Journal of Glaucoma
1:165–173, Raven Press, New York

Age-Related Loss of α-Smooth Muscle Actin in Normal and Glaucomatous Human Trabecular Meshwork of Different Age Groups

Cassandra Flügel, M.D., Ernst Tamm, M.D., Elke Lütjen-Drecoll, M.D., and *Fritz H. Stefani, M.D.

*Department of Anatomy II, University of Erlangen–Nürnberg, Erlangen, and *Eye Hospital of the University of Munich, Munich, Germany*

Summary: The trabecular meshwork of 45 normal human eyes ranging in age from 19 to 81 years and of six glaucomatous human eyes (33–78 years) was studied for the presence of contractile cells. For this purpose, the chamber angle was immunocytochemically stained with antibodies against α-smooth muscle (sm) actin. Within the various ages, the presence of α-sm actin labelled cells showed differences. In younger eyes, α-sm actin filaments were seen in nearly all cells of the trabecular meshwork. With increasing age, fewer cells stained for α-sm actin and were mainly seen in the posterior part of the trabecular meshwork and in the scleral spur. The main filtering portion of the meshwork contained nearly no stained cells. No differences were found between glaucomatous eyes and normal eyes of the same age group. **Key Words:** Human trabecular meshwork—α-Smooth muscle actin—Myofibroblasts—Immunocytochemistry—Ultrastructure.

In previous studies, Kaufman and Bárány (1) have shown that the antiglaucomatous drug epinephrine increases outflow facility even if the ciliary muscle is disinserted from the trabecular meshwork and from the scleral spur. These findings indicate that the cells in the outflow pathways themselves are influenced by the drug.

In a recent study, we have demonstrated myofibroblasts in the bovine chamber angle (2). These cells are present directly adjacent to the outflow plexus and in the transitional region between the reticular meshwork and the ciliary muscle. They stain characteristically positive with antibodies against the smooth muscle specific microfilament actin (α-sm actin) and vimentin, but are negative for the muscle specific intermediate filament desmin. Nerve terminals indicating innervation of these cells are found in close contact with the cells.

Studies by de Kater et al. (3,4) indicate that contractile cells also exist in the human trabecular meshwork. As the glaucomatous disease occurs mainly in older age groups, we were interested in whether or not there are age-related differences in the distribution of such contractile cells in the meshwork and whether or not they are still present in glaucomatous eyes.

MATERIALS AND METHODS

Forty-five human eyes from different age groups ranging from 19 to 81 years were investigated.

Sixteen eyes were derived from eight donors aged 40 to 81 years without history of ocular disease. The

Received March 25,1992. Accepted April 20, 1992.
Address correspondence and reprint requests to Dr. C. Flügel at Department of Anatomy II, University of Erlangen–Nürnberg, 8520 Erlangen, Universitätsstr. 19, Germany.

eyes were enucleated 7–16 h after death. The anterior half of the eyes was dissected in different quadrants and specimens containing the anterior chamber angle (2–3-mm wide) were prepared. Some of these specimens from each quadrant were frozen in isopentane cooled with liquid nitrogen. Similar sectors were fixed in paraformaldehyde-lysine-periodate (PLP; 5) or Zamboni solution (6) for 4 h and then embedded in paraffin or LR Gold (Agar Scientific LTD, Stansted, Essex, U.K.).

Twenty-three eyes from different age groups ranging from 19 to 58 years were obtained from the Eye Hospital, University of Munich; most of these eyes had been enucleated because of posterior choroidal melanoma. The anterior chamber of the eyes was intact, showing no signs of tumor cell infiltration or inflammatory reaction. The material had been fixed immediately after enucleation in formaldehyde for various periods of time and embedded in paraffin wax.

Six eyes were derived from six donors suffering from primary open angle glaucoma (33, 56, 59, 66, 69, and 78 years). The eyes had been fixed in paraformaldehyde and were embedded in paraffin. For ultrastructural investigation, specimens from five of the normal eyes (27, 49, 50, 74, and 81 years) were fixed in Ito's solution (7) and embedded in Epon (Roth, Karlsruhe, Germany).

IMMUNOCYTOCHEMICAL STAINING

Frozen Material

The frozen specimens were cut at 10 μm in sagittal and tangential planes through the chamber angle and mounted on chromalum-gelatine coated glass slides. Following fixation with acetone for 10 min at −20°C, the sections were treated with Blotto's solution (8) for 20 min to reduce nonspecific staining. Incubation with the primary antibody was carried out for 90 min. For the demonstration of α-sm actin monoclonal mouse anti-α-sm actin (Sigma, St. Louis, MO, U.S.A.; 9) diluted in phosphate buffered saline (PBS), pH 7.2–7.4, with 1% bovine serum albumin (1:150) was used. Demonstration of vimentin and desmin was achieved using monoclonal mouse anti-swine vimentin (Dakopatts, Hamburg, Germany) diluted 1:20 and monoclonal mouse anti-human desmin (Dakopatts) in a dilution of 1:5. After incubation with the primary antibody, the sections were given three 10-min washes in PBS, and were then incubated with fluorescein conjugated rabbit-anti-mouse IgG in a dilution of 1:20 for 60 min. After washing in PBS for 30 min, the sections were mounted in Kaiser's glycerin-gelatine (Merck, Darmstadt, Germany).

Paraffin Sections

Sagittal and tangential sections (7 μm) through the chamber angle were cut and mounted on glass slides coated with 1% glue (Pritt; Henkel). After dewaxing, the sections were stained as described above with the primary antibody for 90 min (anti-α-sm actin 1:150, anti-vimentin 1:5, or anti-desmin 1:5) and the secondary antibody (fluorescein conjugated anti-mouse IgG 1:20) for 60 min. Finally, the sections were washed in PBS and mounted with Entellan (Merck) containing 2.5% diamino-bicyclo-octane (Dabco; Merck).

Electron Microscopic Immunocytochemistry

For electron microscopic (EM)–immunocytochemistry ultrathin sections were cut from LR gold embedded specimens, incubated with α-sm actin (1:150) for 8 h, washed, and incubated then with 10 nm gold-labelled anti-mouse IgG (Sigma) for additional 8 h. After washing, the sections were post-fixed with 2% glutaraldehyde, counterstained with uranyl acetate and lead citrate, and viewed with a Zeiss EM 902.

Controls

Control experiments for frozen and paraffin sections were performed using either PBS or a mouse preimmune serum substituted for the primary antibody.

Electron Microscopy

For ultrastructural investigation, sagittal and tangential sections through the different portions of the meshwork were cut from Epon embedded specimens, counterstained with uranyl acetate and lead citrate, and examined with the Zeiss EM 902.

RESULTS

Immunocytochemical Staining for α-sm Actin

Normal Eyes

In all age groups, intense staining was seen in the ciliary muscle cells and in the smooth muscle cells

and pericytes of blood vessels. In the trabecular meshwork, distribution of α-sm actin varied within the different age groups.

Eyes aged 19–42 years. In sagittal sections of the trabecular meshwork from donors between 19 and 42 years of age, α-smooth muscle actin staining was observed in nearly all cells of the cribriform, corneoscleral, and uveal meshwork. Staining was also seen in the cells of the nonfiltering portion of the chamber angle including Schwalbe's line cells at the end of Descemet's membrane. Additionally, staining for α-actin filaments was found within cells of the scleral spur, in the ciliary meshwork which connects the ciliary muscle with the iris root, and in a few single cells adjacent to the outer wall of Schlemm's canal. The endothelial cells of Schlemm's canal remained unstained (Fig. 1A).

Tangential sections through the trabecular meshwork revealed a continuous dense network of α-sm actin cells extending from scleral spur to Descemet's membrane.

Eyes aged 44–59 years. In sagittal sections of all sections of this age group, staining was seen in the scleral spur and the ciliary meshwork. In the corneoscleral meshwork, staining was seen in cells covering the posterior one-third to two-thirds of the lamellae. In most eyes, stained cells were also found in the anterior nonfiltering portion of the meshwork directly adjacent to Schwalbe's line. In the remaining corneoscleral meshwork and the cribriform region, staining was almost completely absent. Within the uveal meshwork, staining was found only in single cells covering the inner uveal lamellae (Fig. 1B). As in the young eyes, single stained cells were seen adjacent to the outer wall of Schlemm's canal.

In tangential sections, in contrast to the young age group, the α-actin labelled cells in the anterior nonfiltering portion and in the posterior part of the meshwork adjacent to the scleral spur were less densely packed and formed only a thin band of stained cells. In the remaining trabecular meshwork, there was only a loose network of stained cells.

Eyes at the age of 70–81 years. In sagittal sections, the specimens from the five oldest donors (70, 74, 80, 80, and 81 years) showed staining in cells of the scleral spur, ciliary meshwork, and single cells adjacent to the outer wall of Schlemm's canal. Within the corneoscleral meshwork, staining occurred only in a very few cells covering the innermost lamellae directly adjacent to the ciliary muscle tips and scleral spur (Fig. 1C). Only in some sections, weak staining was also seen in the posterior portion of the innermost uveal lamellae. No staining was found in the cribriform region. In the most anterior nonfiltering part of the meshwork, staining was found only in one eye of the investigated specimens.

Tangential sections through the inner corneoscleral meshwork showed α-sm actin-labelling restricted close to the ciliary muscle tips. The staining appeared discontinuous and patchy (Fig. 1D).

Glaucomatous Eyes

In the glaucomatous eyes, the staining pattern was the same as in the normal eyes of the same age group.

Staining for α-sm actin was the same in frozen and paraffin sections. Neither the duration of fixation nor the various postmortem time periods affected the stainability for α-sm actin.

Immunocytochemical Staining for Desmin

In all age groups of the normal and glaucomatous eyes, the staining for desmin was only seen in ciliary muscle cells. No staining was found in the different regions of the trabecular meshwork. The staining intensity for desmin filaments was more pronounced in frozen than in paraffin sections.

Immunocytochemical Staining for Vimentin

All cells of the trabecular meshwork showed staining for vimentin. This was not dependent on the age of the donor eyes and was true for the normal and glaucomatous specimens. The staining revealed that even in eyes of the elderly, the trabecular lamellae were always covered by labelled cells.

Similar to the staining for desmin, vimentin filaments were more intensely stained in frozen than in paraffin sections.

Electron Microscopy

Ultrastructurally actin forms 6–8-nm thick filaments. Within the trabecular meshwork of normal eyes, three different cell types could be distinguished with respect to these 6–8-nm filaments, which also stained positively for α-sm actin (Fig. 2A and B).

In cell type I, the filaments were located in all

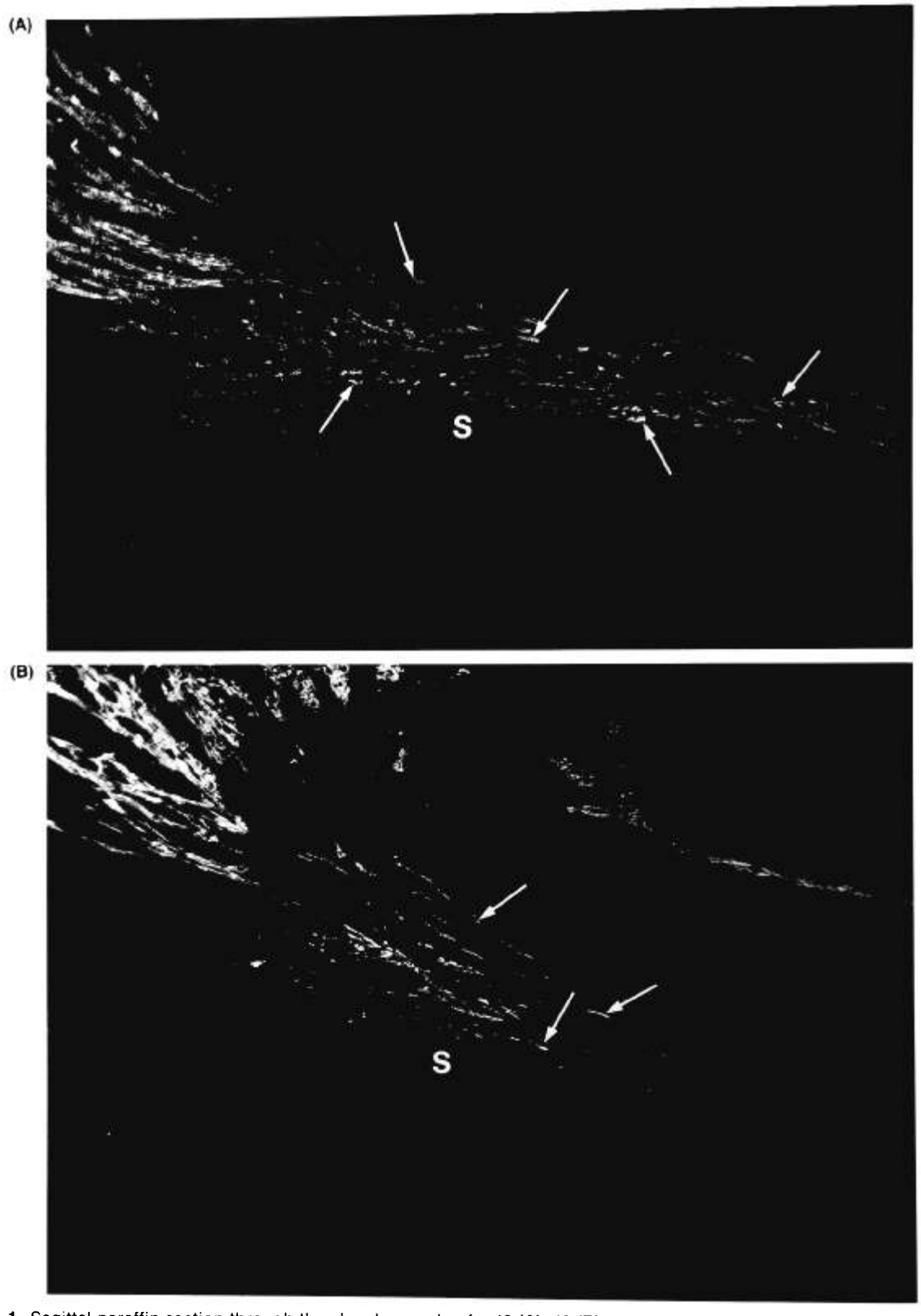
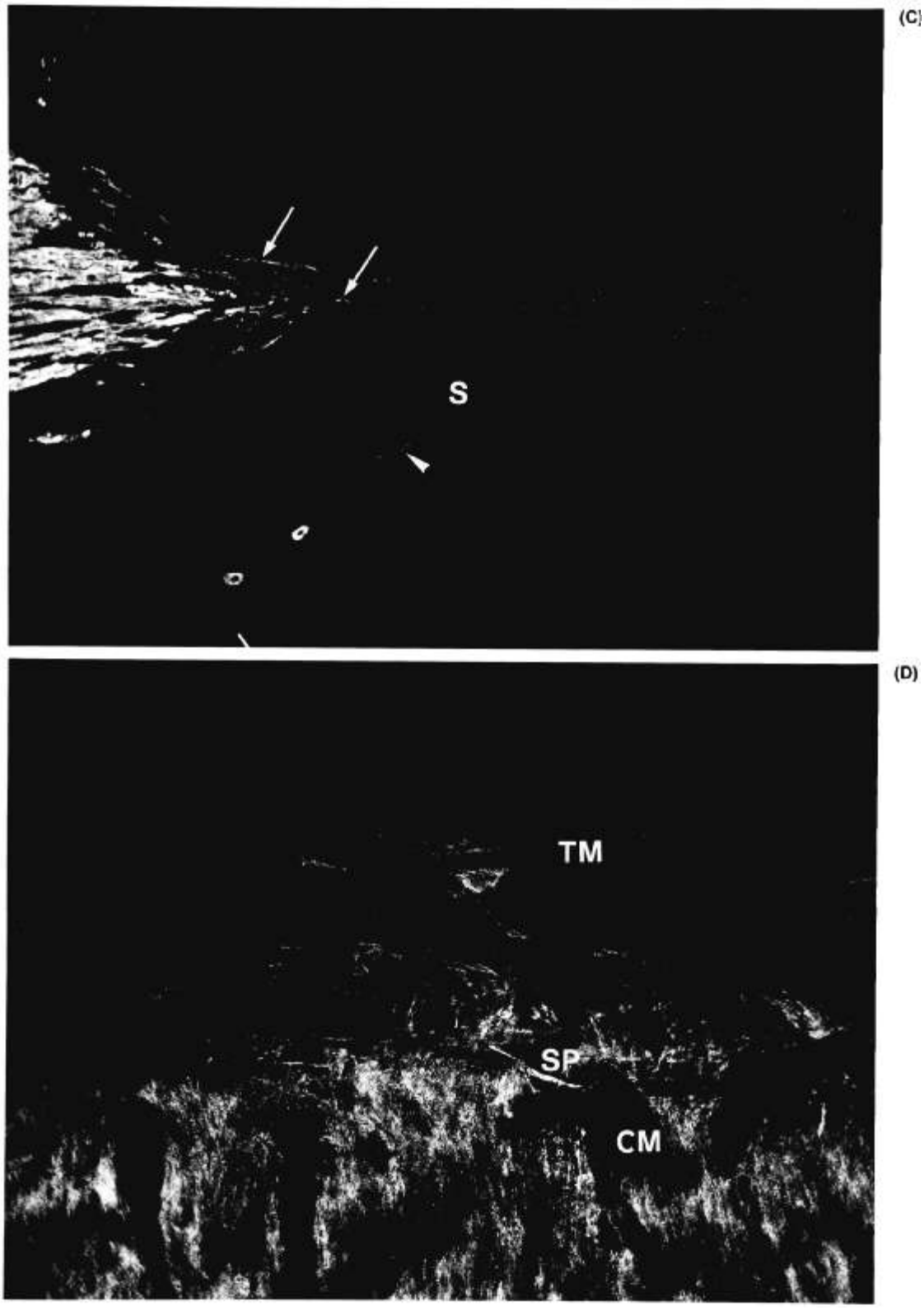


FIG. 1. Sagittal paraffin section through the chamber angle of a 40 **(A),** 49 **(B),** and 74 **(C)** year old donor eye stained for α-sm actin. In **A** α-sm actin positive cells are found within all regions of the trabecular meshwork (arrows). In **B** staining is seen covering still about half of the uveal and corneoscleral lamellae (arrows). In **C** α-actin–labelled cells are only seen within the scleral spur, the adjacent lying uveal and corneoscleral lamellae (arrow), and some cells next to the outer wall of Schlemm's canal



(arrowhead). S, Schlemm's canal; original magnification ×45. In **D** tangential frozen sections (10 μm) through the trabecular meshwork of a 74-year-old donor eye stained for α-sm actin are shown. Labelled cells are only single and scattered, lying adjacent to the ciliary muscle and scleral spur, respectively. CM, ciliary muscle; SP, scleral spur; TM, trabecular meshwork; original magnification ×81.

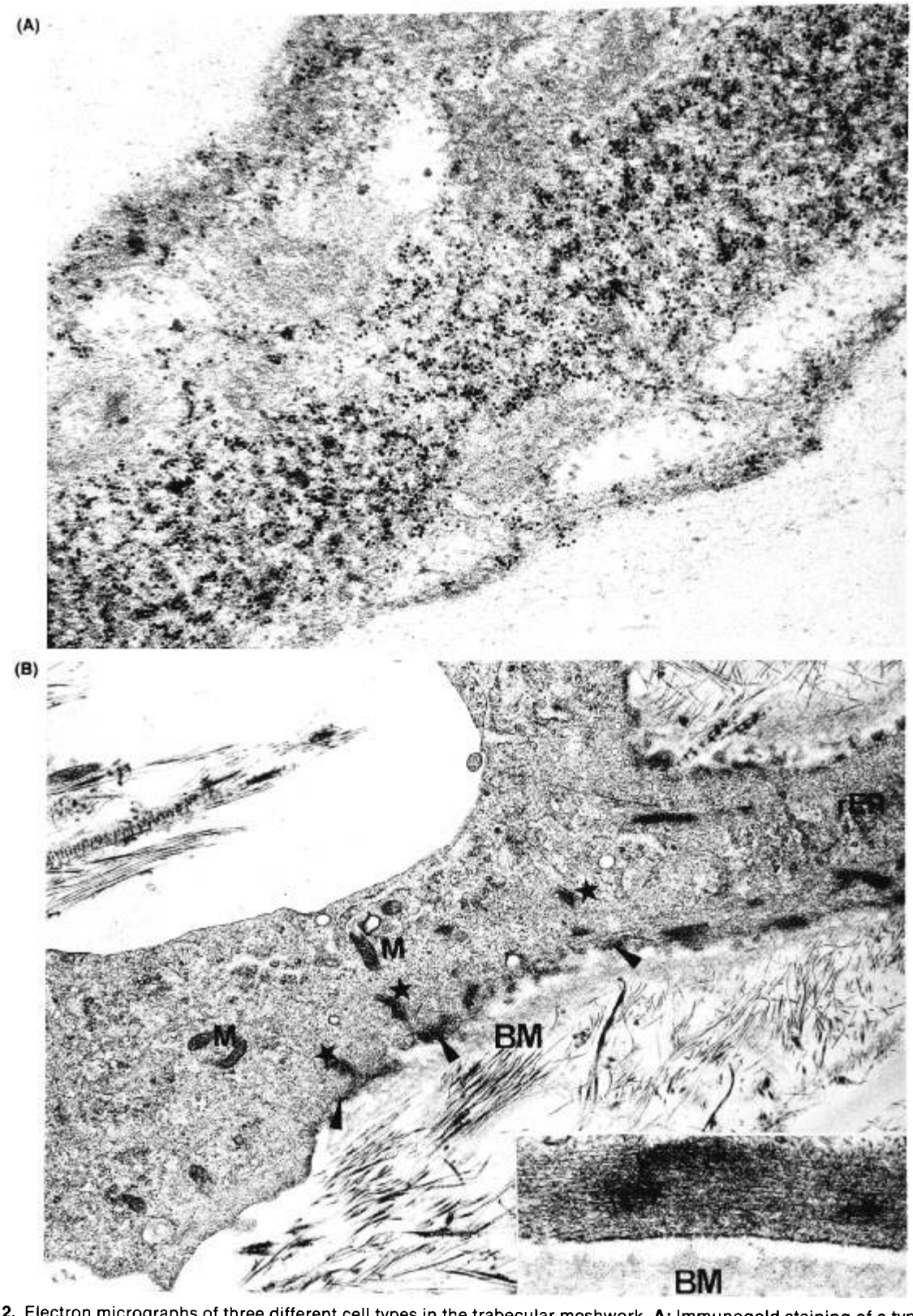
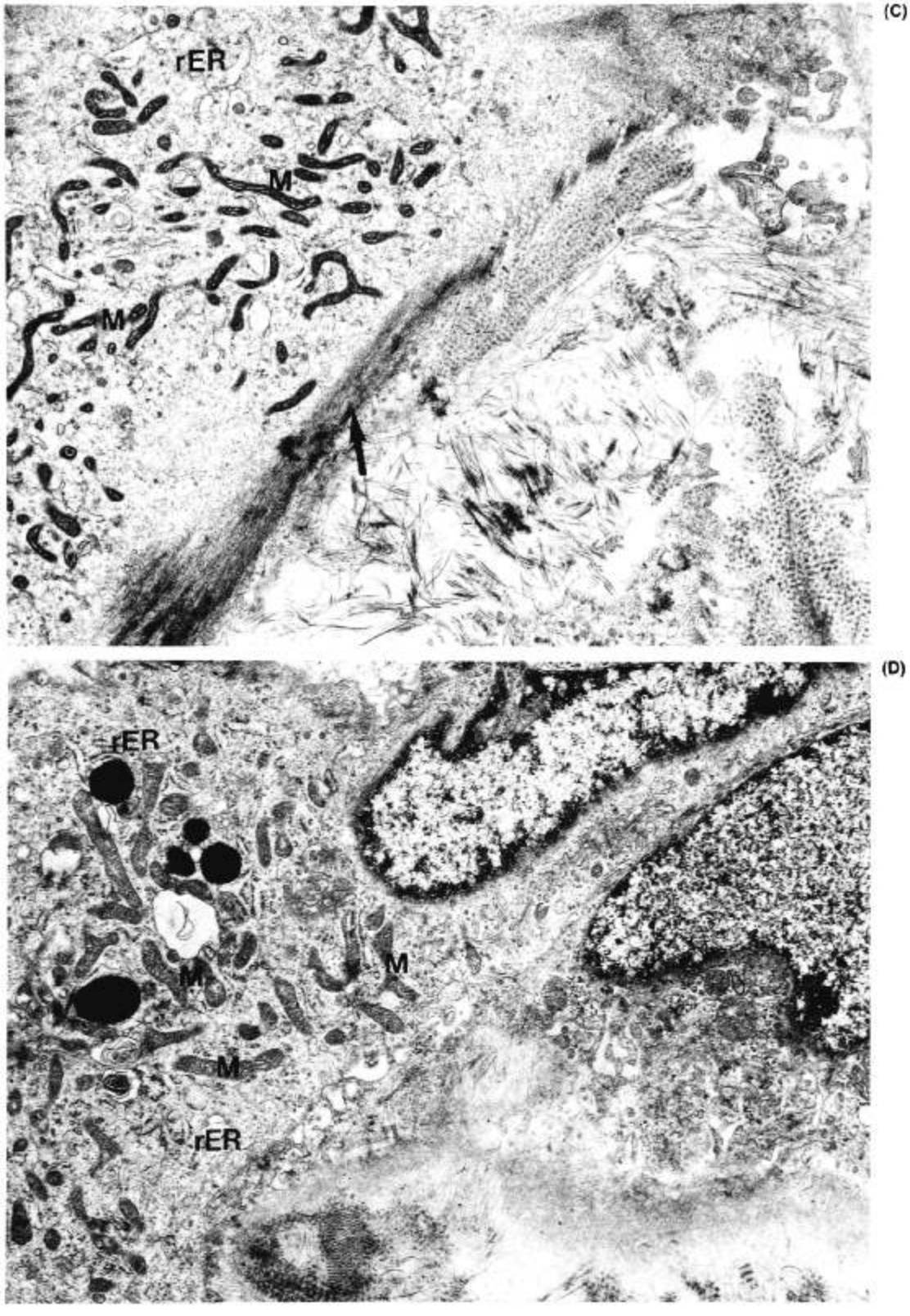


FIG. 2. Electron micrographs of three different cell types in the trabecular meshwork. **A:** Immunogold staining of a type I cell of a 27-year-old donor eye incubated with α-sm actin-antibodies (original magnification ×84,000). The gold particles can be localized to the 6–8-nm filaments that fill large parts of the cytoplasm. **B:** Type I cell of a 27-year-old donor eye (original magnification ×18,200). The cytoplasm is mainly filled with 6–8-nm thick filaments [inset shows a higher magnification (×66,000)] that are connected with each other and with the cell membrane by dense bodies (asterisks) and dense bands



(arrowheads), respectively. Other cell organelles as mitochondria (M) and rough endoplasmic reticulum (rER) are only sparsely present. BM, basement membrane. **C:** Type II cell of a 49-year-old donor eye (original magnification ×12,320). In the type II cells bundles of microfilaments (arrow) are still prominent; however, the other organelles (mitochondria M, rER) fill large parts of the cytoplasm. **D:** Type III cell of a 74-year-old donor eye (original magnification ×14,520). Type III cells are characterized by a bright cytoplasm containing large amounts of rER, vesicles, and mitochondria (M), whereas microfilaments are hardly seen.

areas of the cytoplasm except for a small perinuclear region where other cell organelles were present. Dense bodies were unevenly scattered within the filament bundles and dense band-like condensations appeared between filaments and the cell membrane (Fig. 2B). In the nonlamellar ciliary meshwork, cell type I showed an incomplete basal membrane and at some places the extracellular material seemed to be connected directly to the basal membrane of the cells.

Cell type II contained fewer microfilaments and more other cell organelles such as rough endoplasmic reticulum (rER) and Golgi material in the central cytoplasm. However, thick bundles of microfilaments were still present adjacent to the cell membranes and within the cellular extensions (Fig. 2C).

In cell type III, only very thin bundles of microfilaments were seen localized mainly in the basal cytoplasm adjacent to the trabecular lamellae or in the cellular extensions. The remaining cytoplasm was filled with cell organelles (rER, Golgi material, ribosomes, and mitochondria) (Fig. 2D).

The distribution of the three cell types showed age-related differences. Cell type I was found in all age groups characteristically in the vicinity of the ciliary muscle and was located in the ciliary meshwork and in the scleral spur. In the young aged (27 years) donor eye, single type I cells also were found in the corneoscleral and cribriform meshwork.

Type II cells constituted the majority of cells within the uveal, corneal, and cribriform meshwork in young eyes. In older eyes (45, 50, 74, and 81 years), cell type II was less frequently seen. Here, the cells were mainly found in the posterior parts of the trabecular meshwork adjacent to the scleral spur.

Cell type III was the predominant cell type of the old eyes, whereas in the young eyes type III cells were only occasionally found within the meshwork regions.

DISCUSSION

The presence of filamentous actin in the trabecular meshwork cells of primates has been demonstrated by numerous authors (4,10–15). The functional significance of these filaments in the trabecular meshwork has been discussed mainly with respect to phagocytosis and maintenance of cell junctions.

Two-dimensional gel electrophoresis has shown that filamentous actin consists of different actin isoforms (for review, see ref. 16). The α-actin isoforms are generally considered to be specific for smooth muscle cells and myofibroblasts. This might suggest that α-actin is functionally mainly related to contractile properties.

The present study demonstrates smooth muscle specific α-actin filaments in human trabecular meshwork cells, corroborating the findings of de Kater et al. (4). Our characterization shows that α-actin–labelled trabecular meshwork cells are different from ciliary muscle cells as they do not stain for desmin, which is the intermediate filament predominantly expressed by muscle cells. Although desmin filaments have been described for trabecular meshwork cells in culture by Iwamoto and Tamura (17), in the intact tissue we found staining for this intermediate filament only in ciliary muscle cells (see also ref. 18). Similar results have also been reported by Weinreb and Ryder (15).

In this study, we furthermore demonstrate that the frequency and distribution of α-sm actin positive cells shows age dependent changes within the trabecular meshwork. In the young age group, most of the cells stain for α-sm actin in all regions of the trabecular meshwork. Indeed, some of these cells (the type I cells) are morphologically similar to the recently described myofibroblasts in the scleral spur (19) and in bovine outflow tissue (2). In the bovine meshwork, contractile properties have been demonstrated by various physiological methods (20,21).

Physiological studies on contractility of the human trabecular meshwork are lacking. If in the human, too, α-sm actin positive cells are contractile, in young eyes contraction could influence outflow facility by configurational alterations of the outflow pathway. With increasing age, the number of trabecular cells is reduced (22,23). This is even more pronounced in glaucomatous eyes (24). It might be that there is a specific loss of α-actin positive cells in the trabecular meshwork; on the other hand, in young eyes all cells stained positively for α-sm actin, and in old eyes the lamellae are still covered by trabecular cells in all regions of the meshwork. We, therefore, suggest that the lack of staining in the filtering portion of the meshwork is not caused by loss of cells but is more related to structural changes within the cells. In fact, concomitant with loss of the α-sm actin filaments in the trabecular meshwork cells with age, an increase of synthesizing organelles such as rER can be observed. An increased synthetic activity of the trabecular mesh-

work cells might contribute to the age-related increase of extracellular material in this region, e.g., the thickening of the trabecular beams and the accumulation of plaque material (25,26). Both, increase of synthetic activities and loss of contractile protein might contribute to a decrease in outflow facility happening with age and more pronounced with glaucoma.

Acknowledgment: We are very grateful to Ursi Bechmann, Barbara Teschemacher, and Gerti Link for their expert assistance with the immunocytochemistry. We would also like to give special thanks to Simone Klein for her help in electron microscopy and to Marco Göβwein for his excellent preparation of the photographs. The research reported herein was supported by a grant from the Deutsche Forschungsgemeinschaft Dre 124/6-1.

REFERENCES

1. Kaufman PL, Bárány EH. Adrenergic drug effects on aqueous outflow facility following ciliary muscle retrodisplacement in the cynomolgus monkey. *Invest Ophthalmol Vis Sci* 1981;20:644–51.
2. Flügel C, Tamm E, Lütjen-Drecoll E. Different cell populations in bovine trabecular meshwork: an ultrastructural and immunocytochemical study. *Exp Eye Res* 1991;52:681–90.
3. de Kater AW, Spurr-Michaud SJ, Gipson JK. Localization of smooth muscle myosin-containing cells in the aqueous outflow pathway. *Invest Ophthalmol Vis Sci* 1990;31:347–53.
4. de Kater AW, Shahsafaei A, Epstein DL. Localization of smooth muscle and nonmuscle actin isoforms in the human aqueous outflow pathway. *Invest Ophthalmol Vis Sci* 1992; 33:424–9.
5. Mc Lean IW, Nakane PK. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J Histochem Cytochem* 1974;22:1077–83.
6. Stefanini M, de Martino C, Zamboni C. Fixation of ejaculated spermatozoa for electron microscopy. *Nature* 1967; 216:173–4.
7. Ito S, Karnovsky MJ. Formaldehyde-glutaraldehyde fixatives containing trinitro compounds. *J Cell Biol* 1968;39: 168a–9a.
8. Duhamel RC, Johnson DA. Use of nonfat dry milk to block nonspecific nuclear and membrane staining by avidin conjugates. *J Histochem Cytochem* 1985;33:711–4.
9. Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G. A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol* 1986;103:2787–96.
10. Ringvold A. Actin filaments in trabecular endothelial cells on eyes of the vervet monkey. *Acta Ophthalmol* 1978;56: 217–55.
11. Gipson JK, Anderson RA. Actin filaments in cells of human trabecular meshwork and Schlemm's canal. *Invest Ophthalmol Vis Sci* 1979;18:547–61.
12. Grierson I, Rahi AHS. Microfilaments in the cells of the human trabecular meshwork. *Br J Ophthalmol* 1979;63:3–8.
13. Tripathi RC, Tripathi BJ. Contractile protein alteration in trabecular endothelium in primary open-angle glaucoma. *Exp Eye Res* 1980;31:721–4.
14. Ryder MI, Weinreb RN. The cytoskeleton of the cynomolgus monkey trabecular cell. I. General considerations. *Invest Ophthalmol Vis Sci* 1986;27:1305–11.
15. Weinreb RN, Ryder MI. In situ localization of cytoskeletal elements in the human trabecular meshwork and cornea. *Invest Ophthalmol Vis Sci* 1990;31:1839–47.
16. Skalli I, Gabbiani G. The biology of the myofibroblast relationship to wound contraction and fibrocontractive diseases. In: Clark RAF, Henson PM, eds. *The molecular and cellular biology of wound repair*. New York: Plenum Publishing Corporation, 1988;373–402.
17. Iwamoto Y, Tamura M. Immunocytochemical study of intermediate filaments in cultured human trabecular cells. *Invest Ophthalmol Vis Sci* 1988;29:244–50.
18. Tamm E, Flügel C, Baur A, Lütjen-Drecoll E. Cell cultures of human ciliary muscle: growth, ultrastructural and immunocytochemical characteristics. *Exp Eye Res* 1991;53:375–87.
19. Tamm E, Flügel C, Stefani FH, Rohen JW. Contractile cells in the human scleral spur. *Exp Eye Res* 1992;54:531–43.
20. Coroneo MT, Korbmacher C, Flügel C, Stiemer B, Lütjen-Drecoll E, Wiederholt M. Electrical and morphological evidence for heterogeneous populations of cultured bovine trabecular meshwork cells. *Exp Eye Res* 1991;52:375–88.
21. Lepple-Wienhues A, Stahl F, Wiederholt M. Different smooth muscle-like contractile properties of trabecular meshwork and ciliary muscle. *Exp Eye Res* 1991;53:33–8.
22. Grierson I, Howes RC. Age-related depletion of the cell population in the human trabecular meshwork. *Eye* 1987;1:204–10.
23. Alvarado J, Murphy C, Polansky J, Juster R. Age-related changes in trabecular meshwork cellularity. *Invest Ophthalmol Vis Sci* 1981;21:714–27.
24. Alvarado J, Murphy C, Juster R. Trabecular meshwork cellularity in primary open-angle glaucoma and nonglaucomatous normals. *Ophthalmology* 1984;91:564–79.
25. Rohen JW, Lütjen E. Über die Altersveränderungen des Trabekelwerkes im menschlichen Auge. *Albrecht v. Graefes Arch. Klin. Exp. Ophthalmol.* 1968;175:285–307.
26. Lütjen-Drecoll E, Shimizu T, Rohrbach M, Rohen JW. Quantitative analysis of 'plaque material' in the inner- and outer wall of Schlemm's canal in normal- and glaucomatous eyes. *Exp Eye Res* 1986;42:443–55.