Part II

Molecular Aspects of the Aging Retina

CHAPTER 4

APOPTOSIS IS PRESENT IN THE PRIMATE MACULA AT ALL AGES

ABSTRACT

Purpose: It has become increasingly clear that apoptosis is a main event in photoreceptor cell death in a variety of retinal degenerations. We investigated the role of apoptosis in the physiologically aging primate macula.

Methods*:* Twenty maculae of rhesus monkeys, aged 6 to 34 years, were investigated. Apoptosis was determined in formalin-fixed, paraffin-embedded eyes using the TUNEL (TdT-mediated dUTP-biotin nick end labeling) method and quantitatively analyzed. Morphology of TUNEL positive cells was studied by confocal laser microscopy and transmission electron microscopy. The thickness of the outer nuclear layer (ONL) was determined by image analysis. Furthermore, expression of apoptosis-regulating proteins Bcl-x, Fas and Fas Ligand was studied by immunohistochemistry.

Results: TUNEL positive nuclei showed apoptotic features on confocal laser microscopy. They were scattered and sparsely found in the macula, most frequently in the ONL. The thickness of the ONL decreased with increasing age. Apoptosis was found equally distributed at all ages, although in the two oldest maculae up to 13 times more apoptosis was found. Expression of Bcl-x, Fas and Fas Ligand was equal at all ages.

Conclusion: Our findings indicate that apoptosis in the primate macula occurs at all ages at similar rate, possibly increasing in the oldest age group, and may account for the decreasing thickness of the primate macula with age.

INTRODUCTION

The thickness of the human retina decreases with advanced age, due to loss of photoreceptors and ganglion cells.[26] Photoreceptor loss occurs in atrophic age-related macular degeneration (AMD) as well as in the more severe neovascular form.[90] Apoptosis was demonstrated in 4 out of 16 cases of AMD, neovascular as well as atrophic.[55] Therefore, it was hypothesized that apoptosis is involved in photoreceptor degeneration in AMD.[55] In animal models of retinal degeneration, photoreceptor cell death occurs by apoptosis.[43-45,221-223]

It has become increasingly apparent that apoptosis, a cell suicide program, plays a crucial role in many physiologic processes, such as embryonic development and homeostatic maintenance of tissues, and in many disease processes, for instance malignant tumors. The process of apoptosis is under genetic control and can be initiated by an internal clock (programmed cell death), or by extracellular agents such as hormones, cytokines, natural killer cells, and a variety of chemical, viral, and physical agents. During apoptosis, individual cells show nuclear and cytoplasmic condensation and biochemical analysis reveals internucleosomal DNA fragmentation.[34,35,37]

Apoptosis-regulating genes in the retina have been studied extensively in experimental conditions. The Bcl-2 family consists of many proteins, both inhibitors and stimulators of apoptosis. The apoptosis-protective protein Bcl-2 is expressed widely in the developing neuronal system but downregulated in the adult neuronal system.[58] Bcl-x is the predominant Bcl-2 family member in postnatal neural tissues[57] and adult rat retina.[59] Overexpression of Bcl-2 or Bcl-x is protective for photoreceptor cell death in rd mice.[60] Fas (CD95) receptor mediates apoptosis when triggered by agonistic antibodies or its ligand, Fas Ligand (FasL).[62] Fas and FasL are expressed in normal human retina.[54,64,224,225]

The ocular fundus of humans and rhesus monkeys is almost identical.[226,227] Aged maculae of rhesus monkeys have clinically and histologically detectable pathology associated with age related maculopathy (ARM). Clinically, macular drusen (6 to 74%) identical to human drusen, and alterations of the RPE (18 to 45%) have been demonstrated.[228-232] The maximum life span of the rhesus monkey is one third of humans implying that relative aging occurs three times more rapidly.[227]

The aim of this study was to ascertain whether apoptosis plays a role in the physiologically aging primate macula.

MATERIALS AND METHODS

The animals were procured, maintained and used in accordance with the Dutch law and regulations, the Animal Welfare Act and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources-National Research Council (USA).

Experimental animals

Twenty rhesus monkeys (*Macaca mulatta*) studied were obtained from the Department of Clinical Oncology, University of Leiden, from the project 'Late effects of total body irradiation in rhesus monkeys'.[233,234] The animals used in this study belonged to the non-irradiated control group and were aged 6 to 34 years.

Tissue preparation and histopathology

The eyes of the monkeys were enucleated immediately post mortem and were processed as described before.[235] In short, a portion of the retina of approximately 1 cm^2 containing the macula was sectioned and horizontally divided in two parts. One part was fixed by immersion in formaline for 24 hours. After embedding in paraffin, sections through the centre of the macula were cut at 5 µm thickness and were mounted on silanized glass slides. For light microscopy, sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff and Mallory. The maculae were histologically examined by light microscopy for signs of aging and early macular degeneration, i.e. thickening of Bruch's membrane, basal laminar deposits (BLD), drusen and RPE abnormalities.[112,236]

TUNEL staining

To identify apoptosis, we used the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine (dUTP)-biotin nick end labeling (TUNEL) method, which labels the fragmented DNA ends with biotinylated poly(dU). Paraffin sections, within 1 mm of the foveola, were used for TUNEL staining, according to the method of Gavrieli et al.[39] with the following modifications. After deparaffinization and rehydration, the slides were pretreated with 20 µg/ml DNase-free proteinase K (Gibco Life Technologies, Breda, Netherlands) during 10 minutes at 37ºC. As a positive control one slide was treated by DNase I (Promega, Madison, WI, USA) dissolved in a DNase buffer (30 mM Trizma-base, 140 mM cacodylate, 4 mM $MgCl_2$, 0.1 mM dithiotreïtol, pH 7.2) during 1 hour at 37ºC or overnight at 4ºC to induce chromosomal breaks. Sections were washed in TdT buffer (0.5 M cacodylate, 1 mM $CoCl_2$, 0.5 mM DTT, 0.15 M NaCl, 0.05% bovine serum albumin, pH 6.8) and incubated for 3 hours in a mix containing 25 µl TdT buffer, 5 U TdT (Promega) and

0.5 nmol biotin-16-dUTP (Boehringer Mannheim, Germany). As a negative control, TdT enzyme was omitted. Labeling was done using biotinylated multilink antibodies followed by streptavidin-labeled alkaline phosphatase (Biogenex, San Ramon, CA, USA). New Fuchsin was used as chromogen, and the slides were counterstained with Mayer's hematoxylin. The number of TUNEL-stained nuclei was quantified in four random slides per macula by image analysis.

Confocal laser microscopy

To examine morphology, TUNEL-positive nuclei and apoptotic bodies were localized exactly in TUNEL-stained maculae by computer. Thereafter the slides were unmounted and stained with 5 ng/ml propidium iodide (Sigma, Steinheim, Germany) in Vectashield (Vector, Burlingame CA, USA). The TUNEL-positive nuclei were relocalized and examined using a confocal laser scanning microscope with a He/Ne-laser at 543 nm as an excitation light source. A long pass filter (> 570 nm) was used for the detection of propidium iodide emission light.

Electron microscopy

Another part of the macula was embedded for electron microscopy. The tissue was immersed in 4% paraformaldehyde and, after dehydration, embedded in Lowicryl (Aurion, Wageningen, Netherlands). The blocks were polymerized at -35ºC under ultraviolet light. Ultrathin sections were cut and stained with uranylacetate 6% and lead citrate. The sections were mounted on grids and examined for features of apoptosis using a transmission electron microscope.

Image analysis

Digital microscopic images consisting of 512×512 pixels (0.43 μm/pixel) of each section were recorded with a 40× objective using a Zeiss Axioplan Microscope (Zeiss, Oberkochen, Germany) equipped with a Sony DXC-930P 3-chip color CCD videocamera (with a 0.45× lens). The measurements and estimations were performed with a semi-automatic digital image analysis procedure (software: KS400, Kontron, Germany).

To determine the percentage of TUNEL-positive nuclei, the total numbers of nuclei present in the ONL and INL were estimated in the following way: 1) the mean surface area of individual nuclei that could be identified automatically by the image analysis software was measured ($A_{mean\ nucleus}$); 2) the total nuclear surface area of all nuclei, including the nuclei that were not segmented by the software (A_{total}), was measured, and 3) the total number of nuclei (N_{total}) was estimated as: $N_{total}=A_{total}/A_{mean\ nucleus}$. The number of TUNEL-stained nuclei per 1000 nuclei in the ONL and INL was recorded.

To relate thickness of the macula to age, we chose a section in the perifoveal region of the slides. Because the INL width has a large variance along the foveolar -

parafoveolar - perifoveolar region [237], we restricted this part of the study to the ONL width. We standardized according to the number of ganglion cells, to exclude any possible regional anatomic variation. We ensured that the plane of section was truly axial along the length of the rod inner segments. Therefore, five maculae had to be excluded. The ONL width was measured from the outer limiting membrane to the innermost cell of the ONL.

Immunohistochemistry

Expression of Bcl-x, Fas and FasL was determined as follows. Paraffin slides were deparaffinized and rehydrated. For the Fas antibody, antigen retrieval was performed (pronase treatment for 10 minutes at 37ºC*).* Incubation was performed with polyclonal rabbit anti-human antibodies against Bcl-x and FasL (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and monoclonal mouse anti-human antibodies against CD95 (Fas) (Immunotech, Marseille, France) for 1 hour at room temperature. Labeling was done using biotinylated multilink antibodies followed by streptavidin-labeled alkaline phosphatase (Biogenex, San Ramon, CA, USA). New Fuchsin was used as chromogen. The slides were counterstained with Mayer's hematoxylin, and examined by light-microscopy. Negative controls for immunohistochemistry included (1) omission of the primary antibody, (2) use of irrelevant antibodies of the same isotype, and (3) preabsorption of the Fas and FasL antibodies with a tenfold concentration of the immunizing peptide for 4 hours.

Statistical analysis

To analyse the data, Spearman's correlation was used. A value of $P<0,05$ was considered significant.

RESULTS

Histopathology

BLD and soft drusen were not found in any of the maculae. Hard drusen were noted in 8 maculae. The correlation (r_S=0.57) between number of drusen and increasing age was significant (P=0.01). Diffuse thickening of Bruch's membrane with intercapillary pillars was noted in one macula from a monkey 21 years of age.

TUNEL staining

All maculae were included except for two that after processing did not contain a retina (n=18). TUNEL-stained nuclei, indicating apoptosis, were observed sparsely in the

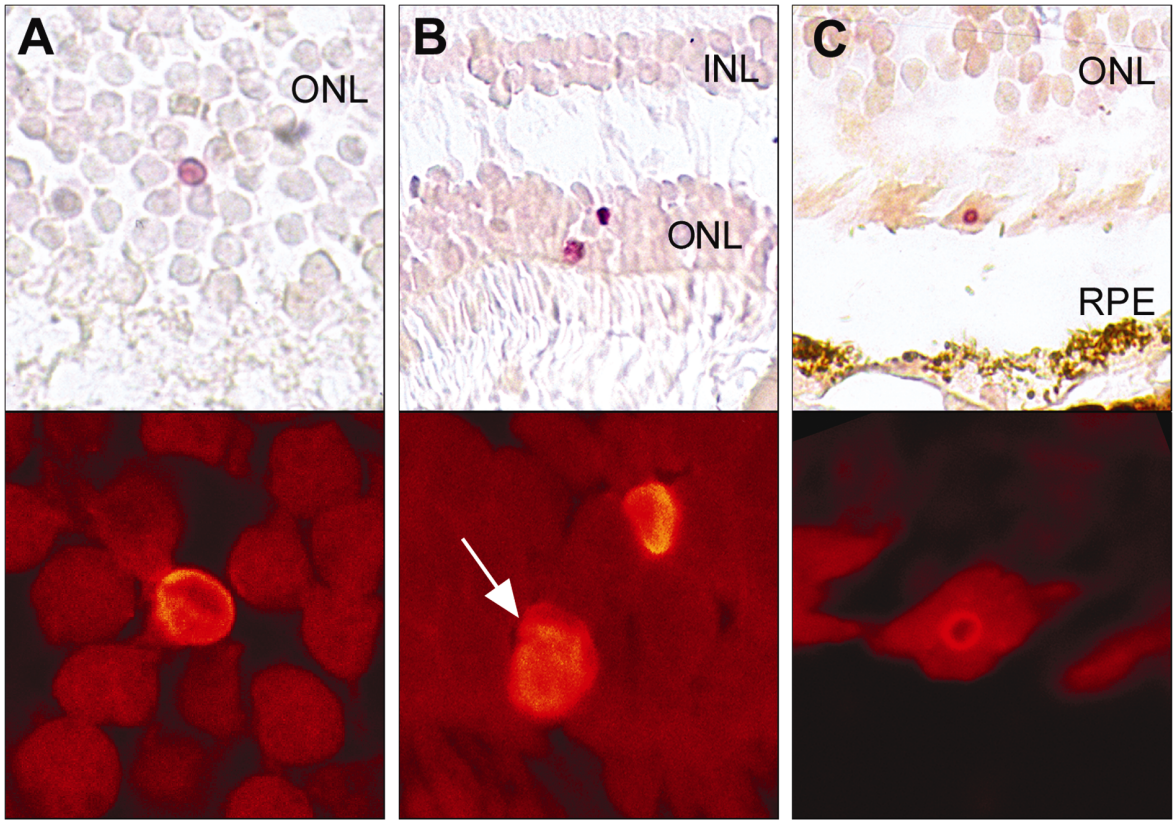

Figure 4.1 ***Apoptosis in rhesus monkey macula****. In-situ 3'end labeling of apoptotic DNA by alkaline phosphatase detection (upper row) and confocal laser microscopy images of same cells (lower row).* ***(A)*** *TUNEL-positive nucleus in ONL of 8-year-old monkey. Nucleus appears shrunken in relation to surrounding cells, and nuclear material appears condensed in periphery of nucleus.* ***(B)*** *TUNEL-positive nuclei in ONL of 34-year-old monkey. One nucleus shows budding (arrow).* ***(C)*** *Apoptotic body in the photoreceptor layer of 18 year old monkey. ONL = outer nuclear layer; INL = inner nuclear layer, RPE = retinal pigment epithelium. Original magnification upper row, 400×; estimated magnification confocal images, 1300× – 1800×.*

nuclear layers of the retina but were most numerous in the ONL (Figure 4.1A, B). TUNEL-stained nuclei were also found in the photoreceptor layer, as well as small labeled particles, consistent with apoptotic bodies[238] (Figure 4.1C). The red-colored apoptotic bodies were clearly distinguishable from RPE pigment. In four maculae TUNEL-stained nuclei were sporadically found in the RPE. The negative controls did not stain, and positive controls showed adequate labeling of DNA fragments.

Confocal laser microscopy

Confocal microscopy of TUNEL-stained nuclei revealed the presence of condensed nuclei with morphologic features of apoptosis: condensation of nuclear chromatin, cell body shrinkage and cell budding (Figure 4.1).

Electron microscopy

In the ultrathin sections of the macula of the oldest monkey we found focally in the ONL a cell, showing phagocytosis of condensed material, consistent with nuclear chromatin (Figure 4.2).

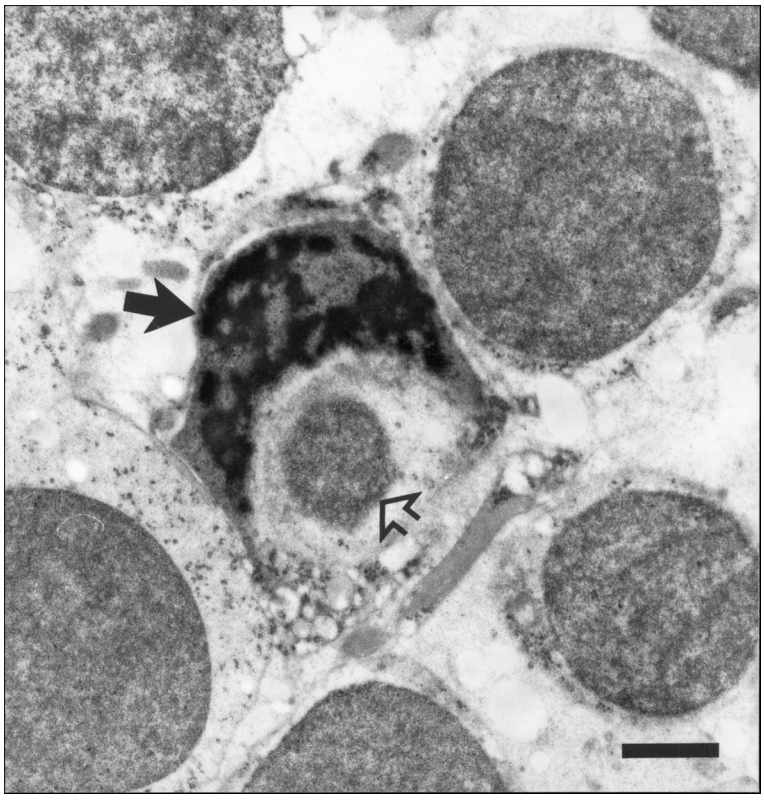

Figure 4.2 ***Transmission electron microscopy*** *of nuclei in the ONL of the oldest monkey (34 years of age) with phagocytosis of condensed nuclear material (filled arrow) by a neighboring cell. The open arrow indicates the original nucleus of the cell. Scale bar represents 1,59 μm.*

Image analysis

TUNEL-positive nuclei in the ONL were found at a rate of 0 to 0.53‰ of the total number of ONL nuclei in each section. TUNEL staining was found equally distributed at all ages, although in the two oldest maculae (32 and 34 years of age) 6 and 13 times more positive nuclei were found in the ONL, respectively (corrected for the amount of nuclei in the ONL) (Figure 4.1B). We found a non-significant correlation of r_S = 0.14 for the ONL and r_S = 0.10 for the INL between TUNEL staining and increasing age (ONL: P = 0.59, INL: P = 0.69; Spearman's correlation coefficient) (Figure 4.3). The thickness of the ONL decreased significantly with increasing age (r_S = -0.56; P = 0.029) (Figure 4.4).

Immunohistochemistry

Expression of Bcl-x was observed in the RPE, in the outer limiting membrane, and in nuclear and plexiform layers, particularly in the outer plexiform layer (Figure 4.5A). Strong expression of Fas was found in ganglion cells and INL, less strong expression in the RPE and variously in choriocapillaris and choroidal vessels (Figure 4.5B). FasL expression was found throughout the neuroretina, in the RPE, and variously in the choriocapillaris (Figure 4.5C). In negative controls, no staining or slightly aspecific

staining was seen (Figure 4.5D, E, F). Expression of Bcl-x, Fas, and FasL in the neuroretina was similar at all ages.

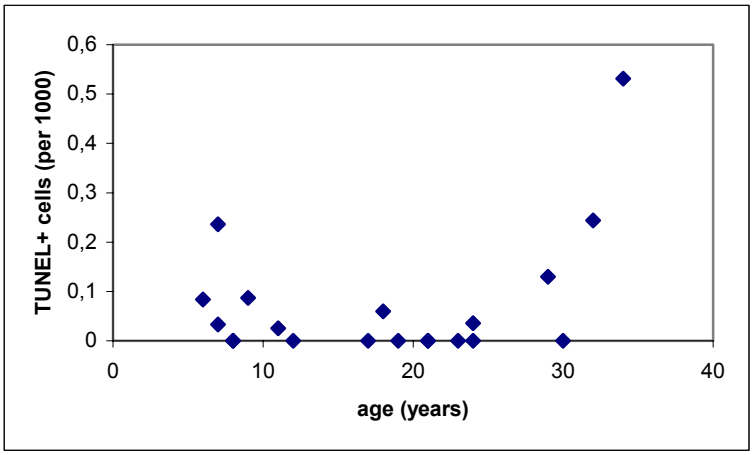

Figure 4.3 *Relationship between TUNEL-stained nuclei in ONL and aging, in years. Number of apoptotic nuclei is quantified per 1000 nuclei of ONL.*

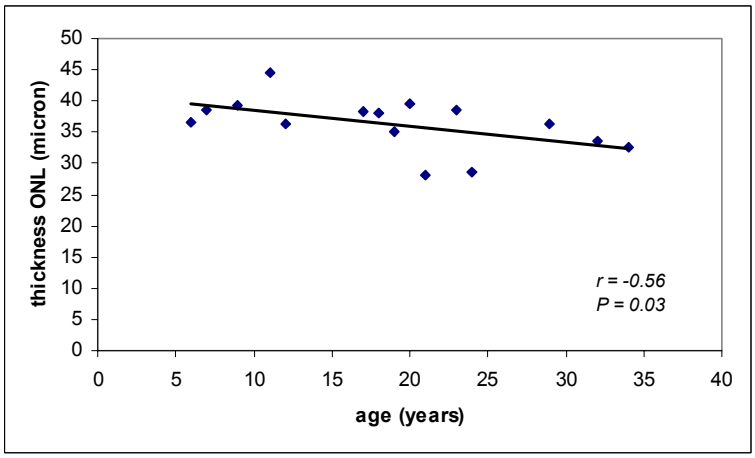

Figure 4.4 *Relationship between age and thickness of ONL (in micrometers) in rhesus monkey, indicating that thickness of the macula decreases with increasing age. r_S = –0.56, P = 0.03 (Spearman's correlation).*

DISCUSSION

We found that apoptosis in the primate macula occurs at all ages at similar rate, even in the youngest age group. In the 2 oldest maculae (>32 years) we found approximately 6 and 13 times more apoptotic nuclei, respectively. However, a significant positive correlation of apoptosis with increasing age could not be demonstrated. This may be due to the relatively small number of aged maculae. Moreover, the occurrence of apoptosis in postmitotic tissues should be a rare event.

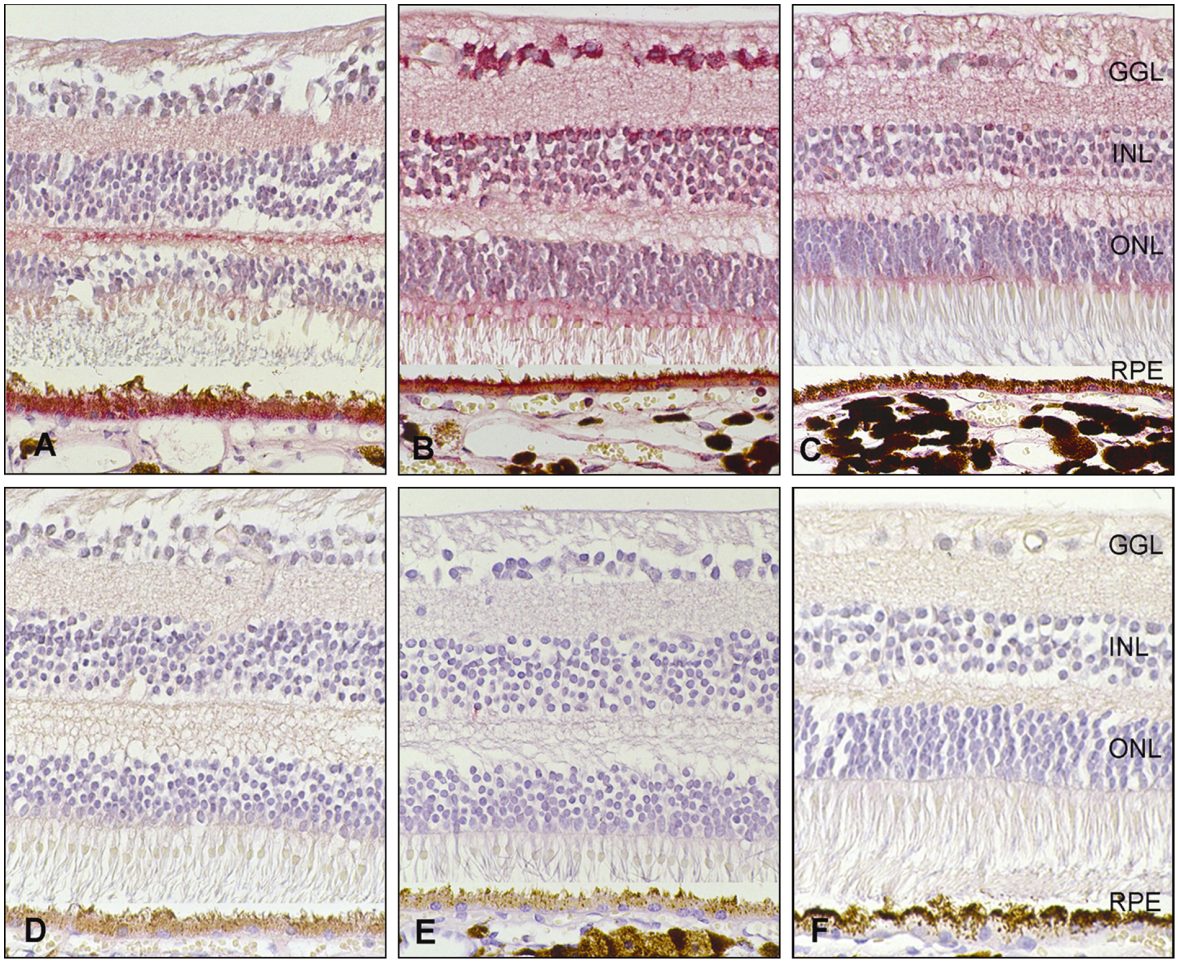

Figure 4.5 ***Immunolocalization of apoptosis-regulating proteins*** *in rhesus monkey macula. Immunohistochemistry was performed on paraffin-embedded tissue and visualized with an alkaline phosphatase system using a red chromogen. Expression of Bcl-x* ***(A)****, Fas (CD95)* ***(B)****, and FasL* ***(C)****. Negative control staining using irrelevant polyclonal rabbit IgG antibodies* ***(D)****, irrelevant monoclonal mouse IgG_1 antibodies* ***(E)****, and peptide blocking of the anti-FasL antibodies* ***(F)****. GGL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer, RPE = retinal pigment epithelium. Original magnification ×400*

With confocal laser microscopy, we demonstrated features of apoptosis, such as nuclear chromatin condensation, cell shrinkage and cell budding in TUNEL stained nuclei. Therefore, we assume that TUNEL positive nuclei in our study represent apoptosis.

The apoptotic process is accomplished quickly[34] and the period through which dying cells can be revealed by the TUNEL method is also relatively short: it is estimated at about 10 hours in rat retinal ganglion cells.[51] Thus, because of the short duration of apoptosis a relatively low incidence of histologic signs of apoptosis can indicate a considerable rate of cell loss.[239] Our findings of constant levels of apoptosis in the ONL might explain the decrease of ONL thickness with increasing age. These findings are in concordance with decreasing of thickness of the human retina with age,[26,27] although a different method was applied in those studies.

Apoptotic cells break up in membrane-bound fragments, so-called apoptotic bodies, that are phagocytosed by neighboring cells and induce no inflammatory response.[34,35,37] On electron microscopy we found phagocytosis of condensed nuclear material by a neighboring cell. Furthermore, we found some apoptotic bodies in the photoreceptor layer by confocal laser microscopy. Therefore it is conceivable that some apoptotic bodies migrate from the ONL and are phagocytosed by the RPE. Apoptosis in RPE cells was a rare event at all ages. However, with a small number of RPE cells present per slide, this result can indicate RPE cell loss by apoptosis. This is in accordance with the observations of decreased RPE cell density in the macular area with age.[27,240] In vitro, cultured RPE cells can be triggered to undergo apoptosis by a variety of agents, such as oxidative stress[25] and lipofuscin components.[241]

Apoptosis in the younger age group may partly be explained by a continuation of the apoptotic process, responsible for the death of redundant cells during development of the retina.[42,242] Another explanation may be that apoptosis resulting from external stimuli is already present at young age and functions in order to remove damaged or dysfunctional cells. Our findings are in concordance with findings of apoptosis in control monkey retinas in other studies[52] and indicate that some apoptosis of the retina occurs as part of normal aging. The equal protein expression of the apoptosis-regulating genes *Bcl-x, Fas* and *FasL* at all ages is in accordance with the steady amount of apoptosis during the aging process.

The apoptotic rate increases in the oldest age group and in maculae with signs of ARM, possibly under influence of other stimuli, internal as well as external. Internal stimuli may be genetic predisposition, as is shown in colonies of rhesus monkeys with high rates of ARM.[229] Likewise, in humans there is evidence for genetic predisposition in AMD.[7,9] A number of external stimuli may be postulated. Hypoxia and ischemia of the outer retina are thought to contribute to the development of AMD.[144] In a recent study on retinal ischemia in rats, apoptosis appeared as late component of neuronal death.[67] Environmental factors such as light are assumed to play a role in retinal degeneration. In albino rats some of the damage inflicted by light may result in apoptosis of retinal photoreceptor cells. [243,244] Apoptosis is also induced by ultra violet light damage.[245] In our study, the investigated rhesus monkeys, being captive, were underexposed to ultraviolet light, and were not exposed to other known risk factors for ARM,[3] although atherosclerosis was present in the older monkeys. In rhesus monkeys, end stage AMD is rare.[231] This may reflect either a slower degenerative process, or may represent an environmentally selective phenomenon.[228] Furthermore, we did not find any BLD in the maculae of these monkeys, which is consistent with findings of other studies.[103,228,231,232] This might implicate a degenerative process in the rhesus monkey eye somewhat different from that in the human eye.

In summary, we found that apoptosis occurs at similar rates at all ages in the primate macula. The process of apoptosis may account for the decreasing thickness of the ONL with age.

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