

## Cell Cultures of Human Ciliary Muscle: Growth, Ultrastructural and Immunocytochemical Characteristics

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*(Received 19 September 1990 and accepted in revised form 13 December 1990)*

Primary ciliary muscle cell cultures derived from human donors (16–91 years) were established and characterized by comparing them with ciliary muscle in tissue sections using immunocytochemical and ultrastructural methods. Monoclonal antibodies against desmin, vimentin,  $\alpha$ -actinin, smooth muscle (sm) specific  $\alpha$ -actin and von Willebrand factor were used. In tissue sections of the ciliary body, ciliary muscle cells, vascular muscle cells, pericytes, endothelial cells and fibroblasts stain for vimentin. Both types of muscle cells and the pericytes stain for  $\alpha$ -sm-actin, but only ciliary muscle cells stain for desmin. For tissue cultures, explants of the meridional and partly the reticular portion of the ciliary muscle were dissected and grown directly or after digestion of the explant with collagenase. Ten primary cell cultures with a typical hill-and-valley growth pattern similar to smooth muscle cells and two with a growth pattern similar to fibroblasts were established. All cultures could be subcultured up to the fifth passage. In fibroblast-like cultures 5–10% of the cells stained for  $\alpha$ -sm-actin. Staining for desmin was not observed. In smooth muscle-like cultures, all cells stained positive for  $\alpha$ -sm-actin. Desmin staining was not seen in growing non-confluent smooth muscle-like cultures. In confluent cultures, about 10% of the cells stained positive for desmin, preferentially in areas where the cells had formed hills. No culture stained for von Willebrand factor. Staining for  $\alpha$ -actinin in smooth muscle-like cultures showed that the dense bands of the myofilaments were arranged in register, similar to the typical ciliary muscle cell morphology seen in tissue sections. Ultrastructurally, the smooth muscle-like cultures showed the typical morphology of cultured smooth muscle cells. We conclude that the smooth muscle-like cultures consist of ciliary muscle cells.

**Key words:** ciliary muscle; smooth muscle; tissue culture;  $\alpha$ -sm-actin; desmin; vimentin;  $\alpha$ -actinin; immunocytochemistry; ultrastructure; human eye.

### 1. Introduction

It is well established that human ciliary muscle undergoes marked structural changes with age (Kerschbaumer, 1888; Fuchs, 1928; Stieve, 1949; van der Zypen, 1970). There is an increase in extracellular material and a pronounced sclerosis and hyalinization of the connective tissue between the ciliary muscle bundles. The age-related changes of the extracellular matrix might well affect the function of the muscle in accommodation and influence uveoscleral outflow. Local treatment of monkey eyes with prostaglandin  $F_{2\alpha}$  causes lysis of connective tissue elements between the ciliary muscle bundles (Lütjen-Drecoll and Tamm, 1988, 1989; Tamm et al., 1989). This might explain why prostaglandin  $F_{2\alpha}$  increases uveoscleral outflow in this species (Nilsson et al., 1989).

Extracellular 'plaque-material' is found in increased amounts in the trabecular meshwork of human eyes with primary open angle glaucoma (Rohen and Wittmer, 1972; Lütjen-Drecoll et al., 1986a). This material is also increased in the anterior portion of the ciliary muscle of glaucomatous eyes (Lütjen-Drecoll et al., 1986b). The reasons for this increase in extra-

cellular material in old and glaucomatous eyes are still unclear.

Cell culture techniques have become an important tool in the study of cellular physiology and the synthesis of extracellular material by smooth muscle cells (Burke and Ross, 1979). To obtain further insight into cellular changes associated with age or glaucoma, we established primary cultures of ciliary muscle cells from donors of different ages. The cultures were characterized by comparing the cells with ciliary muscle cells in tissue sections using immunocytochemical and ultrastructural methods.

### 2. Materials and Methods

#### *Cell Culture*

Ciliary muscle cell cultures were established from post-mortem human eyes, donor ages 16–91 years. The eyes were enucleated within 2–48 hr of death. Immediately after enucleation, they were placed in medium 199 containing 50 U ml<sup>-1</sup> penicillin and 50  $\mu$ g ml<sup>-1</sup> streptomycin (all Gibco Ltd, Paisley, Scotland) for 10 min prior to dissection. Each eye was bisected along the equator and the posterior half removed. The anterior half was placed on a sterile

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TABLE I  
Primary cell cultures derived from ciliary muscle

No.	Age (yr)	Sex	Post-mortem time (hr)	Culturing method
Type A cells (smooth muscle-like-cells)				
7089	16	f	48	Cell suspension†
5489	19	f	12	Whole explant*
8189	30	f	44	Whole explant*
3189	35	m	48	Whole explant*
1548	43	m	10	Whole explant*
3690	49	m	12	Whole explant*
6889	54	m	6	Cell suspension†
4389	69	f	3	Whole explant*
6089	77	f	4	Cell suspension†
6289	91	f	5	Cell suspension†
Type B cells (fibroblast-like cells)				
3089	36	m	48	Whole explant*
7389	60	f	5	Whole explant*

Explants from ciliary muscle were placed as a whole in tissue culture\* or digested with collagenase to obtain a suspension of cells† which were allowed to settle in tissue culture dishes.

Petri-dish, corneal side down. The zonule was cut with fine scissors and the lens removed. A small segment of the anterior half with a width of approximately 5 mm was cut meridionally and further processed for immunocytochemistry (see 'Antibody staining'). The remaining part of the anterior half of the eye was processed for tissue culture of ciliary muscle. One branch of a fine forceps was inserted in the supraciliary or suprachoroidal space of the specimen. The ciliary body was grasped and the ciliary tissue gently pulled away from the underlying sclera and thereby torn from its attachment to the scleral spur. The uveal explant, containing ciliary processes, ciliary muscle and iris was placed in a new sterile Petri dish with the muscle on top. Against the dark background of the pigmented epithelium, the muscle could be easily distinguished, as a broad, pale, circular band. Under the operating microscope ( $\times 40$ ), the outermost parts of the ciliary muscle were cut free with fine scissors and placed in medium 199. Before continuing, two samples approximately 2 mm in length were cut from the muscle to be explanted. One sample was deep frozen and immediately sectioned on a cryostat. The other sample was fixed in Ito's solution (2.5% glutaraldehyde, 2.5% paraformaldehyde and picric acid: Ito and Karnovsky 1968) and embedded in Epon. After polymerization, semi-thin and ultra-thin sections were cut and used as additional controls.

If the frozen sections confirmed that only uncontaminated muscular tissue had been removed, the muscle explants were further processed. Two methods were used (Table I):

*Method 1.* The samples were incubated for 30 min at 37°C in Hank's balanced salt solution (HBSS), supple-

mented with 1 mg ml<sup>-1</sup> collagenase (collagenase type V, Sigma Chemical Co., St Louis, MO). The samples were then placed in a 35-mm sterile, uncoated, plastic Petri dish and covered with a coverslip. One milliliter of medium 199 was added, supplemented with 20% fetal calf serum (Gibco Ltd) penicillin and streptomycin. The explants were incubated at 37°C in humidified air enriched with 5% CO<sub>2</sub>. After 2 days the medium was renewed, thereafter twice weekly. The fresh medium contained the same antibiotics and 10% fetal calf serum. Primary cultures were examined routinely using positive phase contrast optics. Care was taken to make sure that only cells morphologically characteristic of smooth muscle cells in vivo (type A cells, see Results) were kept in culture. If the impression was that fibroblast-like cells (type B) grew out from the explant, the area covered by these cells was marked with a pencil on the underside of the Petri dish. Under sterile conditions, the coverslip was lifted. Then, the marked part of the explant was cut out and removed and the outgrowing fibroblast-like cells scratched away with a sterile needle. The procedure was controlled with phase contrast optics and repeated if necessary. Two cell lines were established by culturing the excised parts of the explants separately. They contained the type B or fibroblast-like cells exclusively.

*Method 2.* The explants were placed in collagenase (1 mg ml<sup>-1</sup>, collagenase type V, Sigma) for 2 hr with occasional gentle agitation. After this time the major part of the explants had been dispersed into single cells. The suspension was centrifuged at 900 rpm for 10 min, resuspended in 2 ml medium 199 with 20% fetal calf serum, penicillin and streptomycin and

injected into a 35-mm sterile, fibronectin-coated, plastic Petri dish. After 2 days, the medium was changed as described above.

Confluent cultures were passaged at a ratio of about 1:4 using trypsin-EDTA.

#### *Antibody Staining*

The specimens were divided meridionally in two parts of equal size. One half was rapidly frozen in isopentane cooled with liquid nitrogen. The other half was fixed in paraformaldehyde-lysine-periodate solution (McLean and Nakane, 1974) for 4 hr at 4°C, washed for 24 hr in phosphate-buffered saline (PBS), then dehydrated in graded alcohols and embedded in paraffin. Ten-micrometer sections were cut from the frozen specimen at a temperature of -20°C and mounted on chrome-alum-gelatine coated glass slides. Fixation of the frozen sections was performed with acetone for 10 min at -20°C. After drying at room temperature, the frozen sections were pre-incubated for 45 min in Blotto's non-fat dry milk solution (Duhamel and Johnson, 1985).

From the paraffin-embedded specimen, meridional sections were cut at 5 µm and mounted on 0.1% poly-L-lysine-coated slides. The sections were deparaffinized and pre-incubated for 20 min in normal rabbit serum (5% in PBS).

After pre-incubation, both frozen and paraffin sections were incubated with the primary antibody for 90 min. For demonstration of the intermediate filament desmin, monoclonal mouse antibodies (Dakopatts, Hamburg, Germany) were used [clone DE-R-11, anti-swine IgG1 (Debus, Weber and Osborn, 1983) and clone D33, anti-human IgG1] both at a dilution of 1:50. For vimentin, a monoclonal mouse anti-swine antibody from Dakopatts (clone V9, IGG1; Osborn, Debus and Weber, 1984) at a dilution of 1:5 was applied to the slides. The antibodies were diluted in 0.1 M PBS, pH 7.2-7.4. Demonstration of  $\alpha$ -smooth muscle (sm)-actin was performed with a monoclonal mouse anti-bovine antibody to smooth muscle specific  $\alpha$ -sm-actin from Sigma (clone no. 1A4, IgG2a; Skalli et al., 1986) diluted in PBS (1:150) with 1% bovine serum albumin (Sigma). Each incubation was performed in a moist chamber at room temperature. The sections were washed three times with PBS, each for 10 min and incubated with fluorescein or rhodamine-labelled rabbit-anti-mouse IgG (Dakopatts) diluted with PBS (1:20). The sections were washed three times in PBS and mounted in Entellan (Merck, Darmstadt, Germany) containing 2.5% 1,4-diazobicyclo-octane (Merck; Johnson et al., 1982). Control experiments were performed using either PBS or a mouse pre-immune serum instead of the primary antibody.

For staining of cell cultures, cells were seeded in tissue culture chambers mounted on Permanox® microslides (Lab Tek®, Nunc Inc. Naperville, IL). Cells

from the first to fifth passages of all established cultures were stained 1, 4, 7 and 21 days after seeding. Culture medium was removed by rinsing three times with PBS. The cells were fixed with methanol for 3 min at -20°C; then incubated for 90 min with the same primary antibodies at the same dilution as used for tissue staining. Additionally, the cells were stained with monoclonal mouse antibodies against human von Willebrand-factor (factor VIII associated antigen) from Dakopatts, clone F8/86, IgG1, (Naiem et al., 1982) at a dilution of 1:50 and bovine  $\alpha$ -actinin from Sigma, clone no. BM-75-2, IgM, at a dilution of 1:200. The cells were rinsed three times with PBS then incubated for another 60 min with a fluorescein-conjugated rabbit anti-mouse IgG (Dakopatts) at a dilution of 1:20. The cells were washed again three times with PBS, then the tissue chambers were removed from the glass slides. The slides were mounted in Entellan containing 2.5% 1,4-diazobicyclo-octane. Control experiments were performed using either PBS or a mouse pre-immune serum instead of the primary antibody.

Stained cells and tissue sections 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 or Ektachrome 400 colour slide film (Kodak Limited, Hempstead, U.K.) were used for photography.

#### *Electronmicroscopy*

The ultrastructure of cells from pre-confluent cultures (1 and 4 days), confluent (7 days) and highly confluent cultures (3 and 4 weeks) of second to fifth passages was investigated from all established cell cultures. The cells were grown in uncoated, plastic Petri dishes or in tissue culture flasks and fixed with Ito's solution for 4 hr. The fixed cells, still in the plastic dish, were post-fixed with 1% osmium tetroxide, dehydrated with graded alcohols and embedded in Epon. Polymerization was done at 60°C. Tangential and perpendicular sections of the cells were cut on an ultramicrotome. The sections were treated with lead citrate and uranyl acetate. For electron microscopic examination a JEOL (JEM 100 B) and a Zeiss (EM 902) electronmicroscope were used.

### **3. Results**

#### *Immunocytochemistry*

*Tissue sections.* Ciliary muscle cells in tissue sections showed a pronounced immunostaining with antibodies against smooth muscle (s.m.) specific  $\alpha$ -actin [Fig. 1(A)]. This was true for all parts of the ciliary muscle. Similar staining was also observed in the vascular smooth muscle cells of the media of the major arterial circle of the iris [Fig. 1(A)] and in pericytes, which were rarely observed around the capillaries of

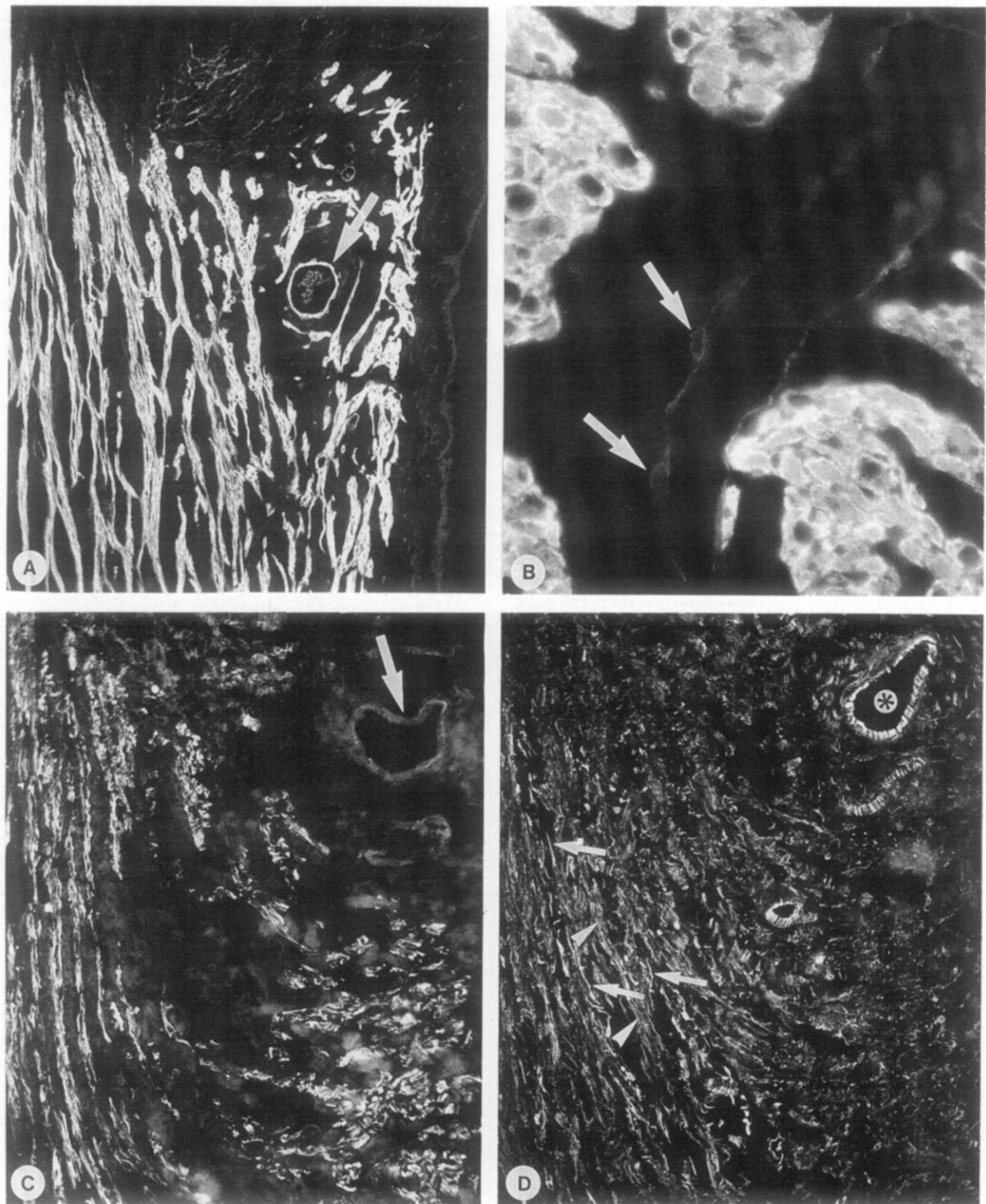


FIG. 1. Immunocytochemistry of ciliary muscle in tissue sections. A, Ciliary muscle cells and vascular muscle cells both stain positive for  $\alpha$ -sm-actin. The vascular muscle cells belong to the vessels of the major arterial circle of the iris (arrows), which pass near the muscle's inner circular portion, (paraffin section,  $\times 230$ ). B, Positive staining for  $\alpha$ -sm-actin is also seen in the pericytes around some of the ciliary muscle capillaries (arrows; paraffin section,  $\times 1000$ ). C, Stain for desmin is seen in ciliary muscle cells only. The vascular muscle cells (arrow) are not stained (frozen section,  $\times 300$ ). D, Endothelial cells, vascular muscle cells around the ciliary body arteries (asterisk) and the fibroblasts (arrows) in the ciliary muscle stain for vimentin. Faint immunostaining for vimentin is also seen in all ciliary muscle cells (arrowhead; frozen section,  $\times 300$ ).



FIG. 2. Histological control section of a ciliary muscle explant. The explant consists of muscle bundles from the meridional and partly the reticular portion. No ciliary epithelium, or stroma from the ciliary processes or from the choroid is present. Vessels with a typical thick media of smooth muscle cells are not seen (paraffin section, Crossmon's strain,  $\times 150$ ).

the ciliary muscle [Fig. 1(B)]. The fibroblasts of the ciliary muscle and of the stroma of the ciliary processes did not stain. There were no differences in intensity or distribution of the stain, when frozen sections were compared with paraffin sections. In contrast, only ciliary muscle cells stained for desmin, not the pericytes, vascular smooth muscle cells or fibroblasts of the ciliary body [Fig. 1(C)]. The intensity of the staining was much more pronounced in frozen sections than in paraffin sections. Endothelial cells, pericytes, vascular smooth muscle cells and fibroblasts stained for vimentin on paraffin sections. On frozen sections, however, faint immunostaining for vimentin was also observed in all ciliary muscle cells [Fig. 1(D)].

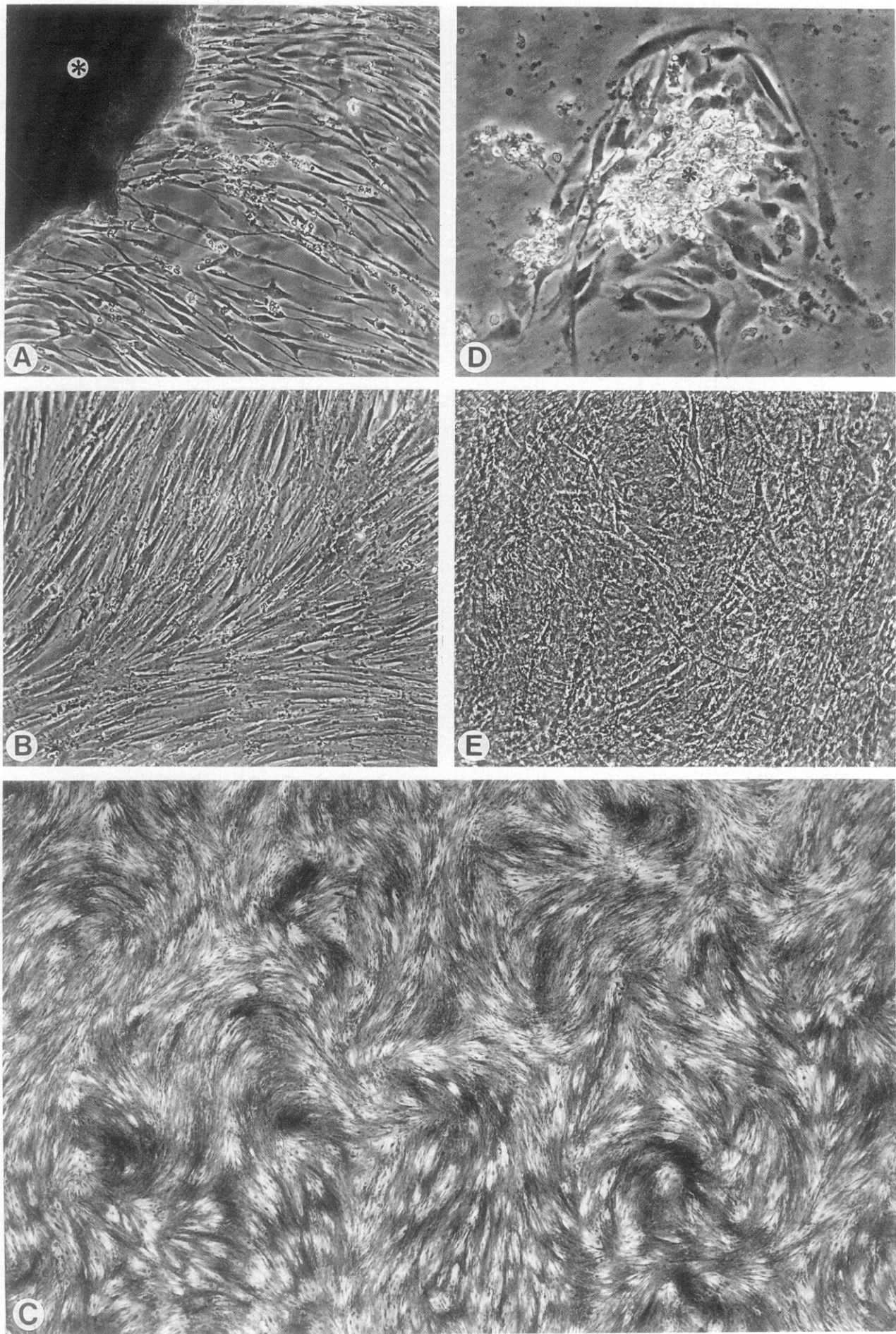
#### Cell Culture

Both light and electron microscopy confirmed that the explants from the ciliary muscle of all donors consisted only of the meridional and partly the reticular portion of the ciliary muscle. No ciliary epithelium, scleral spur, trabecular meshwork and tissue from the stroma of the ciliary processes or from

the choroid were present (Fig. 2). Vessels with smooth muscle cells in the media, which are located in vivo near the circular portion of the ciliary muscle were not observed. The size of the explants varied with the age of the donors. Explants, which were harvested from older donors ( $> 60$  year old) were about half the size of those from younger donors ( $< 35$  year old).

If the explants were cultured as a whole (method 1), initial outgrowth of cells appeared within 2–4 weeks after placing the explants in culture [Fig. 3(A)]. Explants from all dissected younger donors ( $< 35$  year old) grew out even when the post-mortem time extended up to 48 hr (Table I). In contrast, outgrowth of explants from donors more than 35 year old could only be established, if the post mortem time was shorter than 5 hr (Table I). Cells grew from all sides of the explant and were characteristically bipolar, long and cylindric or ribbon-like with oval nuclei. The cells showed long tapering fusiform ends, which usually formed smaller lateral filopodia. When the cells grew to higher densities, they were still elongated but became thinner. At this stage, the ends of the cells usually showed no branching, the cells were closely attached to each other and arranged themselves in a parallel fashion [Fig. 3(B)]. Thus, the culture became organized into a monolayer consisting of longitudinal and slightly curved bands of parallel cells. This growth pattern was seen in all primary cell cultures regardless of the age of the donors. The cells of the primary cultures grew to confluency within 2 months. When the primary cultures were subcultured at a split ratio of 1:4, they usually reached confluency within 7 days. At low densities, subcultured cells were bipolar but broader than the outgrowing cells from the primary cultures. A characteristic feature of the cells was the presence of longitudinal ridges in the cytoplasm. At confluency, the cells showed the same shape and spatial organization as seen in confluent primary cultures. If cells were maintained in the same flask after reaching confluency for up to 4 weeks, the longitudinal arrays of parallel cells displayed a curved pattern terminating in whorls, which consisted of hillocks of multilayered cells and extracellular matrix [Fig. 3(C)]. This growth pattern is similar to the 'hill and valley' growth pattern of smooth muscle cells in culture. Cell cultures showing this growth pattern were named type A cells (smooth muscle-like). All different type A cultures could be subcultured up to the fifth passage, without showing any changes in their growth pattern.

In about one-third of the cases, however, another cell type (type B) grew out from the explants. These cells were of variable size and shape, usually polygonal and broader than those cells previously described. As soon as this cell type grew to higher densities, the cells formed irregular multiple layers, similar to those seen in cultures of skin fibroblasts [Fig. 3(E)]. If both cell types, namely type A (smooth muscle-like) and type B (fibroblast-like) grew out from the same explant, the



type B cells grew out 7–10 days later than the type A cells. If they grew out from different parts of the explants, it was possible to remove the type B cells from the culture as described in Materials and Methods. Without removal, the type B cells overgrew the type A cells quite easily within 1–2 weeks.

In suspensions of ciliary muscle cells (method 2) approximately 10% of the cells settled within 48 hr on the culture substrate. In about 50% of the cases these cells started to divide 1 week after initiation of the culture [Fig. 3(D)], preferably in areas where the cells had settled as cell clumps. Initially, small cell clones of bipolar ribbon or spindle-shaped cells were formed. After several days, these clones also arranged themselves in longitudinal bands of parallel cells and were not distinguishable from the type A cells grown directly from the explants. Clones consisting of the type B cell type could not be observed.

With both methods, ten cell cultures showing the type A (smooth muscle-like) growth characteristics could be established from donors with an age range of 16–91 yr. The growth pattern was the same in all type A cultures, regardless of the age of the donors.

#### *Immunocytochemistry Cell Cultures*

(a) *Type A (smooth muscle-like cells)*. Cells from pre-confluent cultures, which were grown for 4 days, invariably stained positive for  $\alpha$ -sm-actin. The staining revealed typical straight, non-interrupted, cable-like fibers running parallel to each other along the long axis of the cells. When stained with antibodies against  $\alpha$ -actinin, the fibers were decorated in an interrupted pattern with closely spaced beads of  $\alpha$ -actinin. The beads on neighbouring stress fibers were often aligned, giving the cell a striated appearance [Fig. 4(A)]. In regions where the stress fibers terminated near the cell's margin, staining for  $\alpha$ -actinin was seen in uninterrupted lines or bands. These lines extended from the cell margin for some distance towards the interior, running roughly parallel to the straight or gently curved edges of the cells.

At confluency, the whole cytoplasm of the cells was filled with densely arranged parallel fibers, staining brightly both for  $\alpha$ -sm-actin and  $\alpha$ -actinin [Figs 4(B) and (C)]. With antibodies against intermediate filaments, all cells stained positive for vimentin [Fig. 4(D)]. The vimentin filaments formed typical baskets around the nucleus and extended in gently curved arrays towards the cells' periphery. Pre-confluent

cultures, grown for 4 days, did not stain for desmin. In confluent cultures, however, a small amount of cells stained positive for desmin [Fig. 4(E)]. This was most pronounced in highly confluent cultures, which were grown for 3–4 weeks. In these cultures, the desmin was mainly expressed in areas where the cells grew in multilayers and formed hillocks [Fig. 4(F)]. Because of their arrangement in multilayers, quantitation of desmin-positive cells in these cultures was difficult. When the cultures were passaged and the cells allowed to settle on the substratum for 6 hr, approximately 10% of the cells showed perinuclear aggregates of desmin-positive filaments.

(b) *Type B (fibroblast-like cells)*. In both of the two cultures, intracellular fibers stained positive for  $\alpha$ -actinin. Staining for smooth-muscle specific  $\alpha$ -sm-actin, however, was only observed in 5–10% of the cells. In contrast to the type A cultures, the amount of fibers was markedly reduced in most of the cells, when the fibroblast cultures grew to high density. While all cells in the fibroblast cultures stained positive for vimentin, no staining was observed for desmin, neither in pre-confluent nor in confluent cultures.

#### *Electronmicroscopy*

(a) *Type A culture (smooth muscle-like cells)*. In pre-confluent cultures, the perinuclear cytoplasm of the cells was filled with extensive aggregates of rough endoplasmic reticulum and a prominent Golgi apparatus. The rough endoplasmic reticulum was often dilated and filled with amorphous material. Many of the cells had lysosomes with myelin figures or electron-dense material. The cells contained numerous parallel fibers, consisting of bundles of thin (actin) filaments with associated dense bodies [Fig. 5(A)]. The bundles passed through the cytoplasm near the cell membrane and were mainly oriented along the longitudinal axis of the cells. Interspersed between the bundles were numerous elongated mitochondria, free ribosomes, coated vesicles, accumulations of glycogen and lipid droplets. Near the periphery, long parallel rows of membrane bound caveolae were seen. These caveolae were aligned along the bundles of thin filaments. The thin filaments ended in membrane-bound dense bands, which often extended for some distance into the cells. Occasionally, the cells were connected by lateral filopodia, which formed adherens-type or intermediate junctions and gap junctions at their ends. After 4 days

FIG. 3. Cell cultures derived from ciliary muscle. A, Outgrowing type A cells from a ciliary muscle explant (asterisk). The cells are characteristically bipolar, with long tapering fusiform ends (phase-contrast micrograph,  $\times 125$ ). B, After confluency, smooth muscle-like (type A) cultures become organized into a monolayer consisting of bands of parallel cells (phase-contrast micrograph,  $\times 125$ ). C, Confluent type A cultures. The cells grow in longitudinal arrays of parallel cells, which bend and terminate in whorls, which consist of hillocks of multilayered cells (light micrograph, PLP-fixed cells, stained with methylene-blue,  $\times 70$ ). D, After digestion of ciliary muscle explants, small cell clones of ribbon or spindle-shaped cells are formed, preferably in areas where the cells have settled in clumps (asterisk) (phase-contrast micrograph,  $\times 260$ ). E, Fibroblast-like cells (type B) derived from the ciliary muscle grow in irregular multilayers (phase-contrast micrograph,  $\times 125$ ).

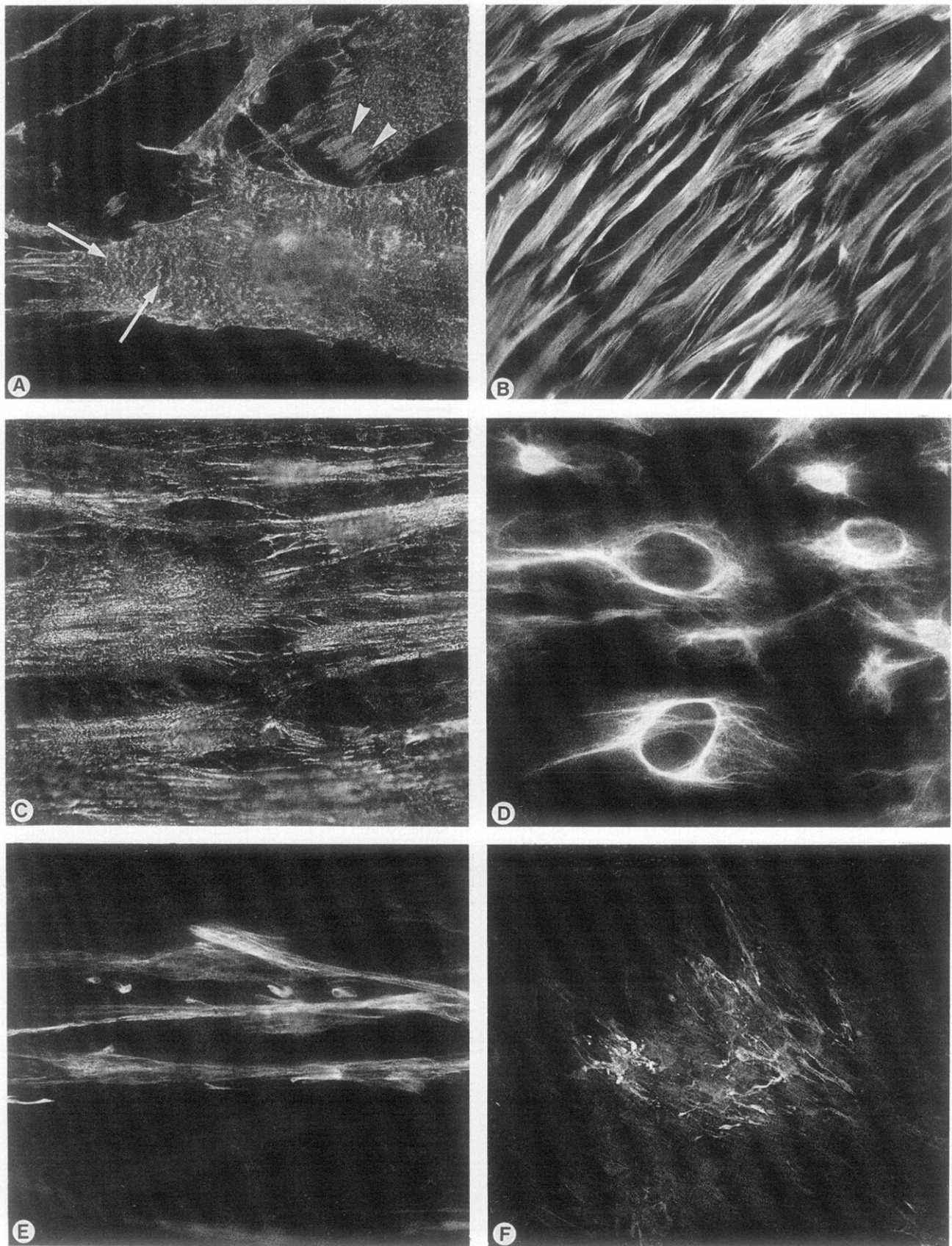


FIG. 4. Immunofluorescence of smooth muscle-like (type A) cultures. A. Stained for  $\alpha$ -actinin, intracellular fibers are decorated with closely spaced beads of  $\alpha$ -actinin. The beads of neighbouring fibers are aligned, giving the cell a striated appearance (arrows). In addition the focal contacts of the muscle cells are stained (arrowheads) ( $\times 1100$ ). B and C. At confluency, the whole cytoplasm of the cells is filled with densely arranged parallel fibers, staining brightly both for  $\alpha$ -sm-actin (B,  $\times 320$ ) and  $\alpha$ -actinin (C,  $\times 900$ ). D. All cultured muscle cells stain for vimentin. The vimentin filaments display typical

in culture, a small and incomplete basal lamina surrounded the cells. In confluent cultures, the elongated cells were connected by intermediate junctions while gap junctions were rare. The rough endoplasmic reticulum consisted of a few juxtanuclear cisternae associated with the nuclear envelope and a small Golgi apparatus. The peripheral cytoplasm was filled with thick bundles of actin filaments densely aggregated with no other cell organelles between them. The bundles were not confined to the superficial region of the cell as in non-confluent cells, but were present throughout the whole cytoplasm.

(b) *Type B cells (fibroblast-like cells)*. The ultrastructure of non-confluent type B cells was remarkably similar to that of non-confluent type A cells. Bundles of thin (actin) filaments, however, were much rarer. In confluent cultures, type B cells showed, in contrast to confluent type A cells, many aggregates of dilated rough endoplasmic reticulum and a pronounced Golgi apparatus, while bundles of thin filaments were still only rarely observed.

#### 4. Discussion

In the present study we report on methods to establish homogenous primary cell cultures derived from human ciliary muscle. Donors of different ages were used. After a careful and histologically controlled dissection procedure, two different cell types, a smooth-muscle like (type A) and a fibroblast-like (type B) can be grown from explants or isolated by enzymatic digestion. These cells retain typical structural characteristics for at least five passages in culture. We conclude that the smooth muscle-like cultures consist of ciliary muscle cells in culture. The fibroblast-like cells are probably derived from fibroblasts in the ciliary muscle.

Ciliary muscle cells from all established cell cultures show a distinct growth pattern seen in phase contrast optics. In confluent cultures, the muscle cells grow in a 'hill and valley' pattern, which is regarded as typical for vascular and visceral smooth muscle cells in culture (Gimbrone and Cotran, 1975; Chamley-Campbell, Campbell and Ross, 1979).

The characteristic growth pattern of ciliary muscle cultures facilitates the discovery of contamination by fibroblasts derived from the ciliary body. The fibroblasts show the same disorganized multilayered growth pattern seen with scleral fibroblasts or keratocytes (Polansky et al., 1979; Hernandez, Igoe and Neufeld, 1988).

The purity and homogeneity of the cell cultures was further confirmed by immunocytochemistry. The

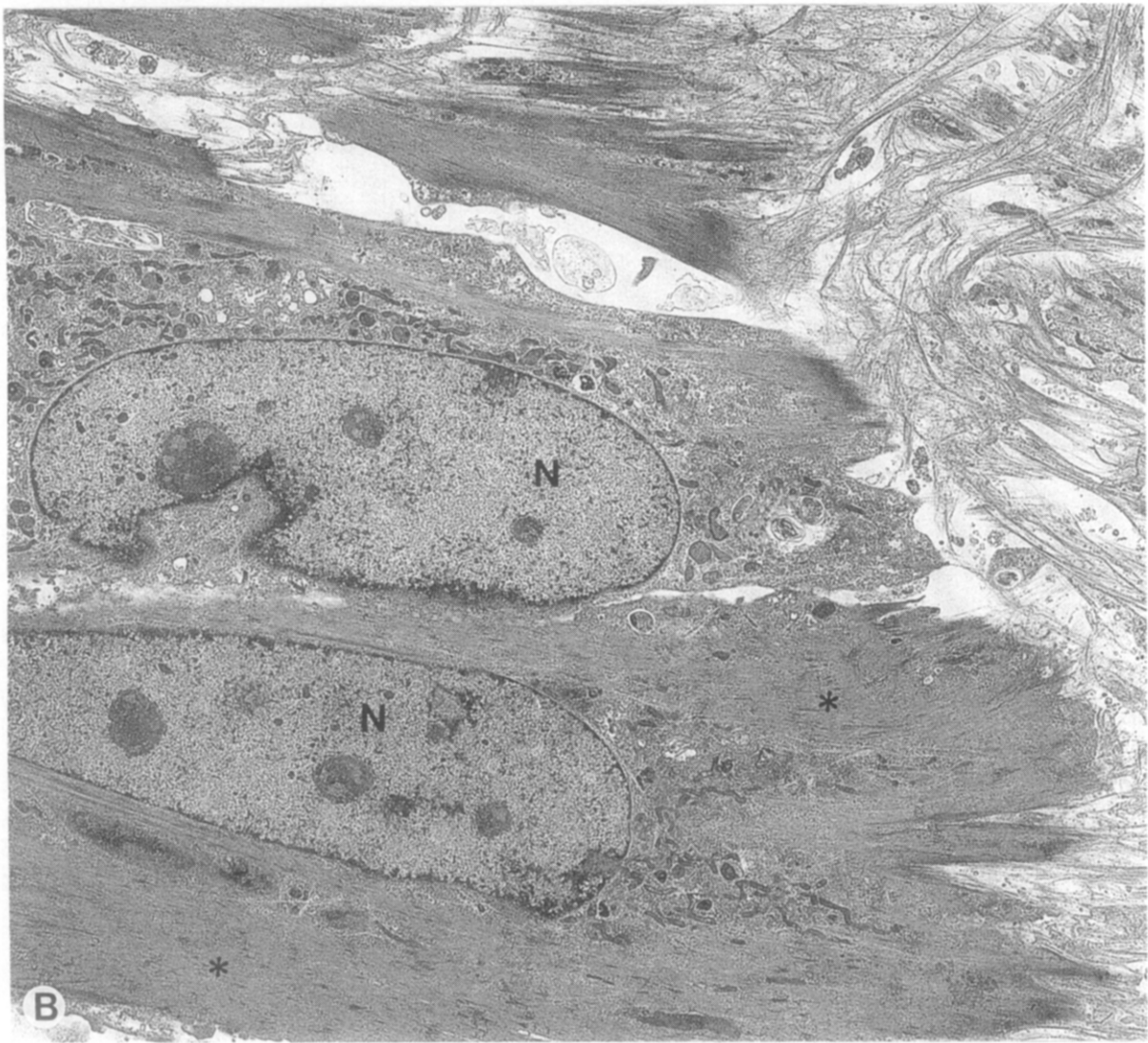
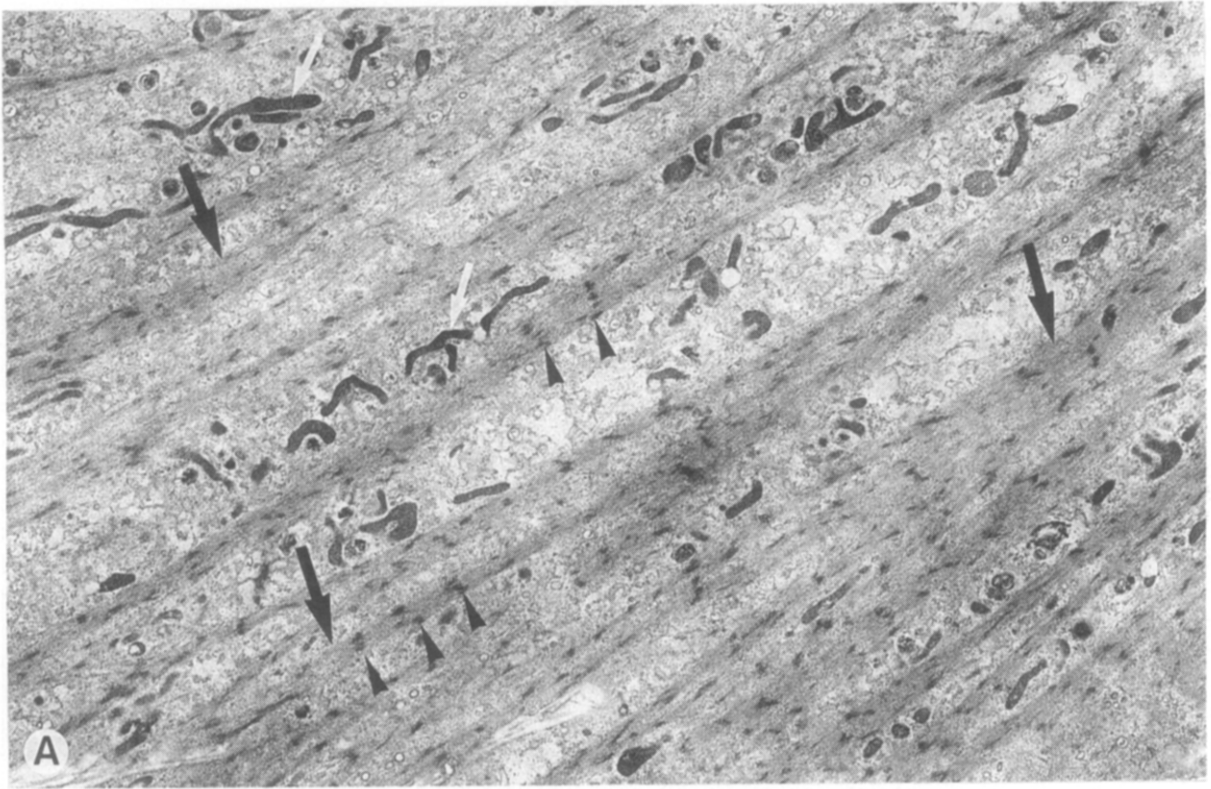
presence of actin isoforms has been proposed as a useful marker for distinguishing between smooth muscle cells and fibroblasts both in vivo and in vitro (Gown et al., 1985; Skalli et al., 1986; Skalli, Vandekerckhove and Gabbiani, 1987). We used an antibody against the  $\alpha$ -actin isoform, which is specific for smooth muscle (Vandekerckhove and Weber, 1979). This antibody has been shown to stain specifically smooth muscle cells of different origins as well as myofibroblasts in tissue sections (Skalli et al., 1986, 1987; Czernobilsky et al., 1989; Darby, Skalli and Gabbiani, 1990; Flügel, Tamm and Lütjen-Drecoll, 1991). Also under culture conditions, smooth muscle cells still express their  $\alpha$ -isoactin (Skalli et al., 1986; Owens et al., 1986), while fibroblasts in culture usually continue to express the non-muscle actin isoforms only (Garrels and Gibson 1976; Gown et al., 1986; Skalli et al., 1986). Our study shows that both the ciliary muscle cells in tissue sections as well as the cell lines derived from them stain uniformly for  $\alpha$ -sm-actin, while the fibroblasts of the ciliary body in situ do not. Additionally, in cultures which are grown from ciliary body fibroblasts, less than 10% of the cells express  $\alpha$ -actin-positive filaments, possibly because of a contamination with muscle cells.

The presence of endothelial cells in our cultures can be excluded by the lack of a typical endothelial cell growth pattern (Gimbrone, Cotran and Folkman, 1974) as well as by the lack of staining for von Willebrand-factor (factor VIII associated antigen: Jaffe et al., 1973). In tissue sections, positive staining for  $\alpha$ -sm-actin is not confined to the ciliary muscle cells. Staining is also seen in the smooth muscle cells of the media of the major arterial circle of the iris as well as, similar to retinal pericytes (Herman and D'Amore, 1985), in the pericytes of some of the ciliary muscle capillaries. The major arterial circle, however, was avoided in the dissection procedure. Compared with our cell lines, retinal pericytes in culture show a very different growth pattern (Buzney et al., 1983). The pericytes of the ciliary muscle capillaries are just a few in number when compared to the vast majority of the ciliary muscle cells. Therefore, it is not very probable that  $\alpha$ -actin-positive cells in our cultures are derived from other sources than ciliary muscle cells.

Cells can further be characterized by their content of intermediate filaments, which are cell-type-specific and differentiation-dependent (for review see Osborn and Weber, 1983). The present study shows that human ciliary muscle not only stains for the muscle-specific intermediate filament desmin, but also for vimentin. The same is the case for skeletal myoblasts in culture (Gard and Lazarides, 1980) and in tissue sections (Van Muijen, Ruiter and Warnaar, 1987), as

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baskets around the nucleus and extend in gently curved arrays towards the cells' periphery ( $\times 900$ ). E and F. In confluent cultures, about 10% of the cells stain for desmin (E,  $\times 800$ ). Desmin-positive cells are predominately located in areas, where the cells grow in multilayers (F,  $\times 240$ ).



well as for vascular smooth muscle in certain arterial vessels (Travo, Weber and Osborn, 1982; Schmid et al., 1982; Kocher et al., 1984; Fujimoto, Tokuyasu and Singer, 1987). In contrast to the ciliary muscle, the muscle cells of the media of the major arterial circle of the iris belong to that kind of vascular smooth muscle cells, which express vimentin only (Frank and Warrne, 1981; Gabbiani et al., 1981; Osborn, Caselitz and Weber, 1981). The fact that in vitro desmin-positive cells were stainable in confluent ciliary muscle cultures only, might be due to a dedifferentiation of the cells in pre-confluent cultures. It is also known for other cultured smooth muscle cells (Skalli et al., 1986; Palmberg and Thyberg, 1986; Ricciardelli et al., 1989) that loss of desmin-positive cells occurs in vitro. Interestingly, in highly confluent ciliary muscle cultures staining for desmin is mostly found in the multilayered hills, where the cells have produced a lot of extracellular matrix. It might be that some components of the extracellular matrix promote the desmin expression of the muscle cells. It has been reported that skeletal myoblasts, grown on laminin substrata differentiate and express more desmin (von der Mark and Öcalan, 1989).

Smooth muscle cells in sub-confluent growing cultures also show other signs of dedifferentiation, e.g. a relative loss of myofilaments together with an increase in rough endoplasmic reticulum and the presence of a large Golgi apparatus (for review see Chamley-Campbell et al., 1979). The same ultrastructural changes are observed in ciliary muscle cultures. But even pre-confluent cultures stain for  $\alpha$ -sm-actin and all cells in confluent cultures recover the typical characteristics of cultured smooth muscle cells (for review see Chamley-Campbell et al., 1979). The morphological grade of differentiation was much higher in these cultures derived from adult ciliary muscle than in a cell line derived from a 1-day-old infant, which was established recently (Korbmacher et al., 1990). This difference might be due to the fact that ciliary muscle of newborn primates is not fully differentiated even in situ and shows a more mesenchymal appearance (Lütjen-Drecoll, Tamm and Kaufman, 1988).

Thus, cultured ciliary muscle cells do not fully preserve their typical in vivo ultrastructure, which has been reported to be very characteristic and different from other smooth muscle cells (Ishikawa, 1962; Lütjen-Drecoll et al., 1988; Flügel, Bárány and Lütjen-Drecoll, 1990). Several structural characteristics, however, seem to be conserved in cultures. Staining for  $\alpha$ -actinin shows that the dense bands of the myofilaments in cultured cells are aligned in register,

a feature, which has also been reported to be characteristic for ciliary muscle cells in tissue sections (Van der Zypen, 1967; Lütjen-Drecoll et al., 1988).

### Acknowledgements

We would like to thank Jutta Gehr and Ute Maurer for their expert assistance with tissue cultures and immunocytochemistry. We would also like to thank Simone Klein for her excellent help in electronmicroscopy and Marco Gößwein for his excellent preparation of the photographs. The study was supported by grants from the Deutsche Forschungsgemeinschaft (Dre 124/6-1 and Ro 81/18-4).

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FIG. 5. Electronmicrographs of cultured smooth muscle-like (type A) cells. A, In pre-confluent cultures, the cytoplasm of the cells is filled with numerous parallel bundles of thin (actin) filaments (arrows) with associated, regular arranged dense bodies (arrowheads). Between the filaments numerous elongated mitochondria are seen (white arrows) ( $\times 6600$ ). B, At confluency, the filaments (asterisks) are densely aggregated. N, Nucleus. ( $\times 3000$ ).

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