Genetic Influences on Susceptibility to Oxygen-Induced Retinopathy

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Purpose. To investigate the inheritance of susceptibility to oxygen-induced retinopathy in the rat with the use of formal backcross analysis.

Methods. Neonatal offspring of inbred albino Fischer 344 (F344) and pigmented Dark Agouti (DA) crosses and F1×F344 and F1×DA backcrosses were exposed to alternating 24-hour cycles of hyperoxia (80% oxygen in air) and normoxia (21% oxygen in air) for 14 days. Retinal avascular area was analyzed by staining with Griffonia simplicifolia isoelectric B4, a marker of vascular endothelial cells. Expression of erythropoietin (EPO) mRNA in retinas was quantified by real-time reverse-transcription polymerase chain reaction.

Results. Oxygen-exposed offspring of two F344×DA F1 crosses showed retinal avascular areas and ocular and coat pigmentation that were similar to those of the DA strain. Mean retinal avascular area was 73%. Offspring of two DA×F1 backcrosses were similar to F344×DA F1 pups, with pigmented eyes and coats and a mean retinal avascular area of 76%. In contrast, offspring of two F344×F1 backcrosses exhibited a range of eye and coat pigmentation. Mean retinal avascular area of pigmented offspring of the F344×F1 backcrosses was 71% (P < 0.001 compared with F344 rats). Mean avascular area of albino offspring of the F344×F1 backcrosses was 27% (P > 0.05 compared with F344 rats). The normalized expression of EPO mRNA was 3.01 ± 1.00 in retinas from pigmented F344×F1 backcross offspring compared with 1.31 ± 0.69 for albino offspring (P < 0.001).

Conclusions. Segregation of the susceptibility trait to oxygen-induced retinopathy in the DA and F344 rat strains is associated with pigmentation and erythropoietin expression and can be modeled using an autosomal dominant pattern of inheritance.

(Risk factors for the development and progression of retinopathy of prematurity (ROP) include demographic factors and the need for supplemental oxygen therapy. Increasing evidence indicates that factors such as racial background and genetic polymorphisms may also be important. However, of the various animal and human studies impli-
UK) for subsequent analysis. An oxygen concentration of 80% ± 1% was maintained for the duration of hyperoxic histochemistry.

**Tissue Processing and Isolectin Histochemistry**

After the 14-day period of cyclic hyperoxia, rats were killed with an inhaled overdose of halothane anesthesia and the eyes were enucleated. Eyes were fixed in 2% wt/vol paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4°C for 90 minutes and dissected according to the method of Chan-Ling. Four equally spaced radially oriented incisions were used to flatten each retina. The microvasculature in retinal whole-mounts was stained with fluorophore-conjugated *Griffonia simplicifolia* isoelectin B4 (GS-B4) (Alexa Fluor 488 conjugate; Molecular Probes, Eugene, OR), which stains vascular endothelial cells, according to a modification of the method of Cunningham, as previously described. In all cases, retinal dissection and histochemistry were performed within 6 hours of enucleation.

**Image Analysis of Labeled Retinas**

The right retina of each animal was used for image analysis. Imaging was performed within 12 hours of lectin labeling using a fluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan) coupled with a CCD-digital camera (Roper Scientific, Trenton, NJ) and image acquisition software (RS Image, version 1.01; Roper Scientific). Sequential, overlapping, high-resolution images of the entire retina were captured using a 4× objective. Images were merged to construct a montage image of the retina (Adobe Photoshop version 7.0, Adobe Systems Inc., San Jose, CA) and were analyzed using image analysis software (ImageJ version 1.30; National Institutes of Health, Bethesda, MD). A masked observer manually outlined and measured avascular areas as a percentage of the total retinal area.

**Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction**

Rat eyes were enucleated immediately after death into chilled dithylypyrocarbonate (DEPC)-treated normal saline, and the retinas were dissected, snap-frozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated using a RNeasy mini-kit (Qiagen, Valencia, CA). Contaminating genomic DNA was removed with DNaseI (DNA-free; Ambion, Austin, TX). Samples free of visible DNA contamination on a 1% agarose gel and with a ratio of 28S:18S rRNA approximating 2:1 were quantified by spectrophotometry. One-microgram samples with a 260:280 nm absorbance ratio of ≥1.9 were reverse transcribed using a first-strand cDNA synthesis kit (SuperScript III First-Strand Synthesis System; Invitrogen, Carlsbad, CA). A reverse transcriptase-free control sample was synthesized in parallel with each cDNA sample, with substitution of DEPC-H₂O for reverse transcriptase. For purposes of normalization, a standard cDNA pool was prepared from pooled retinal RNA extracted from 10 F344, SPD, and DA rats that had been exposed to room air or cyclic hyperoxia.

Primers for rat erythropoietin (EPO) and for the housekeeping genes acidic hypoxanthine guanine phosphoribosyl transferase (*HPRT*) and ribosomal phosphoprotein (*ARBP*) were designed to flank an intron (Primer3 software; Whitehead Institute for Biomedical Research, Cambridge, MA) and tested in silico using specificity against sequences for *Rattus norvegicus* using BLAST software (NCBI, Bethesda, MD). Primer sequences were as follows: ACCAGAGAGTCTCAGGCTCA (EPO forward), GAGGGCATCAATTTCCCTC (EPO reverse); TTGGTGGATATGCCCTTGACT (HPRT forward), CCGCT- GTCTTTAGGCTTTG (HPRT reverse); and AAAGGGTCCTGGCTTT (ARBP forward), GCAAAAATGAGTGGATC (ARBP reverse). Primers were then synthesized (Geneworks Ltd., Thebarton, SA, Australia).

Real-time RT-PCR was performed (RotorGene 2000 Thermal Cycler; Corbett Research, Mortlake, NSW, Australia). Each 20-microliter reaction mixture contained 10 μL SYBR Green master-mix (QuantiTect SYBR Green PCR Master Mix; Qiagen) containing hot-start *Taq* DNA polymerase, SYBR Green I, dNTPs, and PCR buffer (5 mM MgCl₂, Tris-Cl, KCl, (NH₄)₂SO₄, pH 8.7), 2 μL each forward and reverse primer (0.5 μM final concentration), and 6 μL cDNA sample diluted 1/100 with purified water (Ultra Pure; Fisher Biotech, West Perth, WA, Australia). Reaction conditions were initial denaturation (95°C, 15 minutes) followed by 50 cycles of denaturation (94°C, 20 seconds), annealing (50°C, 20 seconds), extension (72°C, 30 seconds), and final extension (72°C, 4 minutes, followed by 25°C, 5 minutes). The standard cDNA pool was included in triplicate in each PCR run, together with a single RT-negative control for each sample and two water (no-template) controls. Melt-curve analysis was used to confirm amplification specificity. The melt-curve of each real-time PCR product was compared with that of the corresponding sequenced product. Real-time RT-PCR products were separated on agarose gels, purified, sequenced, and compared with the predicted amplicon sequence to confirm identity. Purified DNA was labeled (BigDye Terminator version 3.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA) and resolved (ABI 3100 Genetic Analyser; Applied Biosystems).

**Statistical Analyses**

Before statistical analysis of retinal areas, percentages were arc sin-transformed to normalize the variances of the data. Analysis of variance was performed to analyze the transformed data, including repeated-measures designs where appropriate (SPSS, version 11.0.2; SPSS Inc., Chicago, IL). Comparisons between subsets of data were made with pre-planned single degree of freedom contrasts, *Ryan-Finot-Gabriel-Welsch* F tests (REGWF tests), or Bonferroni tests, with significance levels (alpha) set at 0.05 in each case. Summary data were expressed as means with 95% confidence intervals (95% CIs). The Mann–Whitney *U* test corrected for ties was used to compare categorical data. For gene expression data, expression in each sample was determined relative to the standard cDNA pool and normalized to the housekeeping genes *ARBP* and *HPRT* (GenNorm software; Ghent University Hospital, Ghent, Belgium). Normalized expression data were normally distributed; thus, two-way analysis of variance (ANOVA) was used to compare gene expression among different rat strains. The significance level (alpha) was set at 0.05.

**Results**

**Cross-Breeding Experiments**

Heritability of susceptibility to oxygen-induced retinopathy was examined in a series of cross-breeding experiments. The F344 and DA rat strains were selected as relatively resistant and susceptible, respectively, to oxygen-induced retinopathy. Each genetic cross was performed twice, and different gender pairings (male F344 crossed with female DA; female F344 crossed with male DA) were used in each of the two crosses. All the offspring of each cross were exposed to cyclic hyperoxia for the first 14 days of life, before retinal vascular area measurement.

**Susceptibility of F344 × DA F1 Offspring to Oxygen-Induced Retinopathy**

All 16 F1 offspring of the two F344 × DA crosses had ocular and coat pigmentation similar to those of the DA strain. Eye color was dark brown and coat color was agouti, with white patches on the abdomen and paws (Fig. 1A). All neonates exposed to cyclic hyperoxia for 14 days had large avascular regions in the central and peripheral retina (Fig. 2). Mean total retinal avascular area was 73% (95% CI, 69%–77%). The extent of the retinal avascular area of the F1 rats was similar to that previously reported for oxygen-exposed pups of the DA parental strain at the same time point (n = 14; mean difference, 1%; P = 0.02) and substantially larger than for the F344 parental strain (n = 18; mean difference, 24%; P < 0.001).
Susceptibility of Backcross Offspring to Oxygen-Induced Retinopathy

Randomly selected adult rats of additional DA × F344 F1 crosses were mated with parental strain F344 and DA rats to yield backcross offspring. Four backcrosses were performed to accommodate all possible gender pairings. Offspring of the DA × F1 backcrosses were indistinguishable from the F1 pups: all had dark brown ocular pigmentation and agouti coat color. In contrast, the offspring of the F344 × F1 backcrosses exhibited a wide range of coat colors (Fig. 1B). Rats with albino coats had red eyes; rats with coat pigmentation had dark brown eyes.

All newborn offspring of each backcross (45 pups in total) were exposed to cyclic hyperoxia for 14 days, and retinal avascular areas were measured. Offspring of the DA × F1 backcrosses were universally susceptible to the attenuating effects of oxygen on retinal vascularization. Mean total retinal avascular area was 76% (95% CI, 73%–80%) (Fig. 3). Greater variation was found in the extent of retinal vascularization in the offspring of the F344 × F1 backcrosses (mean avascular area, 47%; 95% CI, 38%–57%; Fig. 3).

An association was identified between pigmentation and retinal vascularization in the rat. Highly significant differences were found in the extent of retinal avascular area between albino and pigmented offspring of the F344 × F1 backcrosses ($P < 0.001$; two-tailed Mann–Whitney $U$ test). Accordingly, when the pigmented offspring of the F344 × F1 backcrosses were considered together, the mean retinal avascular area was 71% (95% CI, 65%–78%), compared with 27% (95% CI, 23%–31%) for albino offspring of the same crosses. Pigmented progeny of the F344 × F1 backcrosses had areas of avascular retina that were slightly larger than those of the DA strain (mean difference, 1.6%; 95% CI, 0.1%–5%; $P = 0.003$) and substantially larger than those of the F344 strain (mean difference, 26%; 95% CI, 18%–34%; $P < 0.001$). In contrast, the albino backcross progeny had retinal avascular areas that were similar in size to those of the F344 strain ($P = 1.0$ for difference).

Retinal Gene Expression in Backcross Progeny after Cyclic Hyperoxia

A cohort of seven neonatal rats—four rats from one F1 × F344 backcross and three from another—incorporating the complete spectrum of coat and eye color was selected prospectively for retinal gene expression studies (Table 1). After 14 days of cyclic hyperoxia, the right retinas were used for vascular analysis, and the left retinas were processed for RNA extraction and subsequent quantification of gene expression. The validity of using the left and right eyes of each rat for different analyses was supported by a study that demonstrated significant intereye correlation in retinal vascularization in a rat model of oxygen-induced retinopathy. Before the analysis of gene expression, retinal cDNA samples were grouped according to the retinal avascular areas of fellow eyes. Rats with avascular areas smaller than 50% of the total retinal area were albino and were deemed resistant, whereas those with areas larger than 50% were pigmented and were deemed susceptible to the cyclic hyperoxic exposure. Mean retinal avascular areas of the resistant and susceptible groups were 27% and 68%, respectively. Real-time RT-PCR was then used to quantify expression of the EPO gene in the two retinal cDNA pools. There was significantly less EPO in the cDNA pool from the resistant (albino) rats than in the cDNA pool from the sensitive (pigmented) rats. Normalized EPO expression relative to the standard cDNA pool was 1.31 ± 0.69 for the former and 3.01 ±...
1.00 for the latter \((P < 0.001)\). By comparison, normalized EPO expression was 1.43 \pm 0.23 for the hyperoxia-resistant albino F344 parental strain at the same time point and under the same conditions and 2.24 \pm 0.47 for the hyperoxia-sensitive pigmented DA parental strain.

**DISCUSSION**

Our findings in a formal backcross analysis substantiated the importance of heritable factors in determining the susceptibility of rats to oxygen-induced retinopathy. If such susceptibility is arbitrarily defined as a total retinal avascular area in excess of 50\% and the susceptibility allele is assumed to be dominant, then all offspring of crosses between resistant F344 and susceptible DA rats would be susceptible, as would all progeny of the DA\(\times\)F1 backcrosses (Fig. 4). Furthermore, the ratio of susceptible/resistant offspring of F344\(\times\)F1 backcrosses would be expected to approximate 1:1. Experimental findings closely matched these predictions (Fig. 4). The extent of variation in avascular retinal areas of oxygen-exposed backcross rats argues against a monogenic mode of inheritance. Nonetheless, our results demonstrate that hereditary factors are central to the risk for oxygen-induced retinopathy in the rat and that susceptibility segregates in a manner that is consistent with an autosomal dominant form of inheritance, modified by other genetic influences.

All offspring of F344\(\times\)DA crosses had pigmented eyes and coats similar to those of the parental DA strain, and their retinal vasculature was uniformly susceptible to the attenuating effects of cyclic hyperoxia in the neonatal period. Pups exhibited oxygen-induced retinopathy indistinguishable from that of the DA strain. Similar findings were observed in all oxygen-exposed progeny of DA\(\times\)(F344\(\times\)DA) backcrosses. In contrast, two different patterns of retinopathy were apparent in the progeny of F344\(\times\)(F344\(\times\)DA) backcrosses after exposure to cyclic hyperoxia. Albino offspring with red eyes showed resistance to oxygen-induced retinopathy, whereas the retinal microvasculatures of those with pigmented coats and eyes were more susceptible to the same stimulus. These data provide

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**TABLE 1.** Retinal Phenotypes of F344\(\times\)F1 Backcross Progeny Used to Prepare cDNA Pools for Quantification of EPO Expression in Retina after Cyclic Hyperoxic Exposure

<table>
<thead>
<tr>
<th>Coat Color</th>
<th>Eye Color</th>
<th>Retinal Avascular Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black</td>
<td>Dark brown</td>
<td>55.6</td>
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<tr>
<td>Black hooded</td>
<td>Dark brown</td>
<td>86.4</td>
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<tr>
<td>Black</td>
<td>Dark brown</td>
<td>78.3</td>
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<tr>
<td>Agouti</td>
<td>Dark brown</td>
<td>61.2</td>
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<tr>
<td>Black hooded</td>
<td>Dark brown</td>
<td>57.9</td>
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<tr>
<td>White</td>
<td>Red</td>
<td>37.5</td>
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<tr>
<td>White</td>
<td>Red</td>
<td>17.3</td>
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</table>
hyperoxia. Erythropoietin is an archetypal hypoxia-induced protein. Adding EPO to cultured human vascular endothelial cells triggered receptor phosphorylation, the activation of intracellular cell signaling cascades, and the induction of a proangiogenic factor. Genetic modeling of susceptibility to oxygen-induced retinopathy using a hierarchy of susceptibility among albino rat strains lends support to this notion.

The association between ocular pigmentation and susceptibility to oxygen-induced retinopathy in the DA rat is important for regulating angiogenesis in the cornea. The authors thank Anne-Louise Smith for biomedical engineering expertise and Ray Yates for animal husbandry.

### References

4. Early Treatment for Retinopathy of Prematurity Cooperative Group. Revised indications for the treatment of retinopathy of prematurity: results of the early treatment for retinopathy of pre-

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### Figure 3

Genetic modeling of susceptibility to oxygen-induced retinopathy. S, dominant susceptibility allele; s, recessive resistance allele; unfilled symbols, albino rats; filled symbols, pigmented rats. If susceptibility to oxygen-induced retinopathy is defined as total avascular retinal area greater than 50% after 14 days of hyperoxia, then autosomal dominant inheritance of a monogenic trait can be predicted as shown. All progeny of the F344 × DA F1 cross are susceptible. All progeny of the DA × (F344 × DA) F1 backcross carry at least one susceptibility allele and, therefore, express the susceptible phenotype; 50% of the offspring of the F344 × (F344 × DA) F1 backcross are susceptible and 50% are resistant to oxygen-induced retinopathy. Observed and expected numbers of cross and backcross offspring with either the susceptible or the resistant retinal phenotype are tabulated beneath the figure.


