The use of hyperoxia to induce chronic mild oxidative stress in RPE cells in vitro

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Purpose: To establish a model of mild and chronic oxidative stress using hyperoxia for retinal pigment epithelial (RPE) cells in vitro.

Methods: RPE340 cells and WI38 lung fibroblasts were grown in normal oxygen (20% O_2) and hyperoxia (40% O_2 or 60% O_2). After cell viability was examined, the levels of reactive oxygen intermediates (ROI) by flow cytometry and heme oxygenase-1 (HO-1) mRNA by northern analysis were measured as markers of oxidative stress in both cell types. Proliferative ability and gene expression pattern of growth factors were studied to demonstrate the phenotypic changes induced by mild oxidative stress upon these cells.

Results: While decreased by 60% O_2, 40% O_2 did not affect viability in both cell types, ROI production and HO-1 mRNA expression were elevated in hyperoxia compared to controls, but were inhibited with the antioxidant dehydro-ascorbic acid (DHA). The proliferation of cells by hyperoxia was inhibited in both cell types. The expression of growth factors induced by hyperoxia was cell type dependent. Fibroblast growth factor-2 mRNA was unchanged in RPE cells, but was increased in fibroblasts. Transforming growth factor-β2 was decreased in RPE cells, but unchanged in fibroblasts. Vascular endothelial growth factor was downregulated in RPE cells, while upregulated in fibroblasts. Connective tissue growth factor was decreased in RPE cells, but was unchanged in fibroblasts.

Conclusions: The results demonstrate that hyperoxia induces mild oxidative stress which alters the phenotype of cells in a cell type specific manner.

The retinal pigment epithelial (RPE) cell is exposed to chronic oxidative stress in part from exposure to high partial pressures of oxygen and in part, from its high level of oxygen metabolism which generates large amounts of reactive oxygen intermediates (ROI) [1,2]. Several in vitro models have been developed to study the effects of oxidative stress on RPE cells, but most of these utilize the acute, high level administration of a chemical oxidant [3,4]. While these chemical oxidants induce a specific cellular oxidative stress that is useful for studying cellular responses to individual ROIs, the applicability of these acute chemical effects to the in vivo condition is open to question.

An alternative and perhaps more applicable approach to studying the effects of oxidative stress on RPE cells would apply chronic and lower levels of oxidative stress than the acute levels resulting from chemical oxidants. Recently, von Zglinicki et al. used chronic hyperoxia treatment to induce long term oxidative stress on lung fibroblasts [5]. The purpose of our study was to develop and characterize a model using chronic hyperoxia treatment of the RPE cell that induces mild oxidative stress without causing cell death. Our laboratory and others have studied in depth, the characteristics of one cell line, designated RPE340 [6-9]. In this study, we have utilized RPE340 cells, and compared their response to WI38

lung fibroblasts, a cell line that has been utilized in oxidative stress studies, for comparison.

METHODS

Cell culture: The RPE340 cell line from one globe of a 1-year-old trauma victim was propagated as previously described [6]. While not an established cell line available for purchase, the growth characteristics and mRNA phenotype of this cell line have been characterized in a number of publications [6-9]. Cultures were maintained in Dulbecco's modified Eagle medium/Nutrient mixture F12 with 15 mM HEPES buffer (DMEM/F12; Biowhittaker, Walkersville, MD) and 10% fetal bovine serum (FBS; UBI Upstate, Lake Placid, NY), 0.348% additional sodium bicarbonate, 2 mM L-glutamine solution (GIBCO, Grand Island, NY) at 20% oxygen and 40% oxygen conditioned in 10% CO_2 at 37 °C. The pO_2 in the medium was measured with a YSI 5331 oxygen probe (YSI Incorporated, Yellow Springs, OH) and found to be 162.9±4.6 mm Hg when placed in 20% oxygen, and 296.9±2.9 mm Hg when in 40% oxygen. WI38 cells (ATCC, Manassas, VA) were cultured in Eagle's minimum essential medium with Earle's balanced salt solution, 2 mM L-glutamine (EMEM), 1 mM sodium pyruvate (GIBCO) and 10% FBS at 20% oxygen and 40% oxygen conditioned in 5% CO_2 at 37 °C. This culture medium was selected because it is considered the optimum growth medium for WI38 cells (personal communication, ATCC). In addition, since EMEM has been used in published studies of WI38 cell response to hyperoxia, we selected this medium to allow for comparison of our results with those in

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the literature. For experiments, cells were grown in 75 cm²flasks at an initial seeding density of 10,000/cm². For proliferation assays, cells were passaged before reaching confluence to avoid contact inhibition. At each passage, cell number was counted using a Coulter Counter Z1 (Coulter, Miami, FL) and population doubling level (PDL) was determined as current PDL = last PDL + log₂(collected cell number/seeded cell number).

Cell viability: Viability was quantified using the WST-1 assay according to the manufacturer's recommendation (Boehringer Mannheim, Mannheim, Germany). This assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases in viable cells. Cells were seeded in 96 well plates at 20,000 cells/cm² in DMEM/F-12 (RPE340 cells) and EMEM (WI38 cells) including 10% FBS and grown for 2 days in 20% oxygen before placing half of the cultures in 40% or 60% oxygen for 48 h. WST-1 reagent was added for two h and the absorbance was measured at 485 nm using a HTS 7000 Bioassay reader (Perkin Elmer Corporation, Norwalk, CT). Three independent experiments were conducted.

Flow cytometry: Cells were plated at 10,000 cells/cm² in DMEM/F12 plus 10% FBS (RPE340 cells) and EMEM plus 10% FBS (WI38 cells) and grown for three days in 20% oxygen. At 80-90% confluence, half of the flasks were transferred to hyperoxia (40% oxygen) with or without 150 µM dehydroascorbic acid (DHA; Sigma, St. Louis, MO) as an antioxidant for the duration of the experiments. Cells were rinsed twice with Hanks' balanced salt solution (HBSS) and treated with 10 µM of 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes, Eugene, OR) for 30 min. H2DCFDA is a nonfluorescent compound that readily crosses cell membranes. It is hydrolyzed to 2',7'-dichlorofluorescein intracellularly, and becomes fluorescent when it is oxidized by reactive oxygen species. Cells were then trypsinized and resuspended in 1% paraformaldehyde/HBSS and fluorescence was measured with a FACscan (Becton Dickinson, San Jose, CA) [10,11]. Three independent experiments were conducted.

RNA extraction and northern analysis: Total RNA was extracted using TRIZOL reagent (GIBCO) according to the manufacturer's recommendations. Fifteen-microgram aliquots of each sample were electrophoresed in 1% agarose gels, transferred to nylon membranes, and prehybridized for 3 h at 42 °C in 50% formamide, 5X Denhardt's solution, 5X SSC, 100 µg/ml salmon sperm DNA and 0.1% SDS. The membranes were hybridized for 18 h at 42 °C with 25 ng of the ³²P labeled cDNA probe. Blots were washed in 0.1% SDS/0.1X SSC three times at room temperature, then once at 50 °C before being subjected to phosphorimage analysis (Molecular Dynamics, Sunnyvale, CA). The blots were stripped and sequentially hybridized with the following cDNA probes: heme oxygenase-1 (HO-1), a generous gift from Dr. A. Choi [12], fibroblast growth factor-2 (FGF-2) [13], transforming growth factor-β2 (TGF-β2) [14], vascular endothelial growth factor (VEGF), a gift from Dr. A. Singh (Genentech, Inc., South San Francisco, CA), connective tissue growth factor (CTGF) from Dr. G. Grotendorst [15], and 28S rRNA [16]. Hybridization signals were normalized against 28S rRNA. Three independent experiments were conducted for each growth factor.

Statistical analysis: Since the data were normally distributed, statistical significance at each time point comparing control to the hyperoxia treated condition was determined using the two-tailed Student's t-test. $p < 0.05$ was considered significant.

RESULTS

Cell viability: To determine a concentration of O_2 that would induce chronic oxidative stress to cells without affecting viability, RPE340 and WI38 cells were grown in various concentrations of O_2. When grown in 60% O_2, RPE340 cells showed 77±14% (mean±SEM) viability compared to growth in 20% O_2 ($p < 0.05$) while WI38 cells were 71±13% viable when compared to control ($p < 0.01$; Figure 1). In contrast, RPE340 cells cultured in 40% O_2 for 48 h showed no change in viability (97±7%) compared with growth in 20% O_2($p = 0.7$), while WI38 cells showed 92±9% viability under 40% O_2against control ($p = 0.6$). Thus, the remaining experiments were conducted in 40% O_2.

Reactive oxygen intermediate (ROI) production: ROI production in RPE340 cells became elevated soon after exposure to 40% O_2 (Figure 2A). Within 8 h of incubation, ROI production reached 145% of control ($p < 0.01$), which was maintained throughout the 48 h experimental period. The antioxidant DHA (150 µM) inhibited the hyperoxia induced ROI production after 24 h of incubation ($p < 0.05$). WI38 cells treated with 40% O_2 also showed a rapid increase in ROI production (Figure 2B). This increase in ROI production plateaued at 169% of control ($p < 0.01$) after 24 h of incubation. As with RPE cells, 150 µM DHA inhibited ROI production after 24 h ($p < 0.05$). DHA also tended to decrease the ROI production in both cell types when
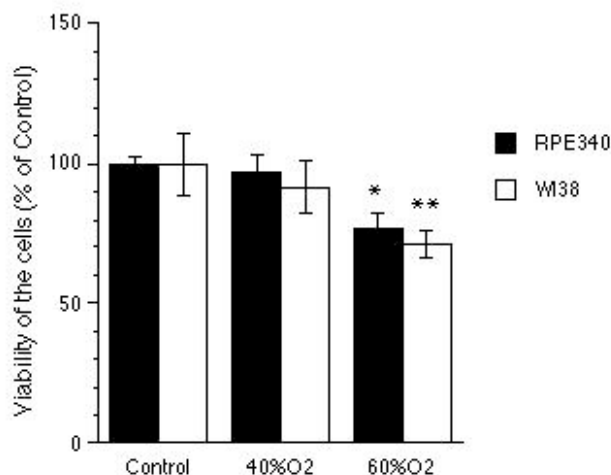

Figure 1. Viability of cells after hyperoxic treatment. Viability of RPE340 and WI38 cells under 20% O_2 (Control), 40% O_2 and 60% O_2. Values are shown as mean±SE from three independent experiments, and the value of control is regarded as 100% in each cell line. Significance levels: *$p < 0.05$, **$p < 0.01$.

grown in 20% O_2, but this change did not reach statistical significance. Further experiments with DHA revealed a dose dependent inhibition of ROI production in both cell lines grown in 40% O_2, reaching significance at ≥ 100 µM DHA in RPE 340 cells ($p < 0.0005$ to $p < 0.05$), and ≥ 10 µM DHA in WI38 cells ($p < 0.01$ to $p < 0.05$; Figure 3A,B). In both cell lines, 150
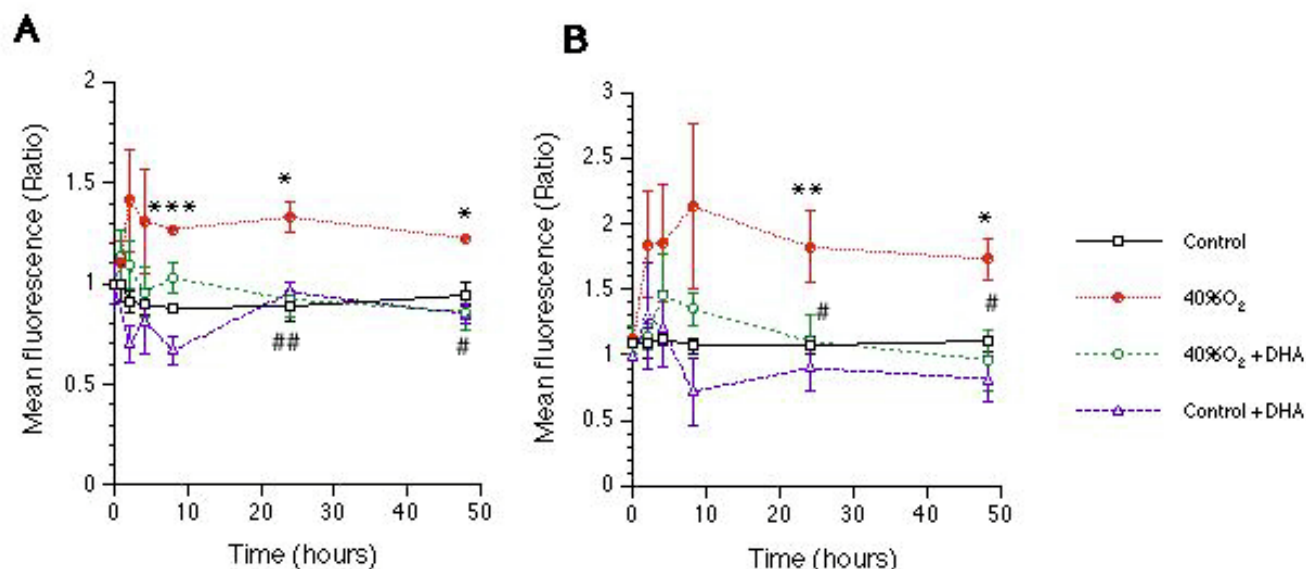

Figure 2. Measurement of intracellular ROI production. Flow cytometric measurement of ROI production in RPE340 cells (**A**) and WI38 cells (**B**) over time. Cells were preincubated at each time point in 20% O_2 (Control) and 40% O_2 with or without 150 µM DHA before adding H2DCFDA. Values are shown as mean±SE of three independent experiments. Data of each condition were compared at the same time point. Significance levels for comparison to controls: *$p < 0.05$, **$p < 0.01$. Significance levels for comparison to hyperoxia treated cells without DHA: #$p < 0.05$, ##$p < 0.01$.
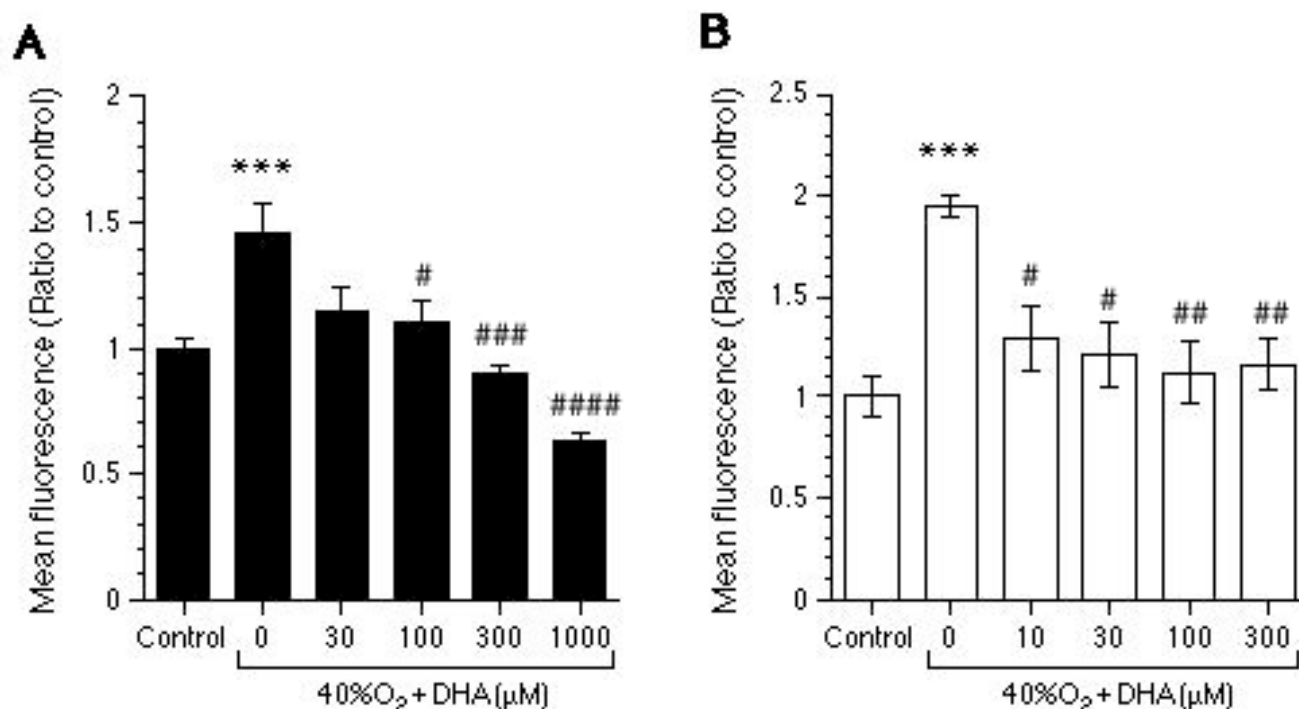

Figure 3. The antioxidant effect of DHA. Dose dependent effect of DHA on ROI production in RPE340 (**A**) and WI38 cells (**B**) grown in hyperoxia. Cells were preincubated in 20% O_2 (Control), and 40% O_2 with the indicated DHA dose for 24 h before adding H2DCFDA. Values are shown as mean±SE of three independent experiments. Significance levels for comparison to controls: ***$p < 0.001$. Significance levels for comparison to hyperoxia treated cells without DHA: #$p < 0.05$, ##$p < 0.01$, ###$p < 0.005$, ####$p < 0.0005$.

μM DHA completely inhibited the ROI production induced by hyperoxia.

HO-1 mRNA expression is induced by hyperoxia: As an alternative measure of the oxidative stress induced by cells grown in hyperoxia, the induction of HO-1 mRNA, a known marker of oxidative stress, was assessed by northern analysis [17]. Cells grown in 40% O_2 showed a modest induction of HO-1 mRNA steady state expression. In general, HO-1 mRNA expression was higher in WI38 than RPE 340 cells (Figure 4A,B). HO-1 mRNA expression gradually decreased in both cell lines with incubation time under both hyperoxic and control conditions, an effect that could be due to increasing cell density during the experimental period [18] (Figure 4A,B) or from the antioxidative effect of HO-1 [19]. We therefore took the ratio of HO-1 mRNA expression from cells grown in 40% to 20% O_2 at each time point to assess the effect of hyperoxia on HO-1 expression. This ratio of HO-1 mRNA expression increased gradually with time in both cell lines (Figure 4C,D). In RPE340 cells grown in 40% O_2, HO-1 mRNA expression increased 146±10% at 24 h (p<0.001) and 187±22% at 96 h

(p<0.005) compared to control conditions. Likewise, WI38 cells grown in 40% O_2 increased HO-1 expression to 185±14% by 24 h (p<0.005), and remained at this level for the duration of the 96 h (p<0.01). Incubation of both cell types with 150 μM DHA, after a short period of enhanced HO-1 induction, inhibited HO-1 mRNA expression. In RPE340 cells, inhibition of HO-1 mRNA induction by hyperoxia treatment reached significance after a 48 h incubation with 150 μM DHA (p<0.05) while in WI38 cells, DHA inhibited HO-1 mRNA induction by hyperoxia after 96 h (p<0.05).

Effect of hyperoxia treatment on cell proliferation: Cell proliferation was used to assess a functional effect of hyperoxic treatment on both cell types. RPE340 cells cultured under normal oxygen proliferated with a constant rate (0.46 PDL/day) until about PDL50, and gradually ceased proliferation by PDL57 (Figure 5A). Hyperoxia inhibited the proliferation of RPE340 cells and shortened their life span to PDL47. WI38 cells grown in 20% O_2 had constant proliferation (0.34 PDL/day) until approximately PDL37, and ceased proliferating by PDL41(Figure 5B). In contrast, hyperoxia rapidly inhibited
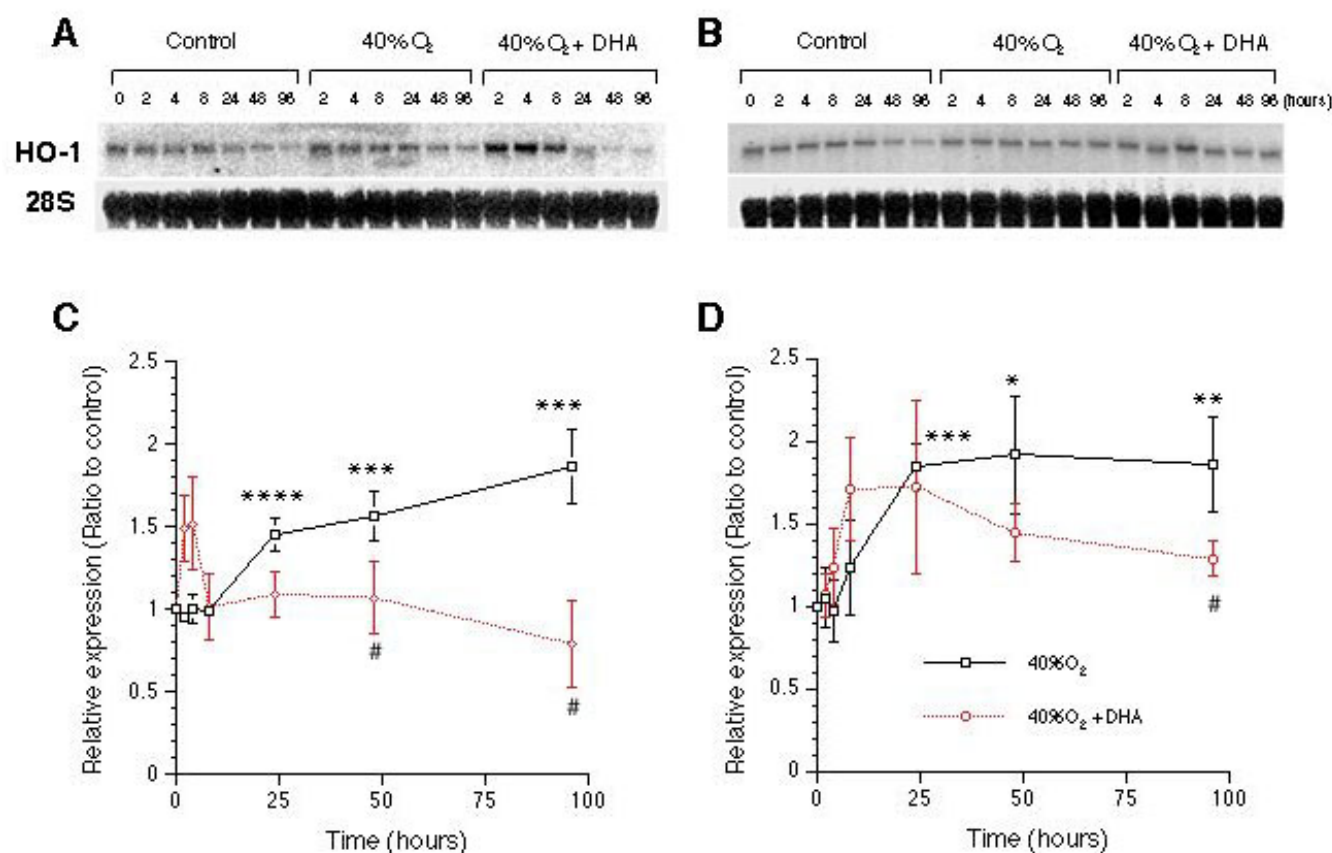

Figure 4. Northern analysis of HO-1 expression. Northern analysis of HO-1 mRNA expression by RPE340 cells (**A**) and WI38 cells (**B**). Cells were cultured in 20% O_2(Control), 40% O_2, and 40% O_2 with DHA (150μM). Signals of HO-1 were normalized against that of 28S rRNA. The ratio of hyperoxia treated: controls at each time point was determined for RPE340 cells (C) and WI38 cells (D) from three independent experiments. Values are shown as mean±SE. Data of each condition were compared at the same time point. Significance levels for comparison to controls: *p<0.05, **p<0.01, ***p<0.005, ****p<0.001. Significance levels for comparison to hyperoxia treated cells without DHA: #p<0.05.

proliferation and shortened the life span of WI38 cells within three PDLs.

Alteration of gene expression: Our laboratory has been interested in a set of growth factors that can alter the phenotype of the RPE-Bruch's membrane-choriocapillaris complex after exposure to oxidative stress. Our initial work treated RPE cells with acute exposure to chemical oxidants [20]. Using this set of genes, we found modest alterations in gene expression that were cell type dependent (Figure 6). FGF-2 mRNA expression in RPE340 cells showed no change after 24 h of incubation, but was upregulated in WI38 cells (126±12%, $p < 0.01$) treated with hyperoxia (Table 1). TGF-β2 mRNA expression was downregulated in RPE340 cells (86±9%, $p < 0.05$), but remained unchanged in WI38 cells grown in hyperoxia (99±11%, p =0.85). VEGF was downregulated in RPE340 with hyperoxia (80±5%, $p < 0.05$), but was upregulated in WI38 cells with hyperoxia (144±18%, $p < 0.05$). CTGF was downregulated with hyperoxia (70±8%, $p < 0.05$) in RPE340 cells, but not in WI38 cells (92±13%, p= 0.31).

DISCUSSION

We demonstrate here that 40% hyperoxia treatment caused detectable oxidative stress in RPE340 and WI38 cells, as measured by ROI production and induction of HO-1 mRNA, without affecting cell viability. ROI production was induced soon after hyperoxic treatment and became significant within 8 h, an effect that was ameliorated by the antioxidant DHA. To test our model, we also assessed functional consequences of

hyperoxia on RPE340 and WI38 fibroblasts, two cell lines which have been characterized in detail [5-9,21,22]. Hyperoxia inhibited cell proliferation and shortened their life span in vitro, an effect that was dramatic in WI38 fibroblasts. Hyperoxia also induced a mild, cell specific alteration in the expression of several growth factors.

Many oxidative stress models have been previously established which use a chemical oxidant like hydrogen peroxide to induce oxidative stress [23]. Chemical oxidants generate significant intracellular ROIs, but most are degraded enzymatically, and consequently, are short-lived in the cell [24]. The dose of oxidant necessary for typical experiments induces peak, temporary effects that might not be relevant to the in vivo condition. It is likely that oxidative stress in vivo is chronic and below levels that cause necrosis or apoptosis. We therefore wanted to establish a model of low grade, chronic oxidative stress in vitro that more closely simulates the situation in vivo. In general, the degree of oxidative stress induced by hyperoxia is lower than that generated by chemical oxidants even when a maximum oxygen tension (95%) is applied [18]. However, chronic exposure to high levels of hyperoxia can induce cytotoxicity, depending on the cell type [24-26]. We sought to use the highest level of oxygen that induced oxidative stress, as measured by intracellular ROI production and induction of HO-1 mRNA, without causing a loss of viability. We chose to use 40% O_2 for our studies because it generated modest levels of ROI production and induced HO-1 mRNA expression without inducing cytotoxicity as was found at
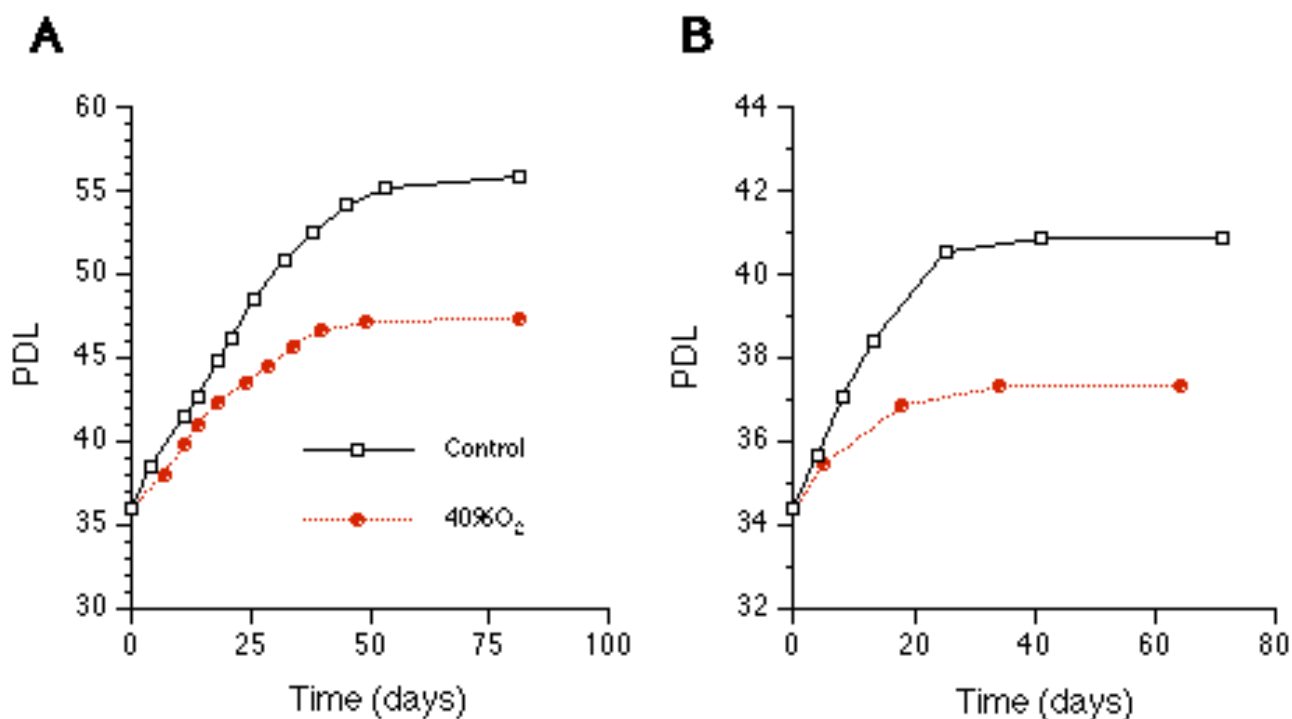

Figure 5. The effect of hyperoxia on cell proliferation. Cumulative growth curve of RPE340 cells (**A**) and WI38 cells (**B**) cultured in 20% O_2 (Control) and 40% O_2. Cells were grown in 75 cm^2 flasks at an initial seeding density of 10,000/cm^2, and were split to 1:2.5 before reaching confluence to avoid contact inhibition.

higher O$_2$ (60% O$_2$) in both RPE340 and WI38 cells. While the intention of our experiments was to document oxidative stress to cells with hyperoxia, we did not perform a detailed analysis of which specific oxidant species were generated. Further studies identifying the specific ROIs could provide insight into oxidative stress mediated changes to RPE cells.

The induction of ROI was consistently lower in RPE340 than WI38 cells with hyperoxic treatment. We presume that this effect can be explained in part, by the considerable antioxidant capability of the RPE compared to WI38 cells, since

RPE cells have abundant glutathione stores and high catalase activity that can reduce oxidative stress [27,28]. Alternatively, differences in the culture media used could have influenced the effect of oxidative stress or the response by the cells. However, our preliminary experiments showed no significant difference in ROI production of RPE340 cells between the two medium types (data not shown). Thus, the influence of medium type may be negligible.

DHA, an effective ROI scavenger [29,30], inhibited ROI production induced by hyperoxia (40% O$_2$) in a dose dependent manner. Interestingly, high doses of DHA reduced the ROI level of cells grown in 40% O$_2$ below that of control cells grown in 20% O$_2$. We furthermore, observed a reduction in ROI production after cells grown in 20% O$_2$ were treated with DHA. Our flow cytometric analysis of DHA treatment on cells grown in 20% oxygen, though not statistically significant, suggests that even 20% O$_2$ produces some ROI in RPE340 and WI38 cells. It is interesting that DHA showed temporal enhancement of HO-1 mRNA expression in the early period of hyperoxia exposure. DHA is rapidly incorporated by the cell and reduced to ascorbic acid via nonenzymatic reaction with glutathione [31]. Since ascorbic acid acts not only an antioxidant but also as a strong reductant [32], this temporal increase of HO-1 mRNA may be related to the reduction of thiol residues in some regulatory proteins (i.e., transcription factors) [18]. N-acetylcysteine, a reducing reagent and a precursor of glutathione, was also reported to enhance the expression of HO-1 mRNA in hepatoma cells cultured in 95% O$_2$ for 6 h [18].

One phenotypic alteration of oxidative stress is the inhibition of cell proliferation without inducing cell death [5,24,33-36]. This effect is probably due to the activation of cell cycle regulatory genes such as p53 and p21 that respond to oxidative stress induced DNA damage [35,37-39]. We showed that hyperoxia had a lower antiproliferative effect in RPE340 than WI38 cells, an effect that correlates with lower oxidative stress levels in RPE340 than WI38 cells when grown in 40% O$_2$. The stimulation of cell growth with an antioxidant and low oxygen tension has been reported in various cell types [36,40-42]. Akeo et al reported that 20% oxygen inhibited the prolif-
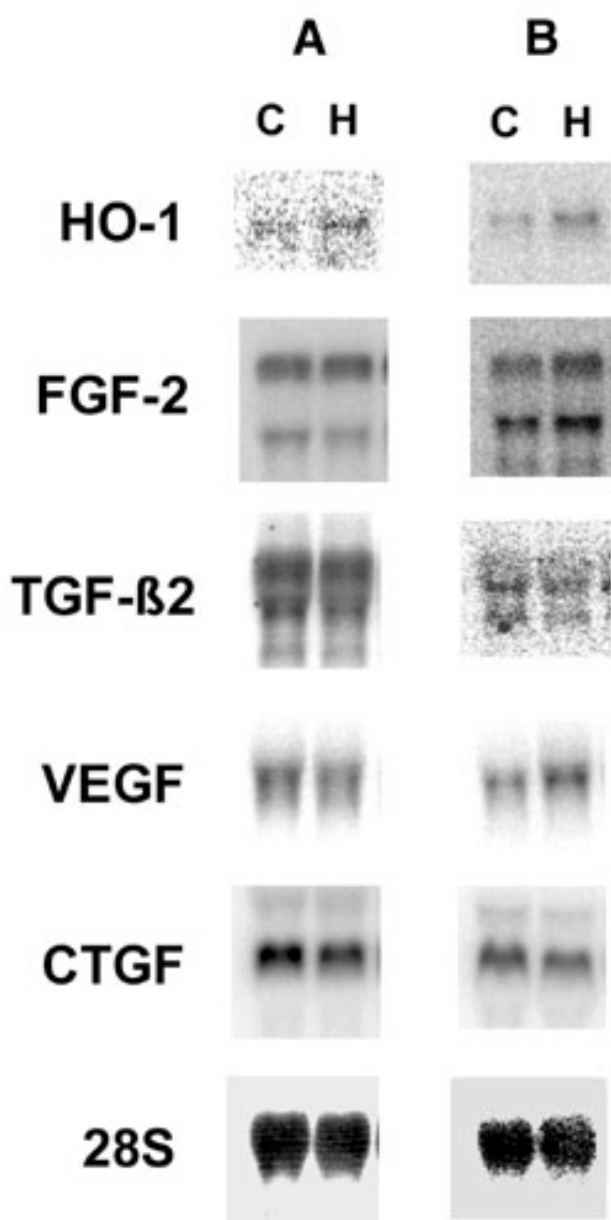

Figure 6. The effect of hyperoxia on gene expression. The change in gene expression by RPE340 (**A**) and WI38 cells (**B**) grown in hyperoxia. RPE340 cells and WI38 cells were grown in 20% O$_2$ (C; control) and 40% O$_2$ (H; hyperoxia) for 24 h, followed by RNA extraction and northern blot analysis. Signals were normalized to 28S rRNA.

TABLE 1. THE PERCENTAGE CHANGE IN GENE EXPRESSION FOR SELECTED GROWTH FACTORS

	RPE340	p	WI38	p
HO-1	145±16	<0.0005	193±33	<0.005
FGF-2	90±10	0.12	126±12	<0.01
TGF-β2	86±9	<0.05	99±11	0.85
VEGF	80±5	<0.05	144±18	<0.05
CTGF	70±8	<0.05	92±13	0.31

Percentage change of the signal from 40% O$_2$ to 20% O$_2$ determined for each blot. Values are shown as mean ± SE from three independent experiments.

eration of porcine RPE cells, and that antioxidant enzymes added to the medium stimulated proliferation [43]. The partial pressure of O_2 in the culture medium under ambient oxygen (20%) was 162.9±4.6 mm Hg in this study similar to published data (130~160 mm Hg) [36,40,44] while that in the RPE layer is 70~90 mm Hg in vivo [1,45], making standard room air (20%) incubation, compared to the environment of the RPE in vivo, a relative oxidative stress environment.

We chose to sample the expression of growth factors known to be important in the maintenance of the RPE-Bruch's membrane-choroidal complex. The response of these growth factors has been studied in RPE cells treated with chemical oxidants [20,46]. In comparison, we found modest changes in gene expression. Changes in age-related disease such as age-related macular degeneration (AMD) occur over a long duration. We hypothesize that chronic, modest changes in gene expression approximates the response to the exposure of the high partial pressure oxygen that occurs in vivo. The pattern of gene expression induced by hyperoxia depends on the cell type, presumably because the basic expression and the importance of each gene varies among cell types. While we found no change in FGF-2 expression in RPE340 cells after hyperoxic treatment, Khaliq et al reported that FGF-2 mRNA was upregulated in human RPE cells grown in hypoxia [47]. Hackett et al reported that FGF-2 mRNA was upregulated in human RPE cells with 100 μM H_2O_2 [48]. These findings may represent a characteristic difference between hyperoxia and chemical oxidant treatment. In contrast, hyperoxia increased FGF-2 mRNA expression in WI38 cells. Care must be taken in interpreting these results since they represent the expression changes in one cell line which may not be applicable to RPE or lung fibroblast cells in general, or to these cells in vivo. Furthermore, since different culture media were used to optimize the growth conditions for each cell type, a direct comparison of gene expression changes may not be valid. However, the gene expression changes found in each cell type do illustrate a phenotypic alteration induced by hyperoxia treatment in each of these cell lines.

It is likely that chronic oxidative stress to the RPE plays some role in the pathogenesis of AMD [1] due to the high oxidative stress environment from the high partial pressure of oxygen, the high metabolic rate of the RPE, chronic light exposure, accumulation of lipofuscin, and the formation of advanced glycation endproducts in Bruch's membrane [1,49]. The accumulated oxidative stress could alter the phenotype of the RPE during aging and AMD [2,50]. Therefore, it is useful to establish a chronic oxidative stress model of RPE cells to investigate the mechanism of how oxidative stress changes the function of the RPE. Further studies may be directed at more fully characterizing the RPE cell phenotype induced by chronic hyperoxia.

ACKNOWLEDGEMENTS

REFERENCES

1. Winkler BS, Boulton ME, Gottsch JD, Sternberg P. Oxidative damage and age-related macular degeneration. Mol Vis 1999; 5:32 .
2. Cai J, Nelson KC, Wu M, Sternberg P, Jones DP. Oxidative damage and protection of the RPE. Prog Retin Eye Res 2000; 19:205-21.
3. Sternberg P, Davidson PC, Jones DP, Hagen TM, Reed RL, Drews-Botsch C. Protection of retinal pigment epithelium from oxidative injury by glutathione and precursors. Invest Ophthalmol Vis Sci 1993; 34:3661-8.
4. Cai J, Wu M, Nelson KC, Sternberg P, Jones DP. Oxidant-induced apoptosis in cultured human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 1999; 40:959-66.
5. von Zglinicki T, Saretzki G, Docke W, Lotze C. Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? Exp Cell Res 1995; 220:186-93.
6. Matsunaga H, Handa JT, Aotaki-Keen A, Sherwood SW, West MD, Hjelmeland LM. Beta-galactosidase histochemistry and telomere loss in senescent retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 1999; 40:197-202.
7. Matsunaga H, Handa JT, Gelfman CM, Hjelmeland LM. The mRNA phenotype of a human RPE cell line at replicative senescence. Mol Vis 1999; 5:39 .
8. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. Extension of life-span by introduction of telomerase into normal human cells. Science 1998; 279:349-52.
9. Shelton DN, Chang E, Whittier PS, Choi D, Funk WD. Microarray analysis of replicative senescence. Curr Biol 1999; 9:939-45.
10. Chen YC, Tsai SH, Lin-Shiau SY, Lin JK. Elevation of apoptotic potential by anoxia hyperoxia shift in NIH3T3 cells. Mol Cell Biochem 1999; 197:147-59.
11. Nelson KC, Carlson JL, Newman ML, Sternberg P, Jones DP, Kavanagh TJ, Diaz D, Cai J, Wu M. Effect of dietary inducer dimethylfumarate on glutathione in cultured human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 1999; 40:1927-35.
12. Shibahara S, Muller R, Taguchi H, Yoshida T. Cloning and expression of cDNA for rat heme oxygenase. Proc Natl Acad Sci U S A 1985; 82:7865-9.
13. Abraham JA, Whang JL, Tumolo A, Mergia A, Friedman J, Gospodarowicz D, Fiddes JC. Human basic fibroblast growth factor: nucleotide sequence and genomic organization. EMBO J 1986; 5:2523-8.
14. Hanks SK, Armour R, Baldwin JH, Maldonado F, Spiess J, Holley RW. Amino acid sequence of the BSC-1 cell growth inhibitor (polyergin) deduced from the nucleotide sequence of the cDNA. Proc Natl Acad Sci U S A 1988; 85:79-82.
15. Duncan MR, Frazier KS, Abramson S, Williams S, Klapper H, Huang X, Grotendorst GR. Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP. FASEB J 1999; 13:1774-86.
16. Gonzalez IL, Gorski JL, Campen TJ, Dorney DJ, Erickson JM, Sylvester JE, Schmickel RD. Variation among human 28S ribosomal RNA genes. Proc Natl Acad Sci U S A 1985; 82:7666-70.
17. Applegate LA, Luscher P, Tyrrell RM. Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. Cancer Res 1991; 51:974-8.
18. Takahashi S, Takahashi Y, Yoshimi T, Miura T. Oxygen tension

regulates heme oxygenase-1 gene expression in mammalian cell lines. Cell Biochem Funct 1998; 16:183-93.

19. Elbirt KK, Bonkovsky HL. Heme oxygenase: recent advances in understanding its regulation and role. Proc Assoc Am Physicians 1999; 111:438-47.

20. Wada M, Gelfman CM, Handa JT, Morse LS, Hjelmeland LM. Tert-butyl hydroperoxide-induced stress response genes and FGF-2 gene expression in human RPE cells in vitro. Invest Ophthalmol Vis Sci 2000; 41:S414.

21. von Zglinicki T. Telomeres: influencing the rate of aging. Ann N Y Acad Sci 1998; 854:318-27.

22. von Zglinicki T, Pilger R, Sitte N. Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. Free Radic Biol Med 2000; 28:64-74.

23. Allen RG, Tresini M. Oxidative stress and gene regulation. Free Radic Biol Med 2000; 28:463-99.

24. Gille JJ, Joenje H. Cell culture models for oxidative stress: superoxide and hydrogen peroxide versus normobaric hyperoxia. Mutat Res 1992; 275:405-14.

25. Barazzone C, White CW. Mechanisms of cell injury and death in hyperoxia: role of cytokines and Bcl-2 family proteins. Am J Respir Cell Mol Biol 2000; 22:517-9.

26. Kazzaz JA, Xu J, Palaia TA, Mantell L, Fein AM, Horowitz S. Cellular oxygen toxicity. Oxidant injury without apoptosis. J Biol Chem 1996; 271:15182-6.

27. Davidson PC, Sternberg P, Jones DP, Reed RL. Synthesis and transport of glutathione by cultured human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 1994; 35:2843-9.

28. Liles MR, Newsome DA, Oliver PD. Antioxidant enzymes in the aging human retinal pigment epithelium. Arch Ophthalmol 1991; 109:1285-8.

29. Mehlhorn RJ. Ascorbate- and dehydroascorbic acid-mediated reduction of free radicals in the human erythrocyte. J Biol Chem 1991; 266:2724-31.

30. Witenberg B, Kalir HH, Raviv Z, Kletter Y, Kravtsov V, Fabian I. Inhibition by ascorbic acid of apoptosis induced by oxidative stress in HL-60 myeloid leukemia cells. Biochem Pharmacol 1999; 57:823-32.

31. Winkler BS, Orselli SM, Rex TS. The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. Free Radic Biol Med 1994; 17:333-49.

32. Carr A, Frei B. Does vitamin C act as a pro-oxidant under physiological conditions? FASEB J 1999; 13:1007-24.

33. Bjerkvig R, Thorsen J, Svendsen E, Holmsen H, Thorsen T. Effects of hyperoxia on human endothelial cell proliferation and morphology in vitro. Undersea Biomed Res 1992; 19:415-26.

34. Dumont P, Burton M, Chen QM, Gonos ES, Frippiat C, Mazarati JB, Eliaers F, Remacle J, Toussaint O. Induction of replicative senescence biomarkers by sublethal oxidative stresses in normal human fibroblast. Free Radic Biol Med 2000; 28:361-73.

35. Shenberger JS, Dixon PS. Oxygen induces S-phase growth arrest and increases p53 and p21(WAF1/CIP1) expression in human bronchial smooth-muscle cells. Am J Respir Cell Mol Biol 1999; 21:395-402.

36. Rosen P, Boulton M, Moriarty P, Khaliq A, McLeod D. Effect of varying oxygen concentrations on the proliferation of retinal microvascular cells in vitro. Exp Eye Res 1991; 53:597-601.

37. O'Reilly MA, Staversky RJ, Stripp BR, Finkelstein JN. Exposure to hyperoxia induces p53 expression in mouse lung epithelium. Am J Respir Cell Mol Biol 1998; 18:43-50.

38. Reddel RR. Genes involved in the control of cellular proliferative potential. Ann N Y Acad Sci 1998; 854:8-19.

39. Imlay JA, Linn S. DNA damage and oxygen radical toxicity. Science 1988; 240:1302-9.

40. Boulton M, Khaliq A, McLeod D, Moriarty P. Effect of 'hypoxia' on the proliferation of microvascular cells in vitro. Exp Eye Res 1994; 59:243-6.

41. Furumoto K, Inoue E, Nagao N, Hiyama E, Miwa N. Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress. Life Sci 1998; 63:935-48.

42. Saito H, Hammond AT, Moses RE. The effect of low oxygen tension on the in vitro-replicative life span of human diploid fibroblast cells and their transformed derivatives. Exp Cell Res 1995; 217:272-9.

43. Akeo K, Curran SA, Dorey CK. Superoxide dismutase activity and growth of retinal pigment epithelial cells are suppressed by 20% oxygen in vitro. Curr Eye Res 1988; 7:961-7.

44. Khaliq A, Jarvis-Evans J, McLeod D, Boulton M. Oxygen modulates the response of the retinal pigment epithelium to basic fibroblast growth factor and epidermal growth factor by receptor regulation. Invest Ophthalmol Vis Sci 1996; 37:436-43.

45. Pournaras CJ, Miller JW, Gragoudas ES, Husain D, Munoz JL, Tolentino MJ, Kuroki M, Adamis AP. Systemic hyperoxia decreases vascular endothelial growth factor gene expression in ischemic primate retina. Arch Ophthalmol 1997; 115:1553-8.

46. Kuroki M, Voest EE, Amano S, Beerepoot LV, Takashima S, Tolentino M, Kim RY, Rohan RM, Colby KA, Yeo KT, Adamis AP. Reactive oxygen intermediates increase vascular endothelial growth factor expression in vitro and in vivo. J Clin Invest 1996; 98:1667-75.

47. Khaliq A, Patel B, Jarvis-Evans J, Moriarty P, McLeod D, Boulton M. Oxygen modulates production of bFGF and TGF-beta by retinal cells in vitro. Exp Eye Res 1995; 60:415-23.

48. Hackett SF, Schoenfeld CL, Freund J, Gottsch JD, Bhargave S, Campochiaro PA. Neurotrophic factors, cytokines and stress increase expression of basic fibroblast growth factor in retinal pigmented epithelial cells. Exp Eye Res 1997; 64:865-73.

49. Handa JT, Verzijl N, Matsunaga H, Aotaki-Keen A, Lutty GA, te Koppele JM, Miyata T, Hjelmeland LM. Increase in the advanced glycation end product pentosidine in Bruch's membrane with age. Invest Ophthalmol Vis Sci 1999; 40:775-9.

50. Hjelmeland LM, Cristofolo VJ, Funk W, Rakoczy E, Katz ML. Senescence of the retinal pigment epithelium. Mol Vis 1999; 5:33.