Synthesis of extracellular matrix components by human ciliary muscle cells in culture

E.Tamm, A.Baur and E.Lütjen-Drecoll

Department of Anatomy, University of Erlangen-Nürnberg, Krankenhausstrasse 9, 8520-Erlangen, Germany

ABSTRACT
The production and spatial organization of connective tissue components in ciliary muscle cell cultures was studied with immunohisto-chemical and ultrastructural methods. Antibodies against collagen types IV and VI, fibronectin and laminin were used.

Laminin stains as pericellular network surrounding individual muscle cells. Type IV collagen shows positive cytoplasmic staining and only small foci of extracellular immuno-fluorescence. Staining for type VI collagen and fibronectin is seen near the ends of the bipolar cells, while the lateral sides of the cells remain unstained.

Electronmicroscopy shows that cultured ciliary muscle cells are surrounded by an incomplete basal lamina. In addition, bundles of 5-20 nm thick extracellular microfibrils are seen. The bundles are oriented parallel to the axis of the cells and are in close contact with the cell membrane in areas where membrane-bound dense bands are formed. Immunoelectronmicroscopy indicates that the bundles contain fibronectin and type VI collagen fibrils. While the fibronectin fibrils approach the cell membrane directly, type VI collagen fibrils are usually separated from the cell membrane by fine fibrillous material of different nature. Quality and spatial organization of the extracellular material in ciliary muscle cell cultures shows marked similarities with the extracellular matrix of ciliary muscle in situ.

INTRODUCTION

A consistent finding in old human eyes is the marked increase in hyalinized connective tissue between the ciliary muscle bundles (1-4). The increase correlates with the onset of presbyopia and might well have influence on the function of the ciliary muscle in accommodation. Moreover, the anterior part of the ciliary muscle exhibits an age-related increase in "plaque material", which is even more pronounced in eyes with primary open angle glaucoma (5). Recently we established and characterized several cell lines derived from human ciliary muscle (6). It is clear that in vitro procedures provide a means of studying the synthetic capabilities of ciliary muscle cells and the factors which influence these activities.

In a previous study we have demonstrated that fibronectin, laminin, and type IV collagen form large parts of the extracellular matrix of the human ciliary muscle (7). In addition, type VI collagen is present in considerable amounts in the "plaque material" in the region of the anterior insertion of the ciliary muscle (7,8). In the present study we investigated production and spatial organization of collagen types IV and VI, laminin and fibronectin in ciliary muscle cell cultures.

MATERIAL AND METHODS

Ciliary muscle cell cultures were established from 10 human donors (16-91 years). The immunocytochemical and ultrastructural characterization of the cultures has been described in detail elsewhere (6). In brief, the cultured cells showed the typical growth pattern of cultured smooth muscle cells, stained for smooth muscle specific α-actin and desmin, and exhibited ultrastructural characteristics of ciliary muscle cells. Application of 10 mmol/l $BaCl_2$ to the cultured ciliary muscle cells resulted in a membrane depolarization with superimposed action potentials (9).

The cells were maintained at 37°C in a 5% CO_2 athmosphere. Culture medium (medium 199

supplemented with 10% fetal calf serum, 50 U ml^{-1} penicillin, 50 µg ml^{-1} streptomycin and 50 µg ml^{-1} ascorbic acid, all Gibco Ltd, Paisley, Scotland) was exchanged twice a week. Confluent cells were passaged at a ratio of about 1:4 using trypsin-EDTA. For the present study 3rd to 6th-passage cultures, one week after confluency were investigated.

Antibody staining

Cells were seeded in tissue culture chambers mounted on Permanox^R microslides (Lab Tek^R, Nunc Inc., Naperville, IL). Culture medium was removed by rinsing three times with phosphate-buffered saline (PBS). The cells were fixed with ethanol/ether (1:1) at -20°C for 3 min. The cells were again rinsed three times with PBS and then incubated for 90 min at room temperature with the primary antibody. For demonstration of type IV collagen, monoclonal mouse antibodies from Heyl, Berlin, Germany (anti-human IgM, Kappa) and from Dakopatts, Hamburg, Germany (anti-human, IgG1 Kappa, CIV22,) (10) were used, both at a dilution of 1:100. For laminin polyclonal rabbit anti-human antibodies from Heyl (11) were applied to the culture chambers at a dilution of 1:10. Demonstration of type VI collagen was performed using a monoclonal mouse antibody (anti-human, Ig1, Kappa) and polyclonal rabbit anti-human antibodies, (both from Heyl) (12) at a dilution of 1:50. Fibronectin was stained with a monoclonal mouse antibody against cellular fibronectin (Sigma Chemical Company, St. Louis MO, clone no. FN-3E2). This product localizes the 240 KD bands of cellular fibronectin. The cells were rinsed three times with PBS then incubated for another 60 min with fluorescein-labelled rabbit anti-mouse IgG or sheep anti-rabbit IgG (both Dakopatts) diluted in PBS (1:20). The cells were washed again three times with PBS, then the tissue chambers were removed from the slides. The slides were mounted in glycerol containing 2.5% 1,4-diazobicyclo-octane (Merck, Darmstadt, Germany). Control experiments were performed using either PBS, mouse or rabbit pre-immune serum instead of the primary antibody. Stained

cultures were viewed with a Leitz Aristoplan photomicroscope (Ernst Leitz GmbH, Wetzlar, Germany) equipped with epifluorescence optics and appropriate filters. A Kodak T-max 400 black and white film (Kodak Limited, Hempstead, England) was used for photography.

Electronmicroscopy

The cells were grown in uncoated, plastic petri dishes or in tissue culture flasks and fixed with Ito's solution (13) for 4 hr. Cells were postfixed with 1% osmium tetroxide, dehydrated with graded alcohols and embedded in Epon. Tangential and perpendicular sections of the cells were cut on an ultramicrotome. The sections
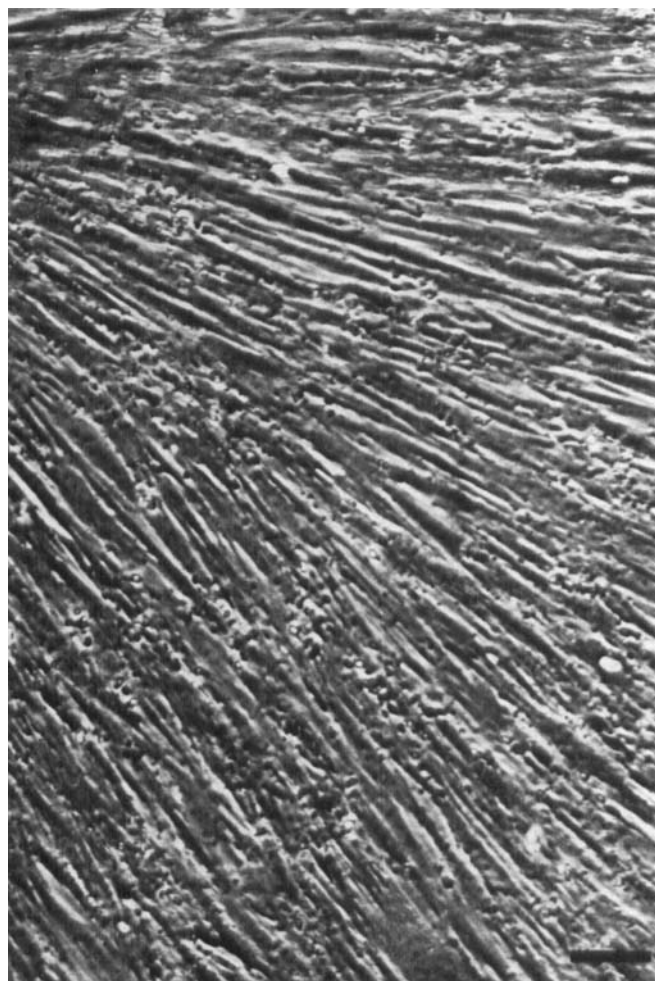

Figure 1: Confluent ciliary muscle cell cultures characteristically consist of longitudinal and slightly curved bands of parallel bipolar cells (donor age 16 years, 3rd passage, phase-contrast micrograph, x 250, bar: 40 µm).

were contrasted with lead citrate and uranyl acetate and viewed using a Zeiss (EM 902) electronmicroscope.

For immunoelectronmicroscopy, cells were fixed in phosphate-buffered paraformaldehyde-lysine-periodate (PLP) (14) solution at room temperature for 3 min. After washing three times 10 min with PBS, the cells were incubated with the primary antibodies (fibronectin, type VI collagen) for 8 hrs at 4°C. The antibody solution contained 1% bovine serum albumin (BSA) and various protease inhibitors (15). After three 30 min changes in PBS to remove unbound antibody, the cells were incubated for 8 hrs in a 1:20 dilution of goat anti-mouse (10 nm, Sigma) or goat anti-rabbit immunogold (5 nm, Sigma), 1% BSA and protease inhibitors. Unbound antibody was removed by three 30 min changes in PBS. The cells were then fixed with 2% glutaraldehyde for 20 min, washed in PBS and embedded in Epon as described above.

RESULTS

Immunohistochemistry

Confluent ciliary muscle cell cultures characteristically consist of longitudinal and slightly curved bands of parallel bipolar cells
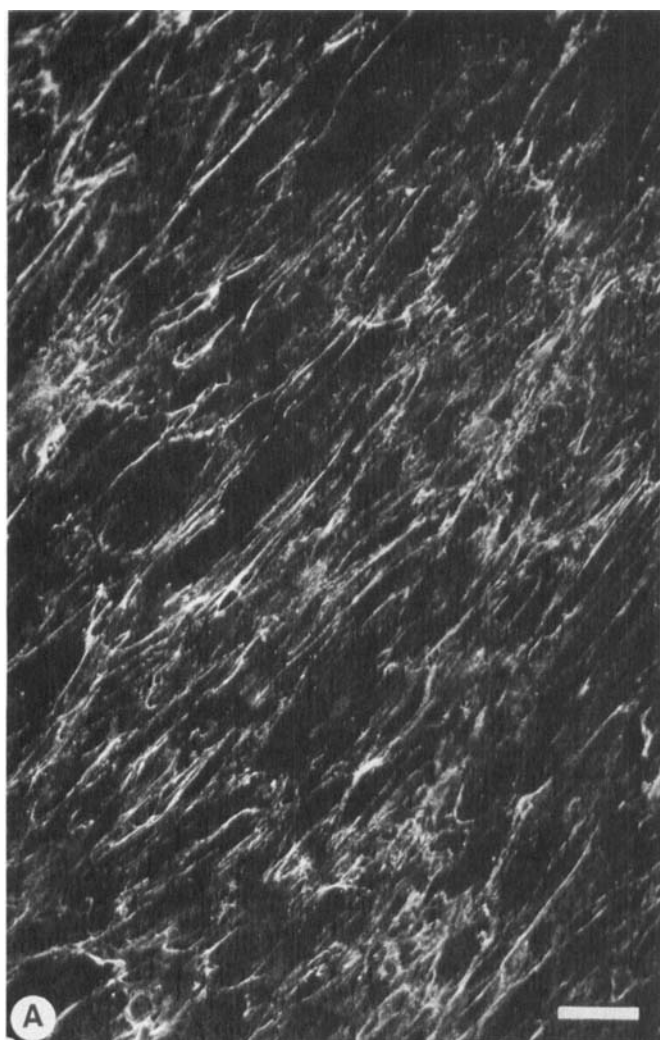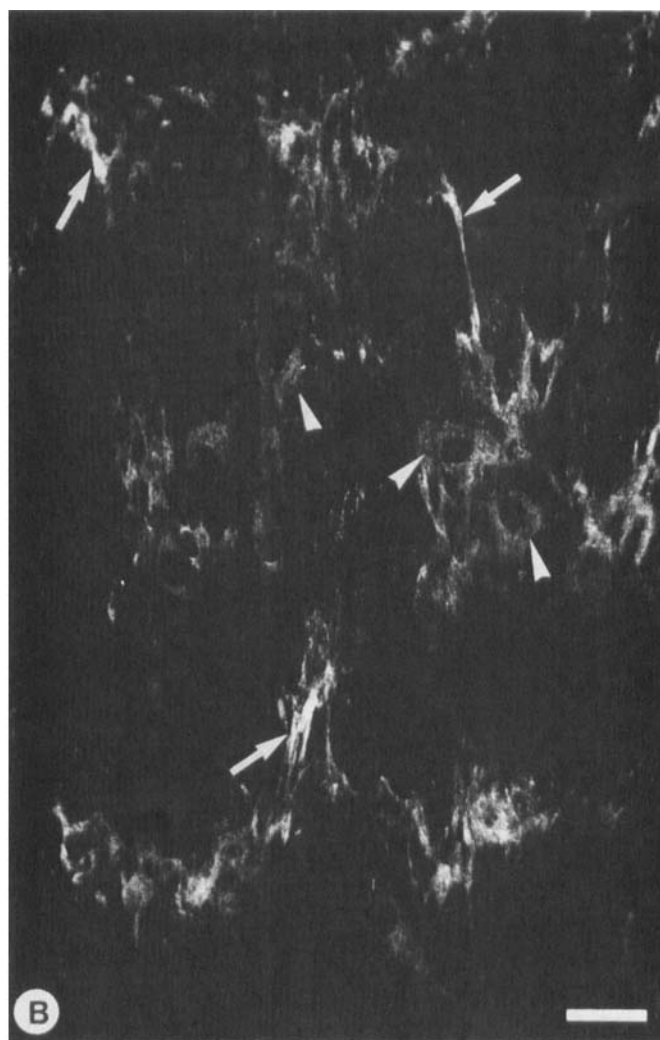

Figure 2: Immunofluorescence of a confluent ciliary muscle cell culture (same donor as in Fig. 1, 3rd passage). A: Staining for laminin reveals a pericellular network which outlines the fusiform shape of the cells. B: Type IV collagen shows positive cytoplasmic staining (arrowheads) and only small foci of positive extracellular immunofluorescence (arrows). (x 300, bar: 33 μm).
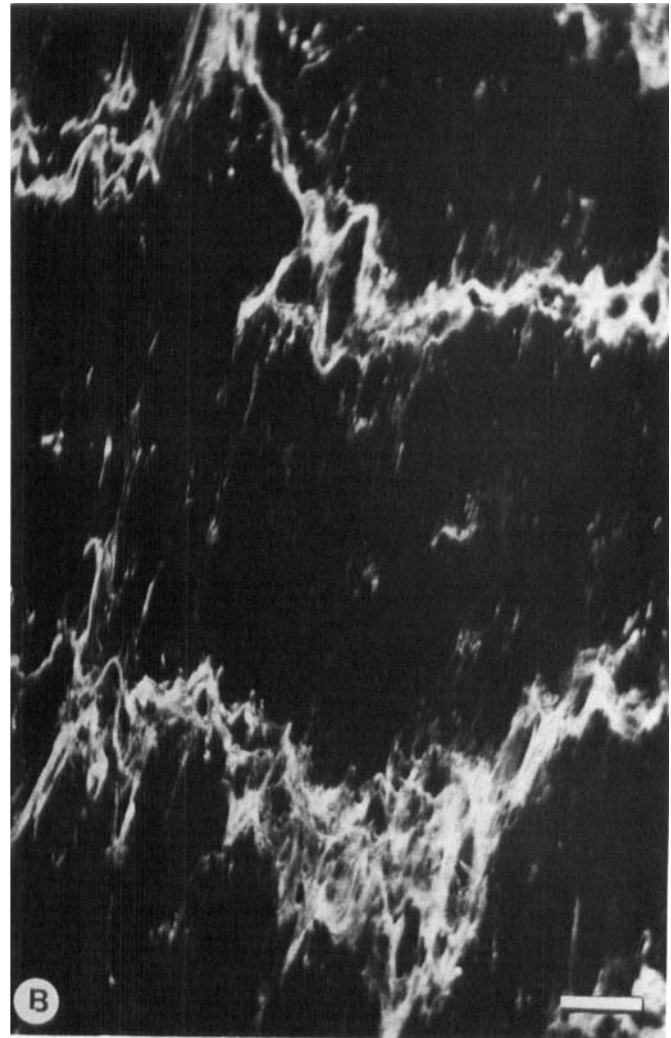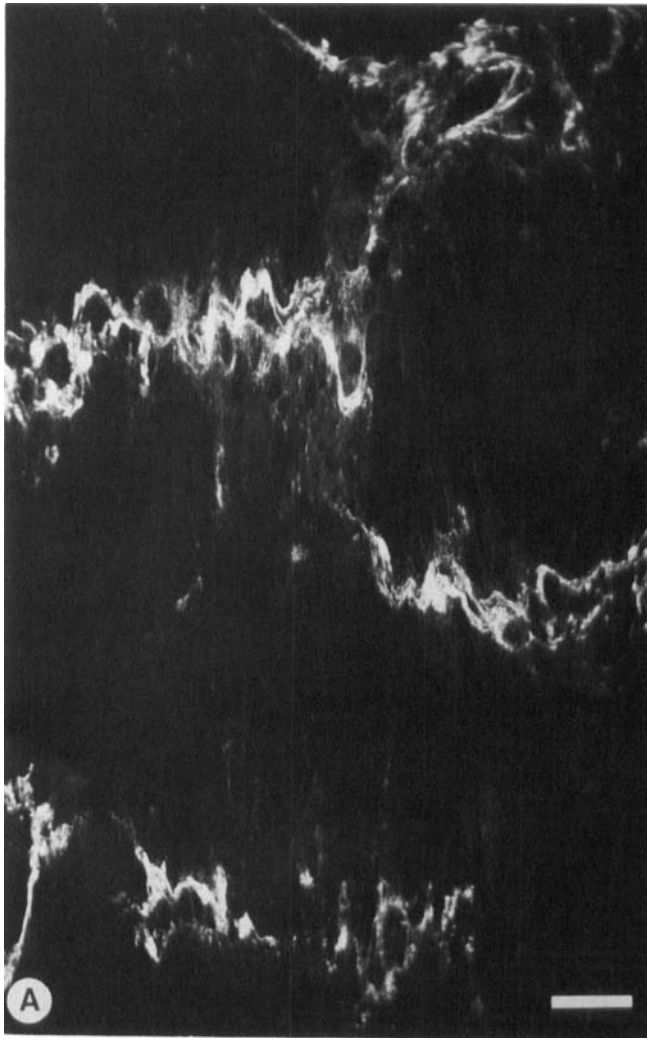
Figure 3: Immunofluorescence of a confluent ciliary muscle cell culture (same culture as in Fig. 2). A: Staining for type VI collagen is confined to the ends of the bipolar cells, whereas the lateral sides of the cells remain unstained. C: Staining for fibronectin shows a similar spatial organization as staining for type VI collagen, but appears to be more pronounced (x 300, bar: 33 μm).

(Fig. 1). Staining of confluent cultures for laminin (Fig. 2A) shows a well established pericellular network which outlines the fusiform shape of the muscle cells. Immunofluorescent staining to demonstrate type IV collagen (Fig. 2B) shows only small foci of extracellular fluorescence. The cells exhibit, however, an intense positive cytoplasmic staining. Type VI collagen is confined to the ends of the bipolar cells, whereas the lateral sides of the cells remain unstained (Fig. 3A). Staining for fibronectin shows a similar spatial organization as staining for type VI collagen, but appears to be more pronounced (Fig. 3B).

Electronmicroscopy

At confluency, the ciliary muscle cells are surrounded by an incomplete basement membrane (Fig. 4A). In addition, the cultures show a matrix of extracellular fine fibrils which appear to be aggregated into loose bundles. The fibrils measure 5-20 nm in cross sections and show no apparent evidence of periodicity. Most of this
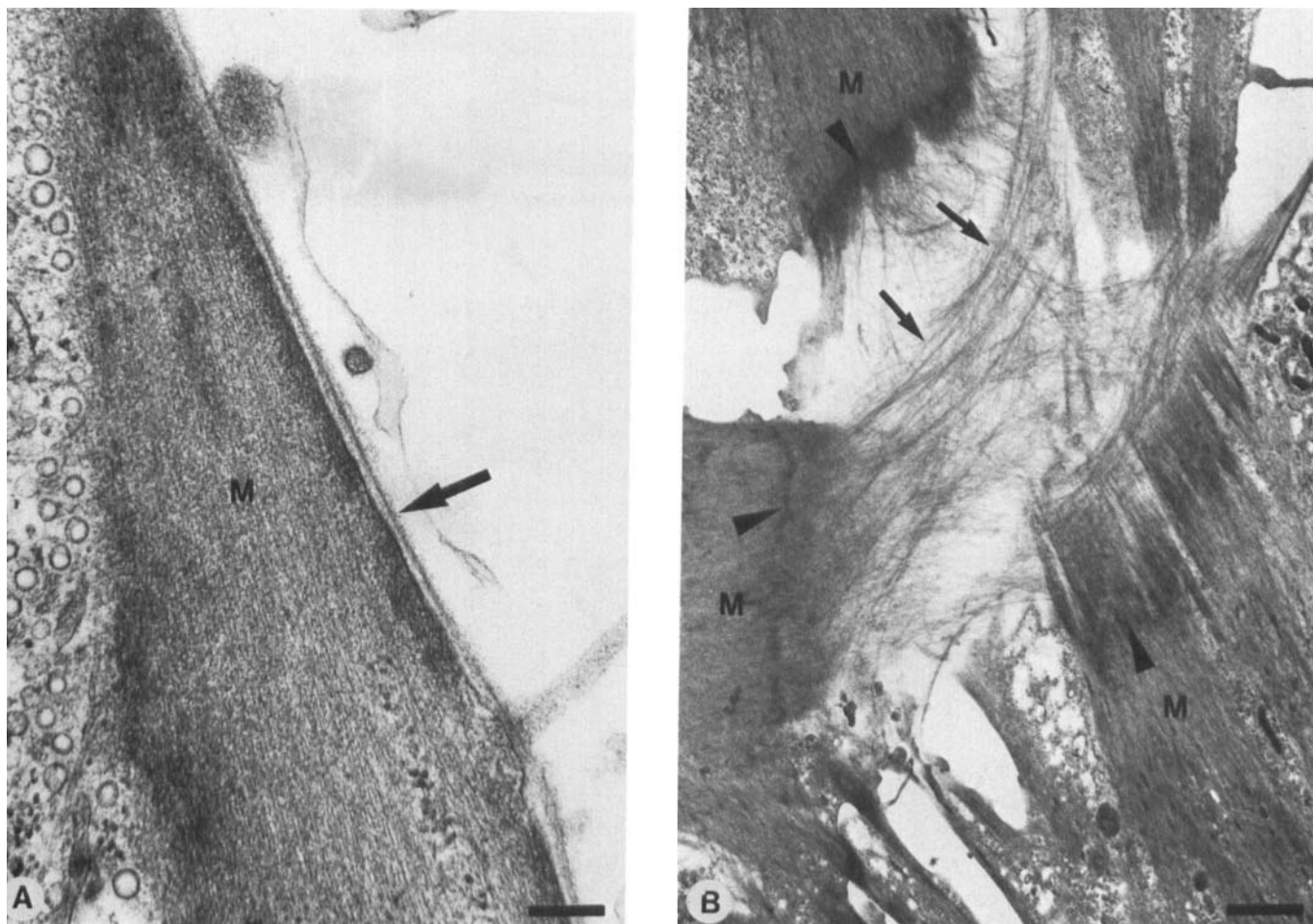
Figure 4: Electronmicrographs of cultured ciliary muscle cells (Same donor as in Fig. 1, 3rd passage). A: At confluency the cells are surrounded by an incomplete basal lamina (arrow). M. Myofilaments (x 60000, bar: 16,6 nm). B. Near the ends of the cells extracellular microfibrils are observed (arrows). The fibrils are in close contact with the cell membrane in areas where dense bands are formed (arrowheads). The orientation of the fibrils is parallel to the myofilaments (M) within the cells (x 8800, bar: 1.13 µm).

material is present near the elongated ends of the bipolar muscle cells (Fig. 4B). Here, the orientation of the extracellular fibrils is parallel to the axis of the individual muscle cells and therefore also parallel to the myofilaments within the cells. The extracellular fibrils are in close contact with the membrane of the muscle cells. In these areas of contact, the cell membrane forms dense bands with adhering myofilaments.

With immunoelectronmicroscopy, intense labelling for fibronectin is seen throughout the whole extracellular fibrillar material (Fig. 5). The fibronectin positive fibrils come in close contact with the cell membrane of the muscle cells. In addition to fibronectin, fibrils which are positively labelled for type VI collagen are present in the bundles of extracellular fibrils near the ends of the muscle cells. In contrast to the fibronectin fibrils, however, the type VI collagen fibrils only occassionally approach the muscle cell membrane directly, but are usually separated from the cells by unlabelled fine fibrillar material of different nature (Fig. 6).
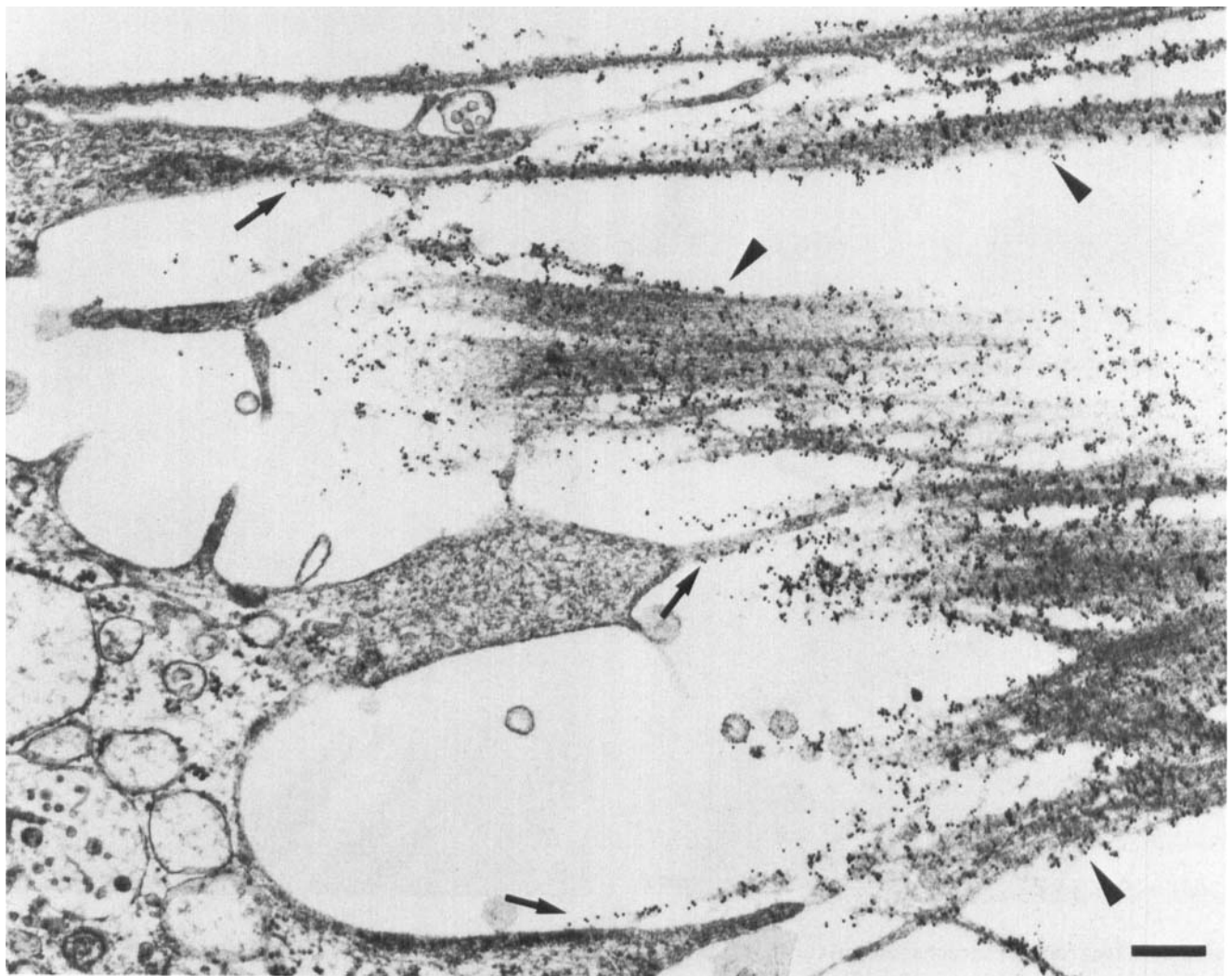
Figure 5: Immunoelectronmicroscopy of cultured ciliary muscle cells (same donor as in Fig. 1, 3rd passage). Intense immunogold labelling for fibronectin is seen throughout the whole extracellular fibrillar material (arrowheads). The fibronectin positive fibrils come in close contact with the cell membrane (arrows) (x 48000, bar: 210 nm).

There were no obvious qualitative changes in extracellular matrix production between cultures derived from donors of different ages.

DISCUSSION

Ciliary muscle cells in culture produce considerable amounts of extracellular fibrillar material. The cultured cells are surrounded by laminin and type IV collagen, both macromolecules essential for basement membranes (16,17). In contrast to ciliary muscle cells in situ (18,19), the basement membrane of ciliary muscle cells in culture is incomplete. Formation of an incomplete basal lamina, however, is regarded as typical for cultured viszeral and vascular smooth muscle cells (20-24).

In addition, immunostaining indicates that fibronectin is a major component of the extracellular fibrillar material synthesized by cultured ciliary muscle cells. The fibronectin-labelled fibrils approach the ciliary muscle cell membrane directly. Indeed, it has been shown that fibronectin mediated cell adhesion occurrs via cell surface proteins (25,26). In tissue culture
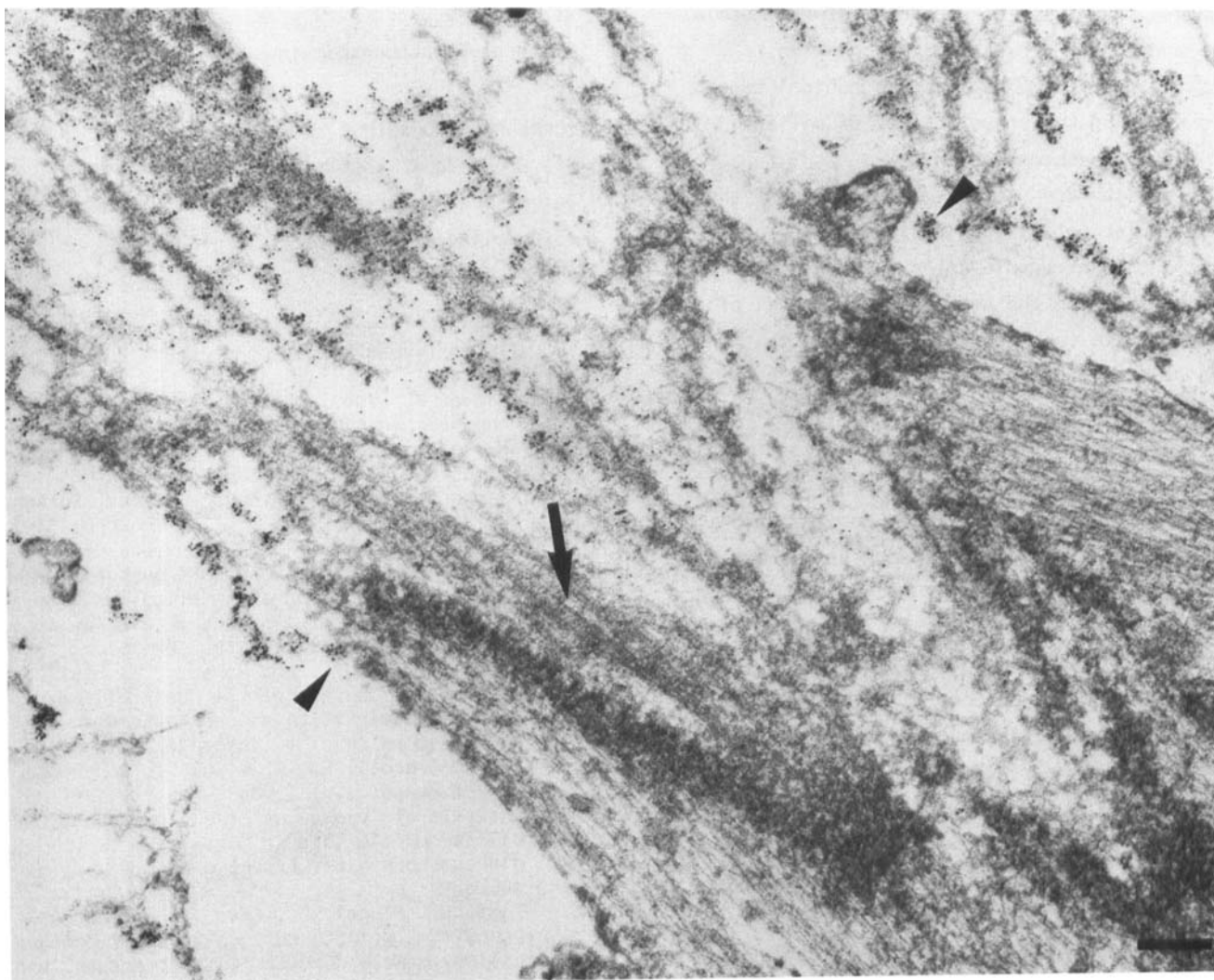
Figure 6: Immunoelectronmicroscopy of cultured ciliary muscle cells (same donor as in Fig. 1, 3rd passage). Immunogold labelling for type VI collagen is also seen in the extracellular fibrils near the ends of the muscle cells. The type VI collagen fibrils only occassionally aproach the cell membrane directly (arrowheads), but are usually separated from the cell (C) by unlabelled fine fibrillar material of different nature (arrow) (x 56000, bar: 180 nm).

conditions, analogous but smaller transmembrane associations which were termed "fibronexus" have been described between fibronectin-containing fibrils and the intracellular stress fibers of hamster and human fibroblasts (27). In situ, these fibronexus have been demonstrated at the surface of myofibroblasts in granulation tissue (28).

The extracellular fibrils synthesized by cultured ciliary muscle cells are characteristically deposited in the region of the elongated ends of the bipolar muscle cells. The fibrils form apparent close transmembrane associations with the cytoplasmic myofilaments and may be regarded as miniature muscle cell-tendon junctions which provide an anchoring function. These structures contain not only fibronectin-labelled fibrils, but also type VI collagen. In contrast to the fibronectin fibrils, type VI collagen fibrils are usually separated from the muscle cell membrane by fine fibrillar material of different nature. Electronmicroscopically, we recently described similar structures between the individual ciliary muscle cells in situ (19) and

suggested that these connections between muscle cells and surrounding stroma may play an important role in maintaining mechanical tension and supporting the shape changes of ciliary muscle during accommodation.

The quality and spatial organization of the extracellular material which is produced by cultured ciliary muscle cells show marked similarities with the extracellular matrix of ciliary muscle in situ. This indicates that the cells have maintained some of their differentiated functions. In situ, type VI collagen is not only found between the individual ciliary muscle cells, but is also a major component of the anterior tendons of the ciliary muscle, which insert to the trabecular meshwork (7). The type VI collagen fibrils form a sheath which surrounds these anterior elastic tendons (7,8). With age, the sheath thickens significantly, thereby forming part of the "plaque material" (5). Quantitative studies have shown that both in the trabecular meshwork (29) and in the anterior part of the ciliary muscle (5), the amount of this type VI collagen containing "plaque material" is significantly increased in eyes with primary open angle glaucoma.

Immunohistochemically, we did not observe obvious qualitative changes between cultures derived from donors of different ages, but quantitative measurements have still to be done. We trust that this cell culture model provides an important tool for such quantitative studies on control mechanisms modulating the production of extracellular matrix by ciliary muscle cells. These studies might be helpful in clarifying the reasons for the increase in extracellular material in the ciliary muscle in old and glaucomatous eyes.

CORRESPONDING AUTHOR

Dr. Ernst Tamm, Anatomisches Institut der Universität Erlangen-Nürnberg, Krankenhausstr. 9, 8520 Erlangen, Germany.

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