Studies on the differentiation of retinal pigmented epithelium cells in culture

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Sephadex G-25 fractions of chick embryo extract affect the differentiation of cloned embryonic pigmented epithelial cells (PE) in culture. Both tyrosinase activity and amounts of melanin are markedly affected by the extract fractions. 5-Hydroxyindole decreased melanin accumulation in PE cells while α-methyl-p-tyrosine had no apparent effect on the process. Melanin disposal as well as biosynthesis may be important in controlling the accumulation of pigment in PE cells under culture conditions. Triiodothyronine greatly increased visible pigmentation and affected the morphology of cultured PE cells.

Key words: pigmented epithelium, tissue culture, triiodothyronine, pigmentation, differentiation.

Little is known about the control of differentiation and melanogenesis in normal pigmented epithelium (PE) cells. A ready tool for such study is available, however, since clones of PE cells from the chick embryo can be maintained in a morphologically differentiated state in cell culture over an extended period of time. Coon and Cahn have demonstrated that Sephadex fractions from chick embryo extract dramatically affect the patterns of growth of cultured PE and cartilage cells. We have previously examined some of the effects of fractions of embryo extract on the morphology of cultured PE cells and have also studied the effects of cyclic-AMP and its derivatives on PE morphology and visible pigmentation. The present study was designed to elucidate some of the factors involved in the control of tyrosinase activity and melanin production in PE cells and the possible role of thyroid hormone in this process.

Methods

Cells from the pigmented epithelium of Gallus domesticus embryos were maintained in 3 ml of Coon's modification of Ham's F-12 medium (F-12) containing 5 per cent fetal calf serum (Gibco,
Fig. 1. Visible pigmentation in cloned pigmented epithelial cells obtained from stage-25 chick embryos. Culture period was four weeks. A, cells grown in H medium; B, cells grown in L medium.

Grand Island, N.Y.) and 0.3 μg of penicillin per milliliter in 60 mm. Falcon plastic culture dishes. Chick embryo extract was fractionated into a high molecular weight (H) and low molecular weight (L) fraction by Sephadex G-25 column chromatography as described by Coon and Cahn. These fractions were added to the basic F-12 medium at a concentration of 5 per cent (v/v) to make either the "H medium" or the "L medium," respectively. Cells were maintained in a 5 per cent CO2, 95 per cent air atmosphere (100 per cent humidity) at 37.5°C. Medium was changed every three days.

For studies of total tyrosine hydroxylase activity, approximately 800,000 d.p.m. of 3H-tyrosine (New England Nuclear Corporation, Boston, Mass.) was added to the medium of the tissue culture dish which was 5.5 × 10−5 M in tyrosine concentration. When appropriate, either α-methyl-p-tyrosine or 5-hydroxyindole was added directly to the incubation medium at a final concentration of 1 × 10−5 M. Isolation of 3HOH formed in the reaction was performed by a modification of the method of Lovenberg and co-workers. Essentially, 1.0 ml of medium from each culture dish was applied to a 2.5 cm. column of Dowex 50 × 8 (200 to 400 mesh) and eluted with distilled water. The first 2.5 ml was collected, sampled, and radioactivity determined in a Packard Liquid Scintillation Spectrometer using 15 ml of Aquasol (New England Nuclear).

For tyrosinase and tyrosine hydroxylase activities determined directly on PE cells, cultures were sucked free of culture medium, the cells were washed several times in a balanced salt solution, cells were scraped from the bottom of the culture dish and transferred quantitatively to small plastic centrifuge tubes (Brinkmann). For tyrosine hydroxyase assay, the reaction volume was 200 μl containing 50 mM potassium phosphate, pH 6.8, 0.25 M mercaptoethanol, 90 mM ferrous ammonium sulfate, and 1 mM dimethyltetrahydropteridine. The reaction was started by the addition of 5 × 10−4 M tyrosine containing approximately 500,000 d.p.m. of radiolabeled tyrosine. Incubation was for three hours at 37°C in a shaking water bath. The reaction was stopped by immersion in ice and the tritiated HOH produced during the reaction separated on a Dowex-50 column as described above. For tyrosinase activity, the same procedure was used except that the final reaction mixture contained only buffer, 1 × 10−4 M dihydroxyphenylalanine (DOPA), and the tyrosine substrate.

Melanin was isolated free of other cellular constituents essentially according to Obika and Negishi. After incubation with 3H-tyrosine, cells were washed and removed from the culture plate by the addition of several drops of 10 N HCl. With repeated washing, the contents of each tube were then transferred to a Millipore filter and washed copiously with water and then 95 per cent alcohol. The filters were dried, placed in counting vials, dissolved with 1 ml of dimethylformamide, and radioactivity determined.

Morphology was observed and documented through a Wild phase-contrast tissue culture microscope fitted with a 35 mm. camera.

Results

The effect of chick embryo extract on visible pigmentation in PE cells after four weeks in culture is seen in Fig. 1. The
addition of the low molecular weight fraction of embryo extract to the medium to form L medium (Fig. 1, B) results in a great deal of visible pigmentation in PE colonies as seen in the right-hand dish. In contrast, very little pigmentation is observed when the medium is supplemented with the higher molecular weight fraction of embryo extract to form H medium (Fig. 1, A). When the medium consisted of F-12 medium alone, an intermediate amount of visible pigmentation was observed. These differences in pigmentation were apparent through long periods of culture and were not merely due to a delay or lag period in the appearance of pigmentation. Also, PE cells cultured in H medium actually proliferated more rapidly than in L medium. Thus, differences in visible pigmentation were not due to greater numbers of cells in L medium.

When tyrosinase activity was measured in PE cells scraped from the culture dish, no difference was observed between the activity in cells grown in F-12 or in H medium while tyrosinase activity was approximately twofold higher in cells grown in L medium (Fig. 2). Cumulative tyrosine hydroxylated during a 13-day incubation period of PE cells under culture conditions was also highest in cultures containing the L fraction of embryo extract. This was assessed by measurement of the total amount of tritiated water released into the tissue culture medium. Total hydroxylation was approximately 35 per cent of this value in cultures containing only the F-12 medium and 50 per cent in ones supplemented with the H-fraction. Incorporation of 1H-tyrosine into melanin was greatest in cells grown in L medium. Cells grown in F-12 medium contained approximately 50 per cent as much radiolabeled melanin while that of cells grown in H medium was only about 15 to 20 per cent as great.

Addition of 1 x 10⁻⁵ M 5-hydroxyindole to the culture medium decreased the accumulation of radioactive melanin by approximately 50 per cent in cells grown in F-12 or H medium and somewhat less in cells grown in L medium. The addition of α-methyl-para-tyrosine had no apparent effect on melanin production. As can be seen in Fig. 3, cells grown over a 13-day culture period in F-12 medium showed approximately equal pigmentation in control (F-12) cultures (Fig. 3, A) and those containing 10⁻⁵ M α-methyl-para-tyrosine (Fig. 3, B). Pigmentation was markedly suppressed, however, with the addition of 10⁻⁵ M hydroxyindole (Fig. 3, C).

The addition of 1 x 10⁻⁵ M triiodothyronine to cultures of cells grown in H medium greatly increased visible pigmentation (Fig. 4). The hormone also had a distinct effect on cell morphology. Fig. 5 compares cells grown in L medium, H medium, and H medium supplemented with triiodothyronine after cessation of log phase growth. Cells in L medium appear well-differentiated, have a regular cuboidal appearance, and have a good deal of visible
Fig. 3. Inhibitor effects on visible pigmentation in cloned pigmented epithelial cells obtained from stage-25 chick embryos. A, F-12 medium alone; B, medium supplemented with $1 \times 10^{-5}$ M α-methyl-para-tyrosine; and C, medium supplemented with $1 \times 10^{-5}$ M 5-hydroxyindole.

Fig. 4. The effect of triiodothyronine on visible pigmentation of cloned pigmented epithelial cells from stage-23 chick embryo. A, H medium; B, H medium plus $1 \times 10^{-6}$ M triiodothyronine.

pigmentation. Cells in H medium are larger in size, have less distinct boundaries and appear to be more randomly oriented. A large amount of extracellular filamentous material can be observed in these cultures. The addition of thyroid hormone reverses this, however, in that cells appear better differentiated as if they were grown in L medium with a more compact, cuboidal appearance and increased pigmentation.

Discussion

Normal development of the embryonic pigmented epithelium is a prerequisite for normal vision in the adult animal. The present system affords a method for studying the differentiation of PE cells in vitro, determining some of the factors affecting differentiation and possibly manipulating conditions to control or maintain normal development. It is apparent that at least one facet of differentiation, i.e., melanation, can be controlled under culture conditions.

It is known that tyrosine can be converted to dihydroxyphenylalanine in melanin-producing cells by two separate enzymes, tyrosinase and tyrosine hydroxylase. To distinguish between these pathways, the inhibitors, 5-hydroxyindole and α-methyl-para-tyrosine, were used in the present study. The former is an excellent inhibitor of tyrosinase while the latter inhibits tyrosine hydroxylase activity. Since 5-hydroxyindole substantially inhibited tyrosine hydroxylation while α-methyl-para-tyrosine did not, only the tyrosinase pathway appears to be operative in the PE.

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cells. Also, since α-methyl-para-tyrosine had no effect on tyrosine hydroxylation, the formation of catecholamines and other biogenic amines by the PE cells seems to be negligible.

Since tyrosinase activity and total melanin content in cells grown in L medium is greater than cells grown in F-12 medium, it appears that a specific promoter is present in this fraction of embryo extract which induces increased tyrosinase activity and subsequent melanin production. In contrast, cells grown in H medium had much less visible pigmentation than those grown in F-12 medium although, significantly, there was no difference observed in cellular tyrosinase activity under the two conditions. This would indicate an increased rate of "disposal" or "degeneration" of melanin in cells grown in the H medium. Alternatively, melanin formation may be blocked in these cells at a site distal to the initial tyrosinase reaction although this appears unlikely. The mechanism of the melanin disposal is not known at present.

The role of hormones in the development and functioning of the retina and pigmented epithelium has not been studied to any great extent. The striking effects of cyclic-AMP and triiodothyronine on PE cells indicate that hormones may have a profound effect on normal development and function both in the embryo and in the adult animal. Not only are morphologic characteristics and pigmentation
dramatically altered but growth rate and cellular adhesion to substratum are also affected by the cyclic nucleotide.  

Thyroid hormones, thyroxine, and triiodothyronine have long been known to affect many aspects of differentiation of embryonic cells as well as normal functioning in the adult animal. Besides general effects on energy utilization, specific effects have been observed on enzyme induction, e.g., hepatic α-glycerophosphate dehydrogenase or glutamine synthetase of the embryonic retina. Effects of thyroxine have also been observed on the plating efficiency and growth rate of cells in culture and on the uptake of uridine into cellular RNA. In the present study, triiodothyronine lead to a more differentiated appearance of PE cells, increased tyrosinase activity, and increased visible pigmentation.

It thus seems clear that many aspects of the differentiation of pigmented epithelial cells can be studied under well-defined culture conditions. It is hoped that such studies will lead to a better understanding of the factors that affect normal or abnormal development of the pigmented epithelium and the neural retina.

The authors wish to dedicate this work to Dr. David C. Cogan and his omnivorous taste in research.

REFERENCES