AGE-RELATED CHANGES OF THE CILIARY MUSCLE IN COMPARISON WITH CHANGES INDUCED BY TREATMENT WITH PROSTAGLANDIN F₂α. AN ULTRASTRUCTURAL STUDY IN Rhesus AND Cynomolgus Monkeys

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SUMMARY

The relationship between individual ciliary muscle cells and the surrounding connective tissue was studied in the eyes of three normal, young (3—4 years) cynomolgus monkeys (*Macaca fascicularis*), three aged (34—36 years) rhesus monkeys (*Macaca mulatta*) and seven young (3—7 years) cynomolgus monkeys topically treated with prostaglandin F₂α (PGF₂α) for 4—8 days. In normal eyes, collagen fibrils and microfibrils are in places in continuity with the muscle cells’ basal lamina, which is connected to the cell membrane by fine fibrillous material. In old eyes, the basal lamina is markedly thickened, masking the connection of fibrils with the muscle cells’ membrane. A distinctive finding in several muscle cells of old eyes are electron-lucent clefts, 60—80 nm wide, between basal lamina and cell membrane, which are not transversed by fibrils or fibrillous material. The cell membrane of these muscle cells shows large folds filled with disarranged myofilaments. Additionally, these cells contain inclusion bodies consisting of concentrically arranged double membranes. Following treatment with PGF₂α, similar changes are seen in young animals, too. Here, the muscle cells have lost their connection to the extracellular fibrils due to a PGF₂α-induced lysis of extracellular material. Lack of attachment between basal lamina and altered muscle cells in aged eyes might indicate an involvement of the extracellular matrix in age-related changes of the individual ciliary muscle cells.

Key words: Ageing; Smooth and ciliary muscle; Collagen; Basal lamina; Prostaglandin F₂α; *Macaca fascicularis; Macaca mulatta*

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INTRODUCTION

With increasing age, the ciliary muscle of the rhesus monkey (*Macaca mulatta*) loses its configurational response to pilocarpine [1] and to electrical stimulation of the Edinger-Westphal nucleus [2,3]. This parallels the progressive decline in functional accommodative amplitude in this species, which is quite pronounced by age 20 and reaches a steady nadir by age 25 [4,5], suggesting that the ciliary muscle is somehow involved, either primarily or secondarily, in the pathophysiology of presbyopia. Morphologically, not only the connective tissue but also the individual muscle cells develop ultrastructural age-related changes [6]. One distinctive finding is an increasing number of inclusion bodies ("fingerprints") within the cytoplasm of the muscle cells, which consist of circular aggregations of double membranes arranged concentrically, resembling a fingerprint or snail shell. Interestingly, similar structures have hitherto not been reported in smooth muscle cells outside the eye, but are often described as degenerative changes primarily within skeletal muscle cells [for review see 7].

The age-related changes in the structure and function of the ciliary muscle might be due to a primary deterioration of the muscle cells. On the other hand, the age changes of the connective tissue could be of primary importance for the ageing process of the ciliary muscle system and the loss of accommodation with higher age, secondarily causing changes in the muscle cells.

In a recent series of experiments we incidentally observed that after topical application of PGF$_2\alpha$ to monkey eyes, the connective tissue of the ciliary body was markedly reduced and deteriorated. PGF$_2\alpha$ is known to reduce intra-ocular pressure significantly in this species [8—11], an effect probably due to loss of extracellular material within the ciliary muscle, which causes an increase in uveoscleral outflow [12]. Additionally, we observed that the changes in the connective tissue after treatment with PGF$_2\alpha$ are accompanied by structural changes within the individual muscle cells, which are, to a large extent, similar to those found in muscle cells of higher age groups. The question arises whether also in normal eyes the continuous deterioration of the ciliary muscle cells with age is primarily caused by changes in the connective tissue and whether PGF$_2\alpha$ might serve as a model for clarifying the mechanism of ageing in the ciliary muscle system.

In the present study we report on the ultrastructure of the ciliary muscle cells and of their surrounding connective tissue elements in normal, young (3—4 years), old (34—36 years) and PGF$_2\alpha$ treated, young (3—7 years) monkeys. Special attention was given to a possible structural connection of the connective tissue with the individual muscle cells.

MATERIALS AND METHODS

Three young adult (3—4 years) cynomolgus monkeys and one 36-year-old rhesus
TABLE I

PROTOCOL OF THE PGF₂α TREATMENT AND QUANTITATIVE EVALUATION OF THE ULTRASTRUCTURAL CHANGES OF THE CILIARY MUSCLE CELLS

<table>
<thead>
<tr>
<th>No.</th>
<th>Duration of treatment (Days)</th>
<th>Dose/treatment (µg)</th>
<th>No. of doses</th>
<th>Fixation</th>
<th>% Ciliary muscle cells (mean ± S.E.M.) with:</th>
</tr>
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<td></td>
<td></td>
<td></td>
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<td>Lyso-</td>
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<td></td>
<td></td>
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<td>somes (mean ± S.E.M.)</td>
</tr>
<tr>
<td>145/85</td>
<td>4</td>
<td>50a</td>
<td>6</td>
<td>Perf.</td>
<td>5.9 ± 2.0</td>
</tr>
<tr>
<td>144/85</td>
<td>4</td>
<td>50b</td>
<td>6</td>
<td>Imm.</td>
<td>12.6 ± 3.4</td>
</tr>
<tr>
<td>31/87</td>
<td>4</td>
<td>4b</td>
<td>7</td>
<td>Perf.</td>
<td>13.7 ± 2.3</td>
</tr>
<tr>
<td>15/87</td>
<td>5</td>
<td>5b</td>
<td>9</td>
<td>Imm.</td>
<td>20.2 ± 5.0</td>
</tr>
<tr>
<td>32/87</td>
<td>7</td>
<td>4b</td>
<td>9</td>
<td>Imm.</td>
<td>16.7 ± 2.5</td>
</tr>
<tr>
<td>33/87</td>
<td>7</td>
<td>4b</td>
<td>9</td>
<td>Imm.</td>
<td>21.0 ± 4.3</td>
</tr>
<tr>
<td>34/87</td>
<td>8</td>
<td>4b</td>
<td>11</td>
<td>Perf.</td>
<td>14.1 ± 1.2</td>
</tr>
</tbody>
</table>

aTreatment with PGF₂α-tromethamine salt.
bTreatment with PGF₂α-isopropylester. Perf., perfusion fixation; imm., immersion fixation. Quantitative morphology: four sections (1 section/quadrant) from each eye were evaluated. The evaluation was restricted to the muscle cells of the anterior meridional portion of the ciliary muscle. The numbers are the percentage of cells showing lysosomes and inclusion bodies (amorphous electron-opaque inclusions with convoluted lamellae or inclusions consisting of spiralling membranes or membranous whorls).

monkey were perfusion-fixed via the heart with Ito’s fixative [13] following perfusion with heparinized NaCl. The eyes of two old rhesus monkeys (34 years) were fixed by immersion immediately after enucleation. Anesthesia for in vivo enucleation and systemic perfusion was i.m. ketamine HCl 15 mg/kg, followed by i.m. pentobarbital Na 30 mg/kg. The animals were killed by a pentobarbital overdose.

The old monkeys were from caged colonies of the Wisconsin (Madison) Regional Primate Research Center. The eyes were sent to us by Prof. P.L. Kaufman, Department of Ophthalmology, University of Wisconsin, Medical School, Madison.

No ocular abnormalities other than senile cataract and peripheral cystoid retinal degeneration in the old animals were seen clinically or histopathologically.

In addition seven young adult (3—7 years) cynomolgus monkeys, were treated topically with PGF₂α (Pharmacia AB, Uppsala, Sweden) in one eye for 4—8 days and diluent in the other eye (Table I). The protocol of the treatment is described elsewhere [12].

For electron microscopic investigation, three of the treated monkeys were fixed by perfusion according to the above-mentioned protocol. In four of the treated animals the eyes were fixed by immersion immediately after enucleation (Table I).

All globes were then divided at the ora serrata and small pieces with a width of 1 mm and containing the whole thickness of the ciliary body, iris, adjacent cornea and sclera were prepared for further electron microscopy. After post-fixation with 1%
osmium tetroxide, the specimens were dehydrated with graded alcohols and embedded in Epon. From each eye at least two specimens from all quadrants were examined. The sections were treated with lead citrate and uranyl acetate. For electron microscopic examination a JEOL (JEM 100 B) and a Zeiss (EM 902) electron microscope were used.

For quantitative evaluation of the PGF₂α-treated eyes one ultrathin section from each quadrant was selected with regard to the technical quality of the sections. These
sections contained the anterior third of the meridional portion of the ciliary muscle (Fig. 1). Particular care was taken to achieve exact sagittal orientation for each section. The examination began at the most anterior tip of the meridional portion and continued further posteriorly until 50 ciliary muscle cells per ultrathin section were evaluated. The evaluation was limited to those muscle cell profiles where the nucleus was included and the overall morphology of the cell and distribution of its organelles (mitochondria and myofilaments) suggested a longitudinal section right through the center of the cell. In each section the percentage of cells showing the following morphological changes were calculated: (i) cells with lysosomes or lipid droplets and (ii) cells containing inclusion bodies (amorphous electron-opaque inclusions containing convoluted lamellae (Fig. 6a) or inclusions consisting of spiralling membranes and membranous whorls (Fig. 6c,d)). For each eye the mean ± S.E.M. was calculated. The investigators evaluating the sections (the authors) were unaware of the experimental protocol of the animals.

RESULTS

Young, untreated animals

Within an elementary bundle the flanks of the muscle cells are separated from each other by their basal lamina. In places, however, nerve axons and nerve endings, located between the muscle cells separate the basal laminae from each other (Fig. 2a). The basal lamina appears as a slightly fuzzy, irregular, electron-dense line 25—30 nm in width, separated from the plasma membrane by a clear space 20—25 nm wide (Fig. 2a). From the basal lamina fine fibrils or fibrillous material spread to the membrane of the muscle cells. Additionally, opposing basal laminae are connected by fine fibrils (Fig. 2a). Occasionally the flanks of adjacent muscle cells are connected by desmosome-like junctions, which resemble the intermediate junctions described by Gabella [14]. The ends of the single muscle cells taper off and show an irregular profile with projections and invaginations (Figs. 2b, 3). Here the projections of the muscle cells can form end-to-end or side-to-end attachments by small desmosome-like junctions. Between the ends of neighbouring muscle cells or from the end of one cell to the lateral side of an adjacent muscle cell (Figs. 2b, 3) bundles of extracellular microfibrils can be found, which have a diameter of 14—16 nm and show a periodicity of 18—20 nm (Fig. 3). The microfibrils seem to be in continuity with the basal lamina of these cells (Figs. 2b, 3).

In those areas where the microfibrils adhere to the basal lamina, the lamina appears to be thicker than around the rest of the cell. Adjacent to this thickened basal lamina, the muscle cell itself forms dense bands. The orientation of the microfibrils and of the fine fibrillous material is parallel to the axis of the muscle cells and therefore also parallel to the myofilaments within the cells (Figs. 2b, 3). Thus the course of the extracellular microfibrils seems to be in continuity with the myofilament arrangement in the muscle cell, via basal lamina and dense bands.
Fig. 2. Sagittal section of ciliary muscle cells within a muscle bundle of the anterior meridional portion. (a) Between the flanks of the muscle cells the connective tissue components are sparse. Only basal lamina as well as numerous nerve endings are seen. The basal laminae are connected to each other by fine fibrils (arrowheads). An intermediate junction (arrow) forms a side-by-side attachment of the muscle cells. (× 18,000, scale bar: 1.16 μm). (b) The ends of the muscle cells form projections (P) and invaginations (I). Extracellular microfibrils spread from the ends of the muscle cells and seem to be in continuity with their basal lamina (arrows). Adjacent to this basal lamina the muscle cell forms dense bands (asterisks). Tangentially-sectioned caveolae of the cell membrane are seen to be surrounded by abundant sacs and tubules of sarcoplasmic reticulum (arrowheads). (× 19,400, scale bar: 1.0 μm).
Fig. 3. The microfibrils near the ends of the muscle cells have a diameter of 14—16 nm and show a periodicity of 18—20 nm. They seem to originate from areas where the muscle cell forms dense bands (arrows). The orientation of the extracellular microfibrils is mainly parallel to the myofilaments within the cell (arrowheads) (× 60 000, scale bar: 350 nm).
The elementary muscle bundles are surrounded by a nearly complete layer of flattened fibroblasts and sparse collagen fibrils. The fibrils measure 30—45 nm in diameter and in longitudinal sections show a cross-striation with a 67-nm period. Most of these collagen fibrils run parallel to the longitudinal axis of the muscle cells and their myofilaments and seem to be linked to the basal lamina of the muscle cells by small microfibrils. These microfibrils differ from the 14—16 nm microfibrils within an elementary bundle. They have an ill-defined contour, no transverse periodicity and a diameter of approximately 8—10 nm. Occasionally the fibroblasts lay close to the basal lamina of the muscle cells with neither 30—45 nm thick collagen fibrils nor microfibrils between them.

In the young cynomolgus monkey the amount of the 30—45 nm collagen fibrils between the muscle bundles is usually rather sparse. However, towards the anterior tip of the meridional portion of the ciliary muscle, near its transition to the trabecular meshwork, the amount of this collagen usually increases considerably.

A similar ultrastructure has also been observed in the eyes of young rhesus monkeys [6, Tamm, E., unpublished data].

**Prostaglandin treatment**

One distinct morphological finding after treatment of the eyes with PGF\(_{2\alpha}\) for 4—8 days is an enlargement of the spaces between the single muscle bundles. This enlargement mainly takes place in the anterior part of the meridional portion of the ciliary muscle and is most clearly seen around the vessels and nerves. With the exception of some myelin figures, the enlarged spaces appear almost empty (Fig. 4). The amount of collagen fibrils separating the bundles is markedly reduced compared with those of normal eyes. The remaining collagen fibrils are dispersed, separated from each other and show an irregular orientation. They have a fuzzy appearance when cut parallel to their longitudinal axis, suggesting their corrosion and resolution into aggregates of microfibrils. This can also be seen in transverse sections through the fibers, where their diameter shows a marked variability. An intimate association with the basal lamina of the muscle cells cannot be seen in the affected areas. In places the microfibrils, normally found between the muscle cells within the bundles can also no longer be observed.

Macrophages are often situated in the widened spaces between the muscle bundles (Figs. 4, 5). They contain electron-lucent vacuoles as well as lipid droplets and phagolysosomes with osmiophilic inclusions. Occasionally macrophages can be found with phagolysosomes containing osmiophilic inclusions with a diameter of approximately 30—50 nm which resemble remnants of phagocytized collagen fibrils (Fig. 5a, b).

In these areas, however, not just the extracellular material but even the muscle cells show distinct changes in their ultrastructure. Frequently cells with lipid droplets and lysosomes containing osmiophilic material are found (Fig. 6b). Additionally, many muscle cells contain amorphous, electron-opaque inclusions, which are not
Fig. 4. Anterior part of the meridional portion of a ciliary muscle after 5 days treatment with $\operatorname{PGF}_{2\alpha}$. The spaces between the muscle bundles are enlarged. Very few collagen fibrils, some myelin figures and a macrophage (M) containing phagolysosomes with osmiophilic inclusions are seen in the spaces. The cytoplasm of a muscle cell shows an amorphous inclusion body (arrow). ($\times$ 2400, scale bar: 8.75 µm).
Fig. 5. (a) Macrophage found in the space between two ciliary muscle bundles of the anterior meridional portion after 7 days treatment with PGF2α. The cell contains a large vacuole filled with long, electron-opaque inclusions. They have a width of approximately 30–50 nm, show a periodicity (arrows) and resemble phagocytized and partly degraded collagen fibrils. (×30 000, scale bar: 500 nm). (b) Higher magnifications of (a). The inclusions show a periodicity (arrows). (×97 500, scale bar: 150 nm).
Fig. 6. Ciliary muscle cells showing various inclusion bodies after 5 days treatment with PGF$_{2\alpha}$. (a) Muscle cell with inclusion body consisting of convoluted lamellae embedded in an amorphous electron-opaque matrix. There is no surrounding membrane visible. (× 15 000, scale bar: 0.93 µm). (b) The myofilaments of a muscle cell are found in close association with an inclusion body and seem to enter or originate in it (arrow). Additionally, the cell contains osmiophilic lysosomes. (× 20 000, scale bar: 0.7 µm). (c) Muscle cell with inclusions consisting of concentric membranous whorls. A connection between a membranous whorl and the lateral sarcoplasmic reticulum is visible (arrow). (× 20 000, scale bar: 0.7 µm). (d) The cytoplasm of a muscle cell shows an agglomeration of irregular aggregates of membranous tubules. (× 16 000, scale bar: 0.94 µm).
Fig. 7. (a) Sagittal section of a ciliary muscle cell in the anterior meridional portion of a 36-year-old rhesus monkey. The cytoplasm of the cell shows concentric membranous whorls (arrowheads) and areas of disarranged myofilaments (asterisk). (× 19 000, Scale bar: 1.3 μm). (b) Higher magnification of the muscle cell's sarcolemm. The basal lamina is thickened, partly laminated and separated by an electron lucent cleft 60—80 nm wide (arrow) from the cell membrane. (× 40 000, Scale bar: 610 nm).
surrounded by a visible unit-membrane (Fig. 4, 6a). Convoluted lamellae, arranged in a fingerprint pattern are occasionally embedded in the amorphous matrix (Fig. 6a). The myofilaments of the muscle cells are often found in close association with these inclusion bodies and sometimes seem to enter the inclusions or originate in them (Fig. 6b). Other cells contain inclusions consisting of systems of spiralling membranes or concentric membranous whorls (Fig. 6c). The concentric arrays consist of membranes, which are arranged mainly circumferentially with a variable periodicity. In the center of the membranous arrays, there are often glycogen particles, mitochondria or lysosomes. Some cells show connections between these membranous whorls and lateral cisterns of the sarcoplasmic reticulum (Fig. 6c). Other cells contain structures which appear as more irregular aggregates of membranous tubules (Fig. 6d). Table I summarizes the number of cells showing either inclusion bodies or lysosomes in the affected areas of the ciliary muscle, i.e. the anterior part of the meridional portion. Regarding the ultrastructural changes in the cells of the anterior meridional portion of the ciliary muscle, no difference was seen between the perfusion- and immersion-fixed eyes. No inclusion bodies could be seen in the muscle cells of the normal, untreated control group.

Old animals

In the 34- to 36-year-old rhesus monkeys, the ciliary muscle showed similar age-related changes as have been described for 26- to 35-year-old animals [6]. Most of the muscle cells contain accumulations of lysosomes or lipofuscin. The number of mitochondria within the center of the cells’ core seems to be increased and the area occupied by myofilaments decreased (Fig. 8). The connective tissue surrounding these muscle cells shows only slight changes. The basal lamina of the muscle cells is conspicuously thickened compared with that of young animals (Figs. 7a, b, 8). Within a muscle bundle, the basal laminae of adjacent muscle cells merge with each other and have a homogenous appearance. It is difficult to distinguish, whether microfibrils within this homogenous material have changed. The homogenous basal lamina extends towards the cell membrane of the muscle cells. In this region, the fine fibrils connecting the basal lamina with the cell membrane seem to be masked, too. Only in those areas where the muscle cells show the characteristic inclusion bodies consisting of circular aggregations of double membranes arranged concentrically (Figs. 7a, b, 8), the shape of the muscle cells, as well as the adjacent extracellular material, show distinctive changes. Most of the cells do not have the normal long-stretched form, but appear more round and show lateral extensions of their cell membrane (Fig. 8). In many places, the basal lamina of the muscle cells is markedly separated from the plasma membrane by clefts 60—80 nm wide (Figs. 7a, b, 8). These clefts are optically empty and not filled or transversed by fibrils, fibrillous or homogenous material. In some areas the basal lamina appears laminated (Fig. 7a, b). Adjacent to these clefts, the plasma membrane of the muscle cells shows the most prominent folds, which contain accumulations of disorganized myofilaments (Fig. 7a).
Fig. 8. Sagittal section of a ciliary muscle cell in the anterior meridional portion of a 36-year-old rhesus monkey. The cell contains an unusual large number of mitochondria while the amount of myofilaments seems to be decreased. The cell membrane shows numerous long extensions (arrows). Within the cytoplasm inclusion bodies are seen (asterisks). Arrowheads = clefts between basal lamina and cell membrane. (× 12,000, Scale bar: 1.75 μm).

DISCUSSION

Our findings show that the collagen fibrils within the ciliary muscle are closely associated with the muscle cells and are connected to them via fibrillous material. Similar structures, suggesting a connection of smooth muscle cells with their stroma, were observed in the guinea pig taenia coli [15—17] and rabbit ileum [18]. In the ciliary muscle these findings indicate that the connective tissue sheaths surrounding the
muscle cell bundles, not just allow a simple sliding of the bundles against each other, but serve as a framework for them, supporting the shape changes of the ciliary muscle during accommodation and desaccommodation. The connection between the individual muscle cells and their stroma may additionally have an important role in maintaining mechanical tension.

Treatment with PGF$_{2\alpha}$ leads to a loss of collagen within the ciliary muscle. Similar findings were observed in the cervix uteri after topical prostaglandin E$_2$ treatment [19]. Prostaglandins, known as potent inflammation mediators may also contribute to the collagenolysis observed in chronic inflammatory processes like rheumatoid arthritis [for review see 20]. In our study, the remaining collagen fibrils show typical changes in their ultrastructure [21,22], suggesting corrosion and digestion. The microfibrils normally situated between the muscle cells within their bundles, are also not seen in these areas after treatment. Additionally we found macrophages with inclusions resembling partly digested collagen fibrils. Similar macrophages are also found in other tissues where marked and rapid destruction of collagen takes place, e.g., the post-partum uterus [23–25]. It has been demonstrated that macrophages secrete specific collagenases [26] and that prostaglandins may regulate macrophage collagenase production [27].

The morphological changes in the muscle cells, like lysosomes and inclusion bodies, might be due to a direct action of PGF$_{2\alpha}$ on the ciliary muscle cell ultrastructure. It is known e.g., that prostaglandin E$_2$ stimulates protein breakdown in skeletal muscle cells through activation of a lysosomal pathway [28].

However, the changes are in fact most pronounced in areas where the extracellular material, respectively, collagen fibrils and microfibrils, is lost. This strongly suggests that the loss of connective tissue elements is somehow involved in the pathogenesis of the ciliary muscle cell changes. Dense bands, supposed to be the site of attachment of actin filaments [29] are clearly located at the site of contact of the microfibrils with the basal lamina. Dense bands, cell membrane and elements of the basal lamina might provide a mechanical link for the microfibrils of the connective tissue and the myofilaments within the cell. The loss of extracellular matrix components might affect the cytoskeletal elements responsible for the orientation of the myofilaments within the cell, or lead to a detachment of the myofilaments from the dense bands. In fact, the connection found between myofilaments and inclusion bodies suggests that these bodies have arisen from the abnormal assembly of degenerated myofilaments, which have lost their connection to the membrane-bound dense bands. The lysosomes found in the affected cells might contribute to the degradation of the detached myofilaments.

A similar interaction between collagen and cytoskeleton in cultured corneal epithelial cells was described by Sugrue and Hay [30]. After removal of the extracellular matrix with trypsin-collagenase, the cells showed a disorganization of their actin filaments, which could be reversed after addition of solubilized collagen.

The membranous whorls may consist of an agglomeration of tubules of sarcoplasmic reticulum normally located between the myofilaments. If the cytoskeletal
elements of the cell are changed, because of the myofilament's detachment, these tubules of the sarcoplasmic reticulum might form irregular aggregates.

The findings in old monkeys support the idea that changes in the extracellular matrix can be responsible for changes in the cytoarchitecture of ciliary muscle cells. The cells with prominent ultrastructural changes, like folding of the sarcolemma, disorganization of the myofilaments and appearance of various inclusion bodies also showed changes in their surrounding basal lamina. The thickened basal lamina was separated by a wide electron-lucent cleft from the plasma membrane of the muscle cells. No fibrils or fibrillar material transversing from the basal lamina to the plasma membrane, could be seen in these clefts. Therefore it seems probable that loss of a structural connection between basal lamina and muscle cells is involved in the observed changes in the muscle cells. We do not know whether the age changes are caused by a lytic process as observed in the PGF$_{2\alpha}$-treated monkeys or are due to changes of receptors involved in the adhesion of fibrils to the muscle cells' membrane.

Other ultrastructural changes, like the increase in lysosomes and mitochondria seem not to be related with the connective tissue changes but might merely reflect age-related changes in the metabolism of the individual muscle cells themselves.

In summary we can say that the connective tissue elements and the muscle cells of the ciliary muscle do not undergo structural changes with age independently. In contrast, during ageing the individual elements of the ciliary muscle influence each other.

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