Characterization of Cholinesterase Activities in Primary Cultures of Retinal Pigment Epithelium

Rocio Salceda, Gustavo Sanchez, and J. M. León-Cázares

This report presents a comparative description of the acetylcholinesterase and butyrylcholinesterase activities and their molecular forms in primary cultures of retinal pigment epithelium (RPE). Acetylcholinesterase activity increases during differentiation of the cells. Sucrose sedimentation analysis of acetylcholinesterase and butyrylcholinesterase molecular forms revealed the presence of A12, G4, G2, and Gt and A8, G4, G2, and Gt, respectively. RPE cells in culture release both cholinesterases into the growth medium, sedimenting as the Gt molecular form. Changes in the molecular forms of both enzymes were observed during differentiation. The results suggest a possible relationship between butyrylcholinesterase activity and cell proliferation and acetylcholinesterase activity and cell differentiation. Invest Ophthalmol Vis Sci 33:1690-1695, 1992

Acetylcholinesterase (AChE) is an enzyme well known for its function in cholinergic synapses, where it hydrolyzes acetylcholine released into the synaptic cleft. Although it is localized in synaptic membranes of cholinergic or cholinoreceptive cells, it has also been detected in cells that have no known association with this neurotransmitter system. In addition, the presence of AChE and butyrylcholinesterase (BChE) in early embryonic stages of development in neuronal and nonneuronal tissues, including retinal pigment epithelium (RPE), has been described. These features suggest that AChE might be involved in other membrane functions as well.

Previous studies have established the existence of multiple molecular forms of both enzymes, characterized by definite sedimentation coefficients and cellular localization. These are the globular forms (G15 G2, G4) and asymmetric forms (A4, A8, A12). The lighter forms appear to be biosynthetic precursors of the heavier ones, although it has been reported that different mRNA species contribute to the formation of the various molecular forms of cholinesterase. However, the functional role of these different forms remains unknown.

A strong correlation between BChE and cellular proliferation and AChE and cellular differentiation has been reported during morphogenesis of neural tissues, including retina and chick retinal cells cultured with retinal pigment epithelial cells.

The RPE that originates from the optic cup is a single layer of specialized cells intercalated between retinal photoreceptors and the choroid. Among other functions, RPE plays a morphogenetic role in the differentiation of photoreceptors and the choroid. The hypothesis that neurotransmitters and their correlated enzymes are involved in maturation processes during morphogenesis has been postulated. Because AChE has been considered an early marker for cell differentiation, we studied the activity and molecular forms of both AChE and BChE activities during differentiation of RPE cells in culture.

Our results establish the RPE as a good model to further understand the relationship between cholinesterase activities and cell proliferation and differentiation.

Materials and Methods

Medium, fetal calf serum, and antibiotics for cell culture were purchased from Gibco (Grand Island, NY). Trypsin, acetylthiocholine, butyrylthiocholine, tetraisopropylpyrophosphoramide (iso-OMPA), and BW 284C51 were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade, and were obtained from standard suppliers.

All investigations conformed to the ARVO Resolution on the Use of Animals in Research.

RPE cells from 7-day-old chick embryos were used. The isolated RPE sheets were incubated at room temperature with 0.13% trypsin in phosphate buffered saline (PBS) for 5 min. Cells were dissociated in TC 199 culture medium supplemented with 10% heat-inacti-
vated fetal calf serum, 2 mmol/l glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded at 4 × 10^4 cells in a micro well culture plate of 0.4 ml capacity and incubated at 37°C.

At different periods of growth, cultures were exposed to 0.1 μCi of 3H-thymidine (2 Ci/mmol; New England Nuclear, Boston, MA) and allowed to grow for six more hours. Then the radioactive medium was removed and cultures were carefully washed three times with PBS. Cells were scraped off the well with 10% trichloroacetic acid, transferred to scintillation vials, and measured for radioactivity after 5 ml of Tritosol in a Beckman (Palo Alto) liquid scintillation counter was added. All determinations were performed in triplicate.

To measure enzyme activities, the cultures were washed with PBS at appropriate times after plating. The cells then were removed with a rubber policeman and homogenized in 0.1 mol/l phosphate buffer pH 8.0, 0.5% Triton X-100 (Sigma, St. Louis, MO). AChE and BChE activities from the extracts and culture media were determined according to the Ellman et al procedure, using 0.5 mmol/l acetylthiocholine or butyrylthiocholine as substrates for AChE and BChE, respectively. 0.1 mmol/l iso-OMPA and 1.5-bis (4-allyl-dimethylammonium phenyl) pentan-3-one-dibromide (BW 284C51) were used as specific inhibitors of BChE and AChE.

Sedimentation through sucrose gradient was performed to separate cholinesterase molecular forms. Cultures were homogenized in 10 mmol/l Tris-HCl (pH 7), 1% Triton X-100, 1.0 mol/l NaCl, and 50 mmol/l MgCl2 (salt homogenization buffer). The homogenate was centrifuged at 5000 × g for 10 min. Aliquots of the supernatant and culture media were loaded on a linear sucrose gradient (5–20%) prepared in salt homogenization buffer. Gradients were centrifuged in a Beckman SW 40 ultracentrifuge rotor at 40,000 rpm for 20 h at 4°C. Fractions (0.6 ml) were collected and assayed for AChE and BChE. Sedimentation coefficients were estimated using catalase (11.3S) and alcohol dehydrogenase (4.8S) as standards.

Protein content was determined following the procedure of Lowry et al.

Results

Under our culture conditions, RPE cells proliferate and reach confluence at 48 hr. Once the culture reached confluence, cells started to differentiate, showing little or no cellular divisions as demonstrated by the 3H-thymidine incorporation experiments (Fig. 1). At 72 hr, the cells became cubical and exhibited a brownish color, becoming a monolayer of pigmented cells at day 4 (Fig. 2).

The time course of production of cell-associated and secreted AChE and BChE activities in RPE cells in culture was investigated over 15 d. AChE activity increased continuously during the days of culture. At one day, in vitro RPE cells showed AChE activity of 6.8 ± 1.6 nmole/min/mg protein, which increased about two-fold at 10 d of culture (Fig. 3A).

The BChE activity was about five times lower than that of AChE and did not show significant differences during the days in culture studied (Fig. 3A). The AChE/BChE activity ratio showed values of 2.4, 4.6, and 9.1 at 1, 4 and, 10 d, respectively.

A parallel increase in cell-associated AChE activity was noted for secreted AChE activity. Figure 3B represents the amount of released enzymes. Released AChE activity was significantly enhanced as a function of the culture’s age, showing an increase of six times from day 1 to day 10 in vitro. On the other hand, the activity of the extracellular BChE remained practically constant through the study period. As a result, the AChE/BChE activity ratio increased continuously from 6.5 to 59 between day 1 and day 10 in culture.

The molecular forms of AChE and BChE were studied at 1 and 4 d of culture, which corresponded to the proliferative and differentiated state of the cells, respectively. Analysis of the cellular proteins by velocity sedimentation gradients revealed four peaks of AChE and BChE activities, which seem to correspond to the A12, G4, G2, and G1 molecular forms for the former and A8, G4, G2, and G1 for the latter (Fig. 4). The predominant molecular form of AChE at day 1 in culture was the A12. However, at 4 d in culture, the activity of the G4 and G2 markedly increased, reaching levels similar to those of the A12.
Changes in the molecular forms of BChE also were observed. The A₄ molecular form is predominant in the cells at day 1 in culture, but is not present at day 4. At four days, the G₄ form is enhanced, leading to an increase in the G₂/G₁ ratio of 1.0 to 3.5 from day 1 to day 4 in culture (Fig. 4).

On the other hand, sedimentation analysis of the culture medium revealed that the released AChE and BChE activities seemed to correspond to the G₄ species, although a small amount of the G₂ and G₁ was observed at day 1 in culture (Fig. 4).

**Discussion**

The results indicate that RPE cells in culture synthesize AChE and BChE, which remain associated with the cells or are released into the medium. The AChE activity is higher than that of BChE, a difference that becomes more significant at progressive ages of the culture (Fig. 1). The amount of released enzyme activities was higher than that of the cell-associated ones. Both enzymes showed different developmental patterns. Cell-associated and secreted AChE activities in-
Fig. 4. Sedimentation profiles of acetylcholinesterase (A and C) and butyrylcholinesterase (B and D) molecular forms from RPE cells in culture. Sucrose density gradient centrifugation was performed as described in Methods. The enzyme activities are plotted on an arbitrary units (optical density 412) as a function of the fractions number, which represents the distance from the bottom of the tube. Arrows indicate the position of catalase (CAT; 11.4S) and alcohol dehydrogenase (ADH; 4.8S). Patterns in (A) and (B) correspond to the cell-associated enzyme activities; (C) and (D) represent the molecular forms released in the medium. At one day of culture: ▲; at four days of culture: O. The values are the average of four different experiments.
increased in parallel with the age of the culture (Fig. 3). These results correlate well with the observed AChE activity increase in RPE cells during chick in vivo development. In contrast, BChE activity was found to be low and did not show significant variations (Fig. 3). Thus, the AChE-BChE activity ratio increased as the cells became differentiated.

Cellular and secreted enzymes differ in the composition of molecular forms. The reported data show that the constituents of the molecular pool of cholinesterases turned out to be complex because we found globular (G1, G2, and G4) and asymmetric forms (A8, A10, and A12; Fig. 4) of both enzymes. Our results agree with those of Martelly and Gautron, who found similar molecular forms for AChE of RPE in developing chick embryo. The G4 and G2 molecular forms appear to be ubiquitous, while the asymmetric species of AChE we found seem to be characteristic of the muscular endplate. However, it also has been reported in rat heart, superior cervical ganglia, chicken ciliary ganglia, chicken optic lobes, tectum, and retina.

Our results indicate that AChE activity is higher in differentiated RPE cells. In addition, the relative proportion of the G4 molecular form of AChE and BChE increased during differentiation. A similar increase in the G4 molecular form has been described during in vivo development and in cultured retinal, neuroblastoma, and pheochromocytoma cells. The release of the G4 molecular form has been reported to occur in several types of cells. The RPE showed a low activity of BChE. Interestingly, however, the asymmetric molecular form was present in proliferating cells but not found at day 4 in culture when the cells were differentiated.

Although the significance of AChE and BChE activities in RPE cells is unknown, they might be related to processes of cell proliferation and differentiation as has been previously suggested, because AChE and BChE are present in early embryonic stages of development in neural and nonneural tissues. Moreover, in RPE and retinal aggregate cultures, Vollmer and Younger found AChE and BChE activities to be strongly correlated with cell proliferation and differentiation, respectively. Whether the increase in AChE activity and in the time-related changes in molecular forms of the enzyme are related to the differentiation of RPE cells remain to be established.

**Key words:** acetylcholinesterase, butyrylcholinesterase, retinal pigment epithelium, culture, proliferation, differentiation.

**Acknowledgements**

The authors would like to thank Ms. Ma. Teresa Martinez for technical assistance.

**References**

21. Gisger V, Vigny M, Gautron J, and Rieger F: Acetylcholines-


