Mitochondrial Abnormalities in Ageing Macular Photoreceptors

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PURPOSE. To evaluate somatic mitochondrial (mt)DNA mutations in the macula during ageing.

METHODS. Ten 30-μm cryostat sections from the macula (foveal and perifoveal regions) and peripheral retina of 14 donors (aged 14–94 years) were cut for cytochrome *c* oxidase cytochemistry. The photoreceptor layer was microdissected and DNA extracted for 4977-bp mtDNA (mtDNA^{4977}) quantification using PCR. Dual cytochemistry for cytochrome *c* oxidase and succinate dehydrogenase allowed the detection of cytochrome *c* oxidase–deficient cones.

RESULTS. Findings showed a progressive accumulation of mtDNA^{4977} from ages 14 to 94 years. From ages 14 to 60 years there was an increase from 0.006% to 0.25%, and from ages 60 to 94 years there was a steeper increase from 0.25% to 5.39%. Counts of cones in the dual-reacted preparations showed more cytochrome *c* oxidase–deficient cones in the foveal region than elsewhere.

CONCLUSIONS. The results show that mitochondrial DNA deletions and cytochrome *c* oxidase–deficient cones accumulate in the ageing retina, particularly in the foveal region. These defects may contribute to the changes in macular function observed in ageing and age-related maculopathy. (*Invest Ophthalmol Vis Sci.* 2001;42:3016–3022)

Ageing is associated with a decline in macular function, with small but significant changes in both visual acuity and contrast sensitivity evident in most elderly individuals. Morphologic changes accompanying this ageing process primarily involve the photoreceptors and the cells of the retinal pigment epithelium (RPE). This is manifested by atrophy and loss of both cell types, depigmentation and hyperpigmentation of the RPE, a progressive accumulation of lipofuscin, drusen formation, thickening of Bruch's membrane, and the appearance of basal laminar deposits.[1] In a proportion of individuals, however, the changes associated with ageing progress to constitute the pathologic features identified with age-related maculopathy (ARM). ARM can be associated with a marked decline in visual function and currently accounts for approximately 50% of all blind or partially sighted registrations in the United Kingdom.[2] Despite extensive research, the cellular mechanisms underlying ARM remain uncertain, and for the majority of patients no treatment is available.[3] Understanding the mechanisms underlying macular ageing may well provide an insight into the etiology of ARM and ultimately lead to prevention and treatment.

Mitochondria are intracellular organelles whose main function is the synthesis of adenosine triphosphate (ATP) through oxidative phosphorylation. This process is dependent on the mitochondrial respiratory chain, an integrated series of five major multisubunit enzyme complexes. Although most of the protein subunits comprising these complexes are encoded by nuclear DNA, some 13 essential polypeptide subunits (7 of complex I, 1 of complex III, 3 of complex IV, and 2 of complex V) are encoded by mitochondrial (mt)DNA.[4] In addition, mtDNA encodes 2 ribosomal (r)RNAs and 22 transfer (t)RNAs, the full complement required for intramitochondrial protein synthesis.[4] Defects in the mitochondrial genome are an important cause of human disease.[5] These mutations give rise to a heterogeneous group of disorders in which the eye is frequently affected, and pigmentary retinal changes are a common ophthalmic manifestation of mitochondrial disease.[6] In approximately 50% to 60% of patients with mitochondrial disease, the pigmentary retinopathy is characterized by predominant posterior pole and macular involvement.[7,8] Histopathologic studies show both hyperpigmentation and hypopigmentation of the macular RPE and abnormalities in the photoreceptors including photoreceptor cell loss.[9,10] These findings can be similar to those seen in the ageing macula.[1]

It is apparent that acquired mtDNA defects may have a role in ageing and age-related disease.[11] mtDNA has at least a 10-fold higher mutation rate than the nuclear genome,[12] due in part to limited repair mechanisms, the absence of protective histones, and its proximity to the free-radical–generating inner mitochondrial membrane. This susceptibility of the mitochondrial genome to damage results in the accumulation of a variety of pathogenic mtDNA deletions with age in a large number of different human tissues.[13]

We were particularly interested in the possibility that acquired mtDNA mutations may play a role in macular ageing. The retina is a prime site for acquired mtDNA mutations, being composed of postmitotic cells that are highly metabolically active.[14] In addition to the factors that contribute to the development of mtDNA mutations in most postmitotic cells, the retina is further exposed to light of variable wavelength, including ultraviolet light,[15-17] which is known to cause mtDNA damage.[18,19]

METHODS

Collection and Preparation of Ocular Tissue for Cytochemical and Molecular Genetic Analysis

We collected eyes from deceased donors either at the time of multiple organ donation or after the harvesting of corneoscleral discs for transplantation. Established techniques were used to preserve tissue integrity and for cytochemical studies.[14]

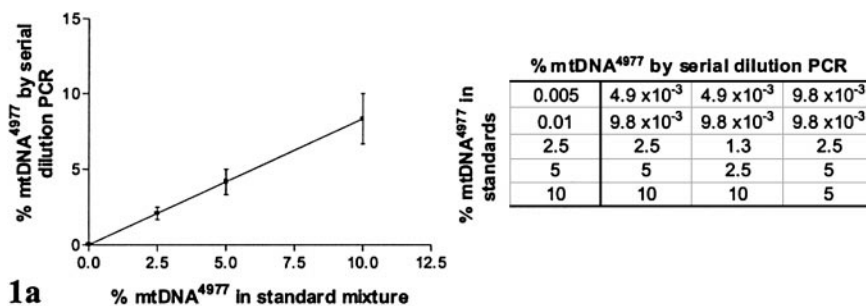| % mtDNA^{4977} by serial dilution PCR			
0.005	4.9 ×10^{-3}	4.9 ×10^{-3}	9.8 ×10^{-3}
0.01	9.8 ×10^{-3}	9.8 ×10^{-3}	9.8 ×10^{-3}
2.5	2.5	1.3	2.5
5	5	2.5	5
10	10	10	5

FIGURE 1. Analysis of deleted mtDNA. (**a**) Comparison of a semiquantitative PCR-based method with Southern blot analysis and serially diluted DNA standards. The correlation coefficient for the data is 1.0, and the associated regression line is $y = 0.833x + 0.0014$. Each concentration of DNA shown was analyzed on three separate occasions and the results are shown in the table. The error bars represent the SE of these three separate experiments. (**b**) Representative analysis of a serial-dilution PCR for mtDNA^{4977} in a 67-year-old donor (perifoveal region). The gels for the total mtDNA (*left*) and mtDNA^{4977} (*right*) are shown with their corresponding dilution values. Graphic analysis resulted in an mtDNA^{4977} level of 0.625%. Two further analyses resulted in a final mtDNA^{4977} level of 0.247% $\pm$ 0.189% (mean $\pm$ SE). The R^2 values for each exponential decay regression line were 0.9401 for total mtDNA and 0.8604 for mtDNA^{4977}.
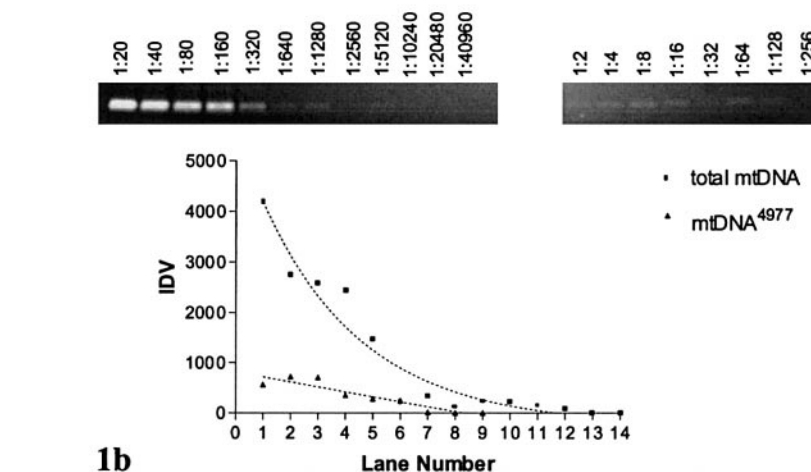
Corneoscleral discs were dissected, and the macular region of each eye was excised en bloc (sclera-choroid-RPE-retina) and frozen. A block of peripheral retina cut from the equator was also frozen in an identical manner. To identify the different anatomic layers, we demonstrated the activity of cytochrome *c* oxidase (COX) cytochemically in 30-μm cryostat (Frigocut; Reichert-Jung, Vienna, Austria) sections (described later).[20] The choroid, RPE, photoreceptors, and inner neural retina (inner plexiform layer plus ganglion cell and retinal nerve fiber layers) were mechanically separated from one another by a micromanipulator. Each layer in turn was then sloughed from the slide with a drawn glass capillary and placed into a separate PCR tube. The products of 10 consecutive sections were pooled before DNA extraction to provide sufficient mtDNA for subsequent quantitative molecular studies. All experiments were performed in triplicate.

Quantification of mtDNA Deletion in Human Retina

As a marker for mtDNA mutations we used the "common" deletion. This 4977-bp (mtDNA^{4977}) deletion, as well as being an important cause of mitochondrial disease, has been observed in many tissues with increasing age.[13] A modified version of the semiquantitative PCR method of Corral-Debrinski et al.[21] was used to estimate the proportion of the mtDNA^{4977} deletion in the total mtDNA extracted from the retinal samples. Samples were digested in 100 μl lysis buffer (500 mM Tris-HCl [pH 8.5]), 1 mM EDTA, 0.5% Tween 20, and 200 μg/ml proteinase K) overnight at 37°C. After DNA extraction, the resultant solution was phenol-chloroform purified and ethanol precipitated using standard methodology[22] and resuspended in 8 μl (pH 7.4) TE buffer (10 mM Tris, 1 mM EDTA) overnight at 4°C. Each sample was initially linearized using the restriction enzyme *Bam*HI (1 μl enzyme and 1 μl commercially supplied buffer) at 37°C for 90 minutes. Using serially diluted standards, we were able to show that the method was semiquantitative under our conditions (Fig. 1a).

Two dilution series were prepared for mtDNA^{4977} (twofold increases in dilution per step) and total mtDNA (an initial 10-fold dilution followed by twofold increases in dilution per step). One microliter from each dilution step was used for PCR amplification using the following primers: for total mtDNA L3108 (nucleotide [nt] 3108-3127) and H3717 (nt3717-3701); and for mtDNA^{4977}, L8282 (nt8282-8305) and H13851 (nt13851-13832).

The reaction was performed in a thermal cycler (Omnigene; Hybaid, Ltd., Teddington, UK) under the following conditions: an initial 2-minute denaturation step, followed by 34 cycles of denaturation (45 seconds at 94°C), annealing (30 seconds at 51°C for total mtDNA or 30 seconds at 56°C for mtDNA^{4977}), and extension (1 minute at 72°C), with a final 8-minute extension at 72°C. All PCR reactions were performed in the following mixture (50 μl): Sample DNA 1 μl, 0.6 μM forward primer, 0.6 μM reverse primer, 0.2 mM dNTPs, 5 μl 10× PCR buffer, (GeneAmp; Perkin Elmer, Norwalk CT), 0.2 μl DNA polymerase (Ampli*taq*; Perkin Elmer), and 35.75 μl sterile nanopure water. Finally, 50 μl mineral oil was added to each tube.

Agarose Gel Electrophoresis and Quantitation

After PCR, 5 μl loading dye (0.25% xylene cyanol, 30% glycerol) was added to each tube. The PCR products were loaded onto a 20 × 20-cm 1.5% agarose gel (Maxi gel tank; Anachem, Ltd., Luton, UK), containing ethidium bromide, in series and electrophoresed at 100 V for 1.5 to 2 hours in 1× TAE buffer (0.8 mM Tris acetate, 0.02 mM EDTA, 0.4 μg/ml ethidium bromide) with a 1-kb DNA ladder. After electrophoresis, the PCR products were visualized on a UV transilluminator (TMW-20; Flowgen, Ltd., Lichfield, UK) and a digital image of the gel obtained using image acquisition apparatus (Alpha Imager 2000: Alpha Innotech Corp., San Leandro, CA). The associated image analysis software (Alpha Ease, ver. 3.3; Alpha Innotech Corp.) allowed the calculation of the integrated density value (IDV) for each PCR product in a dilution series. IDVs were collected until two successive lanes yielded a value of zero. The IDVs were plotted, and the best-fit exponential decay line associated with the data was forced to cross the *x*-axis at zero. The dilution value closest to this was used to calculate the percentage of mtDNA^{4977} in the sample (Fig. 1b):

$$\% \text{mtDNA}^{4977} = \frac{\text{total mtDNA dilution factor}^{(\text{IDV Zero})}}{\text{mtDNA}^{4977} \text{ dilution factor}^{(\text{IDV Zero})}} \times 100$$

TABLE 1. Details of Donors Investigated for Levels of mtDNA Deletion or COX Deficiency in the Photoreceptor Layer

Age (y)	Sex	Eye	Cause of Death	Investigation	Post Mortem Delay (h)
14	M	Unknown	Unknown	P&C	Unknown
22	F	L	Traffic accident	P&C	10
26	M	L	Traffic accident	C	12
26	M	L	Traffic accident	C	10
42	F	R	Subarachnoid hemorrhage	P&C	4
45	F	R	Lung cancer	C	12
45	M	L	Unknown	C	12
48	M	R	Subdural hemorrhage	C	18
50	M	L	Heart failure	P	9.5
53	F	R	Subarachnoid hemorrhage	C	20
53	M	L	Heart failure	P&C	7
54	M	L	Heart failure	C	24
59	M	L	Heart failure	P&C	9
60	F	R	Intracranial hemorrhage	P&C	3
60	M	L	Heart failure	P	10
64	M	R	Chronic obstructive airway disease	P&C	5
65	M	L	Heart failure	P&C	6
67	M	L	Traffic accident	P&C	3
72	M	L	Heart failure	P&C	17
73	F	R	Heart failure	P&C	6
76	F	R	Dementia	P	20
84	F	R	Heart failure	P&C	7
96	M	L	Unknown	P	15

P, PCR; C, cytochemistry.

Demonstration of COX–Deficient Photoreceptors

A dual cytochemical assay was used to detect COX-deficient photoreceptors. Twenty serial 10-μm cryostat sections of retina (peripheral retina and macula) were first incubated in 50 μl COX medium (100 μM cytochrome c, 4 mM diaminobenzidine tetrahydrochloride in 0.2 M phosphate buffer [pH 7.0]) at 37°C for 30 minutes. Sections were then washed in phosphate-buffered saline (PBS) three times for 5 minutes each and then incubated in 50 μl succinate dehydrogenase (SDH) medium (130 mM sodium succinate, 200 μM phenazine methosulfate, 1 mM sodium azide, and 1.5 mM nitroblue tetrazolium in 0.2 M phosphate buffer [pH 7.0]) at 37°C for 40 minutes. After cytochemistry, sections were fixed in formal calcium solution (3.6% formalin, 1.1% calcium chloride [pH 7.4]) for 15 minutes, washed in PBS (pH 7.4) three times for 5 minutes and placed in bleaching solution (3% hydrogen peroxide, 1% disodium hydrogen phosphate) for 16 hours to reveal any SDH reaction product masked by melanin in the RPE.[23] Sections were washed in PBS (pH 7.4) three times for 5 minutes, dehydrated in a graded ethanol series (once at 70% and 95%, twice at 100%), cleared in clearing agent (Histoclear; National Diagnostics, Atlanta, GA) and mounted in DPX (R.A. Lamb, Eastbourne, UK).

The percentage of COX-deficient cones was determined by counting 100 cones per serial section in each of the regions sampled for PCR (Table 1). Additional 10-μm sections were cut for hematoxylin and eosin staining, using standard methods.[24] None of the donors of eyes used in this study had any known history of ocular disease. No histopathologic abnormalities were found in hematoxylin and eosin–stained material from those donors investigated in the ageing series.

RESULTS

Level of Common Deletion in Retina from a Donor with ARM and a Young Control Subject

Initial studies were performed to indicate whether there was an increase in acquired mtDNA mutations with age and, if so, what layers of the retina would be most affected. For these initial studies we chose to determine the level of the common mtDNA deletion in the macular and equatorial retina of eyes obtained from two corneal donors: a 76-year-old with established ARM and a 14-year-old with no known history of ophthalmic disease. The retina from the donor with ARM showed the typical histologic features associated with ARM: extensive drusen formation and degeneration of the RPE. In the young eye, only the macular photoreceptors contained detectable levels of deletion, and then only at low levels (0.006%). In the elderly eye with ARM, the macular photoreceptors had 14% mtDNA deletion. In the inner neural retina a level of 3% deletion was found, whereas in the RPE none was detected. No detectable deletion was found in the choroid. In the peripheral aged retina, low levels of mutant mtDNA were detected only in the photoreceptors and then at levels of less than 0.2%.

Level of Acquired mtDNA Deletions with Age in the Photoreceptor Layer

In view of the results of the initial study, subsequent molecular genetic studies focused on the photoreceptor layer in a series of different-aged individuals (marked P or P&C in Table 1). Our studies show that the common deletion accumulated with age in this layer (Figs. 2a, 2b). In addition, the macular retina (divided into perifoveal and foveal regions, approximately 1.4–2.2 and 0–0.55 mm, respectively, from the foveola) had levels approximately two orders of magnitude greater than the peripheral retina, with the highest levels seen in the foveal region (Figs. 2a, 2b).

COX–Deficient Cells

In other tissues, such as skeletal muscle and the central nervous system, the characteristic cytochemical feature of mtDNA disease and ageing is the presence of COX-deficient cells. We sought to establish whether the presence of the common deletion in the photoreceptor layer was also associated with such cells.

SDH is encoded exclusively by nuclear genes and is therefore independent of mtDNA. Thus, when other components of the respiratory chain are absent because of mtDNA damage, SDH activity should still be present. In the dual COX/SDH assay therefore, COX-deficient cones do not accumulate the brown reaction product during the COX reaction but appear blue after incubation in SDH medium.
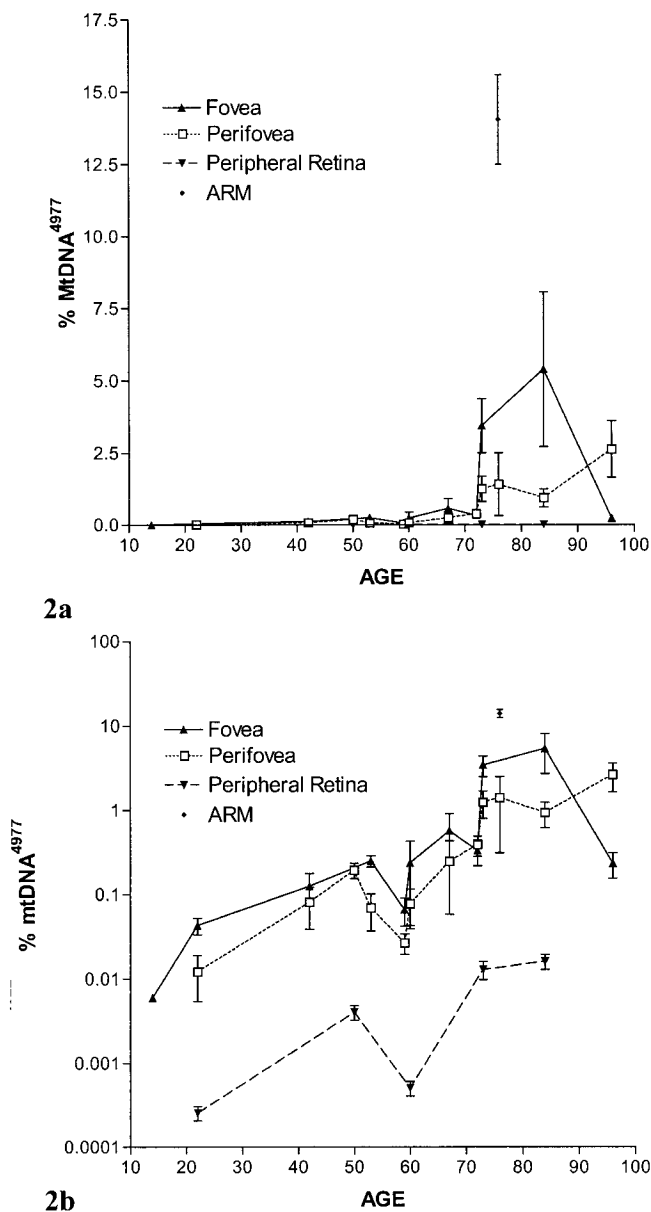
2a

2b

FIGURE 2. Accumulation of mtDNA^{4977} deletion in the photoreceptor layer with age (donors marked P or P&C in Table 1), shown on a linear scale (**a**) emphasizing the steep increase in deletion level after age 65 in the macular regions (foveal and perifoveal). The semilogarithmic plot (**b**) of the same data shows an increase in all regions with age. From ages 14 to 60 years, there was an increase from 0.006% to 0.25% and from ages 60 to 94 years there was a steeper increase from 0.25% to 5.39%. A difference was observed between the two macular sites investigated, with the foveal samples showing a threefold higher level than the corresponding perifoveal sample. The samples of peripheral retina showed levels of deletion generally two orders of magnitude lower than the macular samples, although they too showed an increase with age and in general mirrored the pattern of accumulation in the perifoveal samples. Bars represent SEM for three experiments.

We investigated retinal tissue from 19 subjects (marked C or P&C in Table 1) and showed that COX-deficient photoreceptors were present in the retinas of elderly individuals (Fig. 3). Moreover, the number of deficient cells showed an exponential, age-related increase, much higher in the central retina, reflecting the higher levels of mtDNA deletion found in this region (Figs. 4a, 4b, 4c). In the foveal region COX-deficient cones reached approximately 1% (Fig. 5):

approximately 0.4% in the perifoveal region and approximately 0.2% in the peripheral retina of older donors (≥60 years). In no sections was any COX-deficient cell observed in the underlying RPE. This is in keeping with the molecular genetic studies in which negligible levels of deleted genomes were observed in this layer.

Twelve samples were analyzed using both PCR and cytochemistry (P&C in Table 1). No significant correlation was found between the number of COX-deficient cones and the level of mtDNA^{4977}. The lack of correlation between deletion levels and COX deficiency in our samples may have been due to accumulation of other mtDNA mutations or differences in individual tolerances of COX-deficient cells leading to cell loss.

DISCUSSION

The main purpose of this study was to determine whether acquired mtDNA mutations are present in the human retina with age. Our results have shown the presence of these mutations, which were predominantly present in the photoreceptor layer of the retina. Our previous studies have shown that these cells contain large numbers of mitochondria, reflecting their metabolically active state.[14] Similar to many other tissues the amount of deleted mtDNA increases with age.[25,26]

We speculated that the amount of mutated mtDNA might be greater in the retina than in other tissues, because in addition
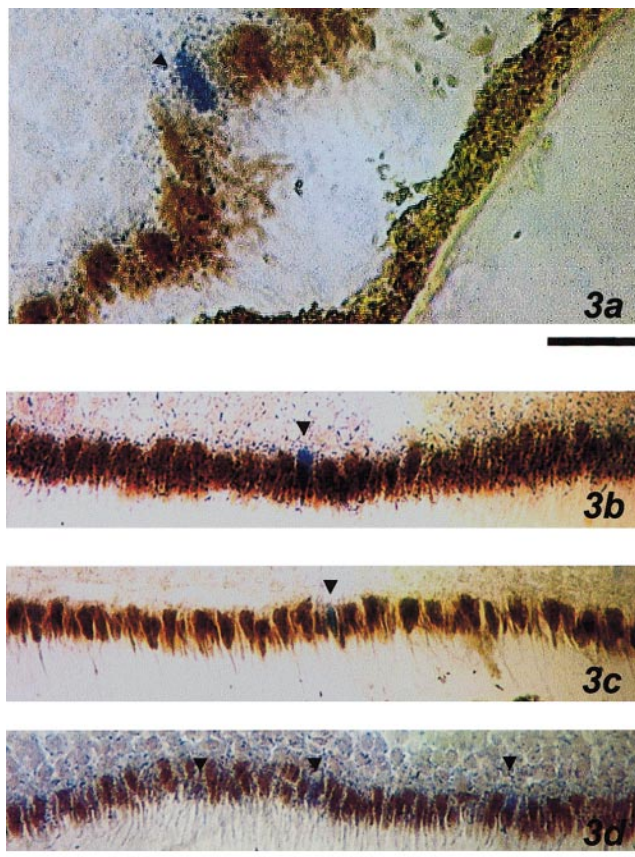

FIGURE 3. COX-SDH dual cytochemistry showing COX-deficient cones (*arrowheads*). (**a**) Single COX-deficient cone in the perifovea (~1.4–2.9 mm from foveola) of a 73-year-old donor. Single COX-deficient cone in (**b**) the parafovea (circa 0.9 to 1.4 mm from foveola) and (**c**) the perifovea of a 67-year-old donor. Multiple COX-deficient cones (**d**) in the perifovea of an 84-year-old donor. Scale bars, (**a**) 20 μm; (**b–d**) 50 μm.
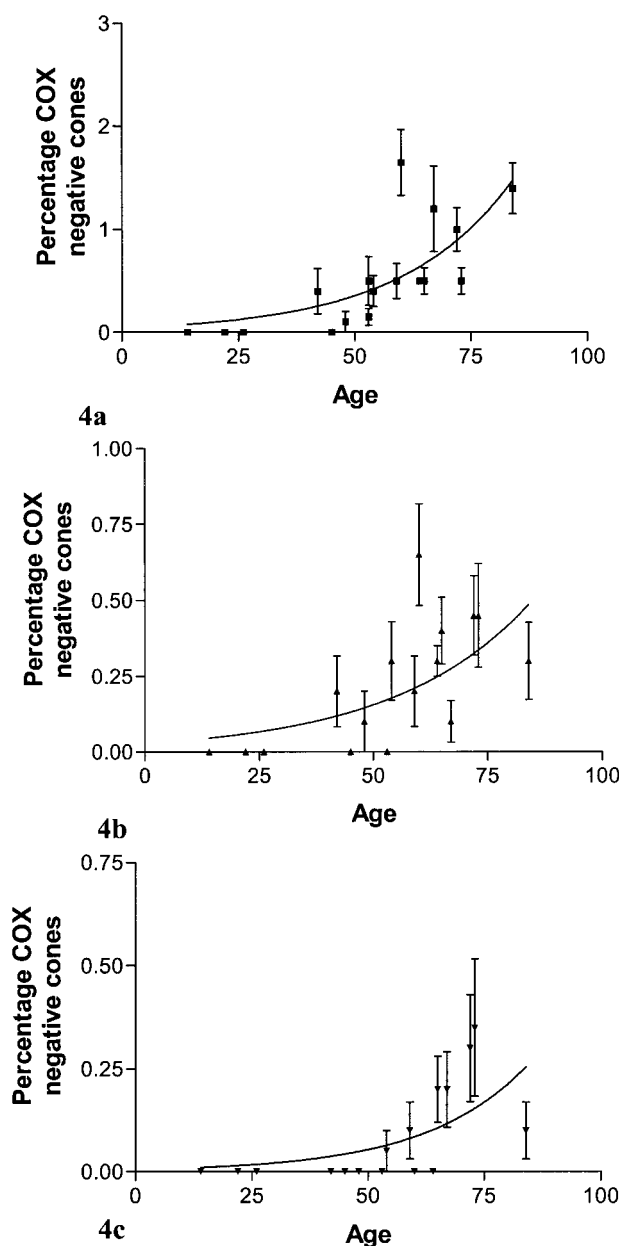
4a

4b

4c

FIGURE 4. Regional differences in percentages of COX-deficient cones. Twenty sections were used and 100 cones counted in each of the three regions of interest per section. These results were used to calculate the mean percentage of COX-deficient cones at each site. The percentages from the 19 donors (C or P&C in Table 1) studied were plotted for each site. An age-related exponential increase in COX-deficient cone frequency was seen at each site studied. The greatest number of COX-deficient cones was generally higher in the foveal region and lower in the peripheral retina. (**a**) Frequencies of COX-deficient cones in the foveal region. The regression line for the data is shown ($R^2 = 0.554$, $y = 0.044e^{0.042x}$). (**b**) Frequencies of COX-deficient cones in the perifoveal region. The regression line for the data is shown ($R^2 = 0.402$, $y = 0.029e^{0.034x}$). (**c**) Frequencies of COX-deficient cones in the peripheral retina. The regression line for the data is shown ($R^2 = 0.400$, $y = 0.005e^{0.046x}$). The error bars represent the SEM.

to other factors that may contribute to causing mutations, the retina is also exposed to low levels of UV light.[16,17] It seems that this may indeed be important, because we observed a much higher percentage of mutated mtDNA in the central than in the peripheral retina. Also, the level of common deletion

was much higher in the photoreceptor layer than has been described in other ageing tissues.[27]

Mitochondrial genetics is complicated by the presence of multiple copies of the mitochondrial genome in individual mitochondria and thus many hundreds or thousands in individual photoreceptors. The presence of both mutated and wild-type mtDNA in the same cell or tissue is termed heteroplasmy.[28] In the presence of heteroplasmy, the mutated mtDNA is functionally recessive, and a biochemical defect is present only if there is more than 60% to 65% mutated mtDNA within an individual cell.[29,30] Thus, the presence of low levels of mutated mtDNA may be of no functional significance. However, previous studies have shown that not only are there many different mutant mtDNA forms in ageing but that individual deletions may clonally expand within individual cells to cause levels of mutant sufficient to result in a biochemical defect.[13,27] The biochemical defect is shown by low activity of COX in individual cells. In our study, we observed COX–negative photoreceptors in the central retinal photoreceptors, the area in which we observed the highest level of deleted mtDNA.

Our observations have extended those in two previous studies, in which the presence of mtDNA deletions in retina with age was investigated.[31,32] These studies suggest relatively low levels of mtDNA[4977] in ageing retina, although neither investigation sampled photoreceptors specifically and especially those in the macular region. The levels of mtDNA[4977] found by us in the peripheral retina are on the same order as those found in the previous reports.[28,29]

In our study, there are undoubtedly mtDNA defects that increase with age, but the significance of this in terms of any age-related decline in retinal function is uncertain. The observation of mitochondrial abnormalities does not mean that there is a causal effect. Furthermore, the mechanism by which cones become COX-deficient is not shown conclusively; age-related
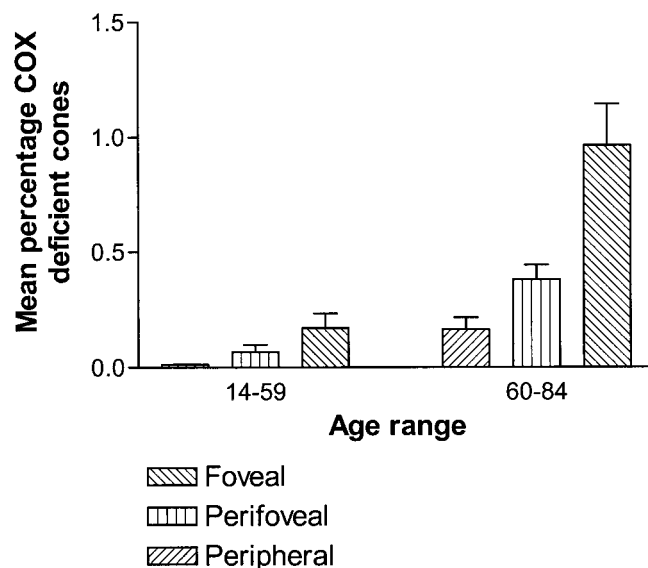

FIGURE 5. Regional differences in the incidence of COX-deficient cones. The data from the counts of COX-deficient cones (Fig. 4) were divided into two broad age groups. These data were pooled and the mean value at each retinal site obtained. A paired two-tailed *t*-test of foveal versus perifoveal and perifoveal versus peripheral retinal regions shows the differences in number of COX-deficient cones to be significant in the older age group. In the younger age group only the difference between foveal and perifoveal regions is significant ($P = 0.046$; peripheral retina versus perifoveal region $P = 0.059$). In the older age group, the differences are significant in both the foveal versus perifoveal region ($P = 0.018$) and the perifoveal region versus peripheral retina ($P = 0.048$).

changes in cellular processes other than those involving the mitochondrial genome may be responsible. However, the current results harmonize with results in studies of skeletal muscle in which the COX deficiency was linked to the clonal expansion of mutant mitochondrial genomes in individual muscle fibers.[27] The objective of future work will be to establish whether there is clonal expansion of mutant mitochondrial DNA in individual cones.

Data derived from human tissue obtained postmortem must also be treated cautiously because of changes that can occur after death and the possible effects of chronic disease. We believe our data minimize such confounding factors. Postmortem delay appears not to affect our results, because there is no obvious relationship between the delay and donor age. In the elderly, there is more chronic disease than in young subjects. However, none of the donors we studied had detectable eye disease, and all donors showed similar changes at the site of maximum change in the retina.

A number of outcomes are possible as a consequence of accumulating mitochondrial damage: (1) The biochemical defect, as shown by COX cytochemistry, would significantly impair the oxidative capacity of these cells with profound consequences on ATP-dependent cellular functions. These include the photoreceptor-specific ATP-binding cassette transporter, which appears to play a crucial role in the cycling of retinoids between the RPE and neural retina.[33,34] There is evidence that inhibition of this transporter results in the accumulation of lipofuscin-like material in the RPE,[34] a prominent feature of macular ageing and ARM.[35] Lipofuscin accumulation is thought to contribute to RPE cell death through light-induced generation of free radicals.[36-39] Loss of RPE cells might be expected to result in death of the overlying photoreceptors. (2) Accumulation of mtDNA damage within photoreceptors may also play a more direct role by inducing apoptosis,[40] a phenomenon observed in a number of ophthalmic disorders.[41] Such a process suggests a possible mechanism for the loss of photoreceptors observed in the ageing retina.[42] It is tempting to speculate that the pathologic processes discussed above in consequence of accumulating mtDNA deletions would be augmented in the ARM retina.

Our studies have clearly shown that mtDNA deletions and COX-deficient cones accumulate in the ageing retina, particularly in the macular region. These defects may contribute to the changes in macular function seen in ageing and ARM.

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