

Non-neural Regions of the Adult Human Eye: A Potential Source of Neurons?

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PURPOSE. Because it is known that both melanocytes and neurons are generated from neural crest stem cells and their derived precursors, the current study was undertaken to evaluate whether adult human ocular tissues, containing melanocytes, have the capacity to generate neuronlike cells in vitro.

METHODS. Choroid and Sclera cells from adult human eyes were separately dissociated and cultivated in the presence of epidermal growth factor and 10% fetal bovine serum. No retinal pigmented epithelial cells were detected. After cell growth, cells were transferred under conditions known to induce neuronal differentiation. Cells were plated on laminin in the presence of fibroblast growth factor-2 or brain-derived neurotrophic factor.

RESULTS. Cells derived from the sclera and the choroid of 15 donors proliferated to attain a 10⁸-fold increase in the number of cells within 4 months. At each passage, groups of cells differentiated into cells with neuronal morphology, expressing neuronal markers confirmed by immunocytochemistry and RT-PCR analyses, such as β -tubulin-III, neurofilament, and tau. Parallel to neuronlike formation, glialike cells, revealed by expression of vimentin and P0, were generated in large amounts. Although, absent from choroid and sclera tissues, nondifferentiated cells appeared in cultures.

CONCLUSIONS. The adult human eye conserves cells able to recapitulate certain neural developmental features. This observation opens new perspectives to study human neurogenesis and to provide an important source of neurons for transplantation studies in the retina and other regions of the central nervous system. (*Invest Ophthalmol Vis Sci.* 2003;44:799–807) DOI:10.1167/iovs.02-0267

Neuroepithelial cells form various ocular tissues during development by successive inductions from adjacent tissues and by intrinsic programs. The retina is derived from the forebrain after evagination and invagination processes, and the retinal pigmented epithelial cell layer appears during the same morphogenic program. The iris is a tissue that is also derived from central nervous system (CNS) neuroepithelial cells. Moreover, this tissue contains melanocytes that are derived from

neural crest stem cells, the neuroepithelial subpopulation that gives rise to all peripheral nervous system (PNS) tissues. Ocular melanocytes are already committed during early migration of neural crest cell precursors. They form between the fore- and the hindbrain and then migrate to reach the ocular choroid.¹ Thus, cells derived from CNS stem cells and from the neural crest stem cells participate in the formation of the retina and adjacent tissues.

Recent work has revealed that the adult mammalian eye conserves neural progenitor cells. Indeed, retinal stem cells were identified in the ciliary margin of adult mice. These cells have the characteristics of renewal and of the generation of all retinal cell phenotypes.² The iris, an area devoid of neurons but derived from the neuroepithelium, maintains the capacity to generate neurons in vitro during adulthood.³ Rat iris explants cultivated in the presence of FGF-2, a factor intervening in different processes of neuron formation, generate cells of which several can differentiate into neurons. Moreover, the foreign expression of the *Crx* gene induces the appearance of the photoreceptor phenotype. Other CNS stem cells have also been identified in different regions of the brain. Transplantations of cells comprising the subventricular zone (SVZ) of *LacZ* transgenic mice into the SVZ area of wild-type mice revealed that a subpopulation of this group of SVZ cells maintains a stem cell function during adulthood.^{4,5} Stem cells are located around mouse ventricles^{6–9} including the spinal cord¹⁰ and the rat hippocampus.¹¹ All these cells show a multipotential capacity for cell generation and an ability to renew. Progenitor cells that have a limited capacity of renewal were also identified in the brain, notably in the cortical parenchyma and even in the optic nerve.¹² This series of results shows that all the tissues that have been investigated so far and that were formed from neuroepithelial cells still contain cells with a potential to generate neurons during adulthood. Moreover, these cells can be isolated in vitro.

In the adult human CNS, various precursor cells and stem cells have been identified in different regions. Neuroblasts have been isolated from the temporal cortex,^{13,14} and the hippocampus using the expression of the specific neural promoter *T α -1*.¹⁵ Multipotent cells able to generate neurons, astrocytes, and oligodendrocytes have been identified in the temporal and the frontal cortex and in the amygdala. These cells have a limited capacity for renewal.¹⁶ Palmer et al.¹⁷ reported that a population of SVZ cells generating neurons and glia is able to proliferate under the stimulation of FGF-2 and cystatin-C,¹⁸ allowing a 10⁸-fold increase in the number of cells. The identity of the proliferating cells, stem cells versus bi- or unipotent progenitors, remains to be discovered. Johansson et al.¹⁹ and Pagano et al.²⁰ described the isolation of stem cells from the adult human SVZ and the olfactory bulb, respectively. However, the investigation of the renewal capacity was not determined to discriminate between a progenitor cell and a stem cell (no data on long-term renewal was presented in these studies). This series of data show that neural progenitors with at least a limited capacity to divide reside in various regions of the human brain and outside the location where they were initially supposed to be.

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TABLE 1. Donor Characteristics

Donor	Age	Cause of Death
1	64	Intestinal adenocarcinoma, liver failure
2	72	Myocardial infarction
3	33	Accident
4	50	Violent death
5	70	Liver failure, duodenal adenocarcinoma
6	63	Hepatic carcinoma
7	81	Pulmonary edema
8	68	Cardiorespiratory arrest
9	60	Diabetes type II (heart bypass graft in 1998)
10	88	Pneumonia, diabetes type II
11	69	Myocardial infarction
12	44	Lung cancer with cerebral metastases
13	18	Cerebral trauma
14	49	Pulmonary embolism
15	22	Cerebral trauma

Cells were processed 10 to 17 hours after death. For all cell cultures derived from the choroids or the sclera, the neuronlike population represented around 0.3% of the total cell number. No correlations with the age of the donor, or with the cause of the death were observed.

Knowing that melanocytes are derived from neural crest stem cells, we evaluated whether adult human ocular tissues, containing melanocytes, possess cells able to generate neuronlike cells *in vitro*. Supporting this hypothesis, Wolter and Bryson²¹ observed by electron microscopy, that certain melanomas may contain cells with neuronal characteristics. In the present study, cells isolated from the adult sclera and the ocular choroid were cultivated, expanded, and submitted to conditions known to induce neuronal differentiation. We observed the appearance of neuron- and glialike cells. Their characteristics and their origin are discussed in the following sections.

MATERIALS AND METHODS

Primary Culture

Cells from the choroid or derived from the innermost layers of the sclera (including the suprachoroid and the lamina fusca) were obtained from organ donors in accordance to guidelines provided by the ethics committee of the Lausanne University School of Medicine and the Declaration of Helsinki. The age of the donors varied from 18 to 88 years. Table 1 shows the age and the cause of death of all donors. The tissues were processed 10 to 17 hours after death. No correlations with the age of the donors or with the cause of the death were observed. The iris, the ciliary body, the pars plana and plicata, and the ora serrata were removed. The retina was then detached, and the RPE cells that remained attached to the Bruch's membrane were eliminated by abundant washing. The rest of the eye was transferred into a well, where the choroid was removed and placed in another well. Again, the eye was placed in a new well, where melanocytes and other cells attached to the sclera were removed by scratching and successive washes (Fig. 1). After harvesting, tissues were placed in NaCl 0.9% containing 0.6% glucose. Pieces of choroid were dissected and minced in PBS containing 0.6% glucose and 1% penicillin and streptomycin (Sigma, St. Louis, MO). An equal volume of a trypsin solution (Life Technologies, Basel, Switzerland) was added, and the final preparation was transferred to a dissociating chamber at 37°C for 45 minutes in O₂ (95%) and CO₂ (5%). The complete solution was centrifuged at 1800 rpm, and the pellet was harvested and then mechanically dissociated, with a fire-polished pipette in the presence of Dulbecco's modified Eagle's medium and F-12 nutrient (Life Technologies) and 10% fetal bovine serum (FBS; Sigma). After centrifugation (1800 rpm), the pellet was resuspended in the same medium containing N1 supplement (Sigma) and epidermal growth factor (EGF; 20 ng/mL; Pepro Tech, Rocky Hill, NJ). Cells were grown in 2 mL of growth medium in six-well plates (TPP; Techno Plastics AG, Trasadingen, Switzerland) at a density of 50,000 to 100,000 cells per well. After the last centrifugation, the viable cells varied between 80% and 90%.

The sclera cells were plated after scratching in the same conditions described for choroid cells. For both preparations, the percentage of

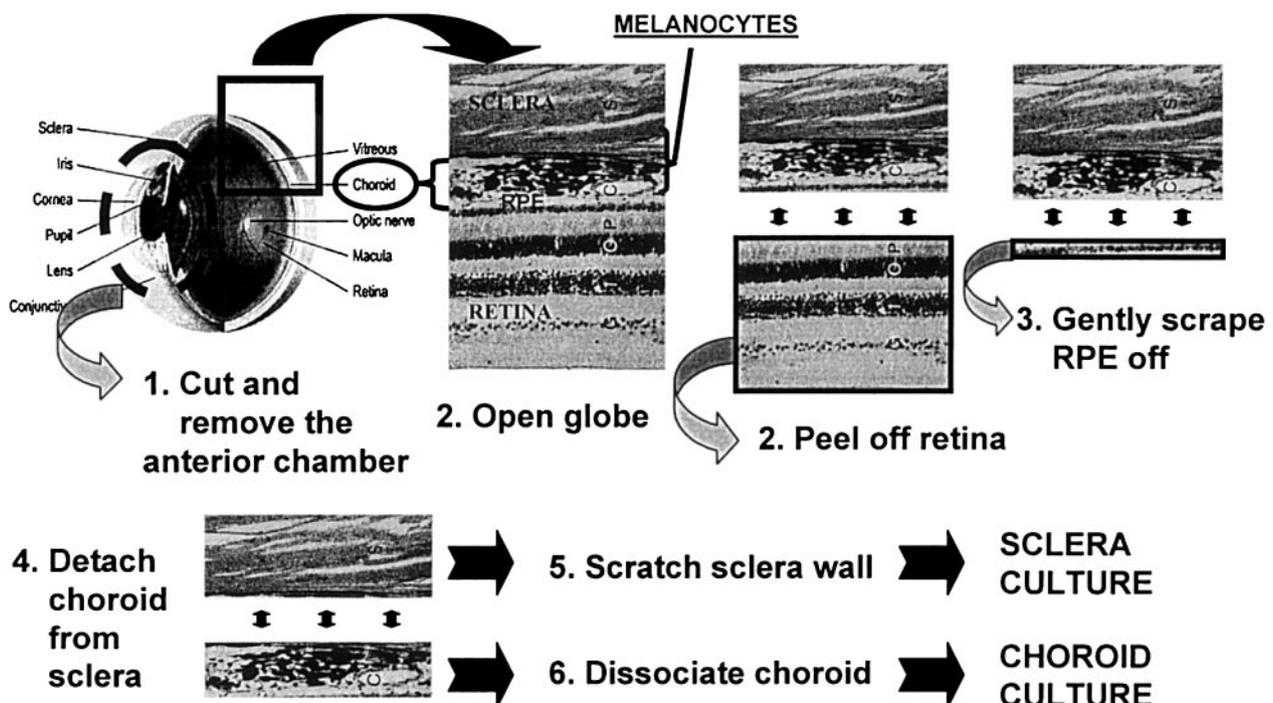


FIGURE 1. Schematic representation of the dissection procedure.

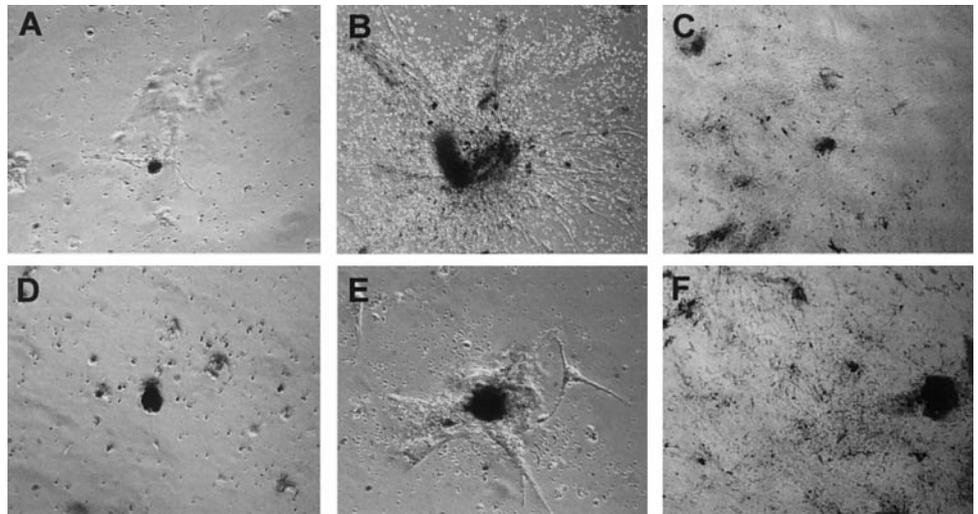


FIGURE 2. Sclera- and choroid-derived cells can proliferate under the stimulation of EGF. (A) In the presence of EGF and serum, adult human melanocytes and choroids cells began to proliferate, forming clusters (B) that generated numerous nonpigmented cells (C). Similar observations were made for cells derived from the sclera wall (D-F). Magnification: (A, B) $\times 100$; (C, F) $\times 40$; (D, E) $\times 200$.

pigmented cells corresponded to 40% to 50% of the total number of dissociated cells. After 1 week, a low percentage of pigmented cells (<3%) formed clusters and generated nonpigmented cells. During the same period, a few primary nonpigmented cells also started to proliferate, representing less than 5% of the total cells plated after trypsinization. Two to 3 weeks later, the cells grew to near confluence and were passed. They were plated at a density of 6667 cells/cm² in 75-cm² flasks. In these conditions, the cells were passaged every 7 days *in vitro* (DIV).

Differentiation of Cells Derived from Sclera and Choroid Tissues

After the cell growth period, cells were transferred to conditions known to induce neuronal differentiation of adult CNS precursors.^{15,16,22} Cells were trypsinized, washed, and then plated at 25'000 cells/cm² onto poly-L-ornithine- and laminin-coated coverslips placed in 24-well (2-cm²) plates. Differentiation was induced by various doses of FBS alone or in the presence of human brain-derived neurotrophic factor (BDNF), human EGF, or human FGF-2 (all from Pepro Tech) during an incubation period of 2 to 15 DIV. Two coverslips were prepared per condition. For all cells, the presence of positivity for the β -tubulin isotype III and GFAP markers (described later) was investigated. The number of total cells as well as β -tubulin-III-positive cells was counted in 10 to 20 40 \times fields. A total of approximately 1000 cells was counted per condition analyzed. Results are expressed as the mean \pm SEM. The Mann Whitney test was used to verify the significance of differences between groups.

Immunocytochemistry

Indirect immunocytochemistry using secondary antibodies conjugated to either cyanine, fluorescein, or 7-amino-4-methyl-coumarin-3-acetic acid (AMCA) was performed on fixed cells or on ocular slices (4% paraformaldehyde) 5 to 15 days after plating, as previously described.²³ Primary antibodies included mouse monoclonal antibodies against β -tubulin isotype III (final concentration, 1:1000; Sigma), neurofilaments (1:100; Sigma), NeuN (1:500; Chemicon, Temecula, CA); S100 β (1:200 Chemicon), vimentin (clone V9, ; 1:200; Dako, Zug, Switzerland), P0 (Juan José Archelos, Universitätsklinikum, Graz, Austria), fibronectin (1:400; Sigma), smooth muscle antigen (1:400; Sigma), CD34 (1:200; Dako, Glostrup, Denmark), and rabbit antiserum glial fibrillary acidic protein (GFAP; 1:400; Genosys, Cambridgeshire, UK); and the mouse IgM monoclonal antibody against O4 (1:20; Roche Molecular Biochemicals, Mannheim, Germany). The rabbit polyclonal antibody against human nestin was a generous gift from Ron D. G. McKay (National Institutes of Health, Bethesda, MD),²⁴ and polyclonal antibodies against RPE65 and RGR were kindly provided by Andreas

Wenzel (University of Zurich, Zurich, Switzerland). The secondary antibodies (Jackson ImmunoResearch, West Grove, PA) included fluorescein or cyanine-conjugated affinity-purified goat antibody to mouse IgG (1:100, 1:200, or 1:500) and AMCA-conjugated affinity-purified goat antibody to mouse IgM (1:100).

Reverse Transcription-Polymerase Chain Reaction

Total RNA from 10⁶ differentiated cells was extracted according to the manufacturer's protocol (TRizol; Life Technologies). Reverse transcription was performed by denaturing 600 ng total RNA with 0.2 μ M oligo NVdT₂₀ in 12.5 μ L water for 10 minutes at 65°C. Samples were then incubated 10 minutes to reach room temperature. Elongation was pursued in a total volume of 20 μ L with 0.5 mM dNTP, 1 \times strand buffer of eAMV-RT (Sigma), 20 μ L of RNasin (Promega, Madison, WI) and 20 μ L of eAMV-RT (Sigma) for 1 hour at 50°C. PCR was performed in a 50- μ L reaction containing μ 2 μ L cDNA, 0.2 μ M of a primer pair and polymerase (AccuTaq; Sigma) according to the manufacturer's protocol. PCR reactions were incubated for 2 minutes at 94°C; for 35 cycles at 94°C for 30 seconds, 70°C for 30 seconds, and 72°C for 1.5 minutes; and for 7 minutes at 72°C. Calbindin primers are defined by the following sequences: 5'-GATGTGACAAGGGAAGGGAGCAC-3' and 5'-GGGGACATTCAAGCTGACCCAG-3', NFM-H primers by 5'-CTG-GAGGGCGAGGCTGCG-3' and 5'-CGCCGGAGCCCTGGATCTG-3', and GFAP primers by 5'-GATTGGAGCCACCATGCCAG-3' and 5'-GGGGCGCCTGGTATGACAC-3'. Ten microliters of each PCR reaction was loaded on a 1% agarose gel and visualized by ethidium bromide staining.

RESULTS

Proliferation Potential of Sclera and Choroid Cells

Melanocytes juxtaposed to the sclera were scratched off, isolated, and incubated in the presence of EGF and 10% fetal bovine serum (FBS, six donors). After 2 to 4 days *in vitro* (DIV), the pigmented cells started to proliferate, first forming brown clusters and then transparent cells, as well as a small number of pigmented cells. After approximately 21 DIV, the cells reached confluence (Fig. 2). The proliferation capacity of these sclera cells was tested by dissociating and plating the cells in growth medium. An increase in the number of cells of up to 10⁸-fold was observed within 4 months. Senescence was observed at approximately passage 11. Similar cell proliferation was observed in choroid cultures ($n = 9$, Fig. 2). The growth curves

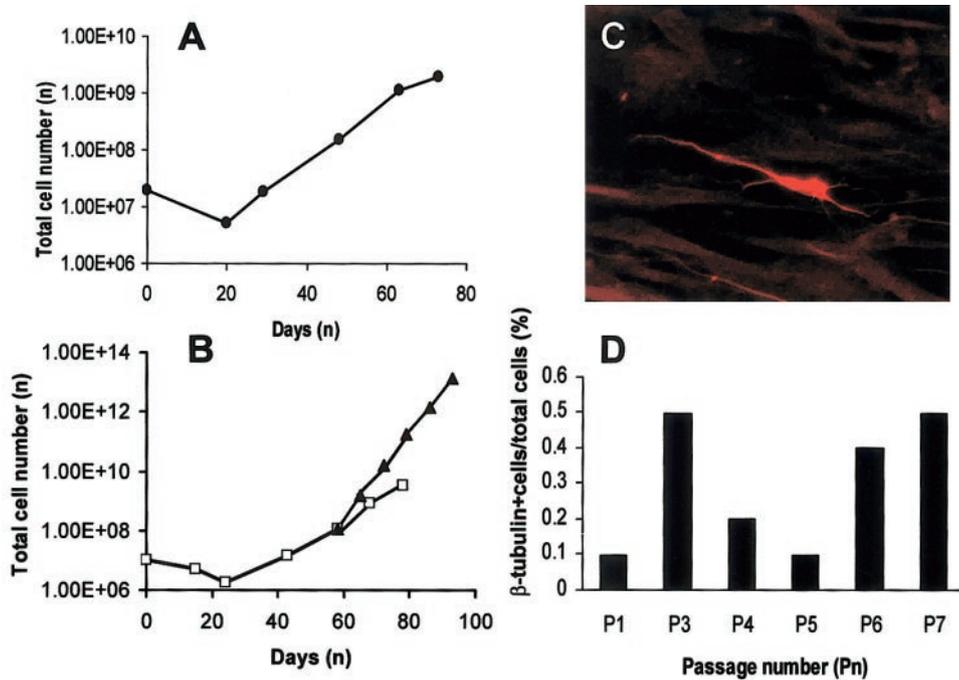


FIGURE 3. Sclera- and choroid-derived cells can be expanded. (A, B) Example of two growth curves of cells derived from the choroids of two different donors reveal that the cells were amplified by five- to seven-fold during each passage and that a total increase of approximately 1000-fold was achieved in four passages, with a constant slope of the growth curve (○). (▲) Growth curve of cells that were frozen and then thawed, showing that these cells can be banked. (C) The morphology of β -tubulin-III-positive cells remained neuronal at a later passage (P6, see Fig. 3 for cell characterization). Magnification, $\times 200$. (D) The generation of neuronlike cells was maintained throughout the passages. The number of β -tubulin-III-positive cells was counted at each passage for cells derived from one donor of choroid cells. Similar observation was recorded for another cell culture. FGF-2 served to induce neuronal differentiation in this series of experiments.

show that, under optimal conditions, an increase of approximately eightfold can be reached (Fig. 3). Freezing and thawing of the cells did not alter the growth capacity (Fig. 3B, black triangles).

We attempted to cultivate RPE cells with the same medium used for sclera and choroid cell growth. For three different preparations, no proliferation of RPE cells was observed. Moreover, primary cells isolated from the sclera or the choroid were negative for antibodies directed against the RPE markers, RPE65 and RGR. These data suggest that our cell cultures did not contain RPE cells. However, other cells were present, together with cells harboring melanocyte morphology (flat and dark pigment). Fibroblasts, as evidenced by the expression of fibronectin, represented approximately 1% of total cells 15 days after plating, 11% at passage 1 ($n = 2$), and 60% to 95% at passage 7 ($n = 4$). No cells positive for the smooth muscle

antigen were present at any time. Endothelial cells were revealed by the antibodies directed against CD34 antigens and represented up to 15% of the total cell population just after plating (choroid culture), but were absent 1 day later and at passages 1 and 2 ($n = 3$). These observations suggest that the culture conditions do not sustain endothelial cell survival and proliferation.

Generation of Neuronlike Cells In Vitro

After 1 week of proliferation, the cells were transferred onto coverslips coated with poly-L-ornithine and laminin in the presence of various factors known to induce neuronal differentiation.^{13,16,23,25,26} In the absence of factors, no neuronlike cells were observed in the presence of 2% or 10% FBS or without FBS. Fibroblast growth factor (FGF)-2 and brain-derived neuro-

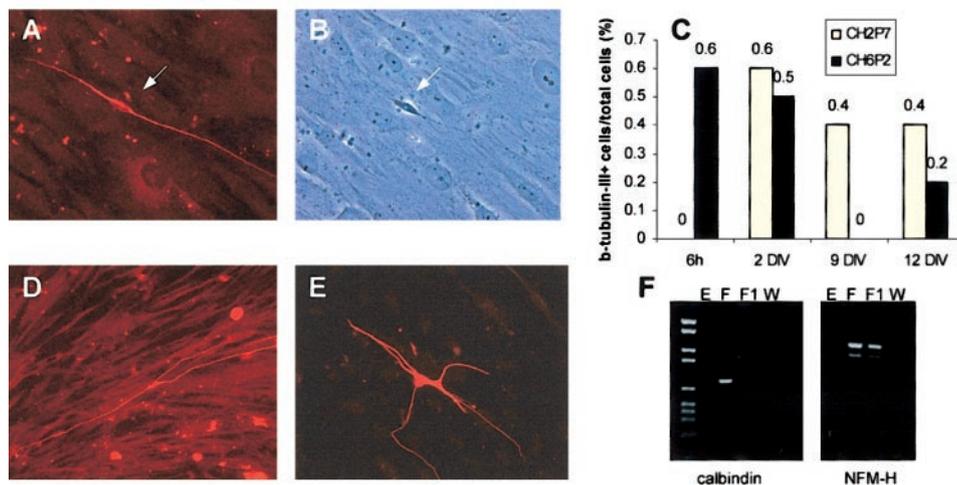


FIGURE 4. Sclera and choroid cells generated neuronlike cells. Proliferating cells derived from the sclera and choroid were induced to differentiate in the presence of laminin and the stimulation of EGF and FBS. After 5 DIV, some cells expressed the neuronal marker β -tubulin-III (A, D, E). The labeled cell in (A, arrow) had a neuronal morphology revealed by phase contrast (B, arrow). Note the bright soma and the dendrites. (C) A time course study of the presence of β -tubulin-III revealed that, in one culture (CH6P2), neuronlike cells were already present at plating. In another culture (CH2P7), they were generated starting at 2 DIV, indicating an early induction of neuronal differentiation. After 2 DIV, the number of β -tubulin-III-positive cells decreased. Different morphologies were observed, depending on the condition used during cell differentiation. (E) The presence of BDNF generated several multipolar β -tubulin-III immunopositive cells. (F) RT-PCR of mRNA extracted from the differentiated cells revealed the presence of mRNA for neuronal markers such as calbindin and neurofilament. The appearance of these mRNAs was related to stimulation of FGF-2. Cells were stimulated by (lane E) EGF and 10% of FBS, (lane F) FGF-2 and 10% FBS, and (lane F1) FGF-2 and 10% FBS. Lane W: control cell preparation. Magnification: (A, B, D, E) $\times 200$.

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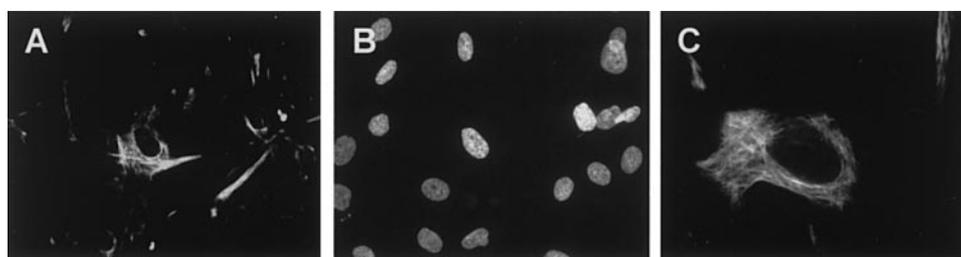


FIGURE 5. Sclera and choroid cultures contained glialike cells. (A) Seven days after induction of cell differentiation, flat cells positive for vimentin were observed. These cells were derived from a sclera culture at passage 4. (B) Nuclei staining with 4',6'-diamino-2-phenylindole (DAPI) revealed that only a subpopulation of cells expressed vimentin. (C) Detection of vimentin labeling at higher magnification revealed the fibrous organization of the vimentin filaments. Magnification: (A, B) $\times 200$; (C) $\times 400$.

trophic factor (BDNF) are known to participate in neuron generation and consequently were tested. Both factors were incubated separately on sclera- and choroid-derived cells plated on laminin. After 7 DIV in the 15 cultures tested, immunoreactive cells for the early marker of neurons, β -tubulin-III (Tuj1²⁷) were detected in all conditions investigated at passage 1 (P1). These cells have a neuronal morphology with a bright soma (Fig. 4). In the presence of EGF and 10% FBS, 0.2% \pm 0.06% of total cells expressed the early neuronal marker β -tubulin-III ($n = 4$). At different passages, cells were tested for their ability to generate neurons. For all cultures ($n = 15$), neuronlike cells were detected at all passages tested and were observed until passage 10. Newly generated neurons constantly represented approximately 0.1% to 0.7% of the total cell population depending on the stimulation and specimen used (Fig. 3). We were successful in generating neuronlike cells from all donors, and we observed no obvious relationship between postmortem delay and donor medical status and with the generation of neuronlike cells. On the other hand, certain cell cultures lost their ability to generate neurons over time, when parallel culture of the same specimen maintained this potential (two observations). These results indicate that neurons can continuously be generated through passages. Cells frozen at different passages maintained their capacity to proliferate and to generate neurons, showing that they can be banked (Fig. 3).

Characterization of Newly Generated Neuronlike Cells

A time-course study was undertaken of two different cultures to determine when β -tubulin-III-positive cells appear and how long they survive. This characterization was performed at two passages (P2 and P7) in two distinct cultures. In both cultures, cells with neuronal markers were present as early as 6 hours after plating, with up to 0.6% of total cells containing the neuronal filament β -tubulin-III. For both cultures, the maximum expression occurred at 2 DIV and had a tendency to decrease between 2 and 12 DIV. This suggests that a population of neuronlike cells was in part produced during the growth procedure.

To characterize the degree of maturity of these neuronal cells, we used different neuronal markers known to appear after the expression of β -tubulin-III. Of the total cells, 0.2% and 0.9% expressed the neurofilament antigens at P1 and P6, respectively (two different donors). We also observed cells with neuronal morphology that contained the tau antigen in the soma (two donors, data not shown). No immunoreactive cells for later neuronal markers such as MAP2 or NeuN were identified. To confirm the immunocytochemistry characterization, we used RT-PCR to reveal the expression of specific neuronal genes. The presence of mRNAs for early neuronal markers,

such as the neurofilament heavy chain (NFM-H), was detected as well as for a marker appearing later in neuronal maturation (Fig. 4), calbindin-1, which was present in cells induced to differentiate by FGF-2. Altogether, these results suggest that sclera and choroid cultures contained or produced newly generated neurons.

Neurotrophic Factors Influence the Morphology and the Number of Neuronlike Cells Derived from the Ocular Sclera and Choroid

In the presence of EGF and 10% FBS, we observed that neurons from eight different cultures were bipolar and elongated (Fig. 4). To reveal whether this neuronal phenotype is intrinsic to cells isolated from the choroid, we used different factors known to act on neuronal differentiation and maturation to change their morphology. We and others have previously observed that neurotrophic factors can induce the differentiation of distinct populations of neurons.²³ We therefore investigated the action of different factors, including FGF-2, EGF, and BDNF. After 5 DIV, the morphology of the neurons derived from the adult human choroid was dependent on the growth factors used. When FGF-2 with heparan sulfate was used, cells with a bipolar form and some neurites were observed. In contrast, BDNF was found to induce the most pronounced change in neuron morphology (Fig. 4), certain immunopositive cells for β -tubulin-III being multipolar. It appears that neuronlike cells derived from ocular sclera or choroid are sensitive to environmental signals in a pattern similar to that observed with other neuronal precursors and neurons localized in the CNS.

In three different cell cultures tested, including two cultures derived from the sclera, we observed an increase of neuron induction in the presence of FGF-2 in comparison to stimulation with EGF and serum. FGF-2 generated 2.67-fold more β -tubulin-III-positive cells ($P = 0.016$; seven conditions tested between P1 and P9).

Glialike Cells

We wanted to assess whether other cells of neural crest lineage were also present in the culture. No cells positive for the smooth muscle antigen were observed at plating and after cell expansion. However, the glial marker vimentin was detected in groups of cells in early and late passages (Fig. 5). The staining corresponds to the filamentous appearance of the antigen. Because expression of vimentin can occur in mesenchymal cells, we tested whether cells containing vimentin express fibronectin. Most cells expressed only one marker, with cells only rarely showing both antigens. These results suggest that vimentin-positive cells represent glialike cells. Sustaining these observations, numerous cells were positive for the glial marker P0. The staining was mainly observed as big patches in the cell

border (data not shown). At passage 4, the percentage of glialike cells varied between 25% and 95% of total cells ($n = 3$, two sclera cultures and one choroid culture). Similar observations were made for cell passage 7. From P3 to P7, P0-positive cells represented 12% to 35% of the total population ($n = 3$, sclera cultures). This indicates that, throughout the passages, the glial population was enriched, whereas the neuronlike cells remained stable. In fact the culture conditions used for cell growth—EGF and serum—favored the proliferation of glialike cells and fibroblasts. Throughout the passages, it a competition seemed to occur between fibronectin and glial cells. No glial cells positive for the GFAP, S100 β , or O4 antigens (oligodendrocyte markers) were detected. We also failed to detect GFAP mRNA by RT-PCR.

Appearance of Cell Precursors during Cell Growth

Different experiments were undertaken to reveal whether the neural precursors were generated in our cultures or are already expressed in the adult human eye. These observations should indicate whether neural precursors already exist in the choroid and the sclera in vivo. The nestin filament antigen, which is expressed during neural development, was therefore tested on ocular slices to identify neural precursors in the adult human eye. In the sclera and the choroid, no cells positive for the nestin filament were observed. These regions investigated in albino mice (BALB/c) were also negative for the presence of nestin. In contrast, the retina of a patient who had had a hemorrhage before enucleation showed nestin-positive cells dispersed in the different layers of the retina (positive control, data not shown). Moreover, primary dissociated cells from the sclera or the choroid and plated on laminin for 12 hours were also negative for the presence of nestin. These results show that the culture medium induced the cell population to regress to a more primitive stage. To reveal precisely the presence of neural precursors in the choroid cell culture, we further investigated the presence of nestin-immunoreactive cells at different time intervals in cell differentiation. In two choroid cell cultures from two different donors in an investigation of cells just after the proliferation process (cells were plated for 6 hours to allow cell attachment), 14.2% (P2) and 8.6% (P7) of the cells expressed the nestin antigen. This number decreased over time to reach 2% and 5.6%, respectively, at 12 DIV (Fig. 6). Some nestin-positive cells presented a nucleus in division, attesting that precursor cells were proliferating (Fig. 6).

Thus, both nestin- and β -tubulin-III-positive cells decreased after the beginning of cell differentiation, suggesting that survival factors were missing in the medium and that nestin cells started to differentiate. These results show that nestin-positive cells are generated in vitro and are not recruited from choroid tissues and then amplified. It appears that a dedifferentiation process occurred in response to the growth medium. However, there is no evidence to link this phenomenon to formation of neurons. These results merely indicate that certain sclera and choroid cells extracted from their original environment were diverted to their initial fate.

DISCUSSION

The results of the present study suggest that the adult human eye contains cells throughout life that have the potential to generate neuron- and glialike cells under specific stimulations. Neuronlike cells derived from sclera and choroid cultures were characterized through three different approaches: morphology, antigen markers, and expression of mRNA. Most neurons expressed early markers of neuronal development, such as β -tubulin-III and neurofilaments, whereas markers of neuronal

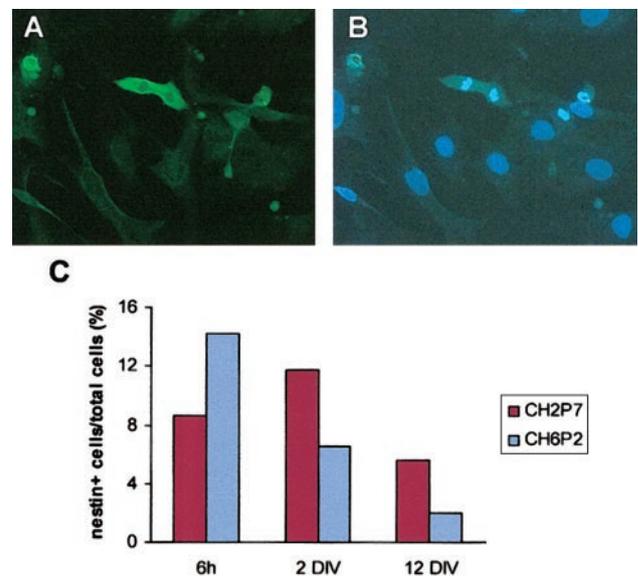


FIGURE 6. Sclera and choroid cultures contained neural precursors. (A) After 7 days of growth and at passages 2 or 7, cells were plated on laminin for 6 hours to allow cell attachment. Nestin-positive cells (green) were present in sclera and choroid cell cultures after the cell proliferation procedure. (B) Their nuclei, stained by DAPI dye (blue), revealed certain nestin-positive cells with chromosomes in telophase, indicating that nondifferentiated cells were in the cell-division process. (C) A time-course study of nestin-positive cells during differentiation in two cultures revealed that a substantial number of precursors was already present at plating and that the number decreased over time. Magnification: $\times 400$.

maturation were revealed only by RT-PCR or by immunostaining (tau) restricted to the cell body. It is interesting to note that in a recent opinion letter, Svendsen et al.²⁸ posited that neurofilament and tau proteins are the most reliable neuronal markers. These observations show that the neuronlike cells found in culture are newly born and exhibit an in vitro development comparable to other neurons generated from other brain areas or from neural stem cells.^{11,27,29,30} The presence of mRNAs specific for neuronal maturation suggests that these neurons started to acquire a complete phenotype, but that our culture conditions did not allow a full maturation and/or long-term survival of these generated neurons. Even if these neurons were generated from an unusual tissue, they expressed a standard pattern of neuronal markers. Moreover, they responded in a similar way to stimulations that were previously described for murine or adult human neural progenitors. For instance, BDNF or FGF-2 was found to induce formation of neurons. In consequence, this culture system provides an in vitro model to study human neurogenesis. Nonetheless, further characterization is needed to reveal whether these neuronlike cells behave in a manner similar to neurons. Are they excitable as are neurons? Are they able to release neurotransmitters? Do they form functional synapses? Can they participate in a neuronal network? It appears that several supplementary studies are still needed to assess that these cells possess all neuronal functions. Nonetheless, sclera cell culture is a source of cells to attempt to answer these questions.

The cultures contained a population of vimentin-positive cells that increased throughout the passages. The absence of fibronectin on vimentin-positive cells suggest that vimentin-expressing cells are glial cells. Moreover, the specific marker for Schwann cells and their precursors, P0, is widely expressed in the culture. P0 is often highly detected in specific locations in the cell periphery, suggesting that cell contact may play a

role in expression of P0. As was true of neuronlike cells, the gliallike cells seemed to have undergone an incomplete cell differentiation in view of the absence of GFAP or S100 β . In the present study, we attempted to find adequate stimulations to induce neuronal differentiation. It is possible that the conditions were not optimal to allow a full differentiation of glial cells. In future studies, we will test the efficiency of factors known to generate PNS glial cells. Glial growth factor and endothelin-3 are good candidates, because they seem to have an effect on both glial cell commitment and glial differentiation.^{31,32} The generation of neuronlike cells from an unusual source is often accompanied by the formation of glia, as was observed in bone marrow stromal cells and skin-derived precursors.^{33,34} It appears that cells derived from the sclera have similar characteristics.

The present study did not reveal whether sclera and choroid cells generate neurons directly or whether different steps are necessary to acquire a neuronal phenotype, but some data seem to indicate a possible pathway. The culture contained precursors revealed by the presence of the nestin filament antigen.^{24,27,35} Therefore, we explored whether nestin-positive cells are inherent to sclera and choroid tissues or are induced by culture conditions. The fact that nestin-positive cells are absent *in situ* in the adult human (and albino mice) sclera-choroid indicates that a population of cells can regress to a more primitive state in our culture conditions, suggesting a dedifferentiation process. We are currently investigating whether the cells isolated by their nestin expression, through the use of cell-sorting techniques based on activation of the nestin promoter, as described by Roy et al.,³⁶ can generate β -tubulin-III-immunoreactive cells. This should help to reveal the different steps necessary for a sclera-choroid cell to become a neuron. This change in cell fate can be related to a transdifferentiation mechanism. The transdifferentiation of non-neural cells into neurons has been previously documented in such cell populations as the PC12 cell line,³⁷ embryonic retinal pigmented epithelial cells,³⁸ and cutaneous precursor cells.³⁴ It is also possible that the sclera and the choroid are composed of a heterogeneous population of functional cells and precursors that do not express nestin and still reside in the adult eye after development. Nonetheless, if this hypothesis is correct, the precursors would have been diverted from their final fate (i.e., non-neuronal).

In mice, retinal stem cells are present in the pigmented epithelium of the ciliary margin (CM) during adulthood.^{2,39} In the present study, the dissection procedure did not include this area, suggesting that the source that generates neuronlike cells is in an area other than the ciliary body. In five specimens, sclera and choroid cells were derived from the posterior part of the posterior chamber, rendering difficult a possible contamination by CM cells. Consequently, the mammalian eye contains additional cells capable of generating neuronlike cells. The sclera and choroid have not yet been described as being sources of neurons during development. Nonetheless, it is possible that our cultures were contaminated by other cell sources. RPE cells could be a candidate, because embryonic RPE cells have been shown to transdifferentiate into neurons.³⁸ However, this phenomenon seems unlikely to have happened in the present cultures, because the percentage of neuronlike cells was very similar in either choroidal or scleral cell cultures. Indeed, we expected to have less contamination in scleral cultures in comparison with choroidal ones, because the sclera is not directly in contact with the RPE. Moreover, after the cells were plated, we did not detect RPE cells by immunocytochemistry. Mesenchymal and endothelial cells could also be at the origin of the neuronlike cells. However, from these two populations, only fibroblasts (mesenchymal cells) populated the cultures. It has been suggested that mesenchymal cells are able

to generate various cell phenotypes, including neuronlike cells.^{40,41} Further characterizations are needed to reveal whether the ocular mesenchyme can generate neurons.

In this study, we did not identify the cell identity that is the source of neuronlike cells. Only clonal analyses will unambiguously reveal the origin of cells having neuron potential. Nonetheless, other groups of cells could be a good candidate. Common cells of the choroid and the sclera are melanocytes and fibroblasts, but only the melanocytes are derived from the neuroectoderm, rendering them the most probable source. Indeed, ocular melanocytes are derived from the neural crest. Neural crest stem cells under the control of intrinsic mechanisms and environmental stimulation produce neurons, glia, cartilage, and melanocytes.⁴²⁻⁴⁵ Throughout the generation of precursors, the cell potentiality is restricted through successive divisions. During this process, neural crest stem cells generate bipotent precursors capable of producing glia and melanocytes.^{31,46} The commitment of melanocytes occurs at the beginning of their migration at the neural tube level. Endothelin-3 has been found to induce regression of embryonic melanocytes to a more primitive state, allowing the generation of glia,³¹ indicating that some melanocytes conserve a certain plasticity. Our cultures contained neuronlike cells and gliallike cells and were derived from tissues containing a large amount of melanocytes.

These observations suggest that a multipotent neural crest precursor, maintaining the capacity to generate neurons, glia, and melanocytes, may reside in the adult human eye, or that certain cells may regress to this state. It is also possible that cells with restricted potential are colocalized in the same tissue. It cannot be excluded that a population of cells can transdifferentiate into neurons or glial cells. Only clonal analysis studies will reveal which cell population is at the origin of neurons and/or glia, whether the adult human eye contains multipotent precursors, and whether the generation of neural cells is due to a transdifferentiation mechanism. To achieve that, it is necessary to determine whether the melanocyte population is homogenous or whether some pigmented cells have the characteristics of neural crest precursors. In summary, the present results show that a cell population maintains, throughout life, the capacity to recapitulate certain developmental features.

The control of cells derived from adult tissues to induce cell proliferation and generate neurons opens new perspectives for therapeutic approaches by *ex vivo* or *in vivo* stimulation. They can be expanded and consequently produce an effective number of neurons. Ideally, approximately 10 billion neurons may be generated by one eye. This important characteristic of proliferation renders the cell accessible to diverse analyses, such as the study of neurogenesis, gene and drug screening, and cell transplantation. The present cultures contained mainly gliallike cells and fibroblasts. Only a low percentage of total cells expressed the nestin or the β -tubulin antigens; the remaining cells are unknown and are currently being characterized. After cell differentiation, this mix of cells may not be appropriate for subsequent transplantation. Neuronal cell isolation should circumvent this problem.

Cell sorting by cellular transfection of plasmids containing the neuronal promoter T α -1 followed by the green fluorescent protein (GFP) sequence allows purification of neuronal cells from the adult human brain.^{15,36} Such a technology can be applied to neuronlike cells of the present study. It is as yet unknown whether these neurons can be differentiated into photoreceptors or other retinal or CNS cells. However, some studies have reported that ocular melanoma can produce neurons with morphologic characteristics of retinal neurons.^{21,47} These observations have to be confirmed with the new molecular and immunocytochemical techniques, by using specific

retinal markers. Nonetheless, they suggest that melanocytes may adopt a retinal fate, rendering these cells potentially useful in therapeutic approaches. Moreover, epigenetic and genetic manipulation may drive sclera cells toward a specific cell phenotype, as was recently shown with CNS precursors.⁴⁸⁻⁵¹ The scleral cells are also a complementary source of neurons to those recently identified in the CNS^{17,48} from bone marrow⁵² or from skin.^{3,4} Indeed, that all these neurons have a different origin may imply that they have different potentials to integrate into the CNS and are consequently important to test in several transplantation paradigms.

Thus, adult human sclera-choroid cells are a new source of neurons for cell replacement in the retina and other CNS regions. Moreover, their adult status is an alternative to the ethical problems raised by the use of fetal or embryonic cells.

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