HERITABLE INFLUENCES IN OXYGEN-INDUCED RETINOPATHY

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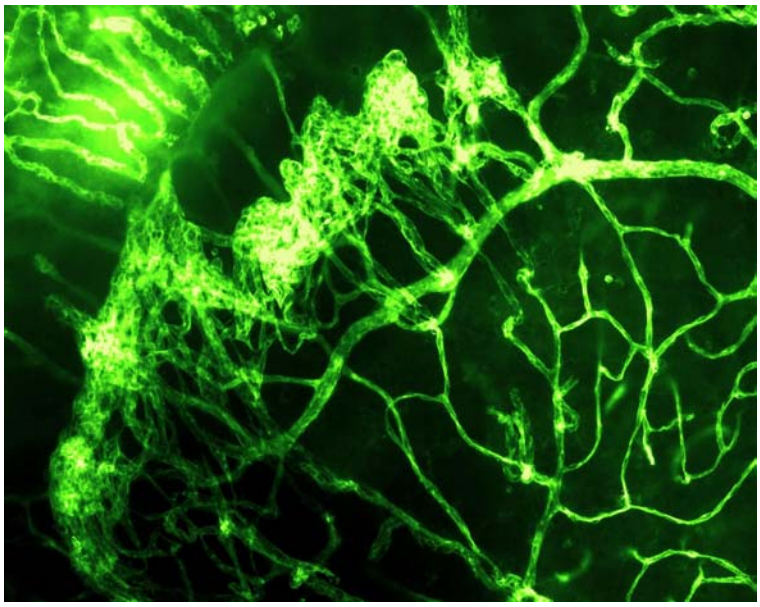
MBBS (Hons)



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SUMMARY OF THESIS

Retinopathy of prematurity, a disease characterised by aberrant retinal vascular development in premature neonates, is a leading cause of blindness and visual impairment in childhood. This work sought to examine differences in the susceptibility of inbred rat strains to oxygen-induced retinopathy, a model of human retinopathy of prematurity. The overriding aim was to identify genetic factors in rats that might be generalisable to humans.

Newborn rats of six different strains were exposed to alternating cycles of hyperoxia and relative hypoxia for fourteen days. Rats were removed to room air and killed for analysis immediately, to assess oxygen-induced retinal vascular attenuation, or four days later to evaluate the extent of hypoxia-induced vasoproliferation. Whole flat-mounted retinae were stained with fluorophore conjugated isolectin GS-IB4, and measurement of vascular area was conducted using fluorescence microscopy and video-image analysis. A hierarchy of susceptibility to the inhibitory effects of cyclic hyperoxia and relative hypoxia on postnatal retinal vascularization was identified for the rat strains studied. Susceptibility to vascular attenuation was predictive of the subsequent risk of vascular morphological abnormalities. Cross-breeding experiments between susceptible and resistant strains demonstrated that the susceptible phenotype was dominantly inherited in an autosomal fashion. These studies confirmed an association between ocular pigmentation and retinopathy risk, however the finding of differential susceptibility amongst albino rat strains implicated factors in addition to those associated with ocular pigmentation.

Quantitative real-time reverse transcription-polymerase chain reaction was used to compare the retinal expression of angiogenic factor genes in susceptible and resistant strains with the aim of identifying a genetic basis for the strain difference. Eight angiogenic factor genes were selected for study: vascular endothelial growth factor (VEGF); VEGF receptor 2; angiopoietin 2; Tie2; pigment epithelium-derived factor; erythropoietin; cyclooxygenase-2 and insulin-like growth factor-1. The most notable difference between strains was the expression of vascular endothelial growth factor

(VEGF) during the cyclic hyperoxia exposure period – higher VEGF expression was associated with relative resistance to retinopathy. Other differences in retinal angiogenic factor gene expression between strains, such as higher expression of VEGF receptor 2 and angiopoietin 2 in resistant strains, appeared to be secondary to those in VEGF. Following cyclic hyperoxia, the expression pattern of angiogenic factor genes changed – messenger RNA levels of hypoxia-induced genes, including VEGF, VEGF receptor 2, angiopoietin 2 and erythropoietin, were significantly higher in those strains with larger avascular areas, than in those strains that were relatively resistant to retinopathy. These findings provide firm evidence for hereditary risk factors for oxygen-induced retinopathy in the rat. Differences in the regulatory effects of oxygen on VEGF expression appear to be central to the risk of retinopathy. The potential relevance of these hereditary factors is discussed in the context of the human disease.

PUBLICATIONS ARISING FROM THIS THESIS

1. van Wijngaarden, P., Coster, D.J. and Williams, K.A, *Inhibitors of Ocular Neovascularization: Promises and Potential Problems.* Journal of the American Medical Association, March 23/30, 2005. **293**(12): p. 1509-1513.

2. van Wijngaarden, P., Coster, D.J., Brereton, H.M., Gibbins, I.L. and Williams, K.A, *Strain-Dependent Differences in Oxygen-Induced Retinopathy in the Inbred Rat.* Investigative Ophthalmology and Visual Science, April, 2005. **46**(4): p. 1445-1452.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed: _____ Date: _____

Peter van Wijngaarden

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ABBREVIATIONS

≤	less than or equal to
≥	more than or equal to
~	approximately
#	number
°C	degrees Celsius
µg	microgram (10^{-6} g)
µl	microlitre (10^{-6} l)
µM	micromolar (10^{-6} M)
µm	micrometer (10^{-6} m)
A	adenine
aa	amino acid
a/bFGF	acidic/basic fibroblast growth factor
Ang 1	angiopoietin 1
Ang 2	angiopoietin 2
AP-1/-2	activator protein-1/-2
ARBP	Acidic Ribosomal Phosphoprotein
ARNT	aryl hydrocarbon receptor nuclear translocator (HIF-1β)
ARVO	Association for Research in Vision and Ophthalmology
ATP	adenosine triphosphate
BM	Bruch's membrane
bp	base pairs
C	cytosine
cDNA	complementary DNA
cGMP	cyclic-guanosine monophosphate
cm	centimetre
COX 2	cyclooxygenase 2
CRYO-ROP	the Multicentre Trial of Cryotherapy for Retinopathy of Prematurity
Da	Dalton
DA	Dark Agouti rat strain
DAG	diacylglycerol

DAO2	Dark Agouti rats exposed to cyclic hyperoxia and relative hypoxia for the first two days of life – day two follows a 24 hour period of relative hypoxia
DAO3	Dark Agouti rats exposed to cyclic hyperoxia and relative hypoxia for the first three days of life – day three follows a 24 hour period of hyperoxia
DAO8	Dark Agouti rats exposed to cyclic hyperoxia and relative hypoxia for the first 8 days of life – day 8 follows a 24 hour period of relative hypoxia
DAO9	Dark Agouti rats exposed to cyclic hyperoxia and relative hypoxia for the first 9 days of life – day 9 follows a 24 hour period of hyperoxia
DAO14	Dark Agouti rats exposed to cyclic hyperoxia and relative hypoxia for the first 14 days of life
DAO18	Dark Agouti rats exposed to cyclic hyperoxia and relative hypoxia for the first 14 days of life, followed by four days of sustained relative hypoxia in room air
DARA14	Dark Agouti rats exposed to room air for the first 14 days of life
ddH_20	double distilled water
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylene-diamine-tetraacetic-acid
EGF	epidermal growth factor
ELM	external limiting membrane
eNOS	endothelial nitric oxide synthetase
EPO	erythropoietin
ETDRS	Early Treatment of Diabetic Retinopathy Study
EtOH	ethanol
ETROP	the Early Treatment for Retinopathy of Prematurity Randomized Trial
$F_{(x,y)}$	F statistic (degrees of freedom, error)
F344	Fischer 344 rat strain
F344O2	Fischer 344 rats exposed to cyclic hyperoxia and relative hypoxia for the first two days of life – day two follows a 24 hour period of relative hypoxia

F344O3	Fischer 344 rats exposed to cyclic hyperoxia and relative hypoxia for the first three days of life – day three follows a 24 hour period of hyperoxia
F344O8	Fischer 344 rats exposed to cyclic hyperoxia and relative hypoxia for the first 8 days of life – day 8 follows a 24 hour period of relative hypoxia
F344O9	Fischer 344 rats exposed to cyclic hyperoxia and relative hypoxia for the first 9 days of life – day 9 follows a 24 hour period of hyperoxia
F344O14	Fischer 344 rats exposed to cyclic hyperoxia and relative hypoxia for the first 14 days of life
F344O18	Fischer 344 rats exposed to cyclic hyperoxia and relative hypoxia for the first 14 days of life, followed by four days of sustained relative hypoxia in room air
F344RA14	Fischer 344 rats exposed to room air for the first 14 days of life
FAK	focal adhesion kinase
FasL	Fas-ligand (CD95L)
FGF	fibroblast growth factor
Fig	figure
flk-1	foetal-liver kinase-1 (VEGFR-2)
flt-1	fms-like tyrosine kinase-1 (VEGFR-1)
g	gram
g	gravity
G	guanine
#G	# gauge
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCL	ganglion cell layer
GH	growth hormone
GOI	gene of interest
GS-IB4	*Griffonia simplicifolia* type I isolectin B4-Alexa 488™ conjugate
HPRT	hypoxanthine guanine phophoribosyl transferase
HIF-1/-2	hypoxia inducible factor-1/-2
hr	hour
HRE	hypoxia response element
HuR	Hu protein R

HW	Hooded Wistar rat strain
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
IGF-1	insulin-like growth factor-1
IL	interleukin
ILM	inner limiting membrane
IM	intramuscular
INL	inner nuclear layer
IP	intraperitoneal
IPL	inner plexiform layer
iU	international units
Kb	kilobases
kDa	kilo Daltons (10^3 Da)
KDR	kinase domain receptor (VEGFR-2)
Kg	kilogram (10^3 gram)
l	litre
LEW	Lewis rat strain
M	molar
m	metre
MAPK	mitogen-activated protein kinase
mg	milligram (10^{-3} g)
MHC	major histocompatibility complex
min	minutes
MIP-2	macrophage inhibitory peptide-2 (IL-8)
ml	millilitre (10^{-3} l)
mm	millimetre(10^{-3} m)
mM	millimolar (10^{-3} M)
mmHg	millimetres mercury
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MW	molecular weight
n	number/sample size

NaCl	sodium chloride
NADPH	nicotinamide-adenine dinucleotide phosphate
NFL	nerve fibre layer
ng	nanogram (10^{-9} g)
NH&MRC	National Health and Medical Research Council of Australia
No.	number
NRP-1	neuropilin-1
NSW	New South Wales
NTC	no template control
OIR	oxygen-induced retinopathy
ONL	outer nuclear layer
OPL	outer plexiform layer
ORP150	oxygen-regulated protein-150
P#	postnatal day #
PAF	platelet activating factor
PBS	Dulbecco's A physiologic balanced salt solution
PCO_2	partial pressure of carbon dioxide
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PECAM-1	platelet-endothelial cell adhesion molecule-1
PEDF	pigment epithelium derived factor
pg	picogram (10^{-12} gram)
pI	isoelectric point
PI3-kinase	phosphatidylinositol 3-kinase
PlGF	placental growth factor
pmol	picomoles (10^{-12} moles)
PO_2	partial pressure of oxygen; oxygen tension
RNA	ribonucleic acid
RNAP2	RNA polymerase 2
ROP	retinopathy of prematurity
RPE	retinal pigment epithelium
rpm	revolutions per minute

rRNA	ribosomal RNA
RT	room temperature
RT1	rat MHC Class II
RT-	reverse transcriptase-free; negative control cDNA
RT-PCR	reverse transcription-polymerase chain reaction
sec	second
SA	South Australia
SD	standard deviation
sFlt	soluble fms-like tyrosine kinase
SNP	single nucleotide polymorphism
SPD	Sprague Dawley rat strain (conventionally abbreviated SD. SPD in this thesis to avoid confusion with the abbreviation for standard deviation)
SPDO14	Sprague Dawley rats exposed to cyclic hyperoxia and relative hypoxia for the first 14 days of life
SPDO18	Sprague Dawley rats exposed to cyclic hyperoxia and relative hypoxia for the first 14 days of followed by four days of sustained relative hypoxia in room air
SPDRA14	Sprague Dawley rats exposed to room air for the first 14 days of life
T	thymine
T_A	annealing temperature
TBE	tris borate EDTA
TGF α / β	transforming growth factor-α/-β
Tie2	tyrosine kinase with Ig and epidermal growth factor homology domain receptor 2: receptor for angiopoietin-1 & -2
T_m	melting temperature
TNF α	tumour necrosis factor-α
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labelling
U	units
USA	United States of America
UV	ultraviolet light
V	volt
v	version; volume
v/v	unit volume per unit volume

VA	visual acuity
VCAM 1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor (VEGF A unless otherwise specified)
$VEGF_x$	VEGF, $isoform_x$ ($_x$ denotes number of amino acid residues)
VEGFR-1	vascular endothelial growth factor receptor-1
VEGFR-2	vascular endothelial growth factor receptor-2
VEGFR-3	vascular endothelial growth factor receptor-3
VHL	von Hippel-Lindau protein
VIC	Victoria
VPF	vascular permeability factor
V_T	tidal volume
w/v	unit weight per unit volume
WA	West Australia
WF	Wistar-Furth rat strain
WG	weeks of gestation
x	times / multiplication factor