patients. The patients were graded by the severity of their disease into four categories: control samples (n=8 eyes), those with DME but no current PDR (n=30 eyes), DME with PDR (n=20 eyes), and those with neovascularization of the iris (NVI) representing the most fulminate version of the disease (n=4 eyes). As predicted by our hypothesis, SDF-1 increases with severity of the diabetic retinopathy in the patients (Figure 3-1). SDF-1 was undetectable by ELISA (sensitivity 18 pg/mL) in vitreous samples from control patients. Patients with fulminate NVI averaged >1,000 pg/mL of SDF-1 in their vitreous, or at least 50 fold the level found in normal eyes. Patients with DME and proliferating diabetic retinopathy averaged >200 pg/mL SDF-1 while those with only DME averaged 75 pg/mL SDF-1 in their vitreous. These results demonstrate that SDF-1 concentrations increase in the vitreous of patients with macular edema and diabetic retinopathy, and that SDF-1 concentration correlates with disease severity ($p<0.005$).

Corticosteriod Treatment Reduces SDF-1 Levels

Corticosteroids have been used for decades to suppress intraocular inflammation and to reduce blood vessel leakage [125, 126]. Triamcinolone or Kenalog (commercial name for triamcinolone acetonide) has been used intravitreally in two recent studies on

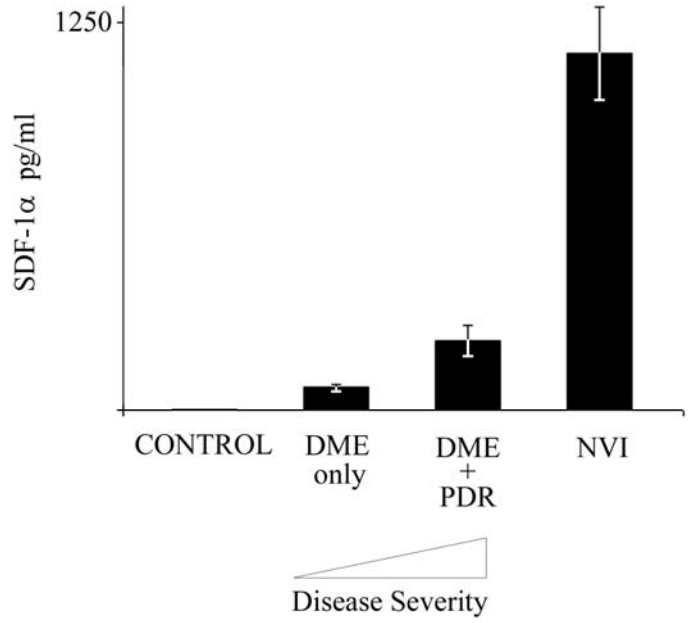

Figure 3-1.SDF-1 expression increases with severity of diabetic retinopathy. SDF-1 concentration in human patients with increasing severity of proliferative diabetic retinopathy. Human SDF-1α specifc ELISA assays were performed in triplicate on vitreous samples from patients with various stages of diffuse macular edema without (DME, n=30), or with (DME + PDR, n=20) proliferative diabetic retinopathy. The most fulminate stage of the disease is represented by patients with neovascularization of the iris (NVI, n=4). Control vitreous samples (n=8) were obtained from non-diabetic patients being treated for other ailments. All control samples were below the level of detection for the ELISA assay (18 pg/mL SDF-1).

DME and has been shown to decrease breakdown of the blood-retina barrier with a significant improvement in visual acuity [127, 128]. The mechanism by which traimcinolone achieves a therapeutic benefit remains unknown. We hypothesized that triamcinolone may reduce the expression of SDF-1 by damaged tissue. To test this hypothesis we assayed vitreous samples from our 46 patients after they received triamcinolone treatment for their DME. DME of our 46 patients was treated by administering 4mg of triamcinolone intravitreally in 0.2 mL balanced salt solution. In select patients with mild disease, repeat intravitreal taps were performed one month post

treatment. Patients with more severe disease such as NVI received multiple triamcinolone treatments with intravitreal samples obtained at each treatment. The vitreous samples were withdrawn, as the standard of therapy, prior to every triamcinolone injection to ensure maintenance of normal ocular pressure. These vitreous samples were from the same patients we previously assayed prior to treatment (Figure 3-1). After triamcinolone treatment the patients showed a uniform drop in SDF-1 levels in the vitreous to near the limits of detection (Figure 3-2). This suggests that reducing SDF-1 levels and the subsequent recruitment of circulating EPC may be one of the mechanisms of action for triamcinolone. Unfortunately, triamcinolone has serious side effects. Nearly one third of triamcinolone-treated patients develop glaucoma that requires treatment to prevent additional visual loss [129]. Therefore, more targeted therapies, such as directly blocking SDF-1 activity may provide optimized patient care.

Role of SDF-1 in Neovascularization

SDF-1 is one of the primary chemokines responsible for the homing of HSC to the bone marrow [122]. SDF-1 expression is induced by a wide variety of cell types in response to stimuli such as stress and injury [123, 130]. SDF-1 signals through its only known receptor CXCR-4, a transmembrane G-protein coupled receptor. VEGF induces increased CXCR-4 [87] expression from endothelial cells while SDF-1 induces VEGF expression in cells that are both hematopoietic and endothelial in origin [131, 132]. Chemotaxis assays have shown that purified endothelial progenitor cells migrate along an SDF-1 concentration gradient *in vitro* [133-135]. Retinal endothelial cells are a more relevant cell type when testing if SDF-1 has any effect in our unique murine model of

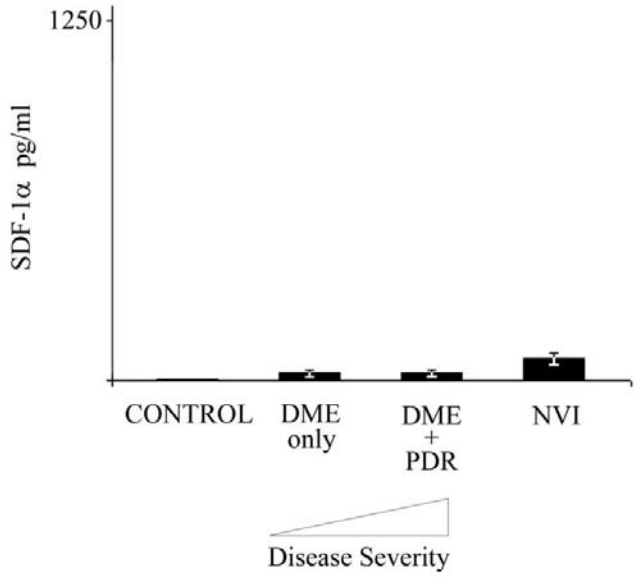

Figure 3-2.SDF-1 concentrations in the vitreous of human patients after treatment with triamcinolone. All patients were treated with at least on round of triamcinolone (4mg) injections intravitreally. Vitreous samples were obtained one month post treatment. Human SDF-1α specifc ELISA assays were performed in triplicate on the vitreous samples. The results are presented according to the severity of the patients original disease. Diffuse macular edema (DME, n=30). DME with proliferative diabetic retinopathy (DME + PDR, n=20). The most fulminate stage of the disease is represented by patients with neovascularization of the iris (NVI, n=4). Control vitreous samples (n=8) were obtained from non-diabetic patients being treated for other ailments. All control samples were below the level of detection for the ELISA assay (18 pg/mL SDF-1).

ischemic retinopathy. We have shown by ELISA that an increase in SDF-1 expression results in a significant increase ($p<0.007$) of vascular cell adhesion molecule (VCAM-1) on retinal endothelial cells (Figure 3-3). An increase in VCAM-1 plays an important role in HSC homing to and mobilization from the bone marrow by allowing for firm adhesion to the activated bone marrow endothelium [136]. We also studied the effects SDF-1 had on retinal endothelial cells and on gap junction proteins. Western analysis indicated that as SDF-1 levels are increased, the expression of occludin by retinal endothelial cells is decreased. Occludin is a gap junction protein responsible for tight junctions between

endothelial cells to prevent leakage of vessel contents into the surrounding tissue (Figure 3-4). These data suggest that SDF-1 acts at several key steps in the process of ischemic repair, such as recruitment of EPC from the marrow, an increase in VCAM-1 expression to promote EPC adhesion, and a decrease in tight junctions to allow EPC to extravagate to the site of ischemia.

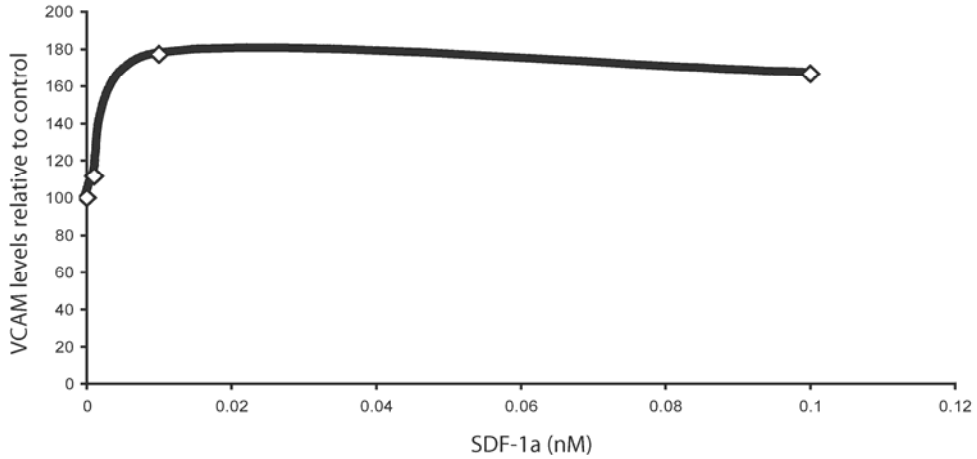

Figure 3-3. SDF-1 increases VCAM-1 expression on endothelial cells. Human retinal endothelial cells (HRECS) upregulate VCAM-1 in response to SDF-1. HREC were isolated from two separate donors (a) 43 year old donor and (b) 53 year old donor. The HREC were cultured in endothelial growth medium containing 10% FCS (EGM) for three weeks in order to establish super-confluent cultures. Control super-confluent HREC cultures were treated with reduced serum (RS) medium or continued in endothelial growth medium (EGM). Test super-confluent HREC cultures were treated with increasing concentrations of SDF-1 protein in RS medium. All treatments were for 48 hours. Cells were harvested in extraction buffer and equal quantities of total protein were used in ELISAs to check for the expression of VCAM-1. No changes in VCAM-1 expression were seen in either control group. Therefore, results were normalized to the combined average of both control groups and are expressed as percent of control. Increasing levels of SDF-1 upregulates the expression of VCAM-1 on HRECs.

SDF-1 Enhances Neovascularization in Ischemic Retinopathy

In order to support our hypothesis that SDF-1 is significant in the progression of proliferative retinopathy, we tested if the administration of exogenous recombinant SDF-1 protein (R&D Systems) could promote neovascularization. To test this hypothesis we

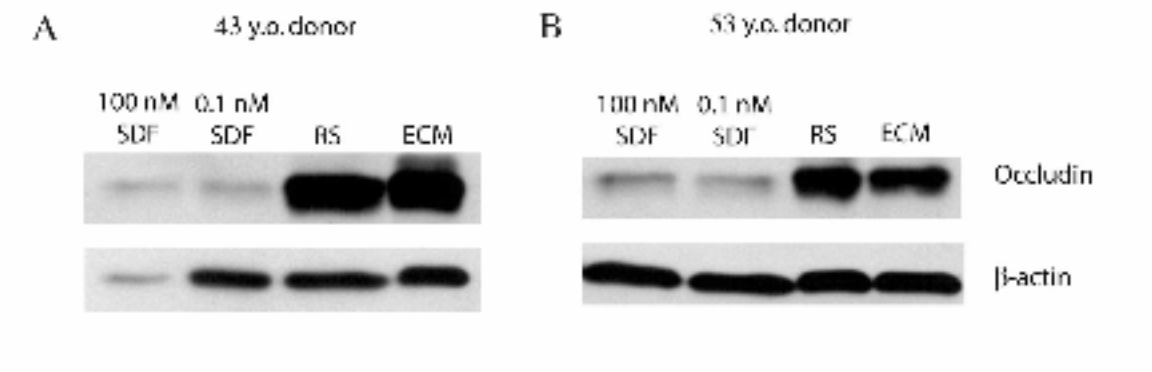

Figure 3-4.SDF-1 reduces occludin expression on endothelial cells. HREC were isolated from two separate donors (a) 43 year old donor and (b) 53 year old donor. The HREC were cultured in endothelial growth medium containing 10% FCS (EGM) for three weeks in order to establish tight cellular junctions. The test cultures were treated for two days with either EGM with reduced serum of 1% (RS), or with EGM, 1%FCS plus either 0.1nM SDF-1 or 100nM SDF. Cells were harvested in extraction buffer and equal quantities of total protein separated by SDS-polyacrylamide gels followed by transfer to nitrocellulose and immuno-blotted for occluding and β-actin (loading control) levels.

utilized our murine model that mimics the pathology seen in PDR in an adult mouse. The model requires the administration of growth factor and injury. The model allows us to tag new vessel formation with Gfp^+ cells and serves as an important tool in investigating the underlying mechanisms of proliferative retinopathy. There is no evidence that cell fusion plays a role in the development of functional blood vessels in our system, but this important point is still being investigated. The basic model has been previously described [112]. The model was modified by replacing the administration of rAAV-VEGF with the administration of rSDF-1 protein at a concentration of 75 pg/ul within the vitreous. The 75 pg/ul dose was chosen to match the lowest concentration of SDF-1 found the vitreous of patients with proliferative diabetic retinopathy (Figure 3-1). Weekly injections were performed up to four weeks post laser in order to sustain the concentration of SDF-1 in the vitreous. Exogenous SDF-1 was able to enhance Gfp^+ HSC-derived EPC migration and incorporation into the sites of ischemic injury (Figure 3-5). We also observed the

recruitment of a large population of Gfp^+ cells that were incorporated outside of the retinal vasculature. They could be inflammatory cells, such as neutrophils, that have an increase in their migratory response towards SDF-1 [137] due to the administration of exogenous rSDF-1 protein, but the time of analysis makes this unlikely. The increase in exogenous rSDF-1 protein could have also recruited a surplus of retinal astrocytes, which are cells that serve as a template for injury-associated retinal angiogenesis [138] and have been shown to promote retinal angiogenesis [139].

Prevention of Neovascularization in Ischemic Retinopathy

We next challenged the postulate that blocking SDF-1 should reduce retinal neovascularization from HSC-derived EPC by blocking their recruitment to the site of injury. To test this hypothesis we once again utilized our unique murine model. The basic model as described above without modification is used. To abrogate SDF-1 activity, we injected a cohort of 10 long-term engrafted animals with a SDF-1 specific blocking antibody in PBS (R&D Systems) into the vitreous at the time of laser injury. The injections were designed to yield a final antibody concentration of 1 µg/µl in the vitreous. Weekly booster injections of SDF-1 blocking antibody were given intravitreally during the ischemic repair phase. Two control cohorts of 10 animals, all with equivalent hematopoietic engraftment, received either no intravitreal injections – model control – or weekly intravitreal mock antibody injections with a PBS + IgG isotype control antibody. Both control cohorts yielded similar levels of HSC-derived contributions to retinal neovascularization. Thus indicating that the isotype control antibody had no effect on HSC derived neovascularization (Figure 3-6). Strikingly, the cohort treated with SDF-1

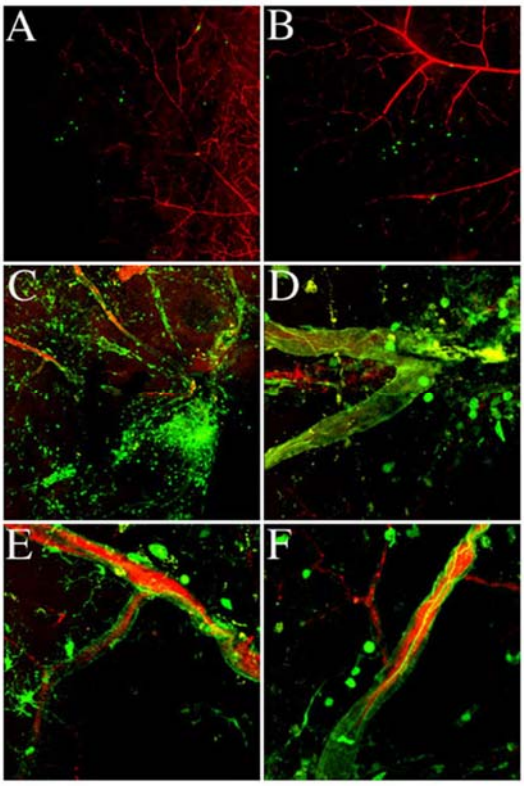

Figure 3-5.Recombinant SDF-1 protein enhances HSC-derived EPC migration and incorporation in sites of ischemia. Animals are perfused with a red fluorescent dye (RITC-dextran, Sigma) to delineate the vasculature. New blood vessels incorporated Gfp^+ HSC progeny thereby forming areas of green/yellow fluorescence. Gfp+ progeny suggestive of astrocytes or glia are also seen incorporated outside of the vascularlture. **(**a,b**)** Left or untreated eyes of two C57BL/6.gfp that were treated in their right eyes to induce retinal ischemia with administration of exogenous rSDF-1 protein (75pg/µl), and without exogenous AAV-VEGF. Note the lack of recruitment and incorporation of transplanted gfp^+ HSC progeny in the control untreated left eyes. (c-f) Right or treated eyes of four representative C57BL/6.*gfp* (including the right, treated eyes of the animals in (a,b)) in which retinal ischemia was induced and were injected intravitreally with rSDF-1 protein as a replacement for the rAAV-VEGF used in our standard model. Note the similar recruitment and incorporation of transplanted Gfp^+ HSC as in Model Control eyes where rAAV-VEGF was used in Figure 8.

blocking antibody had almost no HSC-derived blood vessels produced in response to VEGF bolus and ischemia injury. Confocal microscopy images from four independent test retinas are shown for each of the cohorts (Figure 3-6). Green or yellow vessels indicate the presence of HSC-derived endothelium [112]. Purely red vessels, like those

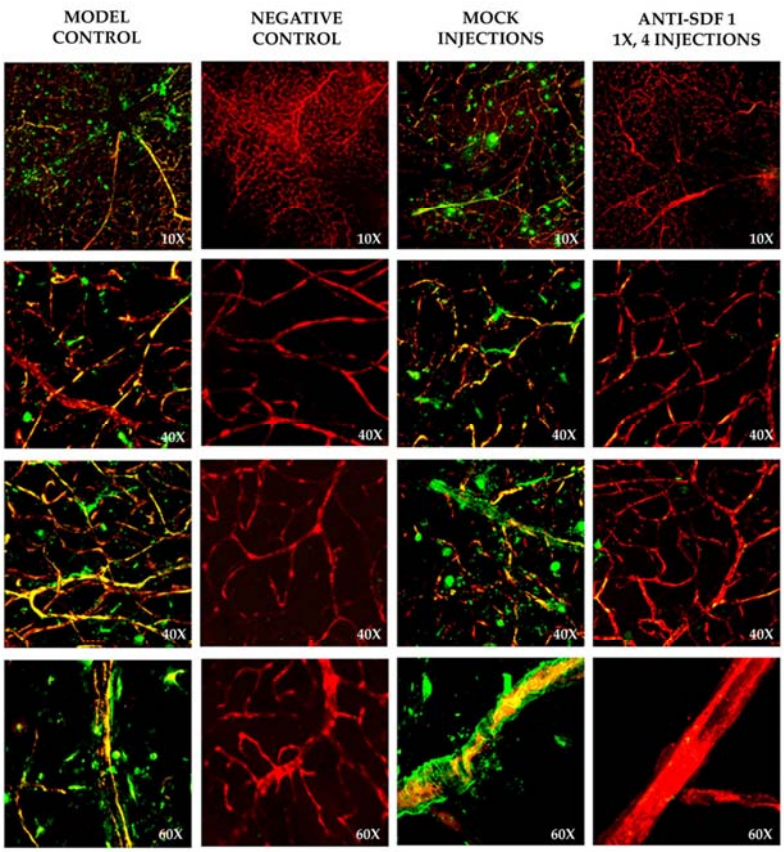

Figure 3-6. Anti-SDF 1 antibody prevents retinal neovascularization by HSC-derived circulating endothelial progenitors. All micrographs are merged confocal images of retinal flat mounts. Animals are perfused with a red fluorescent dye (RITC-dextran, Sigma) to delineate the vasculature. New blood vessels incorporate Gfp^+ HSC progeny thereby forming areas of green/yellow fluorescence. (Model Control) Right or treated eyes from four representative C57BL/6.gfp animals that underwent our standard retinal ischemia model. Gfp+ progeny suggestive of astrocytes or glia are also seen incorporated outside of the vascularture. **(**Negative Control) Left or untreated eyes of the same four C57BL/6.gfp that underwent our retinal ischemia model in their right eyes. Note the lack of recruitment and incorporation of transplanted gfp^+ HSC progeny. (Mock Injections) Right or treated eyes from four representative C57BL/6.*gfp* that underwent our normal retinal ischemia model with the added step of intravitreal injection with PBS containing an isotype control antibody to a final concentration of 1μg/μl. Note the similar recruitment and incorporation of transplanted Gfp^+ HSC as in Model Control. **(**Anti-SDF-1) Right or treated eyes from four representative C57BL/6.gfp that underwent our normal retinal ischemia with the added step of intravitreal injection with PBS containing an anti-SDF-1 antibody to a final concentration of 1μg/μl. Note the absence of newly formed Gfp^+ HSC in the vascular tufts.

seen in the cohort that received antibody and the negative control eyes, indicate no HSC-derived contributions. The negative control eyes in all experiments are untreated left eyes of the animals used in the model. Therefore, they represent the background level of HSC contribution to undamaged vessels after a bone marrow transplant. None of the ten animals that received injections of SDF-1 blocking antibody had significant Gfp^+, HSC-derived contributions to the retinal vasculature above that seen in the control eyes.

The lack of Gfp^+ HSC-derived contribution to the injured eyes that received anti-SDF-1 treatment could result from blocking HSC/EPC derived contribution to neovascularization while still forming new vessels from local endothelial cell proliferation. Alternatively, all new vessel formation could be stopped by the treatment. The confocal imaging analysis suggested the later result when the individual images from differing focal planes along the z-axis used to form the merged images were viewed separately. The remaining red vessels observed in the anti-SDF-1 treated eyes appeared to be the preexisting vessels of the retina. No new preretinal vessels were observed (in the model it is these newly formed preretinal vessels that are Gfp^+). To confirm, we performed cross sectional histological analysis of treated versus non-treated control eyes to better assess total neovascularization (Figure 3-7). Results are depicted for three animals from each cohort of ten. Cross sections of untreated left eyes depict the normal histology of the eye (Figure 3-7, Normal Retina). All of the eyes that underwent the standard model (Figure 3-7, Model Control) exhibited severe preretinal neovascularization, as shown by the gross disruption of the retinal architecture, in response to VEGF administration and retinal ischemia. We have previously shown that

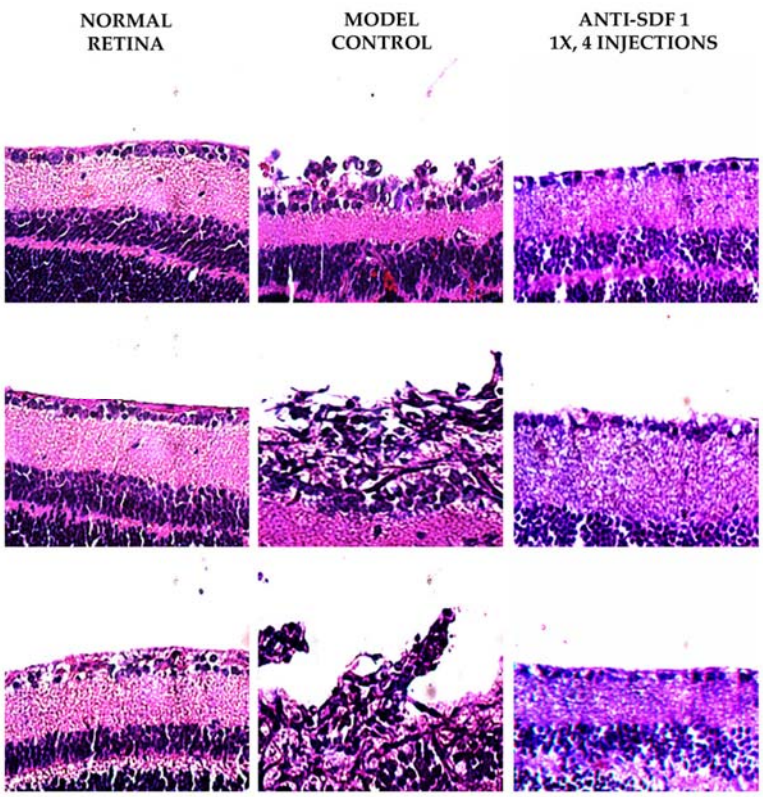

Figure 3-7. Cross sectional analysis of retinal architecture. (Normal Retina) HnE staining of cross sections from three untreated C57BL/6.gfp eyes showing normal retina morphology. (Model Control) HnE staining of cross sections of three C57BL/6.gfp eyes that underwent the neovascularization model showing clearly disrupted retinal architecture and new vessel formation. **(**Anti-SDF-1) HnE staining of cross sections of three C57BL/6.gfp eyes that underwent the neovascularization model and were treated with 1 μg/μl anti-SDF-1 antibody. Note the similar morphology of both the C57BL/6 and anti-SDF-1 antibody treated cross sections. All images were taken at a 20x magnification.

these are the Gfp^{+} vessels in our model [112]. None of the anti-SDF-1 treated eyes exhibited retinal neovascularization and all retained a retinal architecture (Figure 3-7, Anti-SDF 1) that is similar to a normal retina. The anti-SDF-1 treated retinas show disruption to the normal architecture of retina reflective of damage caused by poorly repaired ischemic injury. These results clearly demonstrate that treating the eye with anti-SDF-1 blocking antibody prevents retinal neovascularization in spite of the viral

over-expression of VEGF-189. This suggests that SDF-1 is the more critical proangiogenic factor in our model.

Titration of SDF-1 Antibody

Our initial treatment regime utilized multiple rounds of antibody injection at what was estimated to be a saturating concentration based on the manufacturer's use suggestions. To test the overall effectiveness of the antibody treatment we treated two additional cohorts (n=10). The first treated cohort received one log less antibody per injection (0.1 µg/µl) with four weekly injections beginning the day after laser-induced ischemia as previously described. The second treated cohort received only a single injection, one day after laser coagulation, at the original antibody concentration (1 µg/µl). Both test cohorts, along with a normal model control cohort, were then allowed to recover for one month prior to analysis. Both of the new treatments proved as effective at blocking retinal neovascularization as our original regime (Figure 3-8). The control cohort exhibited a large degree of Gfp^+ HSC-derived neovascularization in their injured eyes with no Gfp^+ contributions in their uninjured eyes (Figure 3-8, Left panel sets). Both treated cohorts showed greatly decreased Gfp^+ HSC-derived contribution to the injured, anti-SDF-1 injected eyes. Almost no Gfp^+ contributions were seen in the vasculature (Figure 3-8, right panel sets). This suggests that easily achievable SDF-1 antibody concentrations may provide effective preventative treatment for diseases such as proliferative retinopathy.

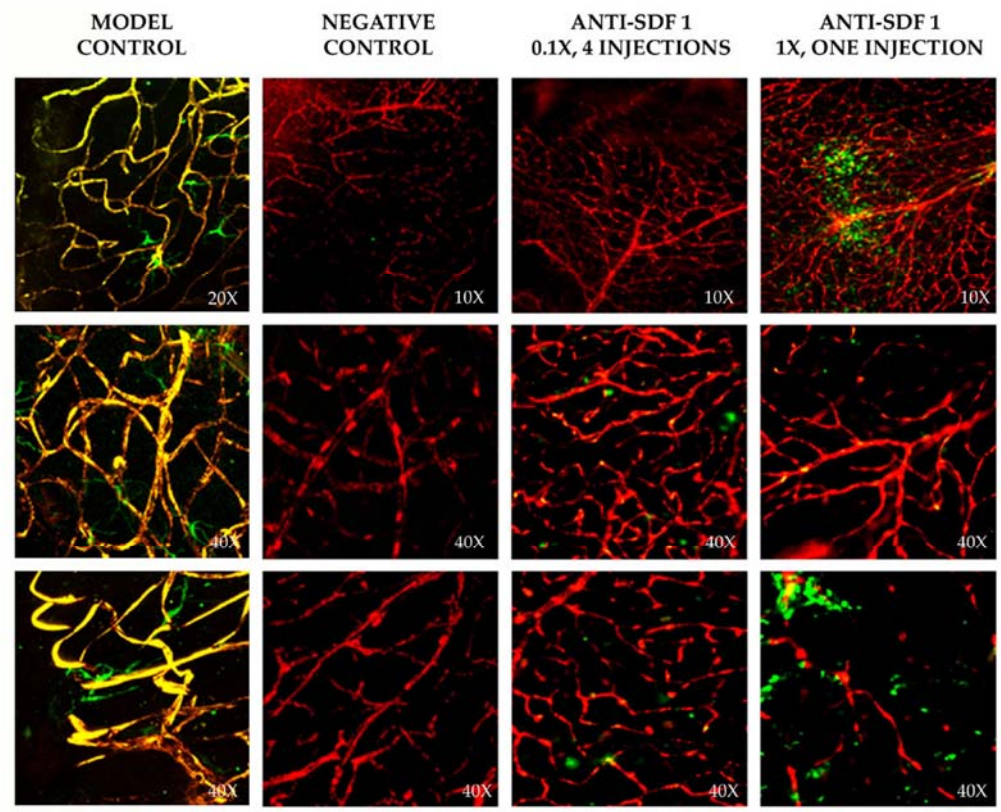

Figure 3-8.Anti-SDF-1 antibody titration. **(**Model Control) Fluorescence confocal micrograph of three C57BL/6.*gfp* retinas that underwent retinal ischemia. **(**Negative Control) Left eyes, untreated, of three C57BL/6.*gfp* that underwent retinal ischemia. (Anti-SDF 1 0.1X, 4 Injections) Three C57BL/6.*gfp* mice that underwent retinal ischemia and were injected with 0.1 μg/μl final concentration of anti-SDF-1 antibody, a ten-fold decrease from the original concentration, intravitreally once a week for 4 weeks. (Anti-SDF 1 1X, One Injection**)** Three C57BL/6.*gfp* mice that underwent retinal ischemia and were injected with 1 μg/μl final concentration of anti-SDF-1 antibody intravitreally the day after injury.

CHAPTER 4
SDF-1 MODULATES PRERETINAL NEOVASCULARIZATION BY RECRUITING CD133+ CELLS FOR THE BONE MARROW

Introduction

Angiogenesis is the growth of new blood vessels from pre-existing blood vessels. This process depends on the proper activation, proliferation, adhesion, migration, and maturation of endothelial cells ECs. Angiogenesis is an important natural process occurring in the body, both in health and in disease, and is highly regulated by angiogenesis-stimulating growth factors and angiogenesis inhibitors [140,141]. Angiogenesis occurs in the healthy body for healing wounds and for restoring blood flow to tissues after injury or insult. When angiogenic growth factors are produced in excess of angiogenesis inhibitors, the body signals for blood vessel growth. When inhibitors are present in excess of stimulators, angiogenesis is stopped. The normal, healthy body maintains a homeostasis of angiogenesis modulators [142].

Until recently, angiogenesis was thought to be the driving force in post-natal neovascularization. The identification and isolation of bone marrow-derived EPC has expanded the way we perceive the underlying mechanisms of post-natal neovascularization from angiogenesis to angio/vasculogenesis [42,43]. This new mechanism is now thought to be the *de novo* vessel formation by incorporation, differentiation, migration, and proliferation of bone marrow-derived EPC [143].

As mentioned above, angiogenesis is a highly regulated process. Recent evidence has shown a pivotal role of CXC chemokines in the control of angiogenesis. Chemokines

are multifunctional proteins that have the ability to promote immune responses, stem-cell survival, development and homeostasis, and the potential to mediate chemotaxis and angiogenesis [84]. It has been shown that endothelial cells express specific receptors for chemokines. CXCR4 was the first angiogenic chemokine receptor identified. CXCR4 binds only one known ligand, SDF-1 [85,86]. The SDF-1/CXCR4 interaction plays an important role during vascular development, as seen by gene deletion experiments. SDF-1 null mice exhibit a phenotype that consists of defects that are lethal, including impaired bone marrow lymphoid and myeloid hematopoiesis [62]. CXCR4 null mice exhibit similar phenotypes, including prenatal death, defects in the formation of gastrointestinal tract arteries, and defects in vessel development, hematopoiesis, and cardiogenesis [64]. More evidence suggesting that the SDF-1/CXCR4 axis is important in vascular development is its interplay with vascular endothelial growth factor-A (VEGF-A). SDF-1 increases VEGF-A production and VEGF-A increases CXCR4 expression. The existence of this regulatory loop generates a circuit that is influenced by hypoxia [87,88].

Using our unique mouse model (Chapter 3) we have shown that SDF-1 is necessary for the recruitment and incorporation of bone marrow-derived EPCs to the site of retinal ischemia, and is also sufficient to promote the process of angio/vasculogenesis and the formation of the preretinal neovascularization seen in the model [112]. In this chapter, we once again utilize our unique mouse model to elucidate into the mechanisms in which SDF-1 is affecting to promote the recruitment and incorporation of bone marrow -derived cells to the sites of ischemic injury. We show that SDF-1 protein levels increases in the bone marrow and vitreal space of the eye immediately following injury to the retina. We also show that there is an increase in bone marrow -derived CD133 cells

circulating in the peripheral blood. These cells also express functional CXCR4, shown by their ability to migrate towards an SDF-1 gradient. We also show that bone marrow -derived CD113 cells directly participate in new vessel formation in the retina. This study further shows that SDF-1 is a primary chemokine in bone marrow -derived angio/vasculogenesis.

Results

Retinal Ischemic Injury Increases SDF-1 Protein Expression in the Eye

We have shown that the HSC can serve as a source of EPC that participate in the formation of neoangiogenic (newly formed) blood vessels, proving that the HSC can have functional hemangioblast activity [112]. We have also shown that the formation of the bone marrow -derived neoangiogenic blood vessels is modulated by SDF-1, a major chemokine involved in the trafficking of bone marrow -derived cells. These data directed us to elucidate into the mechanism in which SDF-1 could be participating in when promoting neoangiogenesis. In order to tackle these questions, we utilized our unique animal model by analyzing the model at different time points (Day 0-pre laser, 1 hr, 12 hrs, day 1, day 3, day 7, and day 28). We harvested whole eyes and performed immunohistochemistry for SDF-1. At every time point, the eyes showed a consistent expression of SDF-1 in the outer nuclear layer (ONL, Fig. 4-1). The reason for this consistent expression may be due to the fact that the retinal pigment epithelium is a source of SDF-1 [144]. The control eyes (Fig. 4-1B) and the day 0 eyes (Fig. 4-1C) appear to have similar SDF-1 expression patterns. We began to see an increase of SDF-1 in the ganglion cell layer (GCL) immediately following ischemic injury. This is important because the GCL contains the ECs that make up the blood vessels of the retina. It appeared that the highest expression level was seen at 1 hr (Fig. 4-1D). We began to

see a slight decrease in expression at 12 hrs (Fig. 4-1E) and with no expression in the GCL by day 1 (Fig. 4-1F) through day 28 (data not shown). Since immunohistochemistry is difficult to make quantitative, we decided to measure the expression levels of SDF-1 in the vitreal space of the eyes by ELISA. Strikingly, the ELISA showed a direct correlation with the immunohistochemistry for SDF-1 (Fig 4-2).

We also performed immunohistochemistry for hypoxia inducible factor-1 alpha (HIF-1α). The C57/BL6 control animals did not show any expression of HIF-1a. On day 0 we see an increase of HIF-1α protein in the GCL (Fig. 4-1C). This increase is probably due to the sensitivity level of the retina. By day 0, the expression levels of VEGF-A are at its highest (data not shown), due to the injection of a recombinant adeno-associated virus (rAAV) that over expresses the murine 188 isoform of VEGF-A (described in the Methods section). We believe that the over expression of VEGF primes the retina for ischemic conditions. The HIF-1α protein is not expressed in the nucleus, suggesting that HIF-1a is not translocating from the cytoplasm to the nucleus for binding to specific promoters (such as the VEGF-A promoter) when tissues are ischemic. By 1 hr (Fig. 4-1D), we see that HIF-1α maintains a consistent expression pattern as in day 0 (Fig. 4-1C) but that there is now expression of the protein in the nucleus. This expression pattern is maintained for every other time point analyzed (Fig. 4-1 D-F). These data suggest that the retina has become ischemic immediately following ischemic injury and remains in an ischemic state for the length of the model.

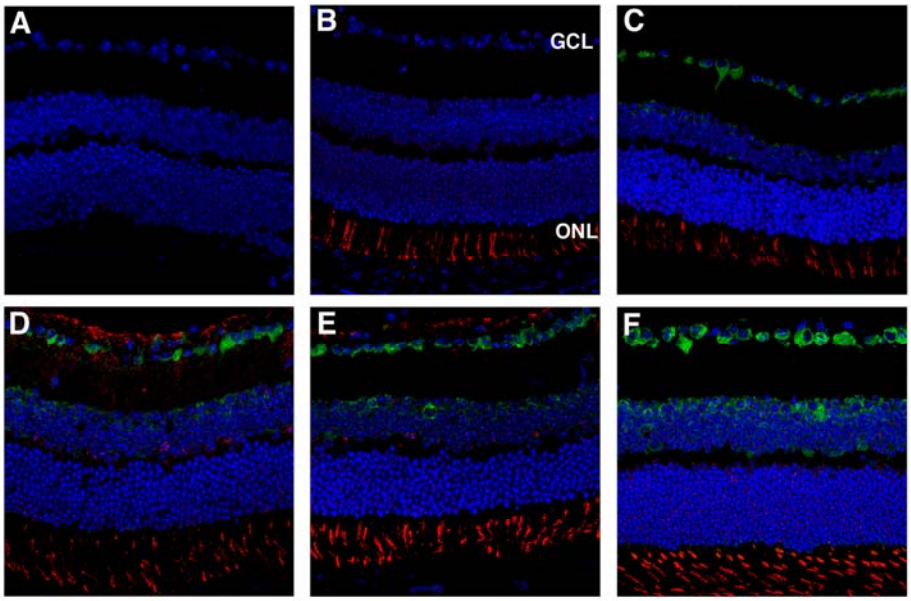

Figure 4-1.SDF-1 localization in the retina after retinal ischemic injury by IHC. Whole eyes were harvested and embedded in paraffin at different time points (n=5) after retinal injury. Tissues were sectioned and IHC was performed for SDF-1 (red), HIF-1α (green), and Dapi (blue). Every time point shows consistent expression of SDF-1 in the ONL. A) IgG isotype control. B) Normal unmanipulated eye. C) Day 0 Pre-laser eyes shows an increase in HIF-1α expression and its expression is maintained throughout all time points. D) As little as 1Hr following laser injury SDF-1 increases in the GCL. E) SDF-1 is still elevated in GCL 12 Hrs post laser. F) By Day 1 SDF-1 is no longer expressed in the GCL, but is maintain in the ONL and HIF-1α maintains present.

Bone Marrow Sinusoids Express SDF-1 following Retinal Ischemic Injury

We believe that the major source of EPCs that participate in the repair/production of blood vessels in ischemic tissues comes from the bone marrow. For this hypothesis to hold true, bone marrow -derived cells must migrate from the bone marrow to the peripheral blood. This process is accomplished by transendothelial migration of BM-derived cells through the sinusoids that are present throughout the vascular niche of the bone marrow compartment. We wanted to test if SDF-1 was playing a role in this process. We once again utilized our unique animal model and analyzed bones harvested at the same time points as the eyes. We performed immunohistochemistry for SDF-1

protein expression. The over expression of VEGF in the eye by rAAV-VEGF had no effect on the expression pattern of SDF-1 (Fig. 4-3 C), as compared to the control bones (Fig. 4-3 B). Similar expression patterns were seen in all time points (Fig. 4-3 B-D, Fig. 4-3 G,H), except for 12 hrs (Fig. 4-3 E) and day 1 (Fig. 4-3 F). At 12 hrs we began to see

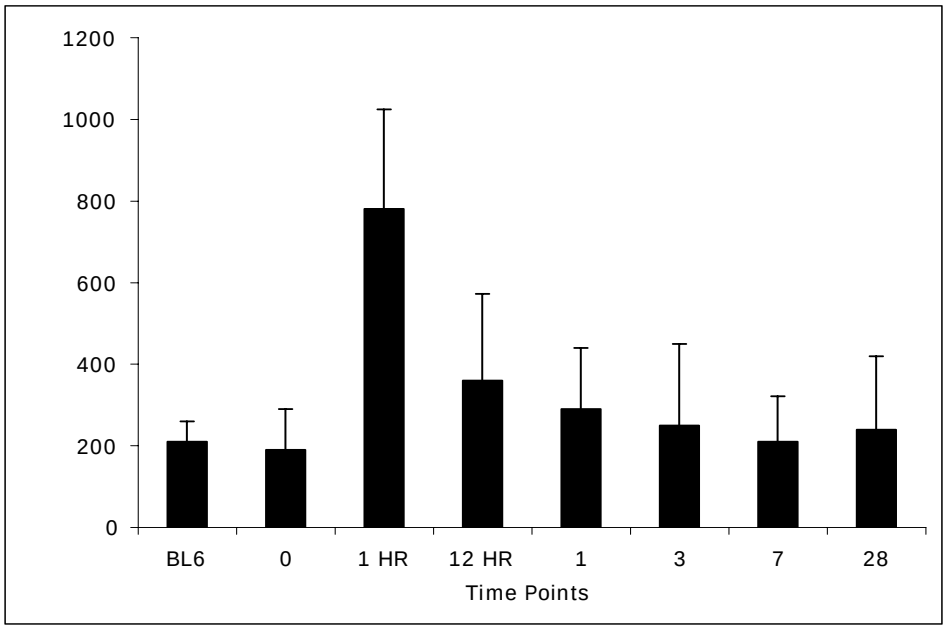

Figure 4-2.SDF-1 ELISA quantifying an increase of SDF-1 protein in the retina following retinal ischemic injury. Vitreal fluid was obtained from mice (n=5) at different time points following retinal injury, same as in Figure 4-1. ELISA was performed for SDF-1 and the quantification showed a direct correlation with the IHC in Figure 4-1. ($p<.005$)

the endothelial cells that make up the sinusoids expressing SDF-1. By day 1, almost all sinusoids were expressing SDF-1, with a return to control levels (Fig. 4-3 B, C) by day 3 (Fig. 3 G). In order to quantify the expression of SDF-1 we performed ELISA (Fig. 4-4). Once again, we saw a direct correlation with the IHC expression pattern and actual protein levels of SDF-1.

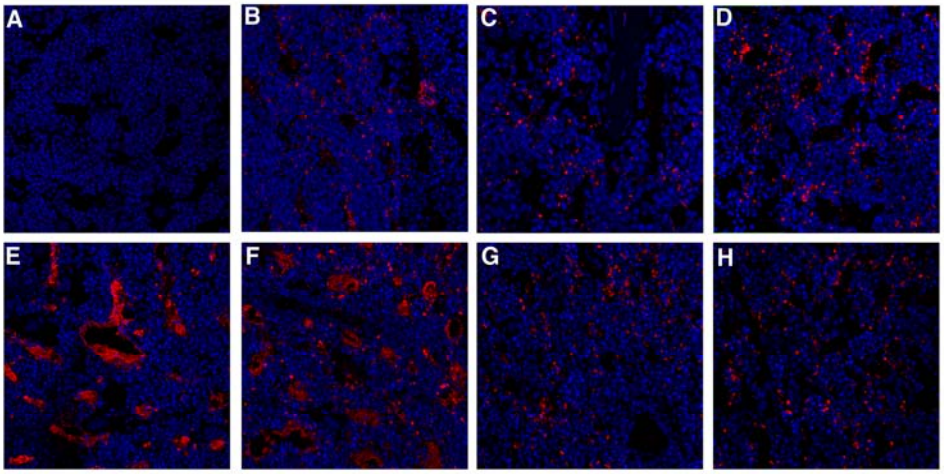

Figure 4-3. SDF-1 localization in the bone marrow after retinal ischemic injury by IHC. Long bones of hindlimbs were harvested and embedded in paraffin at different time points (n=5) after retinal injury. Tissues were sectioned (5um) and IHC was performed for SDF-1 (red) and Dapi (blue). A) IgG isotype control. B) Normal unmanipulated bone marrow. C) Day 0 Pre-laser bone marrow. D) 1 Hr following retinal ischemic injury. Note the widespread, sporadic distribution of SDF-1 throughout the bone marrow compartment in B-D. E,F) By 12 Hrs and day 1 following retinal ischemic injury SDF-1 expression appears to be increased and localized to the sinusoids within the bone marrow compartment. G, H) By day 3 and 7 the SDF-1 expression returns to a similar pattern seen in B-D.

Circulating Bone Marrow -derived CD133+ Cells Increase following Ischemic Injury

We next wanted to test if murine bone marrow -derived cells that express the cell surface marker CD133 could participate in new vessel formation seen using our model. CD133 is a very promising stem cell marker that can be used to isolate a subpopulation of cells that consists of EPC. This is the case because CD133 is expressed only on very immature endothelial cells and its expression is lost as the endothelial cells mature. In order to determine if bone marrow -derived CD133+ cells had the potential to participate in neoangiogenesis, we needed to test if there was an increase in circulating bone marrow -derived CD133+ cells in the circulating peripheral blood following ischemic injury. Analyzing the peripheral blood at the same time points mentioned above, we see that there is a rapid increase circulating bone marrow -derived CD133+ cells with a sustained

increase from 12 Hrs to 3 days following ischemic injury (Fig 4-5). The increase in circulating bone marrow -derived CD133+ cells suggests that these cells have the potential to participate in neovessel formation following ischemic injury to the retina.

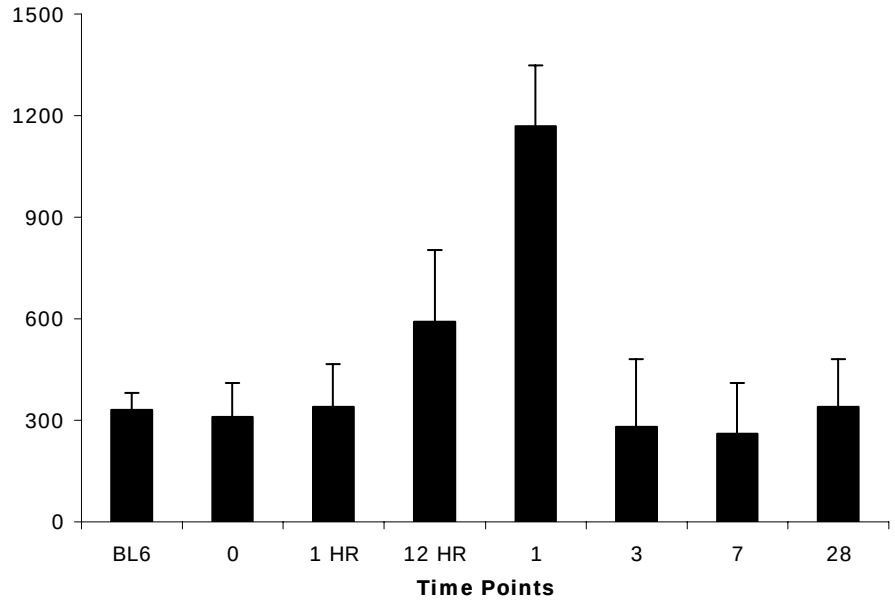

Figure 4-4. SDF-1 ELISA quantifying an increase of SDF-1 protein in the bone marrow compartment following retinal ischemic injury. 2.5×10^5 whole bone marrow cells were obtained from mice (n=5) and lysed at different time points following retinal injury, same time points as in Figure 4-3. ELISA was performed for SDF-1 and the quantification showed a direct correlation with the IHC in Figure 4-3. ($p<.005$)

Bone Marrow -derived CD133 Participate in New Vessel Formation *in vivo*

We hypothesize that bone marrow -derived CD133+ cells are responsible for the new vessel formation found in our model. In order for this hypothesis to be valid, we need to first determine if SDF-1 could modulate their involvement in blood vessel repair. We first decided to determine what percentage of murine bone marrow -derived CD133+ express CXCR4, SDF-1's only known receptor. Bone marrow cells were isolated and analyzed using FACS (Fig. 4-6). Nearly all cells that were positive for CD133 were also positive for CXCR4. In order to determine if the CXCR4 receptor is functionally active

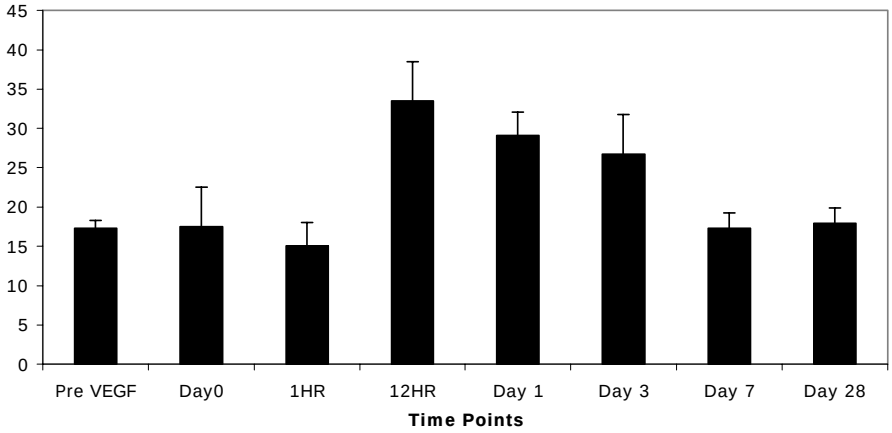

Figure 4-5. Bone marrow-derived CD133+ cells increase in the peripheral blood following retinal ischemic injury. Peripheral blood was isolated from the tail vein of mice (n=5) at various time points following retinal ischemic injury. There is an increase in the percentage of CD133+ cells in the peripheral blood as early as 12 Hrs post retinal ischemic injury and the increased lasted until 3 days post injury; until shifting back to homeostatic levels.

on bone marrow -derived CD133+ cells, bone marrow marrow cells were isolated and sorted for CD133 and CXCR4 and used in a chemotaxis assay (Fig. 4-7). Bone marrow -derived CD133+/CXCR4+ cells migrated towards a SDF-1 gradient. These data suggest that SDF-1 has the potential to recruit bone marrow -derived CD133+ cells to the sites of new vessel formation in the ischemic retina.

We next wanted to determine if bone marrow -derived CD133+ cells could actively participate in neovessel formation *in vivo*. We utilized our unique murine model, which previously showed that SDF-1 is necessary and efficient to drive new vessel formation, with slight modification. Mice were not lethally irradiated and transplanted with GFP+ bone marrow donor cells. Instead, healthy C57/BL6 mice were injected with rAAV2-VEGF-A 188 in the right eye. Four weeks were allowed for peak VEGF-A 188

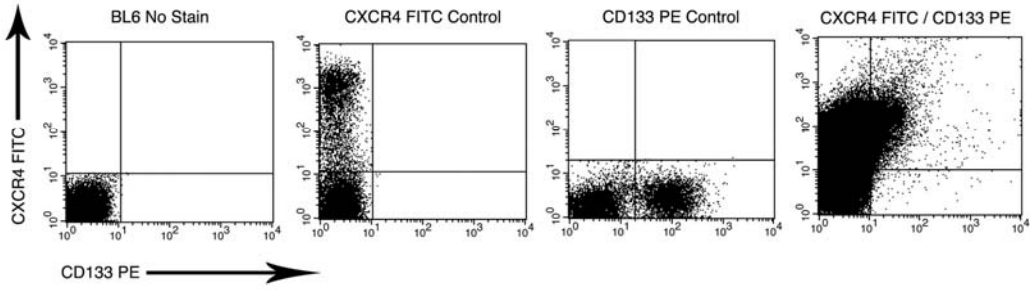

Figure 4-6. Percentage of bone marrow cells that coexpress the markers CD133 and CXCR4. Whole bone marrow was isolated and stained with antibodies to CD133 (PE) and CXCR4 (FITC). Cells were then analyzed using the FACs Calibur. 7.4% of total bone marrow was positive for both CD133 and CXCR4, as shown in the upper right quadrant of the FACs plot marked CXCR4 FITC / CD133 PE.

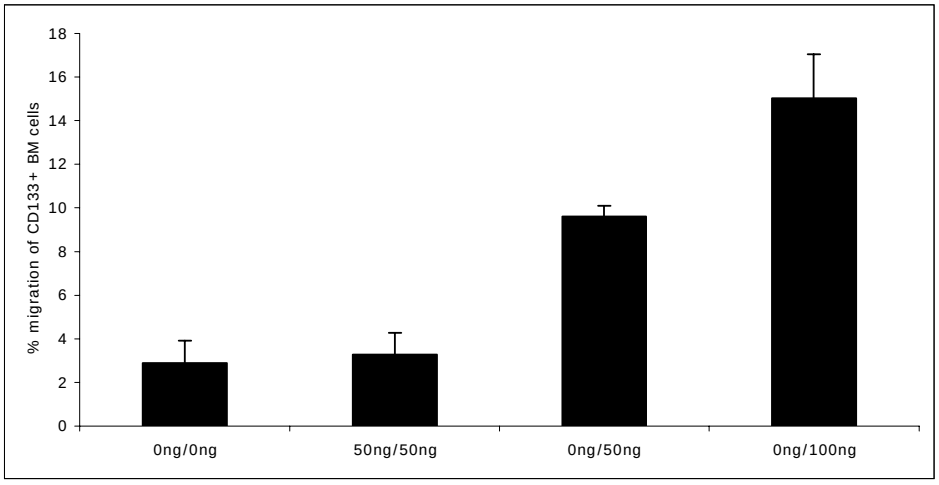

Figure 4-7. Migration of CD133/CXCR4+ bone marrow cells to a SDF-1 gradient. Whole bone marrow was isolated and stained with antibodies to CD133 (PE). Cells were then sorted using the FACs Diva. $4x10^4$ CD133+ cells were then placed in the upper transwell insert of a Boyden chamber, with or without rSDF-1 protein in 100 μl of media. The lower chamber contained various concentrations of rSDF-1 protein in 600 μl of media. Cells were placed in 37^{o}C incubator for 2 Hrs. Cells that migrated to the lower chamber were collected and stained with CD133 PE and quantified using the FACs Calibur.

expression, and then laser photocoagulation was performed on the right eyes in order to promote neovessel formation by causing ischemia in the retina. The day following ischemic injury, CD133+/GFP+ cells were isolated from the bone marrow of donor mice

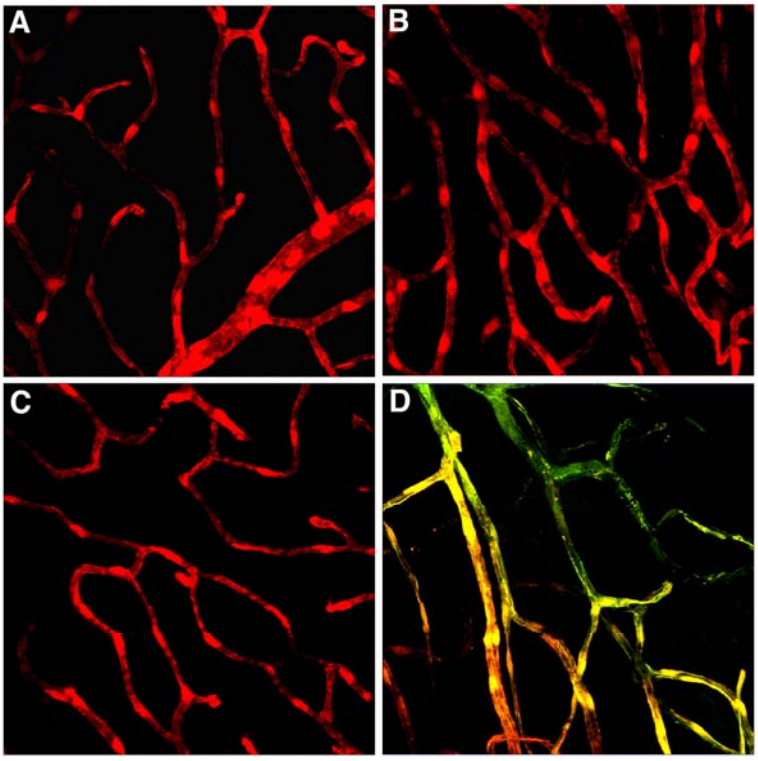

Figure 4-8.Bone marrow-derived CD133/GFP+ participate in neovessel formation following retinal ischemic injury. A) Left retina (n=3), negative control. B) Right retina (n=3) that underwent retinal ischemic injury and did not receive 2,000 bone marrow-derived CD133/GFP+ cells following laser injury. C) Right retina (n=3) 2wks post retinal ischemic injury. These animals received 2,000 bone marrow-derived CD133/GFP+ cells following laser injury and show no sign of incorporation or homing of donor cells. D) Right retina (n=4) 4wks post retinal ischemic injury. These animals received 2,000 bone marrow-derived CD133/GFP+ cells following laser injury. Note the incorporation and homing of donor cells to the retina.

and 2,000 cells were injected intravenously via the tail vein. Right and left eyes were enucleated and retinas were flat mounted at two weeks and four weeks. None of the left eyes showed any contribution from the CD133+/GFP+ donor cells (Fig. 4-8A). At four weeks, right eyes showed contribution from the CD133+/GFP+ donor cells (Fig. 4-8D). Interestingly, the right eyes from two weeks showed no contribution from CD133+/GFP+ donor cells (Fig. 4-8C). Recent evidence has shown that the source of GFP+ cells that participate in blood vessel repair is not actually GFP+ donor cells but autofluorescence from platelets involved in clot formation. In order to get around this possibility, we

simply had control animals that went through the model as mentioned above without the injection of CD133+/GFP+ donor cells (Fig. 4-8B). The right eyes of these animals showed no evidence of autofluorescence. These data suggest that bone marrow -derived CD133+ act as long term EPCs and can participate in new vessel formation *in vivo*, and that the source of perceived GFP is not due to autofluorescence but due to the incorporation of GFP+ donor cells.

CHAPTER 5
USE OF AN ESTABLISHED PRIMATE MODEL OF PROLIFERATIVE RETINOPATHY TO DETERMINE THE EFFICIENCY OF USING AN ANTI-SDF-1 ANTIBODY-BASED THERAPY IN NONHUMAN PRIMATES

Introduction

In Chapter 3 we used a previously established the adult mouse model that simulates much of the retinal pathology that is associated with diabetic retinopathy in humans. The studies in Chapter 3 monitoring the levels of the chemokine, SDF-1, in human patients with diabetic retinopathy noted that the severity of the disease correlated with higher concentrations of SDF-1. SDF-1 is a potent chemotactic factor, and we hypothesized that SDF-1 could represent a key factor recruiting endothelial progenitors to areas of retinal ischemia and driving the aberrant neovascularization associated with the proliferative stage of diabetic retinopathy. We tested our hypothesis by demonstrating that an anti-SDF-1 MAb completely blocked the aberrant neovascularization observed in our adult mouse model of proliferative retinopathy. The results of this study clearly established a link between SDF-1 and proliferative retinopathy, and allowed us to propose that SDF-1 represents a new drug target for the treatment of proliferative retinopathy in humans afflicted with diabetes.

The aim of this body of work is to further the preclinical development of anti-SDF-1 antibody-based therapy to treat diabetic humans with proliferative retinopathy. The successful development of this approach may complement or replace current methods for the treatment of diabetic retinopathy, including retinal laser photocoagulation.

Based on our recent successes in establishing an adult mouse model to study proliferative retinopathy and identifying key regulatory molecules controlling the process of pathologically neovascularization, we propose that we will be able to determine the efficiency of the anti-SDF-1 antibody-based therapy by utilizing an established nonhuman primate model for proliferative retinopathy. In this study we show that the anti-SDF-1 antibody is indeed efficient at blocking newly formed blood vessels within the retina and that this antibody-based therapy may prove to be useful in human patients.

Anti-SDF-1 Antibody is Efficient at Blocking Neovascularization in a Nonhuman Primate Model of Neovascularization

The need for new treatment regimes for diabetic retinopathy has prompted considerable research into the pathogenesis of diabetic retinopathy, with much of the focus on identifying growth factors controlling retinal neovascularization . The prime candidate for mediating retinal neovascularization is thought to be VEGF. VEGF was originally described as being a potent modulator of vascular permeability and inducer of angiogenesis (new blood vessel formation) by acting on VEGF-receptor expressing endothelial cells to induce their proliferation [145, 146]. Patients with proliferative diabetic retinopathy were found to have elevated levels of VEGF in their vitreal fluid and their levels increased as they progressed from the nonproliferative to proliferative stage of diabetic retinopathy [147, 148]. The source of the elevated vitreal VEGF may be a number of cell types in the eye known to produce VEGF including endothelial cells, pericytes, glial cells, Muller cells and ganglion cells [149, 150]. Antibody-, oligonucleotide-, and aptamer-based therapies targeting VEGF have been developed [151-153]. Adequate testing of VEGF-targeted therapies has been hampered by the lack of an adequate adult rodent animal model that mimics the retinal pathology associated

with proliferative retinopathy in diabetic humans. To date, the mouse retinopathy model of prematurity, in which neonatal mice are exposed to high levels of oxygen to induce ischemic conditions for promoting neovascularization (Retinopathy of Permaturity Model), has been the workhorse for testing these therapies [154]. These anti-VEGF therapies are being tested for their ability to block neoangiogensis in the neonatal developing retina but not aberrant neovascualarization as observed in diabetic retinopathy found in adults.

In contrast to mice, nonhuman primates, such as monkeys, contain a macula with a foveal avascular region and therefore represent a better experimental system for establishing an animal model of diabetic retinopathy. As is the case for the mouse, naturally occurring or experimentally induced diabetes will not lead to diabetic retinopathy in nonhuman primates. However, the prominent role played by VEGFA in controlling both normal physiological angiogenesis and pathological neovascularization has prompted several groups to induce ocular neovascularization by intravitreal injections of recombinant VEGFA [155-158]. Depending on the study, iris neovascularization and neovascular glaucoma, commonly associated with extreme cases of diabetic retinpathy in humans were detected, while others detected variable levels of intraretinal (within the retina) and preretinal (extruding into the vitreous) neovascularization. A second approach involving laser retinal vein occlusion with growth factor administration has been successful in inducing iris neovascularization in monkeys [159]. Both models of ocular neovascularization in adult nonhuman primates have been used to test anti-VEGF therapeutics [160, 161].

We have combined two existing models of retinal neovascularization in *Rhesus macaque* monkeys by combining intravitreal administration of recombinant VEGF with laser-induced retinal vessel photocoagulation. Combining exogenous VEGF with laser injury should provide the most stringent test for the efficacy of anti-SDF-1 MAb treatment to block proliferative retinopathy in non-human primates. In this model we see an increased amount of intraretinal neovascularization, but we do not see any preretinal neovascularization. In Figure 5-1 we show that the anti-SDF-1 antibody is efficient at blocking intrarentinal neovascularization. This is depicted by the absence of neovascular lumens (black arrows) in the eyes that were treated with the antibody. We quantified the effects of the treatment by blindly counting the intraretinal neovascular lumens in all experimental cohorts (Figure 5-2) and showed that eyes that did not receive any antibody treatment had approximately 17 intraretinal neovascular lumens and eyes that received the anti-SDF-1 antibody had approximately 3 intraretinal neovascular lumens, similar to unmanipulated eyes. These data suggest that an anti-SDF-1 antibody-based treatment may be used in concert with other treatments or individually to help treat diabetic retinopathy in human patients.

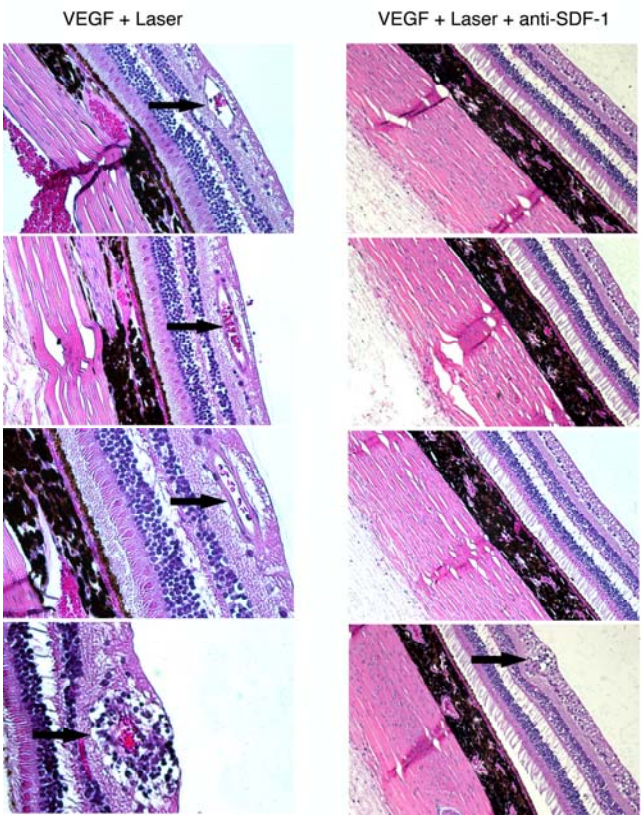

Figure 5-1.Efficiency of anti-SDF-1 antibody-based therapy in an established model of proliferative retinopathy in nonhuman primates. The cohort that received exogenous VEGF and laser injury we see an increase in intraretinal neovascularization. The black arrows indicate representative intraretinal vascular lumens that have are newly formed following the experimental procedures. The cohort that received exogenous VEGF, laser injury, and anti-SDF-1 antibody shows a marked decrease in the amount of newly formed intraretinal neovascular lumens.

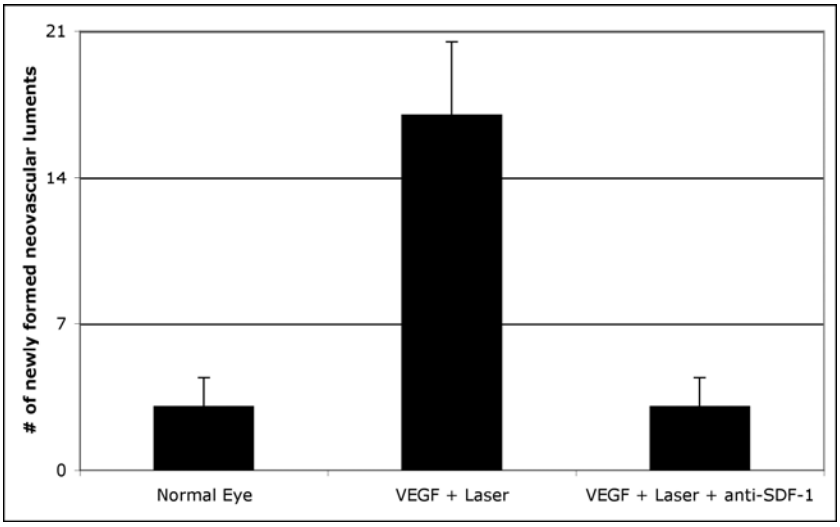

Figure 5-2.Quantification of intraretinal neovascular lumens. Serial sections of OCT embedded whole eyes were H & E stained. Intraretinal neovascular lumens were blindly counted and placed within proper groups was completed. Cohorts that received exogenous VEGF and laser injury developed 17 +/- 3.4 neovascular lumens. Cohorts that received exogenous VEGF and laser injury, and anti-SDF-1 antibody developed 3 +/- 1.4 neovascular lumens, similar as what was seen in the normal eye.

CHAPTER 6
GENERAL CONCLUSIONS

Diabetes mellitus is common endocrine disorder characterized by chronic hyperglycemia with the end result being vascular dysfunction in the eye, kidney and central nervous system Diabetic retinopathy is a major cause of visual impairment and blindness in the United States. The early stage of diabetic retinopathy, termed nonproliferative retinopathy, is characterized by increased vascular permeability, thickening of the basement membrane and loss of pericytes from retinal capillaries. The pericytes form an outer sheath around the endothelium and play a critical role in regulating capillary blood flow. The retinal capillaries begin to hemorrhage creating microanurysms that disrupt regional blood flow, leading to localized areas of ischemia. These hypoxic areas containing low oxygen levels trigger signals that induce the proliferation of new blood vessels from the existing vasculature. The outgrowth of new vessels is known as neovasculogenesis and typifies the proliferative stage of diabetic retinopathy. The newly formed blood vessels are fragile and have disastrous consequences if they extrude into the vitreous cavity of the eye thereby destroying the normal architecture of the outer retina and potentially hemorrhaging into the vitreous, causing loss of vision.

When proliferative diabetic retinopathy (PDR) is left untreated, about 60 percent of patients become blind in one or both eyes within five years. For three decades, laser photocoagulation has been the mainstay in the management of diabetic retinopathy [161]. Laser treatment for PDR breaks down the blood retinal barrier and can cause or worsen

diffuse macular edema (DME) [161, 162]. Surgical treatment of PDR and DME has visual consequences and is not always effective. Corticosteroid treatment can lessen the impact of macular edema and PDR, but also has serious side effects, such as glaucoma, that require additional treatment [163]. A highly selective therapy that could prevent new vessel formation within the vitreous without serious side effects would represent a significant improvement over the current standard of care for proliferative retinopathy.

There are currently two large scale Phase III clinical trials underway for the treatment of AMD and DME. Both involve blocking the activity of VEGF by binding to it and inhibiting its signaling with its receptor [164, 165]. The preliminary studies have been very promising, with an increase of visual acuity in approximately 26% of the patients who have been treated. Though anti-VEGF treatments may be a great advancement in alleviating the effects of ocular diseases, there may be other cytokines/chemokines that, when blocked, may improve visual acuity by augmenting anti-VEGF treatments. Our clinical data provide the first evidence that SDF-1 may play a major role in the pathology of proliferative retinopathy. Our murine data have also shown that SDF-1 is both necessary and sufficient to promote the incorporation of bone marrow-derived endothelial cells within an ischemic retina. Blocking SDF-1 activity in our murine model completely abrogated recruitment of HSC-derived endothelial precursors and local endothelial cell driven ischemic repair, thus effectively preventing preretinal neovascularization. The murine model system we employ uses an acute injury to promote a proliferative retinopathy that has a similar pathology of preretinal neovascularization to that seen in the proliferative stages of diabetic retinopathy in humans. The assumption and caveat is that similar pathologies result from similar

mechanisms. This assumption may not hold absolutely true. Given the lack of a true animal model for diabetic retinopathy, we cannot fully validate our assumption short of performing clinical trials. We have recently completed our first experiment with anti-SDF-1 antibody treatment in nonhuman primates. The data are similar to the data seen using our murine model of proliferative retinopathy, with an anti-SDF-1 antibody blocking the formation of new neovessels. Our nonhuman primate and patient data strongly correlates with the murine model and further suggests that targeting SDF-1 may serve as a safe, alternative approach in treating proliferative retinopathies.

Blocking SDF-1 activity within the vitreous via immunoglobulin injections or other means could potentially provide such an improved treatment for PDR and DME. Single antibody injections have already been shown to be effective for up to one month in our murine model. We are currently testing how long a single antibody injection can provide effective preventative therapy in this model. Antibodies are stable proteins which should be able to persist for extended times in the relatively protease-free environment of the vitreous [124]. Since the eye is self-contained, high antibody concentrations are easy to achieve and maintain. We are also hopeful that we can titer the amount of SDF-1 blocking antibody to such a point where destructive pre-retinal neovascularization is prevented while allowing ischemic repairs within the retina.

We have also began elucidating in how SDF-1 could be working mechanistically in the formation of preretinal neovascularization by bone marrow-derived EPC in our unique animal model. In recent years, there has been an increasing amount of evidence showing that the EPC exists as a unique subtype in the circulating peripheral blood. The EPC has been shown to express various endothelial markers, and incorporate into

neovessels at sites of ischemia. These data have made EPC a very attractive cell type for uses in therapeutic applications, such as the neovascularization or regeneration of ischemic tissue. The therapeutic potential of EPCs has been explored by many preclinical and clinical studies, particularly in the treatment of ischemic cardiovascular disease. Animal studies have shown that using transplanted bone marrow-derived cells as a source of proangiogenic tissue can be efficacious in the treatment of acute myocardial infarction [13, 14] chronic myocardial ischemia [166,167] and peripheral vascular disease [168].

Though many of the studies mentioned above have had promising results, there are still limitations for the therapeutic applications of postnatal EPCs. One such limitation is the source of EPCs. Many studies use a heterogeneous population of BM-derived cells. The isolation of EPCs based on phenotypic characterization is a controversial topic. The attempt to characterize the EPC has been clouded by the presence of other circulating endothelial cells in the peripheral blood. The failed attempt to accurately characterize the EPC has further been clouded by the extreme overlap of cell surface markers shared between the EPCs and the cells of the hematopoietic lineages. Such markers include CD31 [169] and VEGFR2 [170]. A promising cell surface marker that is being used to isolate subpopulations of cells that represent an EPC is CD133. CD133 (also known as AC133 or Prominin-1) is a 5-transmembrane glycoprotein whose function is still unknown [171]. Interestingly, CD133 appears to define a subpopulation that contains long-term hematopoietic stem cell properties and is only expressed on EPC, not on mature EC [171, 172]. These data suggest that CD133+ bone marrow-derived

cells may prove to be a useful population for transplantation and regeneration of ischemic tissue.

Another limitation for the therapeutic application of postnatal EPC is their low number in the peripheral blood, particularly in patients at risk for cardiovascular disease [166,167]. An approach to solve this problem is the mobilization of EPC into the peripheral blood by cytokines. In a model of hindlimb ischemia, systemic administration of GM-CSF enhances the number of EPC found in the peripheral blood and helps increase the amount hindlimb neovascularization [173]. Although cytokine therapy used to increase circulating EPC numbers looks promising, many safety concerns have been raised, mostly relating to the augmentation of generalized inflammatory responses [174,175].

Another strategy to help augment the amount of EPC contribution to sites of ischemic tissue is the local administration of proteins that enhance EPC homing. One such protein is the potent chemokine SDF-1. SDF-1 belongs to the CXC family of chemokines and binds to one known receptor, CXCR4. The expression of SDF-1 in bone marrow stromal cells is critical for the maintenance of the bone marrow microenvironment [72]. Animals deficient in both SDF-1 and CXCR4 are embryonic lethal and display multiple defects, including impaired bone marrow lymphoid and myeloid hematopoiesis and impaired vasculogenesis in the gastrointestinal tract [62,64]. EPC express CXCR4 and migrate towards an SDF-1 gradient [135]. SDF-1 protein levels have been shown to increase in the heart following myocardial infarction [176,177] and in the brain following a stroke [178]. Recently, the local administration of SDF-1

into the ischemic hindlimb of a rat and into a rat heart after myocardial infarction has shown that SDF-1 can augment EPC-mediated vasculogenesis in ischemic tissues.

In summary, our data suggest that SDF-1 may be a key player in angiogenesis and in the progression of proliferative retinopathy. SDF-1 clearly has the potential to give EPC the directional cues necessary to reach sites of ischemia. SDF-1 can increase the expression of VCAM on endothelial cells, suggesting that SDF-1 may promote firm adhesion of HSC-derived endothelial cells to the vasculature endothelium and may also facilitate in the migration and homing of the EPC. SDF-1 appears to have an impact on the ability of gap junction proteins to form tight junctions, making it possible for EPC to enter sites of ischemia. By analyzing our model at various time points, we were able to elucidate into the mechanism by which SDF-1 uses to promote neovessel formation in the retina. We have shown by IHC and ELISA that SDF-1 protein expression rapidly increases in the bone marrow and retina following laser photocoagulation. We have also showed that the retina is in an ischemic state following ischemia-induced injury. This is shown by the translocation of HIF-1a from the cytoplasm to the nucleus, suggesting that HIF-1a is binding to specific promoters of proangiogenic factors, such as VEGF. There is also an increase of bone marrow -derived CD133+ cell numbers in the circulating peripheral blood following laser injury of the retina. The bone marrow -derived CD133+ cells express functional CXCR4 and migrate towards and SDF-1 gradient. Most importantly, CD133+/GFP+ donor cells can participate in long-term neovessel formation in ischemic retinas, suggesting that CD133 may prove to be an important marker when isolating EPC that will be used for cell-based revascularization therapies or for the enhancement of endothelial repair. Our human clinical data show that the corticoid

steroid, triamcinolone, decreases the severity of diabetic retinopathy. Triamcinolone may be working in part by reducing the levels of SDF-1, as shown by ELISA. Unfortunately, triamcinolone treatment comes with serious side effects, such as glaucoma. Our murine data suggest that as little as one intravitreal injection of a blocking antibody to SDF-1 can work to block neovascularization in our acute injury model for up to 1 month. These data suggest that using antibodies to block SDF-1 activity may provide a safe and effective alternative treatment for ischemic diseases, such as PDR and DME.

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BIOGRAPHICAL SKETCH

Jason Mathew Butler was born on August 22nd, 1978, in Amittyville, NY. After graduating from Cooper City High School in Cooper City, FL, in 1996, he attended Broward Community College where he received his A.A. In August 1999, Jason transferred to the University of Florida in Gainesville, FL, where he earned his Bachelor of Science degree in zoology and met his future wife, Diana Elizabeth Hewitt. After a year working as a transgenic technician in the laboratory of Dr. Edward W. Scott, Jason entered the University of Florida's College of Medicine Interdisciplinary Program in Biomedical Sciences. After becoming a graduate student in the laboratory of Dr. Edward W. Scott, Jason began studying the role of chemokines in hemangioblast activity. He presented a poster at the Keystone Symposium in 2003 and was first author of an article published in the Journal of Clinical Investigation.