Nonvascular Contractile Cells in Sclera and Choroid of Humans and Monkeys

Vadims Poukens,1,2 Ben J. Glasgow,1,2 and Joseph L. Demer1,3

PURPOSE. To investigate by histochemistry and immunohistochemistry the distribution and innervation of nonvascular contractile cells in the sclera and choroid of humans and monkeys.

METHODS. Globes were obtained from 2 macaque monkeys and 19 human cadavers that ranged in age from fetal life to 94 years. Immunohistochemistry was performed using monoclonal antibody against human smooth muscle (SM) α-actin and tyrosine hydroxylase (TH). The nicotinamide-adenine dinucleotide phosphate (NADPH)-diaphorase reaction was used as a marker for nitric oxide synthase.

RESULTS. The scleras of all but fetal, newborn, and infant globes exhibited myofibroblasts,amelanotic, fibroblastlike cells having SM α-actin immunoreactivity. In the choroid of all but fetal eyes, SM cells were present in the suprachoroidal layer, forming a reticulum of flattened laminae, and in the choriocapillaris where ovoid-to-spindle-shaped SM cells were arrayed in parallel layers immediately adjacent to Bruch’s membrane. Contractile cells in the sclera and choroid were most concentrated subfoveally and were sparse anteriorly. Nerve terminals positive for NADPH-diaphorase were colocated with SM α-actin-positive cells in the sclera and choroid, whereas TH-positive nerve terminals colocaled with SM cells in the choroid. Clusters of ganglion cells were present on the posterior surface of globes near SM cells.

CONCLUSIONS. The posterior choroid and sclera of humans and monkeys contain nonvascular contractile cells. The presence of nerve terminals and adjacent ganglion cells suggests neural control of these contractile cells. The absence of such contractile cells in fetal, newborn, and infant eyes is an argument against a major role of these cells in promoting ocular enlargement. These contractile cells may instead participate in regulation of refractive state by maintenance of ocular size in the face of intraocular pressure or in intermediate-term regulation of choroidal thickness.

The finding of apparent accommodation in pseudophakic humans suggests that there may exist a mechanism for altering the refractive state of the eye by altering axial length.1 Studies performed in chickens by Wallman et al.2,3 showed that ocular length changes associated with spectacle-lens-induced hyperopia and myopia are mediated first by changes in the thickness of the choroid, followed later by changes in the growth rate of the sclera. Wallman and colleagues2,3 proposed that the extravascular smooth muscle (SM) found to be present in the avian choroid by other investigators4 might participate in modulation of choroidal thickness to regulate refractive state. Such a choroidal mechanism for regulation of refractive state would be of considerable interest if present in humans.

The human uveal tract has long been recognized to contain SM, particularly in the iris dilator, iris sphincter, and ciliary body. There is conflicting literature regarding the presence of SM elsewhere in the human eye. As early as 1859, Müller5 described light microscopy of single SM cells in the human choroid, but this finding was disputed by anatomists at the turn of the century.6,7 Only a few years later Fuchs8 described “unstriped muscle fibers” around the human optic nerve. Although in subsequent light microscopic studies other investigators interpreted SM in the human choroid to be heterotopic,9,10 the most recent electron microscopic and immunohistochemical studies have shown a network of SM α-actin-positive cells throughout the human choroid.11

Myofibroblasts (MFBs) have a phenotype similar to fibroblasts but also contain extensive contractile actin and myosin.12 Fibroblasts may be induced to differentiate into MFBs13,14 and are present in granulation tissue.15 However, stable populations of MFBs also occur.12 The presence of MFBs in normal sclera has been described in the human scleral spur,16 in the vicinity of Schlemm’s canal in the rat,17 and in the aqueous outflow region in the bovine,18 but has not been found elsewhere in the normal sclera of humans or animals. If widely present in the sclera, MFBs might have an important role in the regulation of ocular size or shape and thereby, refractive error.

The purpose of the study was to investigate the distribution of nonvascular contractile cells in the sclera and the
choroid of humans and monkeys and to assess their innervation using histochemistry and immunohistochemistry.

**MATERIALS AND METHODS**

**Specimens and Fixation**

Globes were obtained from 19 human cadavers without known ocular diseases and 2 adult macaque monkeys. The human specimens were obtained at autopsy 6 to 36 hours after death. The two monkeys underwent intra-aortic perfusion with neutral-buffered paraformaldehyde after they were euthanatized by barbiturate overdose in conformity with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the American Veterinary Medical Association. After fixation in 10% neutral-buffered formalin and paraffin embedding, eight human globes were sectioned horizontally through the lens and posterior pole (specimens 3, 5, 11, 13, 14, 15, 16, and 17), and seven globes were serially sectioned in the corneal plane (human specimens 1, 2, 5, 12, 14, and 19 and macaque monkey specimen 20). Eyes from five human cadavers and one macaque monkey (human specimens 4, 7, 8, 9, and 10 and macaque monkey specimen 21) were fresh-frozen, sectioned horizontally through the lens and posterior pole, and then fixed in ethanol.

**Histochemistry**

Stepwise and serial 10-μm-thick sections were mounted on gelatin-coated glass slides. Masson’s trichrome stain was used to show collagen, and van Gieson’s stain was used to indicate elastin. The nicotinamide-adenine dinucleotide phosphate (NADPH)-diaphorase reaction was used as a marker for neuronal nitric oxide synthase, a gaseous neurotransmitter and neuromodulator commonly associated with SM. To show the NADPH-diaphorase reaction, mounted frozen sections were incubated in 0.1 M phosphate buffer, pH 7.4, containing 0.3 Triton X-100, 0.1 mg/ml nitroblue tetrazolium (Sigma Chemical, St. Louis, MO) and 1.0 mg/ml b-NADPH (Sigma) at room temperature for 30 to 60 minutes. Some sections were lightly counterstained with nuclear fast red.

**Immunohistochemistry**

Myofibroblasts and SM were confirmed using monoclonal antibody raised in the mouse against human SM α-actin (Dako, Copenhagen, Denmark) applied at 4°C overnight at dilutions varying from 1:200 to 1:400. Tyrosine hydroxylase (TH) immunoreactivity was shown using a monoclonal mouse antibody (Instar Corporation, Stillwater, MN) applied overnight at 4°C at a dilution of 1:300. Sections of cadaveric human adrenal medulla served as a positive control. For each antibody, non-specific peroxidase was blocked using 3% H2O2 for 5 minutes. A secondary antibody, biotinylated anti-mouse IgG (Vector Laboratories) were used as chromogens. Vascular SM provides an internal positive control in each section stained. Using a micrometer, we compared the thickness of the zone of immunoreactive cells with scleral thickness in the same section.

**RESULTS**

The monoclonal antibody against human SM α-actin was specific for vascular and uveal SM in human specimens but slightly cross-reacted with the monkey’s striated muscles and labeled SM. As described below, some fibroblastlike cells in human sclera were also immunoreactive to SM α-actin.

We examined globes from 19 humans ranging in age from 11 weeks' gestation to 94 years old (8 females, 11 males) and from 2 male monkeys, 34 months and 7 years old. The characteristics of subjects and the immunoreactivity of their scleral and choroidal tissues to human SM α-actin are shown in Table 1. Many specimens within sclera and choroid amelanotic, nonvascular fibroblast-like cells with dendriform processes have SM α-actin immunoreactivity (Fig. 1). These cells are considered to be MFBs. Immunoreactivity in MFBs was shown using both the blue alkaline phosphatase chromogen (Fig. 1A), avoiding ambiguity with endogenous melanin in specimens, and with the brown diaminobenzidine chromogen (Fig. 1B), which provides superior cytologic detail.

Nine globes (from humans ages 59, 65, 68, 68, 70, 72, and 79 years and both monkeys) showed a substantial distribution of MFBs within the inner sclera that were reactive for SM α-actin. Eight globes (from humans ages 4, 17, 31, 37, 37, 48,
Choroid

B

FIGURE 1. Coronal 10-μm-thick section of globe from 59-year-old male human (specimen 11) taken tangential to the scleral surface near the posterior pole. (A) Staining for smooth muscle (SM) α-actin shows positive immunoreactivity (blue chromogen) in inner sclera and laminated structures of the suprachoroid. Counterstained with nuclear fast red. (B) Scleral section adjacent to the fovea and parallel to the scleral surface stained using diaminobenzidine (brown) chromogen. Note fibroblastlike cells positive for SM α-actin.

61, and 94 years) showed scattered scleral MFBs, whereas the four globes from the youngest subjects (both fetuses, the 13-day-old infant, and the 17-month-old child) exhibited no SM α-actin reactivity within the sclera. Most of the MFBs were concentrated posteriorly in the inner sclera adjacent to the fovea, where they were distributed across as much as 20% of scleral thickness in humans and across as much as 80% in monkeys. The concentration of contractile cells gradually decreased anteriorly and was absent a few millimeters anterior to the equator. The MFBs were always oriented parallel to the scleral surface. We did not quantify the number of MFBs because it is difficult to separate MFB cell bodies from cell processes in sections perpendicular or tangential to the sclera (as in the majority of our specimens). Cell bodies of MFBs are best seen in sections parallel to the scleral surface as shown in Figure 1B.

In the choroid, SM α-actin-immunoreactive cells were present in two distinct distributions. The suprachoroid, the external layer of the choroid, contained SM α-actin-positive cells as a part of a reticulum of flattened laminae (Fig. 2A). These laminae alternately interdigitated at acute angles oblique to the choroidal surface and then separated to form flat spaces within the suprachoroid layer. These flat spaces within the reticulum did not contain erythrocytes and lacked an endothelium. The laminae also contained dendritic melanocytes, elastin fibers, and collagen bundles (Fig. 3). The organization of the suprachoroidal reticulum into flat spaces delimited by interconnected laminae was observed better in the globes that were fixed and processed whole...
FIGURE 2. Choroid and sclera from 59-year-old male human (specimen 11) immunostained for smooth muscle (SM) α-actin. (A) Suprachoroid containing SM α-actin-positive cells (blue chromogen) that form reticulum of flattened laminae, counterstained with nuclear fast red. (B) Internal choriocapillaris contained ovoid-to-spindle-shaped single cells (brown chromogen) located immediately below and parallel to Bruch’s membrane. Melanin in retinal pigmented epithelium (RPE) cells is darker than the brown immunoreaction product. The myofibroblasts (MFBs; arrows) are distinct from the vascular SM of blood vessels, which have ovoid lumina. Several leukocytes in a blood vessel stain lightly with hematoxylin and also with DAB despite peroxidase blocking. Counterstained with hematoxylin.

(specimens 1, 2, 5, 11, 13, 19, and 20), without cutting before embedding (Figs. 2, 3), compared with the remainder of specimens that were cut before embedding. The internal choriocapillaris contained ovoid-to-spindle-shaped fibroblastlike cells immunoreactive to SM α-actin located in a single cell layer immediately below and parallel to Bruch’s membrane (Fig. 2B). Thirteen globes, including those of both monkeys, showed a substantial number of cells with at least focal labeling of all laminae of the full thickness of suprachoroid for SM α-actin. Six globes, including those of the newborn infant, showed scattered labeling of cells with SM α-actin, whereas two globes (both from stillborn fetuses) exhibited no extravascular SM α-actin reactivity. The nonvascular SM cells in the choroid were mostly concentrated in the area of the fovea centralis and gradually decreased anteriorly. Scattered nonvascular SM cells were noted in the laminae of the suprachoroid anteriorly up to the ciliary body where they made connections to the ciliary muscle fibers in a few specimens. Some nonvascular SM cells in the suprachoroid were connected directly to the MFBs of the inner sclera, especially in the foveal region (Fig. 1A).

The number of MFBs in the sclera increased with age. There were no extravascular contractile cells in fetal posterior sclera or choroid. Myofibroblasts were increasingly prevalent in the sclera of specimens from older subjects, although this tendency was not apparent in the choroid. Both monkey specimens contained numerous SM α-actin-positive cells in the sclera and choroid (Fig. 4). The older monkey’s globe contained more SM α-actin activity than did that from the younger, especially in the posterior sclera where SM cells were distributed across as much as 80% of the scleral thickness in the subfoveal area.

To show innervation of contractile cells, double labeling was performed on frozen sections of globes from humans and
FIGURE 3. Adjacent 10-μm-thick sections of sclera and choroid cut from a whole embedded eye of an adult human (specimen 11). Note laminated structure of suprachoroid. (A) Suprachoroid laminae and inner sclera contain cells positive for smooth muscle (SM) α-actin (blue chromogen, counterstained with nuclear fast red). Most SM α-actin-positive cells in the suprachoroid were myofibroblasts. (B) Staining with van Gieson's stain shows elastin fibers (black) in laminae of suprachoroid. (C) Staining with Masson's trichrome stain shows collagen (blue) in laminae of suprachoroid. RPE, retinal pigmented epithelium.
monkey. By staining with antibody against TH, the rate-limiting enzyme for catecholamine neurotransmitter synthesis, we were able to show varicose terminals colocalizing with SM α-actin-positive cells in the choroid (Fig. 5). There was no TH activity in scleral contractile cells of specimens from humans or monkeys.

The NADPH-diaphorase reaction was also performed on SM α-actin-labeled frozen sections from humans and monkeys.
Findings were similar in both. Positive NADPH-diaphorase reactivity was shown in fine axons and terminal varicosities within scleral (Fig. 6A) and choroidal (Fig. 6B) cells that had been stained with SM α-actin antibody, highly suggestive for the presence within nonvascular contractile cells of autonomic nerve terminals containing neuronal nitric oxide synthase. These neural structures resembled the innervation in vascular SM seen in the same sections (Fig. 6B). We carefully examined paraffin-embedded globes that had been serially sectioned and observed several clusters of ganglion cells on the posterior surface of globes from monkeys and humans near the cells with SM α-actin reactivity (Fig. 7). It was not possible to immunohistochemically identify neurotransmitters in these episcleral ganglion cells, because this would have required...
Ganglion Cells

FIGURE 7. Masson’s trichrome staining of ganglion cells in section of posterior sclera of globe from macaque monkey adjacent to the smooth muscle α-actin-positive cells (not shown).

serial sectioning of frozen specimens that was not practical to perform.

DISCUSSION

This study confirms and extends to monkeys the finding of extravascular SM α-actin-immunoreactive cells in the choroid of humans. Extravascular SM α-actin immunoreactive cells were present in the choroid of all globes from adults and newborn infants but not globes from the fetuses. The concentration of SM cells in the choroid did not vary dramatically over the adult age range. We also report the novel finding of fibroblastlike cells, also immunoreactive to SM α-actin, in the internal part of the posterior sclera in humans and monkeys. We interpret these cells to be MFBs. The absence of MFBs in the posterior sclera of globes from rapidly growing fetal and newborn subjects is an argument against a major role of these cells in promoting ocular enlargement. These cells evidently appear some time between 17 months and 4 years of age. Although the function of the MFBs in posterior sclera is unknown, they might participate in regulation of refractive state by changing the shape of posterior globe or maintenance of ocular size. It is interesting that eyes from the youngest subjects, the eyes lacking in MFBs, are from the age range in which excessive intraocular pressure produces ocular enlargement, whereas eyes from subjects older than 3 years of age do not undergo enlargement when intraocular pressure is high. Perhaps in the older individuals the MFBs, in conjunction with collagen and elastin, enable eyes to resist the lifelong distending effects of intraocular pressure in a manner analogous to the way that the SM and elastic tissues of the great arteries resist the constant effects of arterial pressure. Additional studies are required to determine whether the MFBs appear about the age of 2 years, the end of the rapid phase of human ocular growth.

Fibroblasts can differentiate into the MFB phenotype under the influence of many factors, including heparin, granulocyte macrophage-colony-stimulating factor, and helium-neon laser treatment. Cardiac MFBs express SM α-actin during right ventricular pressure overload. It is possible that intraocular pressure or another stimulus induces transformation of scleral fibroblasts to MFBs.

The organization of the suprachoroidal reticulum into flat spaces delimited by interconnected laminae has been what controversial. The appearance might be a result of shrinkage artifact from tissue processing. As an alternative, we propose that the spaces between SM laminae in the choroid are real. Most ophthalmic pathologists typically embed and section globes that have been cut open into callottes, a practice that alters the normal distribution of elastic forces in the sclera and choroid. We noted that the grill-like organization of the suprachoroid was observed best in the globes that were fixed and processed without cutting before embedding (Figs. 1, 2). In the globes opened before fixation or tissue processing, the flat spaces were not evident or were difficult to recognize, possibly because fluid was squeezed out of the spaces under the pressure of a knife or scissors (Fig. 5). The organization described here of the human and monkey suprachoroidal reticulum into flat spaces is similar to large lymphatic lacunae and the multilayered membra fusca of the suprachoroidea of a chicken’s eye and also similar to the lymphatic sinuistyle structures in the outer choroid of the Japanese monkey. However, the flat spaces observed here in the suprachoroidal reticulum lacked an endothelial lining, at least as could be discerned by light microscopy.
The finding of TH-positive, meandering, and bifurcating nerve terminals mingling with the SM in the choroid suggests a sympathetic innervation, as is generally the case for orbital SM.28-29 The observed morphology is typical of autonomic innervation to SM.30 Such sympathetic innervation was not observed in scleral MFBs.

The finding of NADPH-positive nerve terminals within the SM and MFB cells suggests a nitroergic,31 parasympathetic innervation. Many SM systems, including vascular, intestinal, biliary,32 and bladder,33 are known to be innervated by non-adrenergic, noncholinergic neurons that utilize the gaseous neurotransmitter and neuromessenger nitric oxide (NO), which induces SM relaxation and mediates actions of other neurotransmitters. Nitric oxide is formed by the conversion of L-arginine to citrulline and conversion of NO by NO synthase, an enzyme identical to neuronal NADPH diaphorase.31 The pterogalpatine ganglion seems a possible source of nitroergic innervation to choroidal and scleral SMs and MFBs, because it contains numerous nitroergic cells.34-36 Another source of nitroergic innervation to choroidal and scleral contractile cells could be choroidal NO synthase-positive ganglion cells comprising a plexus predominantly localized adjacent to the fovea.37-38

The continued influence of optical factors on ocular growth even after optic nerve section implies that a local signal from the neurosensory or pigmented retina must be transmitted directly to the sclera and choroid.3 We suggest here that one potential messenger compound might be the low-molecular-weight gas NO. The outer retina is rich in NO synthase, which is present in the photoreceptor ellipsoids, horizontal cells, amacrine cells, bipolar cells, and Müller cells.39-42 Nitric oxide is involved in multiple local feedback loops involving mechanical forces. In response to local mechanical forces, vascular endothelium releases NO to relax vascular SM and regulate local blood pressure.43 Nitric oxide synthase in the macula densa of the kidney functions as part of a local negative feedback loop to control the SM sphincter of the afferent arteriole and thus to regulate local glomerular capillary pressure and glomerular filtration rate.44 It is thus plausible that local mechanical feedback loops, using local release of NO, might regulate scleral and choroidal tissue tension via SM cells and MFBs. Such feedback loops might include the nitroergic ganglion cells we observed adjacent to areas of concentration of MFBs in the sclera; these neurons probably form part of the retro-ocular plexus, generally considered to be an orbital extension of the pterogalpatine ganglion.45 The nitroergic choroidal ganglion cell plexus, which is structurally similar to the intrinsic ganglia of the enteric nervous system,38 is another likely candidate for participation in neural-mechanical feedback loops.

Could contractile cells in the sclera and choroid participate in regulation of the refractive state of the eye? In more than one animal species, optical manipulations in young eyes can influence local ocular growth,46-47 even after section of the ciliary,48 or optic49 nerves. In chicks, the change in refractive error is eventually mediated by scleral growth but is initially mediated by changes in choroidal thickness of as much as threefold.2-3 Wallman and colleagues50 have proposed that anteroposterior movement of the retina by changing choroidal thickness provides a mechanism for altering refractive state that has a time course intermediate between accommodation, which is mediated by the action of the ciliary body and lens, and the process of emmetropization, which is mediated by scleral growth. Fenestrated capillaries in the choroid are very permeable to low-molecular-weight substances,50 resulting in rapid turnover of fluids and substances in the choroidal extravascular compartment. Smooth muscle tone in choroidal extravascular spaces might thus be able to alter choroidal thickness, and thus refractive error, by changing the accumulation of fluid or proteoglycans.2 However, the changes in proteoglycan incorporation seen with optically induced ocular size change, approximately 30%, are small compared with the twofold changes in choroidal thickness they observed.5 Weisman and colleagues51 have hypothesized that avian extravascular SM may directly participate in the regulation of choroidal thickness. This suggestion is consistent with the predominantly subfoveal distribution of choroidal extravascular SM observed here and the observed reticular distribution demarcating extravascular spaces. In humans, extravascular SM contraction would thin the choroid, whereas relaxation would permit the choroid to thicken. It is interesting that the nitroergic choroidal ganglion cell plexus, a potentially rich source of innervation of the colocated choroidal extravascular SM, is found only in humans and fowate primates. The nitroergic choroidal ganglion cell plexus is absent in avestate animals,38 which nevertheless have choroidal ganglion cells that use other neurotransmitters.52-53 The axial length and thus the refractive error at the fovea is most critical to visual acuity, whereas the peripheral retina is much more tolerant. Perhaps the predominantly subfoveal choroidal SM, neurally regulated in part by the underlying choroidal ganglion cell plexus, acts as if in parallel with lenticular accommodation to accommodate the eye to focus on near targets. This might explain the modest accommodation present in pseudophakic humans.1 Scleral MFBs are also most numerous in the foveal region, and scleral MFBs and choroidal extravascular SM were both absent in the newborn human eyes before foveal maturation.

We propose that a choroidal mechanism may mediate intermediate changes in ocular focus at the fovea and perhaps residual accommodation after the onset of lenticular presbyopia. Long-term changes in ocular size, such as emmetropization in the growing eye, might be partially mediated by the neurally regulated tone of posterior scleral MFBs, opposing the force of intraocular pressure. The hypothesis of intermediate changes in axial ocular length by the SM of the choroid and long-term changes in axial ocular length by the MFBs of sclera is supported by the observed difference in innervation. The choroidal SM is innervated more richly by sympathetic and parasympathetic terminals, whereas the scleral MFBs are innervated less richly and only by NO.

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