INTERLEUKIN 10 (IL-10) SELECTIVELY INHIBITS NEOVASCULARIZATION IN THE MURINE MODEL OF RETINOPATHY OF PREMATURITY

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by

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This thesis was prepared under the direction of the candidate's thesis advisors, Dr Janet Blanks and Dr Kathleen Dorey, Department of Biomedical Science and has been approved by the members of his supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Biomedical Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

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Ocular neovascularization (NV), the development of new blood vessels in the eye, occurs when excessive vascular endothelial growth factor (VEGF) is produced. Eventually NV may lead to photoreceptor loss and or blindness, as it does in age-related macular degeneration (AMD), retinopathy of prematurity (ROP), and diabetic retinopathy.

We tested the hypothesis that the anti-inflammatory cytokine, interleukin-10 (IL-10); can reduce inflammation and block NV in the affected areas of the retina. The mouse ROP model was used for this study of NV. Seven day old neonates stayed in 75% oxygen for five days, then were given intraocular injection of IL-10 and NV was evaluated after seven days in room air. Controls were uninjected contralateral eyes. IL-10 strongly inhibited NV without affecting intra-retinal vessels. The selective inhibition of IL-10 on

NV suggest a possible therapeutic use in infants with ROP, in diabetic retinopathy, and possibly, in AMD where inflammation is a risk factor.

To my beautiful and loving wife **Nicole** and our two children, **Christina** and **Daniel.**

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Chapter 1

Introduction

1.1. Neovascularization

Neovascularization (NV) is defined as the growth of new blood vessels into a tissue (e.g. lens, vitreous, primary tumor) resulting in pathology. Neovascular diseases of the retina are major causes of blindness worldwide. Age Related Macular Degeneration (AMD), is a progressive and chronic eye disorder. In the western world it is the leading cause of blindness in adults over the age of 60. Globally, it ranks third only behind cataract and glaucoma as a cause of blindness (WHO, 2004). In 2002, the global estimate for visual impairment was 161 million; AMD accounted for 8.7% of this estimate (Rsenikoff, et al., 2004). There is a loss of central vision in AMD, therefore fine vision required for activities such as reading and driving is impaired. Peripheral vision however, is usually retained. AMD occurs in two forms. Dry AMD (atrophic) generally develops slowly affecting both eyes simultaneously. This accounts for about 80% of cases. Extra-cellular deposits behind the retina called drusen characterize this form of the disease. With time the presence of a high density of drusen leads to photoreceptor death (Johnson et al, 2004). The wet form, (neovascular AMD) is characterized by neovascularization growing from the choroidal layer of the eye into the subretinal space causing rapidly deteriorating vision and severe impairment (Fig.1). These new blood vessels have a propensity to leak, damaging the retina (Mitchell, Bradley, 2006). Diabetic retinopathy is the leading cause of new cases of blindness among working adults in the U S. In this disease, NV grows into the vitreous (Fig.1). NV is a final common pathway leading to

vision loss in diabetic retinopathy, retinopathy of prematurity (ROP) and AMD (Fong, et al., 2004).

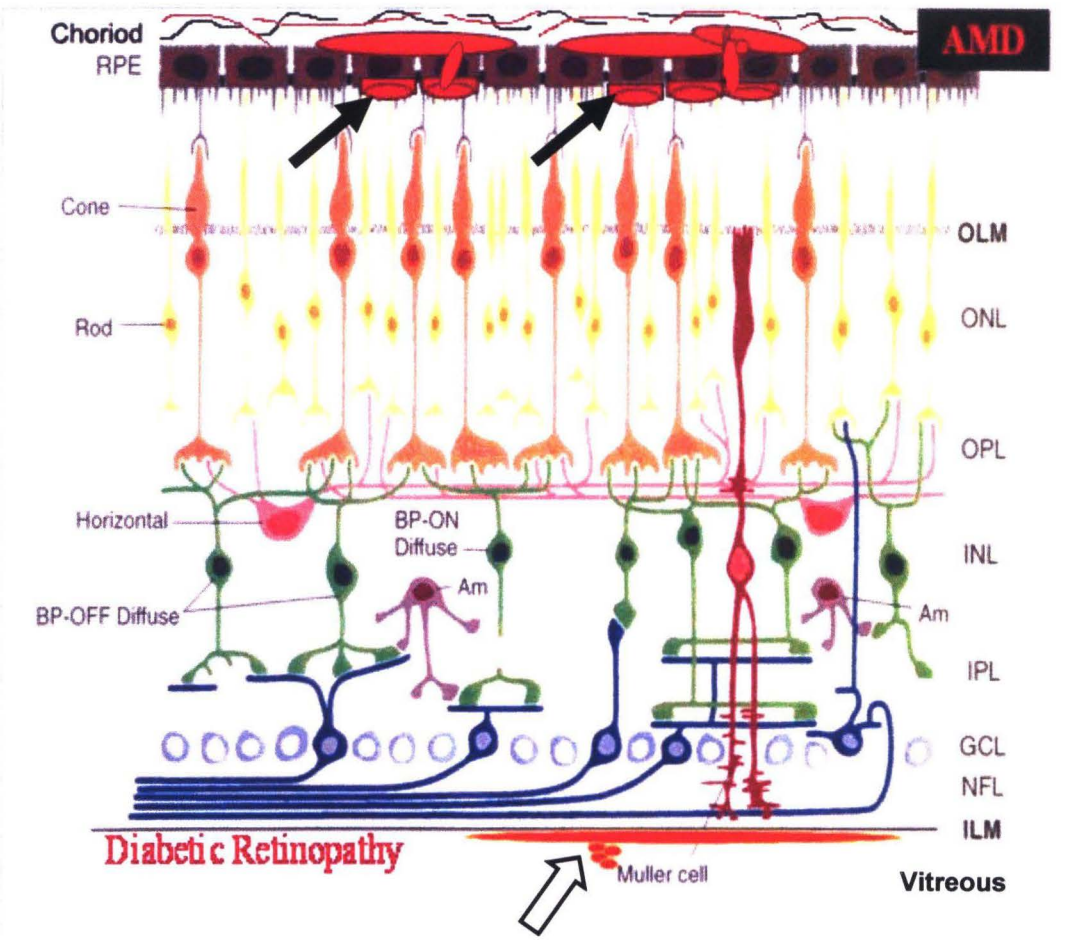

Figure 1*. Diagram of the retina. In AMD, NV grows from the choroid above the retinal pigment epithelial layer (RPE) into the subretinal space (black arrows). In diabetic retinopathy and retinopathy of prematurity NV originates from vessels in the inner retina and grows into the vitreous (open arrow). [Modified from original figure by John Dowling].*

In normal physiology, angiogenesis is involved in growth and development of the vasculature of an organism as well as an important aspect of wound healing. In females, NV occurs during the monthly cycle to regenerate the endometrium. NV also occurs in the pathogenesis of arthritis and atherosclerosis. NV also plays a vital role in the transition of

tumors from the benign to the malignant state. For a tumor to grow beyond a few millimeter in diameters it has to recruit new blood vessels. The body is able to maintain homeostasis by producing a number of angiogenic growth factors e.g. vascular endothelial growth factor (VEGF), as well as inhibitors of these factors (e.g. transforming growth factor, pigment epithelium derived factor (PEDF), and thrombospondin-1). When angiogenic growth factors are produced in excess of the angiogenic inhibitors, the balance is tipped in favor of neovascularization. This delicately balanced interaction between pro-angiogenic and antiangiogenic factors is fundamental to the maintenance of normal vasculature and hence visual integrity.

Ocular NV is characterized by extensive proliferation of new blood vessels by sprouting from preexisting ones into areas that should remain avascular, (e.g. the vitreous and subretinal space). NV is based on vascular remodeling and involves many stages summarized in Fig. 2. (Eichler, 2004).

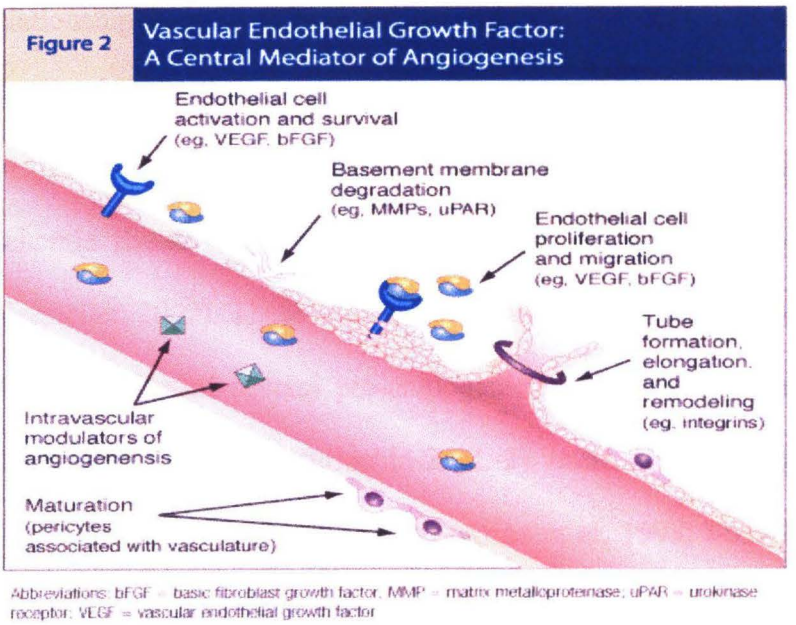

Figure 2*. Illustration of the process of angiogenesis. Angiogenic factors such as VEGF and basic fibroblastic growth factor (bFGF) are bound to receptors on the surface of endothelial cells, supporting their survival; in high concentration, they stimulate angiogenesis. The endothelial cells release matrix metalloproteinases (MMPs) and urokinases that degrade the basement membrane surrounding the capillary. The cells proliferate and migrate toward the source of the VEGF- finally forming tubes that are the precursors of the first branch of the vessel (Parikh et al, 2004).*

In diabetic retinopathy and ROP, the growing blood vessels leak proteins and fluid into the retina resulting in edema and blurred vision. They also break easily resulting in a hemorrhage and permanent vision loss occurs if not treated immediately. Research has long shown that hypoxia is one of the main driving forces for the upregulation of VEGF, a major factor promoting neovascularization (reviewed by Ferrara et al, 2003). In the rat ROP model, Reynaud and Dorey (1994) found that the risk and intensity of NV was strongly correlated with the avascular area of the retina. This avascular area is an area of high expression of VEGF (Dorey et al., 1996). The hypoxia induced upregulation of VEGF expression by retinal glial cells plays a vital role in initiation and progression of retinal NV (Hata, et al, 1995). In 1994, Miller et al demonstrated not only a causal

relationship between VEGF and retinal NV, but also a potential therapeutic value of VEGF inhibition in ischemic retinal diseases.

An inflammatory reaction elicited by macrophages and monocytes occurs in ischemic tissues and seems to be another major factor in the development of NV (Carmeliet, 2002). These cells accumulate in the ischemic tissue and are associated with the production of several pro-angiogenic substances including VEGF, tumor necrosis factor alpha (TNF-α), interleukin-2 (IL-2), basic fibroblastic growth factor (bFGF), and matrix metalloproteinases (MMPs) (Arras et al, 1998; Sunderkotter et al, 1994). It has been demonstrated that inflammation also plays a vital role in the ROP model (Paropia, 2001). Another pro-angiogenic substance produced by macrophages *in vitro* can inhibit the degradation of HIF-1α that causes the up-regulation of VEGF (Li et al 2000). Given the role of inflammation in NV and AMD Anti-inflammatory agents (e.g. IL-10) might be significant in future therapeutic approaches to ocular NV.

1.2. Vascular endothelial growth factor

Vascular endothelial growth factor, VEGF, is a heparin-binding hydrophilic glycoprotein with a molecular weight of 45kDa (Ferrara and Henzel, 1989). It belongs to a large family of growth factors which include placenta growth factor (PlGF), as well as six isoforms of VEGF. (Eriksson and Alitalo, 1999). These isoforms have different numbers of amino acids: 121, 145, 165, 183, 189 and 206, which arise from alternative splicing of mRNA. The small molecular weight proteins are relatively soluble whereas the larger ones, (e.g. $VEGF_{206}$ and $VEGF_{189}$) are almost completely sequestered in the extracellular matrix (Stalmans, 2005).

VEGF-A, the most studied of the group, is produced by a number of cells including neurons, retinal glial (Müller) cells, astrocytes, retinal pigment epithelial cells, epithelial cells and fibroblasts (Yi et al,1998; Herve et al, 2006). VEGF plays an important role in the development of the vasculature of animals during embryological development (Gogat et al. 2004). Elevated levels of VEGF occur in skeletal muscle during exercise, which stimulates capillary growth (Richardson et al, 2000). Studies show that basal levels of VEGF are always present in the retina, and are important in the development and maintenance of normal retinal vessels (Stone et al, 1995; Provis et al, 1997) as well as neural progenitors of the retina (Yang and Cepko, 1996; Hashimoto et al, 2006). In the CNS, VEGF is seen as both a neurotrophic as well as a survival factor.

VEGF is also associated with pathology and is considered the main factor promoting neovascularization, which occurs in cancers, ischemic and inflammatory diseases (Yancopoulos et al., 2000). It is also involved in the onset and progression of both AMD and diabetic retinopathy (Witmer, 2003). VEGF has a high specificity for VEGF receptors on endothelial cells, which proliferate, migrate and form new blood vessels when stimulated (Ferrara et al., 2003). Hypoxia causes an upregulation of VEGF production, which stimulates a signaling cascade in endothelial cells. These endothelial cells are first stimulated to degrade their basement membrane, then they proliferate, migrate, invade extracellular matrix and finally form new tubes.

1.3. VEGF Receptors

Three glycosylated tyrosine kinase receptors of VEGF have been identified. These include VEGFR-1 (Flt-1), VEGFR-2 (KDR) and VEGFR-3 (Flt-4). Flt-1 and KDR are

specific to VEGF-A, while Flt-4 is a receptor for VEGF-C and VEGF-D (Joukov et al., 1996). Researchers consider KDR, exclusively expressed in endothelial cells, to be the receptor involved in the mediation of VEGF signaling in endothelial cell.

When VEGF binds to KDR, a tyrosine kinase signaling pathway is initiated leading to MAP-kinase activation and gene expression. A variety of proteins are produced, including endothelial cell nitric oxide synthase. This enzyme stimulates production of nitric oxide which helps to promote blood vessel permeability, proliferation, migration and differentiation into new mature blood vessels (Hood J.D, 1998). It has also been reported that KDR plays an important role in activation of the phosphatidylinositol 3-kinase pathway, a pivotal signal transduction pathway in the process leading to endothelial cell survival induced by VEGF-A (Becker et al, 2001). Antibodies raised against VEGF can suppress tumor growth in vivo and NV in eyes with AMD (Kim et al., 1993). Blocking VEGF with specific antibodies is the treatment of choice for NV in AMD. The literature suggest many antiangiogenic substances, including IL-10, hold huge promise for treatments of cancer, arthritis, and ocular neovascularization.

1.4. ROP mouse model

When mice, at 7 days of age (P7), are placed in 75% oxygen the levels of hypoxia-inducible factor-1α (HIF-1α) falls (Ozaki et al, 1999). This decrease in HIF-1α levels causes a down-regulation of VEGF levels with subsequent damage to, and regression of the still delicate retinal vasculature, resulting in large areas of non-perfusion (Pierce et al 1996). When the mice are returned to room air, the large areas of non-perfusion result in hypoxia (Zhang et al, 2003), which triggers the up-regulation of HIF-1α (Ozaki et al,

1999). As summarized in figure 3, an increase in the levels of HIF-1α causes an increase in the levels of VEGF mRNA expression and finally VEGF (Pierce et al, 1996).

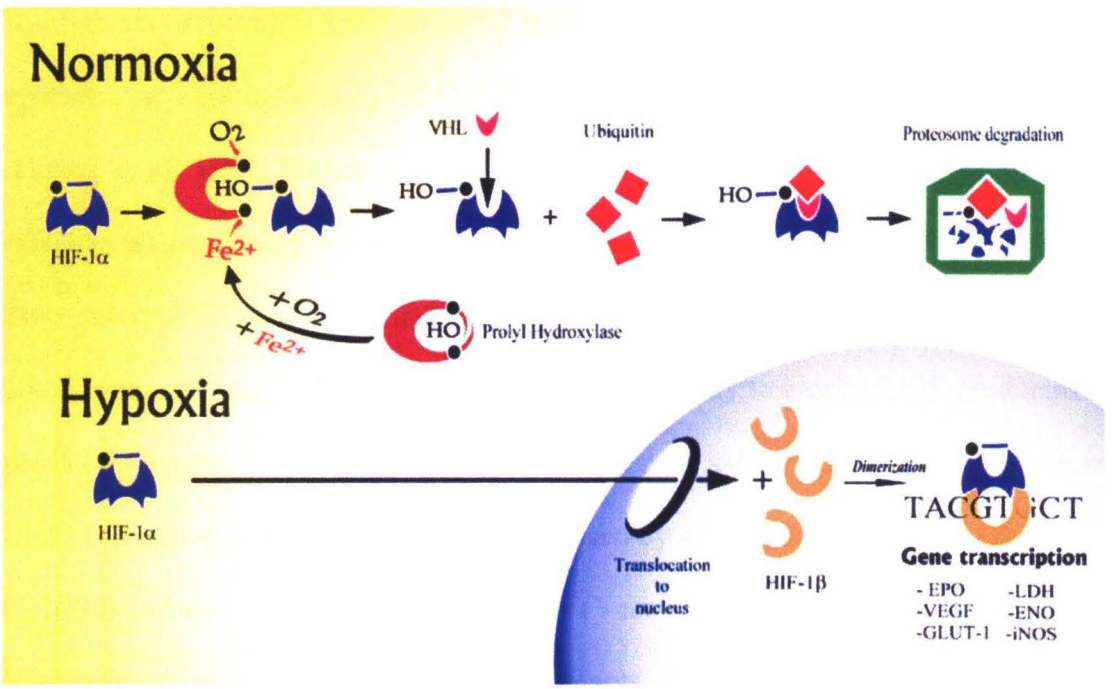

Figure 3. *HIF-1α regulation of VEGF expression. In normoxic conditions, HIF-1α is continuously produced and, in the presence of Fe^{2+} and oxygen, is continuously degraded through the Von Hippel Lindau protein (VHL)/Ubiquitin/Proteosome pathway. During hypoxia, prolyl hydroxylase, which catalyzes the hydroxylation of proline residues in HIF-1α under normal O_2 conditions (Schroedl et al, 2002), is inhibited and HIF-1α accumulates. After translocation and combination with the constitutive HIF-1β, the transcription factor binds to hypoxic response element (HRE) sequences to upregulate a number of downstream genes such as VEGF. (Figure 5, LaManna et al, 2004).*

1.5. Interleukin-10

Our experiments tested the possibility that IL-10, an anti-inflammatory cytokine, would inhibit NV. IL-10 is a pleiotropic cytokine that is produced mainly by macrophages, Th2 lymphocytes and B-lymphocytes. It is a powerful anti-inflammatory and immunosuppressive protein that can suppress production of interferon gamma (IFN-

γ), IL-2 and pro-inflammatory cytokines. IL-10 can also inhibit proliferation and development of Th1 cells, monocyte/macrophage cells as well as natural killer (NK) cells and their subsequent cytotoxic effects.

IL-10 is also reputed to have powerful antiangiogenic effects and block expression and synthesis of TNF-α, VEGF, MMP-9 and hence neovascularization and tumor growth (Huang et al, 1999; Stearns, 1999). Cervenak et al, (2002) showed IL-10 to be quite effective in abolishing angiogenicity and tumorigenicity of Burkitt's lymphoma, and Matsumoto (1997) found that IL-10 inhibited VEGF production by leukocytes of patients with kidney disease. If IL-10 can be shown conclusively to block VEGF production, the potential therapeutic implications are vast. The risk of side effects would be low because IL-10 is a native protein endogenously produced by microglia of the retina (Broderick et al, 2000) and shown to be anti-inflammatory (Verwaerde et al, 2003) and neuroprotective (Koeberle et al, 2004). Current developments in this field are exciting for both researchers and sufferers of various kinds of neovascular diseases of the eye. Given these possibilities, research was undertaken to determine the efficacy of IL-10 to prevent ocular NV. The objective was to create NV in the murine model ROP and to inject IL-10 intraocularly into 12 day old mice and, finally, to analyze NV in treated and untreated eyes.

Chapter 2.

Materials and Methods

2.1. Mice

For this study, twenty normal C57BL/6J mice from the Jackson Laboratory (Bar Harbor, ME) were used. These mice were housed in the vivarium at Florida Atlantic University where the neonates were born. Of the twenty animals used, three expired prematurely and three had cataract (Table 1).

2.2. Oxygen exposure

The oxygen induced ROP model was used for this experiment. At postnatal day 7 (P7), neonates were divided among three cages, each with one nursing dam and several pups. Cages were placed in a hyperoxia chamber (designed and constructed by Dr. Dorey) for five days. A control group was placed in the same room and exposed to room air (21%O_2). Both groups were exposed to 12 hours on / 12 hours off light cycle at 23°C (room temperature) and given food and water ad *libitum.*

The O_2 level in the chamber was carefully maintained at 75% ± 1% with medical oxygen using a PROOX Model 110 gas Oxygen controller (BioSpherix, Ltd. Redfield, NY). Leakage was adjusted to permit a high flow rate (1.5 to 2.0 l/min) to minimize CO_2 levels. The chamber was not opened during the five day period but was checked regularly to ensure proper functioning. The P12 experimental animals were removed five days later and placed normoxia. At this time, one eye received either an intravitreal injection of IL-10 (1μL of 10μg/ml) or phosphate buffer solution (PBS). Uninfected contralateral eyes served as a controls.

Prior to injection the animals were anaesthetized by an intra-peritoneal injection of ketamin/xylazine solution, 3-3.5µl/gram of body weight. [For 5ml of anesthetic: 3.5 ml 0.1M PBS + 1.2 ml Ketamin (100mg/ml) + 0.3ml xylazine (20mg/ml)]. One drop of Atropine sulfate (1%) was applied to the mouse eyes to dilate the pupils to facilitate the injection. A topical solution of 2.5% methylcellulose was applied to prevent drying of the cornea. Toe clipping allowed animal identification. Intravitreal injections were performed using a Hamilton 801RN 10µl syringe. All animals recovered on a heating pad and antibiotic ointment (Neomycin + Polymyxin B + Dexamethasone ophthalmic ointment) was applied to the site of injection to prevent infection. Both controls and experimental animals were returned to the vivarium and left in room air (normoxia) for another five days.

Table1. Treatment of animals with IL-10 or PBS.

Mouse	Hyperoxia-75%	Normoxia	Treatment	Comments
M1	×		IL-10-L	Cataract-L
M2	×		PBS-R	Cataract-L
M3	×		IL-10-L	
M4		×	IL-10-L	
M5	×		IL-10-L	
M6	×		PBS-L	Cataract-L
M7		×	IL-10-L	
M8	×		IL-10-L	
M9		×	PBS-L	
M10	×		IL-10-L	
M11		×	No injection	
M12	×		IL-10-L	
M13	×		No injection	
M14	×		PBS-L	
M15	×		PBS-R	
M16		×	No injection	
M17		×	No injection	
M18				Expired
M19				Expired
M20				Expired

Total number of animals used in this experiment and treatment received. Animals were either exposed to hyperoxia or room air. One eye received an injection of IL-10 and the contra-lateral eye received either an injection of PBS or no injection.

2.3. Phosphate Buffer Solution

The PBS solution was made from two freshly prepared stock solutions as follows: Stock A (27.6g/1000ml of monobasic sodium phosphate), Stock B (28.4g/1000ml of dibasic sodium phosphate).To make 200mls of a 0.01M buffer solution required, 1.9mls of stock A, 8.1mls of stock B, and 1.7g NaCl in 200mls total. The pH was adjusted to 7.4 with hydrochloric acid using a pH meter. After the pH adjustment, 0.04g $MgCl_2$ and 0.02g $CaCl_2$ (hydrous) were added. For PBS with bovine serum antigen (PBS-BSA), .02g BSA was added to the freshly prepared batch of the above. All solutes were properly dissolved and solutions were stored at 4°c.

2.4. Tissue Processing

Twenty animals at P17 were sacrificed by exposure to CO_2 followed by decapitation. The eyes were enucleated with the aid of a dissecting light microscope using iridectomy scissors. The cornea was punctured using a stainless steel needle. Eyecups were placed in 4% paraformaldehyde (prepared in PBS). Paraformaldehyde fixation was chosen since it does not affect lectin binding patterns compared to those of frozen sections and has relatively low levels of auto-fluorescence. After two hours, a slit was made in the cornea and the eyecup returned to the fixative (4°C).

Following overnight fixation, the corneas were removed, the lenses excised and the eyecups rinsed in several changes of PBS for 12 hours. Thorough rinsing reduces background fluorescence associated with formaldehyde fixation. The eyes were embedded in acrylamide following the procedure of Blanks and Johnson (1984) using freshly prepared ice-cold acrylamide monomer solution to which was added 5µl/ml (vol.: vol.) of freshly prepared 10% ammonium per sulfate solution to induce polymerization.

A small amount of this mixture was placed in 1.5ml tubes and allowed to polymerize to form a "pad". An eyecup was carefully placed on each "pad" and more freshly prepared ammonium persulfate/acrylamide mixture added to cover the tissue. Tubes were covered and placed in the fridge for two hours to enhance hardening of the gel. Holes were punctured in the cap of each tube to facilitate closure without moving the specimen.

After acrylamide polymerization, the tissue was carefully cut from the tube using a moistened razor blade and placed in a small (15mm×15mm×5mm) plastic freezing mold and surrounded by O.C.T. (an embedding medium for frozen tissue specimens to ensure optimal cutting temperature). Care was taken to properly position the eye cup. Each tube was then gently immersed in liquid nitrogen to freeze the tissue and stored at -80°C.

2.5. Cryostat sectioning

Tissue equilibrated for an hour in the cryostat (20°C) to increase the ease of sectioning and reduce cracking. Blocks were then removed from the molds and mounted on standard cryostat chucks using O.C.T. Blocks were trimmed until the optic nerve was barely visible, then every 10^{th} section was taken. The first section taken was labeled number 1, and then the next was number 11, the next number 21 and so on. For each retina, about six sections (1, 11, 21, 31, 41 and 51) were mounted on glass slides. Tissues were sectioned at 7-10μm in thickness using disposable microtome blades (Richard-Allen Scientific, Kalamazoo, MI) and melted on Poly-L-Lysine coated microscope slides (NewComer supply, Middleton, WI) in serial order. Slides were stored at 4°C. There were three sections per slide.

2.6. Lectin Binding

Sections were incubated in a humid chamber for 15 minutes in PBS containing 1mg/ml of bovine serum albumin (PBS-BSA) followed by rinsing for 10 minutes in PBS. Excess buffer was drained off the sections. Prior to staining, each section was encircled using liquid-repellent slide marker pen to reduce the possibility of the lectin solution losing contact with tissue. Sections were covered with a solution of 20μg/ml of fluorescein-conjugated Griffonia simplex lectin. All lectin stocks were kept at 4°C, diluted in PBS-BSA, and filtered before use. Following lectin binding, sections were rinsed in PBS for 15 minutes and coverslipped using Fluoromount-G (Southern Biotech, Birmingham, Al). The mounted sections were dried before microscopic examination.

2.7. Fluorescence microscopy

An Olympus Provis AX70 fluorescent microscope fitted with an Olympus PMC35DX digital camera was used to examine and photograph each retinal section using MagnaFire 2.1 software. An UPlan F1 20×/0.50Ph1 objective lens was used. Images were taken sequentially to obtain a complete montage of each section. These were labeled alphabetically (e.g.1a, 1b, 1c, 1d etc). The lectin-positive vascular profiles in each retinal section were clearly observable. Each image taken was properly labeled e.g. (retina number, experiment number, date). In order to ensure accurate measurements when using Metamorph© analytical software (used to quantify changes in NV), areas were identified on each image and served as a landmark to reduce the possibility of counting the same vessel twice. Records of each section were kept by sketching areas of overlap. Digital images were copied to compact discs for analysis.

2.8. Image analysis

Metamorph© analytical imaging software version 6.0 (Universal imaging Corporation, Downingtown, Pa 19335) was used to interactively analyze the retinal sections. Archiving of the retinal images was achieved using Adobe Illustrator®.

2.9. Determination of retinal vascular area

The retinal vascular area was analyzed by first outlining the area between the outer plexiform layer and inner limiting membrane (Fig.4). Isolation of the blood vessels was done by interactive adjustment of a threshold until only the brightly stained fluorescent blood vessels were included in the measurement (Figs. 5 & 6). The retinal blood vessel area and retinal area were obtained and saved to Microsoft Excel data spreadsheets. The percent retinal vascular area was calculated by [(blood vessel area/retinal area ×100)]. Each image of every retina was examined and the average retinal vascular area and standard deviation (SD) determined.

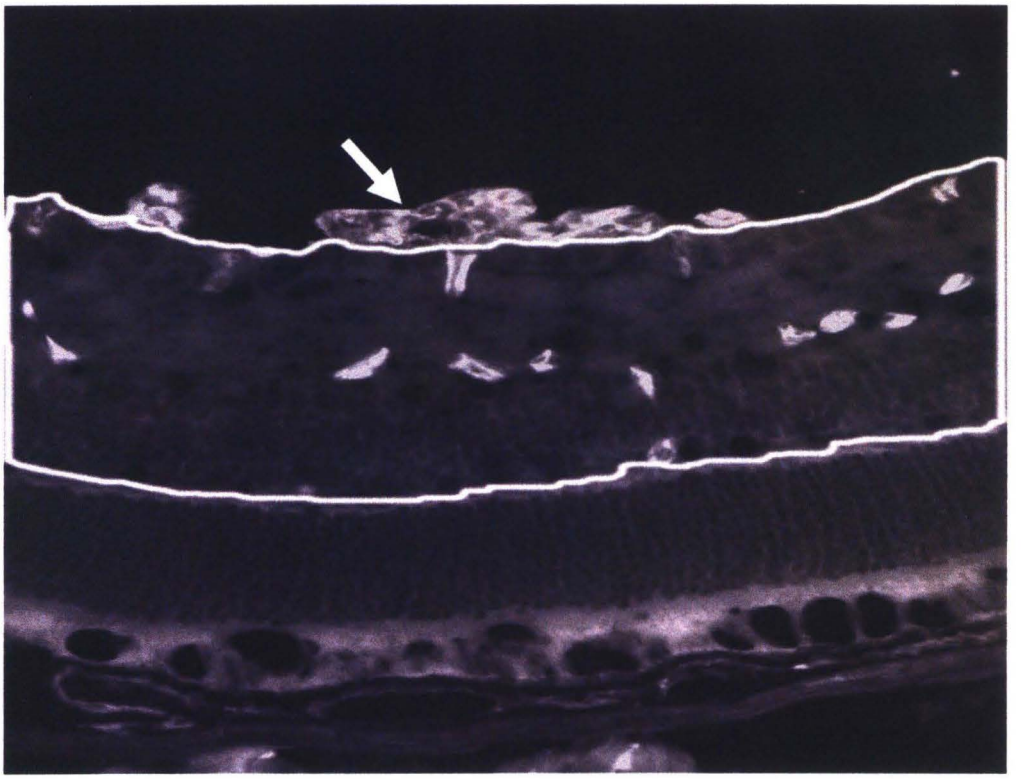

Figure 4. *Retinal section showing measurement of total area. Arrow indicates NV extending from the inner retina into the vitreous.*

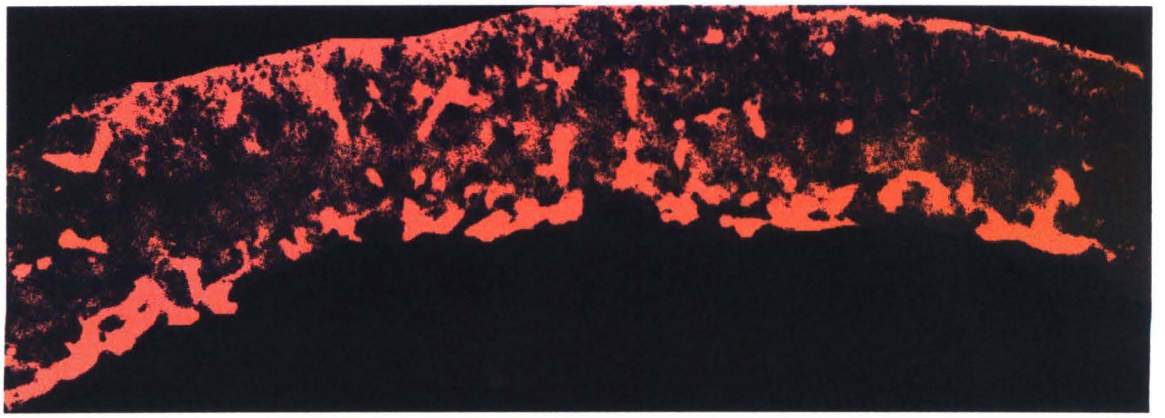

Figure 5. *Retinal section as it appears in the MetaMorph program before thresholding.*

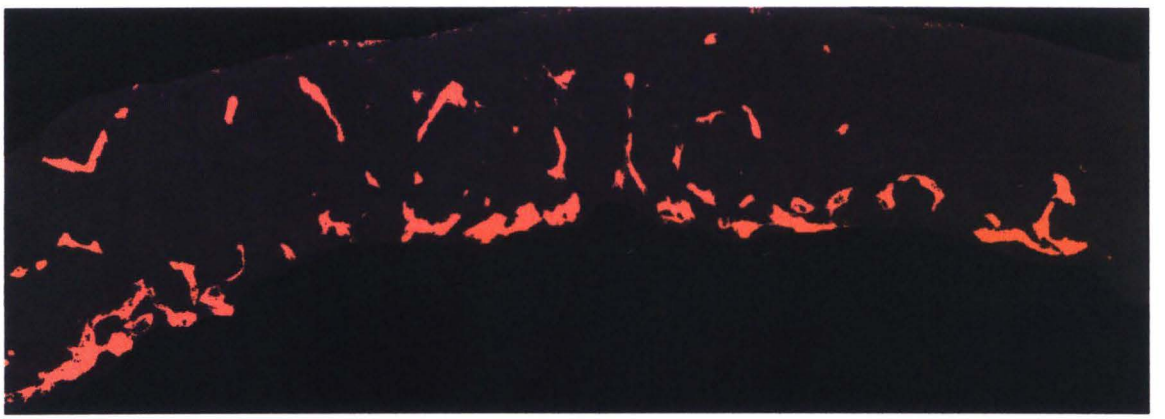

Figure 6. *Retinal section after thresholding. Only fluorescent vessels shown.*

2.10. Determination of NV Area.

Neovascularization (NV), in this study, is defined as the presence of lectin-stained blood vessels within or external to the inner limiting membrane, i.e., within the vitreous. For those sections which showed NV, the total area of NV was determined by encircling the NV and measuring the area of fluorescently tagged vessels. The percent of all blood vessels in the section that were truly NV was calculated [(NV blood vessel area/total

blood vessel area) ×100]. Total retinal blood vessel area was obtained by adding the retinal blood vessel and NV areas (zeros denoted areas without NV). Data for each retina was obtained by adding the individual sections.

2.11. Statistical analysis of data.

Each retina had about six sections and for each section, there were several images. For each retina, the average of all the images from each section was determined so that each animal was represented by a single data point. The standard deviations were also determined. Statistics was performed in Excel using the standard student's unpaired t-test.

Chapter 3

Results

Of the eleven animals exposed to hyperoxia, NV occurred in all. Representative examples are shown in Figure 7. The oxygen exposed animals developed abundant intra-vitreal neovascularization, as expected, whereas animals in room air showed no NV (Fig. 8). As summarized in Figure 8, IL-10 inhibited NV area by 79.05 % ($p < 0.00004$). The IL-10 treated left eyes consistently had less NV area than the untreated contra-lateral right eye (Fig. 9).

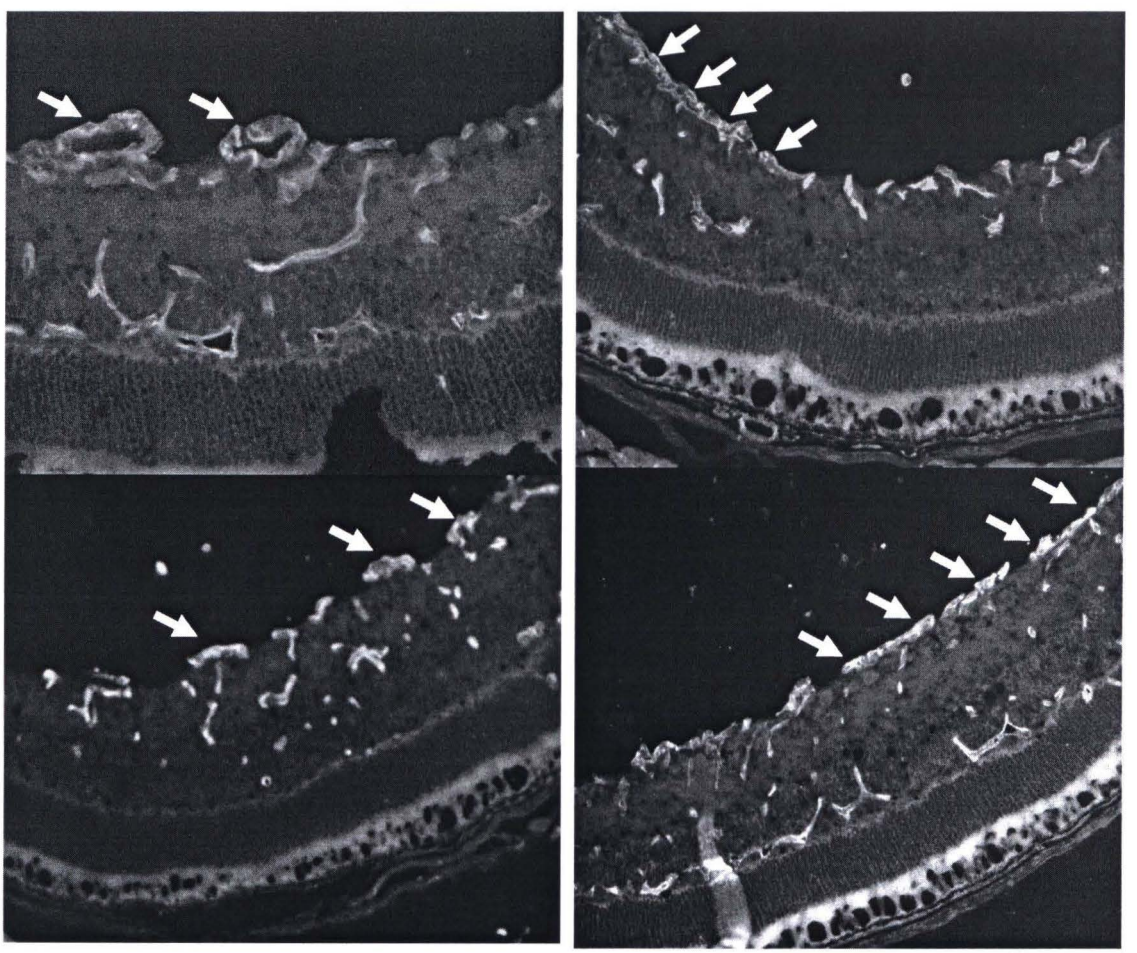

Figure 7. *Representative images showing neovascularization in retinas from four different mice that had been exposed to oxygen. In each image, NV fronds (white arrows) are found on the inner surface of the retina, extending into the vitreous. Original magnification 200X*

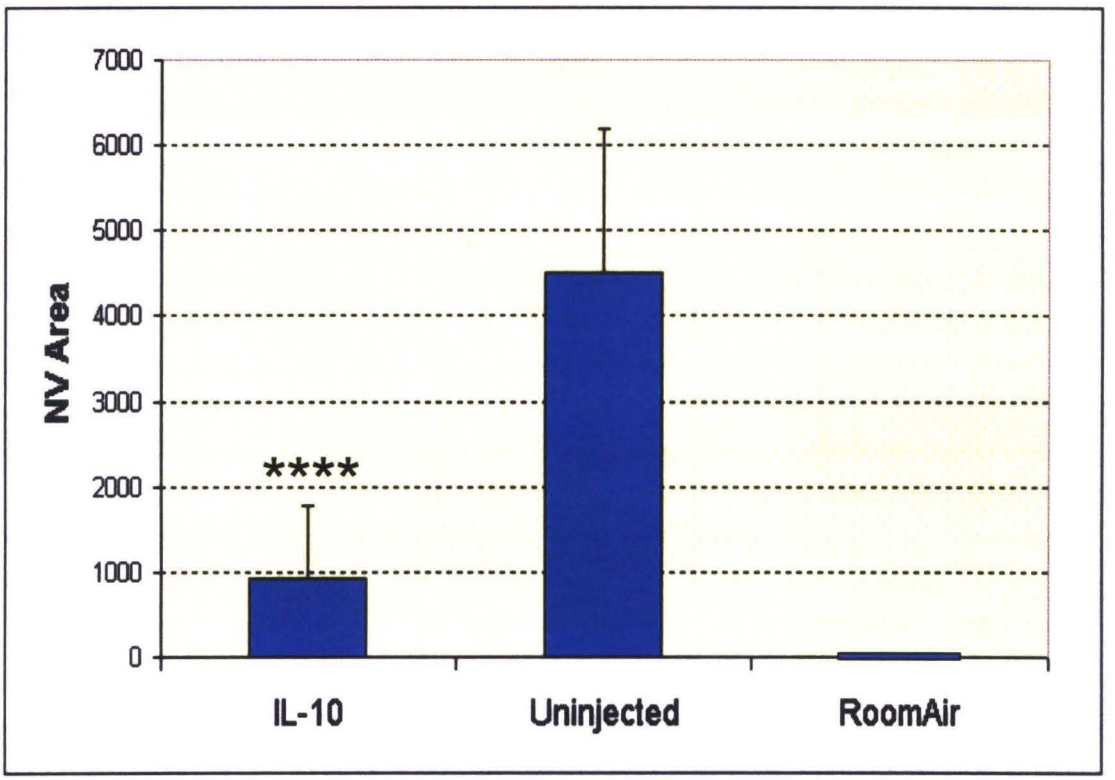

Figure 8. *Effect of IL-10 on the NV area. IL-10 injection reduced NV by 79.5 %. The room air animals showed no NV. (Means and Standard deviations; ***** = $p<0.00004$).*

To compare the relative effect of IL-10 on normal vessels and on NV, we examined the percent of retina occupied by blood vessels and the percent of vessels that were NV. The area of retina occupied by blood vessels (percentage vascular area) and the percent of vessels that were neovascular (NV) for each of the oxygen-exposed animals are presented in Table 2. The IL-10 injected animals had a NV score of 1.75%± 1.7%, considerably lower than the NV score of 8.3 %± 4.0% seen in the uninjected animals (Fig. 10).

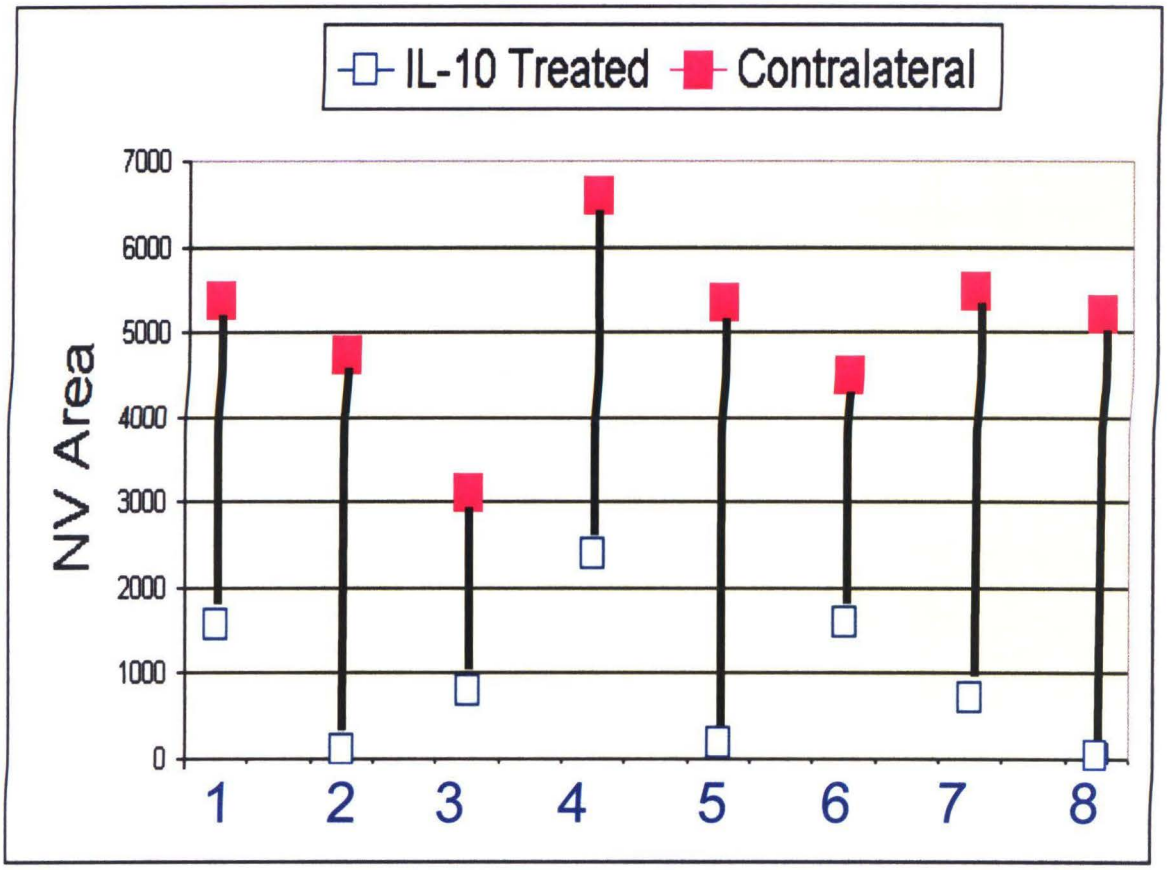

Figure 9. *Comparison of NV area in the IL-10-injected right eyes and the contralateral uninjected left eyes of eight mice.*

IL-10 treatment did not alter the area of the intra-retinal vessels (Fig.11) or the percent of retinal area occupied by blood vessels (Fig. 12), suggesting that IL-10 acted selectively. Selectivity was further supported by the evidence that neovascularization represented a significantly lower percent of the total blood vessel area (in the retina and vitreous) in treated eyes than in the untreated eyes (Fig. 10). If both types of vessels were affected equally, the percent occupied by NV would not change.

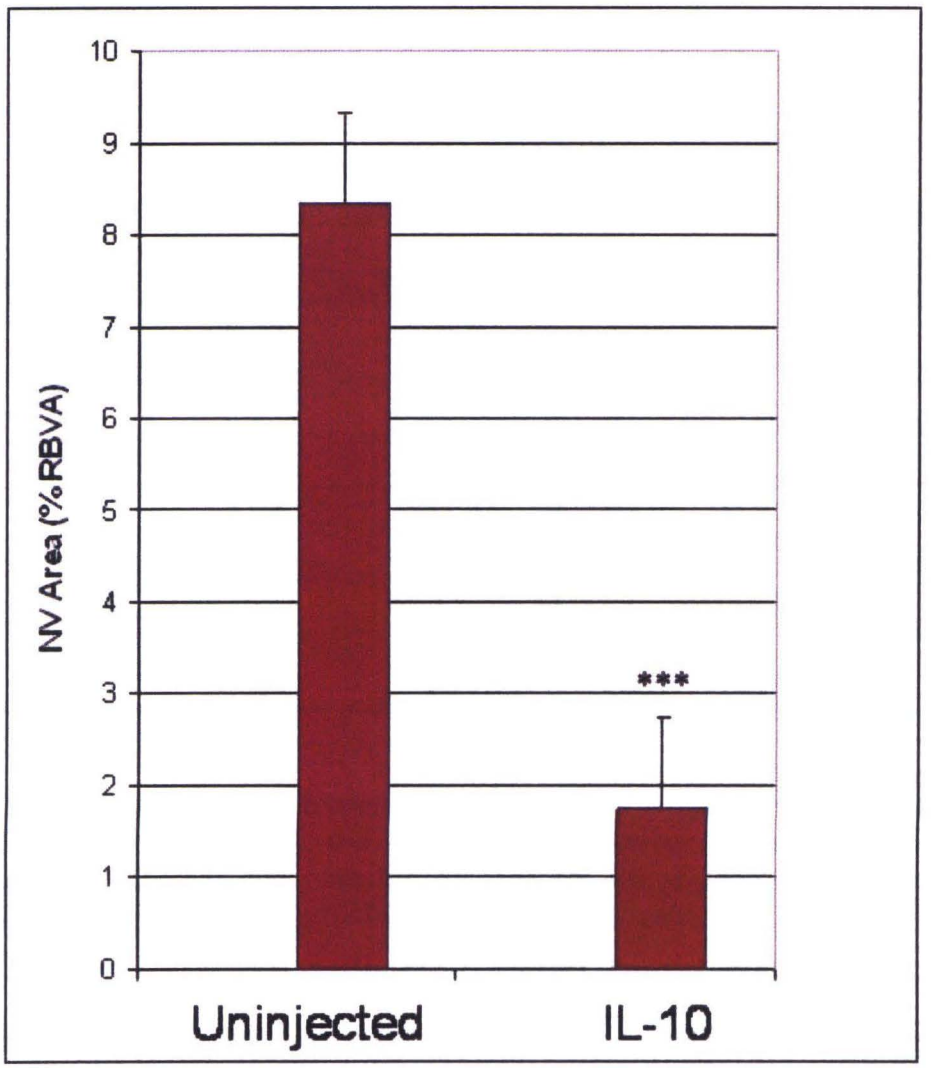

Figure 10. *Effect of IL-10 on the NV Area; (Expressed as a percent of total retinal blood vessel area; %RBVA) in animals exposed to hyperoxia. IL-10 significantly reduced the %RBVA ($P<0.002$; Means and Standard Deviation).*

Table 2. *Vascular areas in hyperoxia-exposed animals.*

	Contralateral uninjected eyes		Injected eyes		
Animal #	% vascular area	% NV area	Injected	% vascular area	% NV area
1	0.23	0.47	*		
2	1.99	5.28	*		
3	1.15	8.32	IL-10	1.27	1.12
5	2.92	10.25	IL-10	0.36	4.41
6	1.56	6.72	PBS	0.64	8.02
8	3.67	11.47	IL-10	1.72	2.35
10	1.76	15.86	IL-10	3.14	0.6
12	3.02	7.42	IL-10	1.78	0.24
13	1.64	9.91	*		
13	0.46	5.29	*		
15	1.36	10.70	PBS	0.01	0

The percent of the retinal area occupied by blood vessels (percentage vascular area) and the percent of all vessels that were within the vitreous (percentage NV area) determined in IL-10 injected and contralateral eyes of mice exposed to hyperoxia (= Retinal sections rejected due to problems during cryosectioning).*

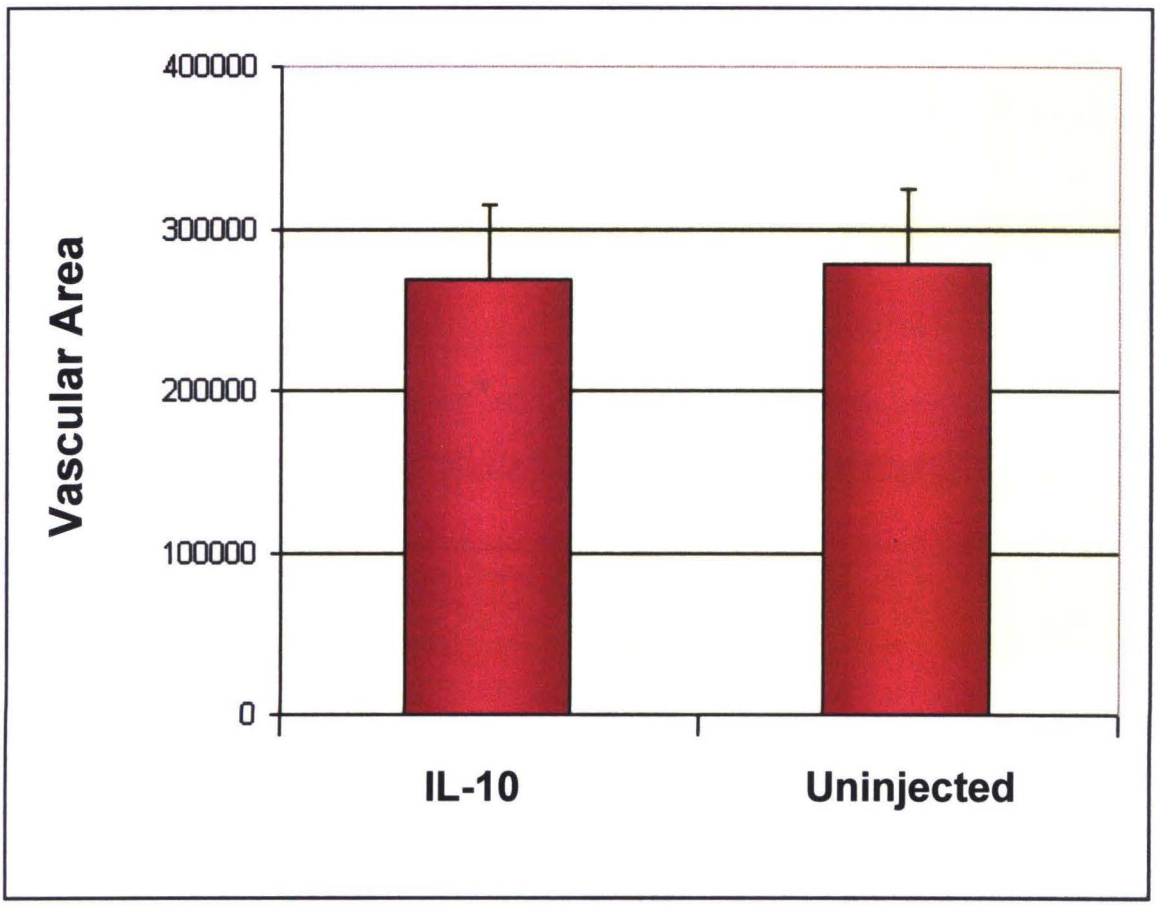

Figure 11. *Effects of IL-10 on total area of blood vessels in retinas from mice exposed to hyperoxia (Means + Standard Deviation).*

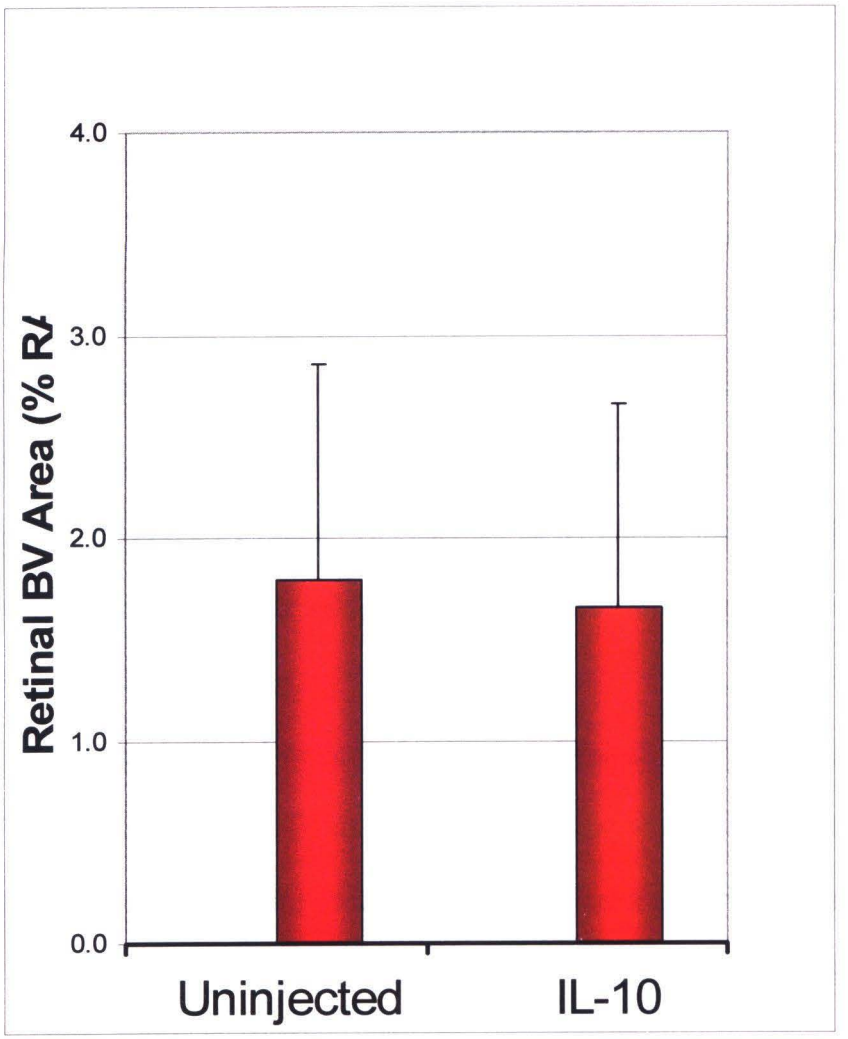

Figure 12. *Effects of IL-10 on the percent of retinal area occupied by blood vessels from mice exposed to hypoxia (Means + Standard Deviation).*

Chapter 4.

Discussion

The murine ROP (retinopathy of prematurity) model provides a useful tool to investigate the effects of IL-10 on ocular neovascularization. Neonatal mice exposed to hyperoxia develop ocular NV when returned to room air (Smith, 1994). Of the twenty animals in this experiment, three developed a cataract and three died prematurely during elevated oxygen exposure. The cataract development may have been caused by damage to the lens as a result of the intraocular injection. Despite these minor problems the results are encouraging.

Using this model we have demonstrated that IL-10 treatment caused reduction in NV (Table 2 and Fig. 10). Of the eleven animals exposed to hyperoxia all developed NV. A comparison of IL-10 injected right eyes with contra-lateral uninjected eyes of eight mice (where data was available for both eyes) showed that IL-10 consistently lowered the NV area (Fig. 8). Our results are significant and consistent with the findings of Kohno et al, (2003) who demonstrated that IL-10 reduced angiogenesis in tumors formed by two ovarian cancer cell lines. Earlier, similar reports demonstrated that IL-10 can inhibit tumor development in Burkitt's lymphoma (Cervenak et al 2000). Cervenak further suggested that this inhibition of tumor growth was due to IL-10's direct anti-angiogenic activity. It has also been showed that IL-10 significantly reduced ischemia-induced angiogenesis in a mouse hind limb model of NV (Silvestre, 2000). Huang (1996) demonstrated that IL-10 suppressed tumorigenicity in nude mice by inhibiting NV, and this anti-angiogenic effect was due to down-regulation of VEGF.

IL-10 might have a direct effect on VEGF by down regulation of its production. Alternatively IL-10 may not act by completely blocking VEGF production, but instead by reducing VEGF to levels that no longer support NV. However, IL-10 is known to not only reduce VEGF expression, but also to act as an anti-inflammatory agent suppressing macrophage activation and cyclooxygenase-2 (Cox-2) expression. Inhibitors of Cox-2 are known to suppress NV. Mertz (1994) and later Tsujii (1998) have suggested that IL-10 may inhibit Cox-2 which is known to stimulate the angiogenic process by up-regulating VEGF. In addition, Stearns et al (1999) found that IL-10 inhibits secretion of matrix metalloproteinases (MMP2 and MMP9) which are considered essential for vessel growth. In order to fully examine the promising therapeutic potential of IL-10, further research is needed to determine the targets of its antiangiogenic action.

In addition to its effect on NV, we observed that IL-10 treatment had essentially no effect on total area of retinal blood vessels in animals exposed to hyperoxia. Anti-angiogenic agents, especially those targeting VEGF, act on both developing blood vessels and NV. If IL-10 works by suppressing VEGF, it would be expected to have no effect on mature blood vessels, but would cause regression of newly formed vessels and would inhibit further growth of developing vessels. During the post oxygen-exposure period, intra-retinal vessels repaired equally well in the IL-10 treated and untreated eyes (Figs.11 and 12). In contrast, IL-10 inhibited NV growth. The fact that IL-10 appears to reduce NV in affected areas of the retina while leaving normal vessel repair intact, is of potential therapeutic significance. This is especially important in infants with ROP. When premature infants are placed in hyperoxia, there is down-regulation of HIF-1α and subsequently VEGF. This down-regulation of VEGF results in damage to and regression

of the retinal blood vessels resulting in areas of non-perfusion (Ozaki et al, 1999). When infants are returned to room air, these areas of non-perfusion become ischemic and trigger VEGF production, resulting in NV growing into the vitreous. The current treatment strategy for babies with ROP is to apply several laser burns to the peripheral avascular region of the retina. This ablates the ischemic area of the retina, reducing VEGF and resulting in regression of the vessels growing over the central macular region of the eye. While it causes severe loss of peripheral vision, this procedure preserves more functional vision and prevents total blindness. (Hurley et al., 2006).

NV in diabetic retinopathy also results in vessel growth into the vitreous. Clinical treatment involves placing a grid of small laser burns over the entire retina to reduce the oxygen required by the retina, and thus reducing hypoxia.

An optimal anti-angiogenic agent for both ROP and diabetic retinopathy would treat the NV, while leaving the other retinal vessels intact. Our data on IL-10 suggests this is feasible. The fact that IL-10 is produced in the retina and helps maintain immune regulation in the eye further enhances the significance of this work.

If IL-10 is functioning as an anti-inflammatory agent in suppressing NV, our data strongly suggest that IL-10 could be an effective treatment for the "wet" form of AMD. Interestingly IL-10 is reported to be neuroprotective and might also help preserve photoreceptors. In both forms of AMD, NV occurs after long periods of the disease and recurrent episodes are common. A single injection of IL-10 with its very short half life of three to five hours would not work; sustained release would therefore be desirable. Slow release pellets could be surgically placed into the eye to prolong the treatment period for diabetic retinopathy. Gene therapy also shows potential as an effective treatment strategy.

For example, cells transfected with the IL-10 gene could be encapsulated and placed in the vitreous to express IL-10. These cells would secrete IL-10 continuously hence solving the problem of the short half life of IL-10. Gene therapy is likely to have an advantage over repeated intravitreal injections, which carry the risk of causing infection and cataract development.

In summary, we have demonstrated that IL-10 significantly reduced NV in the murine model of ROP. We have also shown that IL-10 has no effect on the total retinal blood vessel area of hyperoxia-exposed animals, indicating that IL-10 is acting selectively. Although the exact mechanism of action of IL-10 is still not certain, these findings suggests that the use of IL-10 as an antiangiogenic agent may be beneficial in the treatment of patients with diabetic retinopathy, wet AMD and/or ROP.

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