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Distribution of erythroid spectrin (α, β) immunoreactivity in the human retina

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Spectrin (α and β) is a tetrameric, key membrane skeletal protein initially reported to be present in erythrocytes. It is also present in other cells, neurones and the retina. This study reports immunoreactivity (IR) to erythroid spectrin in human retina at different ages (56-91 years), as examined by immunohistochemistry and immuno-electron microscopy (IEM). Spectrin IR was localised moderately in ganglion cell layer, inner- and outer plexiform layers and predominantly in photoreceptor inner segments and macular fibres of Henle. As the latter are interleaved by outer processes of Müller cells, IEM was used to see which cellular processes contained spectrin. IEM revealed that gold particles were associated with microtubules of cone inner segments and in fibres of Henle, but were absent in Müller cell processes, supporting immunohistochemical data on the absence of IR in Müller cells (stained with vimentin antibody). In fibres of Henle, a steady expression of spectrin was seen until eighth decade, and then a decreased expression beyond ninth decade, which paralleled with degenerative microtubule changes (seen by transmission electron microscopy) in them. These changes could be responsible for age-related down-regulation of spectrin expression in fibres of Henle. It is likely that one specific role of spectrin in the human retina could be maintenance of the macular fibres of Henle. Its reduction with age may cause alterations in cone membrane, which may be responsible for their vulnerability in ageing.

Keywords: Human retina, Fibres of Henle, Müller Cell, Parafovea, Microtubules

Spectrin is a key membrane-skeletal protein of erythrocytes. It is a tetramer of two alpha chains (240 kD) and two beta chains (220 kD), forming αß-spectrin (also called erythroid spectrin). It is attached to erythrocyte membrane proteins *via* interaction with ankyrin and F-actin[1,2]. Studies have indicated that αß-spectrin is essential for the maintenance of erythrocyte shape and strength, since in diseases and mutations, its loss results in altered erythrocyte shape, giving clinical presentations like hereditary spherocytosis and hemolytic anemia. Besides erythrocytes, αß-spectrin is also present in certain non-erythroid cells, such as muscles, keratinocytes, spermatocytes and neurones. Another related protein, called brain spectrin or fodrin (240/235 kD) has been reported in the literature. While both proteins are present in the vertebrate nervous system, there is a marked cell-specific distribution of both proteins in neurones. For example, in the retina, erythroid spectrin is localized in ganglion, bipolar and photoreceptor (cone) inner segments[3-5], while brain spectrin is clearly present in the optic nerve, and diffusely in inner and outer nuclear layers (INL, ONL), and ganglion cell layer (GCL)[6]. αß-spectrin is essential for the development of photoreceptor polarity in Drosophila[7]. In diabetic retinopathy, an altered αß-spectrin structure in erythrocytes has been reported[8].

The aim of this study was to examine the cellular and subcellular distribution of αß-spectrin immunoreactivity (IR) in the human retina with ageing. αß-spectrin appeared to down-regulate with ageing and this altered expression was correlated with other molecular markers and age-related electron microscopic changes seen in the retina.

Materials and Methods

Eyeballs

The eyes (Table 1) were obtained from cases of eye donations from postmortem individuals (n=10) who had no history of systemic or eye diseases. They were obtained from National Eye Bank, Dr Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, India. The eye bank authorities received written consent for eye donation from relatives of the deceased. The eyes were collected and utilised in research *via* ethics

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Table 1 — Information about the donors (n=10)

Age*	Sex	Cause of death**	Delay in fixation (h)	Methods used
50	M	Heart attack	3	IHC
56	M	Myocardial infarction	2	IHC, TEM
62	F	Heart attack	1	TEM
67	F	Heart attack	2	TEM, IHC, IEM
70	M	Myocardial infarction	2	TEM, IHC, IEM
72	F	Heart attack	2	IHC, IHC, IEM
78	F	Heart attack	2	TEM,
81	F	Myocardial infarction	1	TEM, IHC, IEM
85	M	Myocardial infarction	3	TEM
91	M	Cardiac arrest	2	TEM, IHC

*in years; ** obtained from registry; M, male; F, female

IHC, immunohistochemistry; IEM, immuno-electron microscopy; TEM, transmission electron microscopy

approval from the Institute human ethics committee (IHEC No. As-207/2008), adhering to the tenets of Helsinki declaration.

Tissue fixation

The right eye was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 24 h at 4°C for use in immunohistochemistry (IHC). The left eye was immersion fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M PB (pH 7.4) for 6 h at 4°C for use in transmission electron microscopy (TEM) and in 0.2% glutaraldehyde for 30 min at room temperature and later in 4% paraformaldehyde for 2 h at 4°C for immuno-electron microscopy (IEM).

Immunohistochemistry

The tissue samples were washed and cryoprotected in 15 to 30% sucrose overnight, cut into 14 µm thick transverse frozen sections and mounted onto gelatin-coated slides. Before immunolabeling, adjacent retinal sections were stained with hematoxylin and eosin to see the tissue characteristics and degree of postmortem changes. The sections were labeled with monoclonal mouse primary antibodies against αß-spectrin (Sigma-Aldrich Inc., MO, USA; catalog number: 30021010000; dilution: 5 µg/mL), actin (Dako Inc, Denmark; catalog number: M0851, smooth muscle specific; dilution: 3 µg/mL) and vimentin (Sigma-Aldrich Inc., MO, USA; catalog number: V6630; dilution: 3 µg/mL). Vimentin is an intermediate filament protein known to be present in Müller cells[9]. The reason for its inclusion in this study was explained in the results section. Sections were first quenched of endogenous peroxidase activity by treating in 0.3% hydrogen peroxide and incubated in the respective primary antibodies for 48h at 4°C. After washing, they were incubated in biotinylated anti-mouse IgG (dilution: 1 µg/mL; Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA) for 6h at 4°C. The antigen-antibody binding sites in tissue sections were visualised by employing the avidin-biotin immunoperoxidase method, using 0.05% 3, 3′ diaminobenzidine tetrahydrochloride hydrate (Sigma-Aldrich Inc., MO, USA) as the chromogen. The slides were dehydrated in ethanol and coverslipped with DPX. Few slides were counterstained with hematoxylin. For negative control, sections were omitted with the primary antibody treatment.

Transmission electron microscopy

IHC for spectrin revealed positive labelling in the fibre layer of Henle (FH, the oblique, long photoreceptor axons of the macula). They are interleaved with the outer processes of Müller cells at the level of outer plexiform layer (OPL). Transmission electron microscopy was aimed to see the fine structural organisation of this zone in young as well as aged retinas. The retinas were separated from the adherent choroid and the macular and mid-peripheral regions (eccentricity: 6-12 mm) were cut. The samples were postfixed in 1% OsO_4 for 1 h at 4°C, dehydrated in acetone and embedded in araldite CY 212 (TAAB, UK). Thick Sections (1 µm) were cut, stained with toluidine blue and observed under a light microscope. Thin sections (60-70 nm) were contrasted with uranyl acetate and alkaline lead citrate and observed under a Morgagni 268D transmission electron microscope (Fei Company, The Netherlands).

Immuno-electron microscopy

To see the distribution of αß-spectrin at the level of OPL (the FH versus Müller cell processes), Immuno-electron microscopy was done. The aldehyde fixed samples were dehydrated in ascending grades of ethanol, embedded in LR White resin (TAAB, UK) and polymerized overnight at 55°C. Thin sections (70-80 nm) were cut and mounted on to nickel grids. For labelling, the grids were first treated in 1% cold water fish gelatin and 1% bovine serum albumin in 0.1 M tris buffer (pH 7.4) for 1 h to avoid non-specific reactions. These grids were then

incubated in anti αß-spectrin antibody (dilution: 10 µg/mL) overnight, washed in buffer for 1 min X 3 changes and again incubated in goat-anti mouse secondary antibody conjugated to 15 nm colloidal gold (TAAB, UK) for 3 h. After washing, the grids were contrasted with uranyl acetate and observed under a Morgagni 268D transmission electron microscope (Fei Company, The Netherlands). Digital images were acquired using Megaview III CCD camera, using iTEM software (Soft Imaging Solutions, Münster, Germany). For control, the primary antibody treatment was omitted in few grids.

Results

Immunohistochemical observations

The general organization of the human retina, stained with hematoxylin and eosin, is shown in (Fig. 1). A thick fibre layer of Henle (FH) is present in the parafoveal retina (Fig. 1A), which is absent in the mid-peripheral retina (Fig. 1B). IHC revealed αß-spectrin to be localised in GCL of macular (Fig. 2A) as well as mid-peripheral retina. Few neurones of the INL were weakly immunopositive. A moderate level of IR was present in the inner and outer plexiform layers (IPL, OPL; Fig. 2B-D). In the

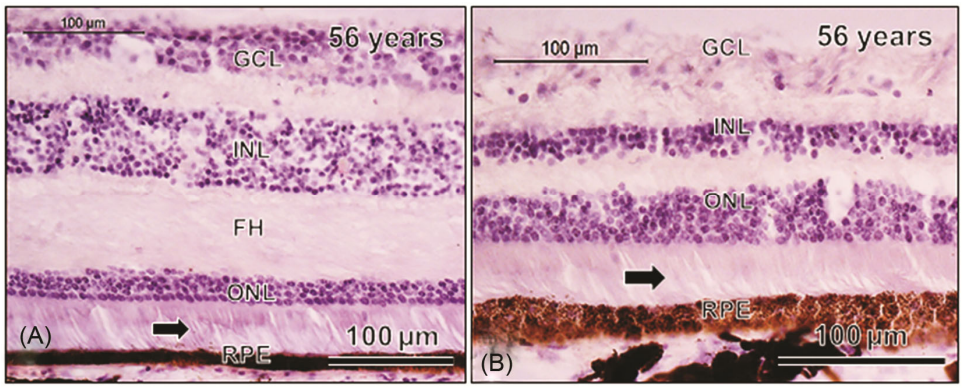

Fig. 1 — General organization of human retina in the macula, stained with hematoxylin and eosin. Parafoveal retina (A) shows a thick fibre layer of Henle (FH) beneath the inner nuclear layer (INL); and (B) It is absent in the mid-peripheral retina. Arrows denote the photoreceptor cell layer. RPE, retinal pigment epithelium. From 56-year-old donor retina

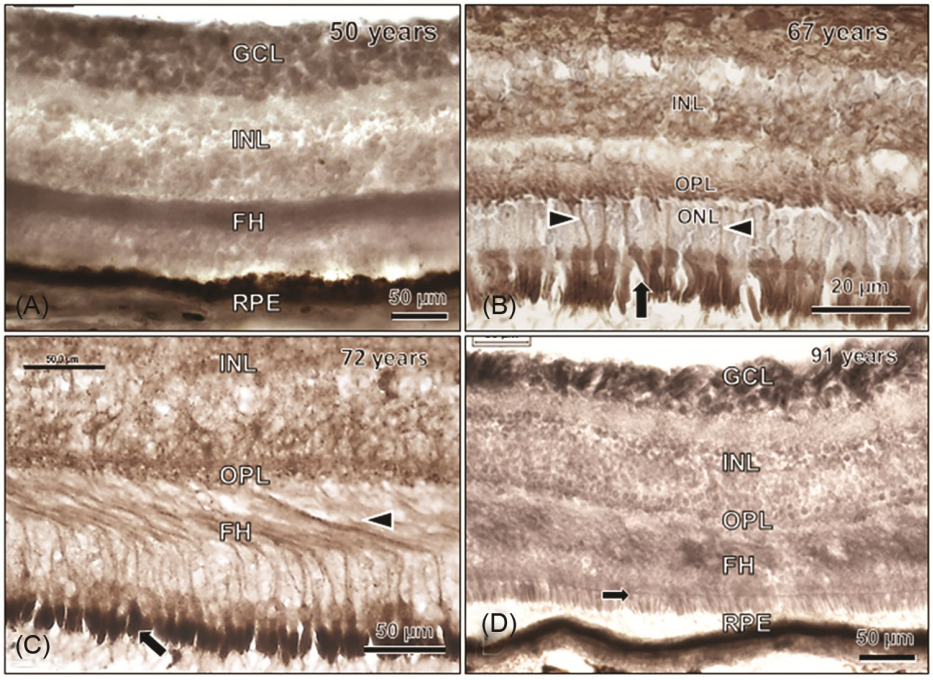

Fig. 2 — αß-spectrin IR and its changes with ageing of the retina. (A) Parafoveal retina shows strong IR in GCL and FH. B-D: IR is clearly present in OPL and IPL and photoreceptor axons (arrowheads; B & C), but weak in FH; and (D). In all, IR is variably present in photoreceptor cells (arrows). Donor ages are indicated on the images

outer retina, the FH was strongly αß-spectrin immunopositive (Fig. 2A), as was also the case in photoreceptor inner segments (Fig. 2B & C). A reduced IR pattern was found in the FH and photoreceptor cells of the elderly (*e.g.*, Fig. 2D). No IR was seen in outer segments elsewhere in the retina. Similar to αß-spectrin staining pattern, a strong IR for actin was found in the FH (Fig. 3A-C), which, however, did not show any age-dependent change (until 91 years). The control retinas did not show any reactions for either for αß-spectrin or actin (not shown).

Electron microscope observations

IHC revealed that αß-spectrin was distributed in the macular FH. The latter is unique in the primate retina: it is composed of elongated photoreceptor axons that course obliquely towards the parafovea (Fig. 4A). This layer also receives the outer processes of Müller cells that are interlaid with the macular photoreceptor axons[10-12]. The FH in transverse sections showed numerous microtubules, each of which had a diameter of 24 nm. They were surrounded by electron-dense, thin outer processes of Müller cells (Fig. 4B). Longitudinally oriented microtubules were present in the photoreceptor inner segments (Fig. 4C) and axons (Fig. 4E). To confirm which cellular elements (*i.e.*, FH versus outer processes of Müller cells) showed αß-spectrin IR, vimentin was immunolocalised in sections from the parafovea and mid-peripheral retina. The results showed

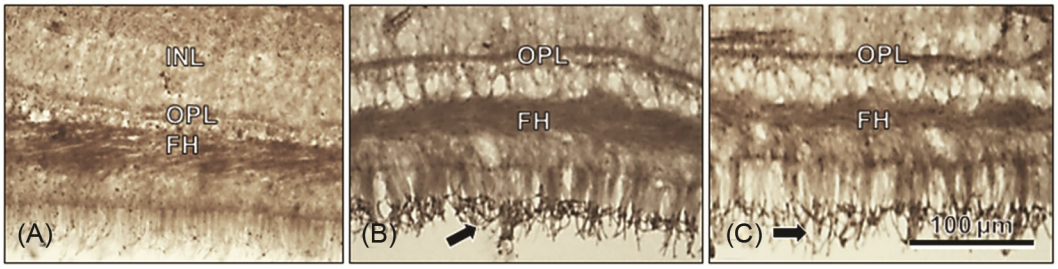

Fig. 3 — IR to actin in human retina. Parafoveal retina shows strong IR in FH and OPL. In Fig. B and C, IR is variably present in photoreceptor outer segments (arrows). From (A) 70-year; (B) 81-year; and (C) 91-year-old donor retinas

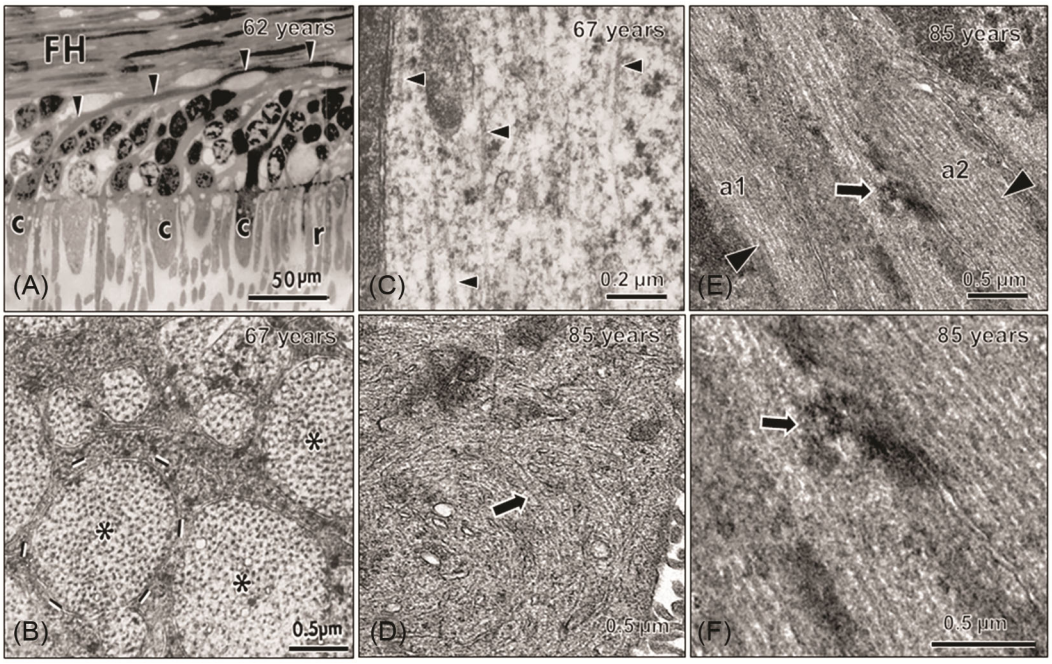

Fig. 4 — Transmission electron micrographs showing the fiber layer of Henle (FH) and its changes with age. (A) A long, oblique axon of FH (arrowheads) is indicated. c, cones, r, rods; (B) MC processes (dashed lines) surround the axons (asterisks) in FH; (C) Straight, discrete microtubules (arrowheads) of cone inner segment; (D) Disorganised bent microtubules (arrow) in portion of a cone inner segment (myoid); (E) Normal, straight microtubules (arrowheads) in two axons (a1, a2, cut longitudinally) of FH. Portion of theaxon a1 shows disorganized microtubules (arrow), which is shown in enlarged view in (F) (arrow), appearing as dense amorphous remnants

vimentin to be localised prominently in the inner, vertical processes of Müller cells and in their outer processes aligned at the same level as for the FH (Fig. 5). Double localisation of vimentin with αß-spectrin by immunofluorescence method was performed, but since both markers were present in the FH (not shown), it was difficult to identify and interpret the exact localization of individual markers. Since this did not resolve the issue of identification, IEM was performed. It was revealed that the gold particles were present in cone inner segments (Fig. 6A) and axons of the FH (Fig. 6B-D). In photoreceptor cells, gold particles were clearly associated with the microtubules (Fig. 6A). The axoplasm of the FH showed gold particles (Fig. 6B-D), which were absent in the relatively dark Müller cell processes that surrounded them. No labeling was found in negative control sections omitted with the primary antibody treatment (not shown).

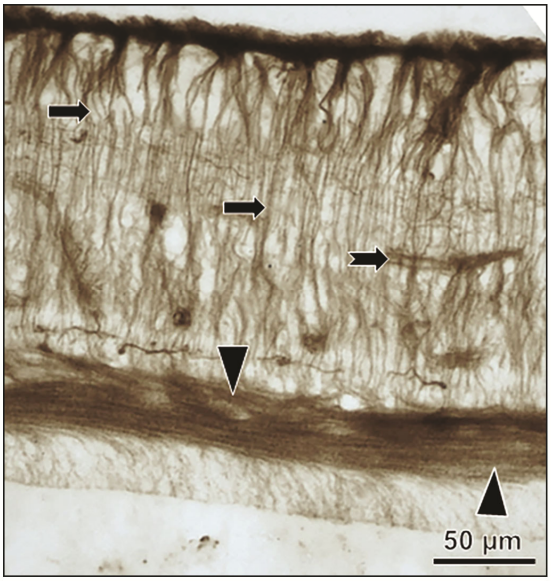

Fig. 5 — Vimentin IR of human macula (parafovea) of a 67-year-old male. IR in Müller cell inner processes (arrows) as well as outer processes (arrowheads) intermingled in the FH is indicated. Capillaries are also labelled (notched arrow)

Observations on age related changes in the FH

The FH axoplasm was filled with microtubules and few long mitochondria. In aged retinas, there was evidence for structural alterations in them as well as in Müller cell outer processes that surrounded them. The parafoveal region revealed occasional dark fibres, suggesting early signs of degeneration. While most of the outer processes of Müller cells appeared normal, in many instances, vacuolated Müller cell processes surrounding them were seen (not shown). The microtubule content of the photoreceptor inner

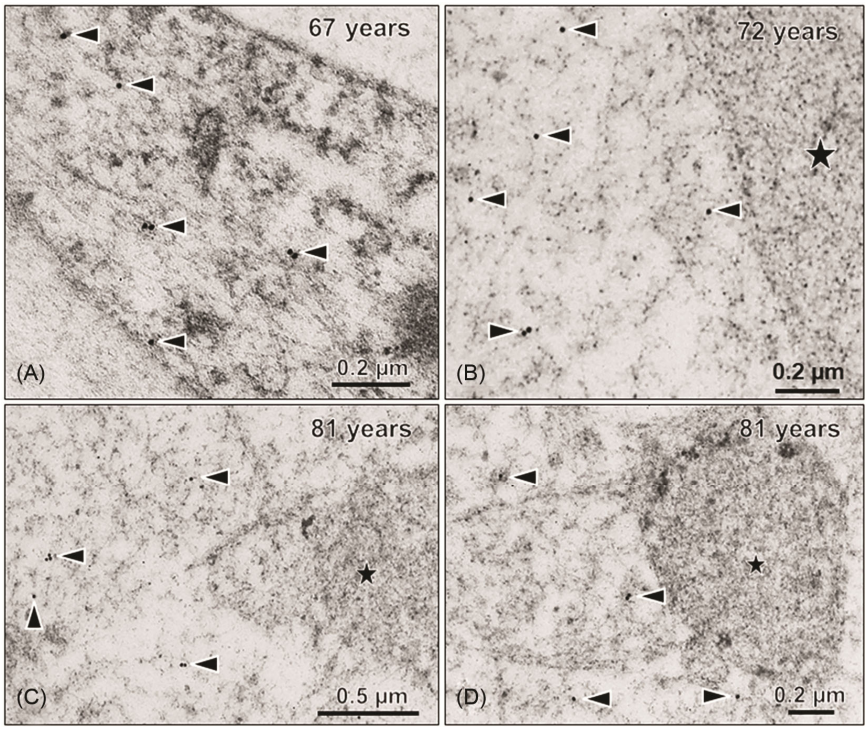

Fig. 6 — Immunogold localisation of αß-spectrin in cone ellipsoid (A) and axons of FH (B-D). A: Shows gold particles (arrowheads) in association with microtubules. B-D: Show gold particles (arrowheads) in axoplasm in FH, but absent in the relatively dark Müller cell processes (stars) that surround them. From (A) 67-year;(B) 72-year; and (C& D) 81-year-old donor retinas

segments (ellipsoid and myoid zones) and FH was found to be altered to some extent; in photoreceptor cells, microtubules were bent and highly disoriented (Fig. 4D), an anomaly compared to the normal pattern of straight, discrete microtubules (Fig. 4C) seen in lower ages. In the FH, at focal points along the axonal fibres, they appeared to be disorganised (Fig. 4E & F), which led to the appearance of highly electron-dense substances in those axons (Fig. 4F).

Discussion

The present study reveals that in the human retina, an αß-spectrin signal is moderately present in GCL, INL (most probably in bipolar cells), IPL and OPL and strongly in macular photoreceptor axons and inner segments (ellipsoids). Its robust expression in the FH indicates that it is a predominant component of membrane skeleton in macular photoreceptor axons.

Possible association of αß-spectrin with microtubules of the FH

Earlier studies indicated spectrins as components of the membrane-skeleton proteins in retinal photoreceptor cells, and are held responsible for their shape maintenance. Similarities in the distribution of membrane-skeleton proteins in erythrocytes as well as retinal cells were reported[4,5]. In adult retina, for example, αß-spectrin (along with actin, protein 4.1 and ankyrin) is detected in photoreceptor cells, bipolar cells, and ganglion cell axons[3,5]. The authors speculated that the membrane-skeleton of erythrocytes and retinal cells share functional similarities. An erythroid spectrin-like protein (240 kDa, alpha chain) in retinal rod outer segments has been reported by Wong and Molday[13]. They found an anti-erythroid spectrin antibody (whole molecule) to label predominantly a 240 kDa polypeptide of rod outer segment membranes. Two faintly labelled bands in the molecular weight range of 210-220 kDa were also observed. According to them, these components may represent variants of the beta-chain of spectrin that are weakly cross-reacting or present in smaller quantities than the alpha-chain in rod outer segment. This study did not detect any IR for αß-spectrin in human photoreceptor outer segments. Immuno-electron microscopy revealed the association of αß-spectrin with microtubules of photoreceptor inner segments and FH. It appears that in these photoreceptor compartments, this arrangement forms the basis for stabilisation of their membrane skeleton components. It remains to be seen how microtubules of photoreceptor axons interact with αß-spectrin. Although actin filaments were not detected by transmission electron microscopy, immunohistochemistry revealed significant IR for actin in the FH in the macula. Thus, the interaction of spectrin with microtubules and actin might play a role in the maintenance as well as stabilisation of photoreceptor inner segment and long FH, so that they are not deformed during wave propagation or in pathology.

Microtubule changes and implications of age-related reduction of αß-spectrin IR in the FH

With ageing, there are clear signs of some degeneration of the microtubules of the FH[14,15], which is likely to form the basis for apparent loss of αß-spectrin IR in aged human retina, *via* loss of interaction between the two cytoskeletal components. The microtubule degeneration was not due to fixation artefacts: the vast majority of the microtubules appeared to be intact in most FH examined and it was only in patchy areas that such disorganisation of microtubules was found along the axonal fibres in the FH. Further, the donor retinas from the lower ages did not show such microtubule changes in the FH fixed in the same tissue preservation method.

The reduced level of αß-spectrin IR in the FH of aged retina was a notable finding of this study. This indicates a decreased expression of αß-spectrin in this cell compartment, which tends to suggest that photoreceptor membrane-associated proteins are vulnerable to be changed with ageing of the human retina. This may alter photoreceptor membrane skeleton and hence should influence their survival. Such a pattern of reduced αß-spectrin IR with ageing may be associated with age-related alterations of photoreceptor axonal morphology (present study) and shape. So, this factor (in the light of membrane skeleton alterations in cone cells) may be somehow responsible for the vulnerability of photoreceptor cells and death with ageing of the human retina[16-18]. With regard to other proposed functions, Koenig *et al*.[19] suggested that αß-spectrin along with actin may play a role in axoplasmic transport of membranes concerned with plasma membrane recycling. Spencer *et al*.[20] reported a non-erythroid α-spectrin to colocalise with protein 4.1 in cone myoids of lower vertebrate retinas, and both proteins were contiguous to f-actin bundles of the myoid, extending up to the outer limiting membrane. The authors proposed that interactions of protein 4.1 with α-spectrin and f-actin in cones may play a role in the maintenance of cone inner segment

morphology[20], as proposed for the maintenance of erythrocyte shape.

Acknowledgement

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Conflict of interest

The author declares no conflict of interest.

References

1 Bennett V, The membrane skeleton of human erythrocytes and its implications. *Annu Rev Biochem*, 54 (1985) 273.
2 Bennett V, The spectrin-actin junction of erythrocyte membrane. *Biochim Biophys Acta*, 988 (1989) 107.
3 Drenckhahn D & Bennett V, Polarized distribution of Mr 210,000 and 190,000 analogs of erythrocyte ankyrin along the plasma membrane of transporting epithelia, neurons and photoreceptors. *Eur J Cell Biol*, 43 (1987) 479.
4 Lazarides E & Nelson WJ, Expression and assembly of the erythroid membrane-skeletal proteins ankyrin (goblin) and spectrin in the morphogenesis of chicken neurons. *J Cell Biochem*, 27 (1985) 423.
5 Spencer M, Giebelhaus DH, Kelly GM, Bicknell J, Florio SK, Milam AH & Moon T, Membrane skeleton protein 4.1 in developing Xenopus: expression in postmitotic cells of the retina. *Dev Biol*, 139 (1990) 279.
6 Isayama T, Goodman SR & Zagon IS, Spectrin isoforms in the mammalian retina. *J Neurosci*, 11 (1991) 3531.
7 Chen TW, Chen G, Funkhouser LJ & Nam SC, Membrane domain modulation by Spectrins in Drosophila photoreceptor morphogenesis. *Genesis*, 47 (2009) 744.
8 Petropoulos IK, Margetis PI, Antonelou MH, Koliopoulos JX, Gartaganis SP, Margaritis LH & Papassideri IS, Structural alterations of the erythrocyte membrane proteins in diabetic retinopathy. *Graefes Arch Clin Exp Ophthalmol*, 245 (2007) 1179.
9 Kivelä T, Tarkkanen A & Virtanen I, Intermediate filaments in the human retina and retinoblastoma. An immunohistochemical study of vimentin, glial fibrillary acidic protein, and neurofilaments. *Invest Ophthalmol Vis Sci*, 27 (1986) 1075.
10 Hogan MJ, Alvarado J & Weddell J, Histology of the Human Eye. (Philadelphia: WB Saunders Company) 1971.
11 Borwein B, The retinal receptors: A description. In: Vertebrate Photoreceptor optics (Enoch JM and Tobey FL Jr, eds., Springer-Verlag, Berlin, Heidelberg) 1981, 11.
12 Nag TC & Wadhwa S, Ultrastructure of human retina in aging and various pathological states. *Micron*, 43 (2012) 859.
13 Wong S & Molday RS, A spectrin-like protein in retinal rod outer segments. *Biochemistry*, 25 (1986) 6294.
14 Nag TC, Kathpalia P, Gorla S & Wadhwa S, Localization of nitro-tyrosine immunoreactivity in human retina. *Ann Anat*, 223 (2019) 8.
15 Nag TC, Kathpalia P & Wadhwa S, Microtubule alterations may destabilize photoreceptor integrity: Age-related microtubule changes and pattern of expression of MAP-2, Tau and hyperphosphorylated Tau in aging human photoreceptor cells. *Exp Eye Res*, 198 (2020) 108153.
16 Gao H & Hollyfield JG, Aging of the human retina. *Invest Ophthalmol Vis Sci*, 33 (1982) 1.
17 Aggarwal P, Nag TC & Wadhwa S, Age-related decrease in rod bipolar cell density of the human retina. An immunohistochemical study. *J Biosci*, 32 (2007) 893.
18 Shelley EJ, Madigan MC, Natoli R, Penfold PL & Provis JM, Cone degeneration in aging and age-related macular degeneration. *Arch Ophthalmol*, 127 (2009) 483.
19 Koenig E, Kinsman S, Repasky E & Sultz L, Rapid mobility of motile varicosities and inclusions containing alpha-spectrin, actin, and calmodulin in regenerating axons *in vitro*. *J Neurosci*, 5 (1985) 715.
20 Spencer M, Moon RT & Milam AH, Membrane Skeleton Protein 4.1 in inner segments of retinal cones. *Invest Ophthalmol Vis Sci*, 32 (1991) 1.