Predominant melanogenesis and lentoidogenesis *in vitro* from multipotent pineal cells by dimethyl sulfoxide and hexamethylene bisacetamide

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ABSTRACT Pineal cells of the 8-day embryonic quail are multipotent cells which differentiate *in vitro* into skeletal muscle fibers, pigmented epithelial cells (PECs), lens cells and neurons. However, it was not yet clear whether precursor cells which gave such a wide repertoire of differentiation were single type or not. The present culture studies revealed that pineal cells were exclusively directed to ocular differentiation pathways by dimethyl sulfoxide (DMSO) and hexamethylene bisacetamide (HMBA), suggesting a single type of precursor cell in the pineal body. DMSO directed pineal cells to differentiate into PECs. Co-administration of basic fibroblast growth factor (bFGF) with DMSO partially inhibited PEC differentiation and promoted lens cell differentiation. Northern blot analysis using cDNAs specific to PEC and lens cell confirmed this morphological observation. HMBA completely inhibited pigmentation of cultured pineal cells and markedly promoted lens cell differentiation. Ocular differentiation of pineal cells was accompanied with the loss of myogenicity. We discuss three possible pathways of lens cell differentiation from pineal cells. The agents which affect pineal cell differentiation seemed to modulate the cell-substrate interaction. And the interaction was suggested to be one of the environmental cues in the differentiation.

KEY WORDS: *pineal cells, multipotency, melanogenesis, lentoidogenesis, dimethyl sulfoxide, hexamethylene bisacetamide*

Introduction

The pineal body of avian and mammalian species is an endocrine organ which transduces light information from eyes. In the process of ontogeny, the pineal body in these species has transiently rudimentary photoreceptor cells (Zimmermann and Tso, 1975; Omura, 1977). Furthermore, the pineal body in lower vertebrates is a photoreceptive organ like lateral (usual) eyes (Eakin, 1970). The endocrine pinealocyte has been thought to be a direct sequence from the photoreceptor cell and rudimentary photoreceptor cell (Oksche and Hartwig, 1979).

It was already known that pineal cells of the embryonic quail retain oculopotency, which includes differentiation ability of pigmented epithelial cells (PECs) and lens cells (Watanabe *et al.*, 1985). In the previous conditions, however, expression of oculopotency was accompanied by other types of cells such as skeletal muscle fibers and fibroblastic cells. Also differentiation of lens cells was inconsistent and needed a prolonged culture period. In this study, we show improved culture conditions which conduct pineal cells to differentiate exclusively into ocular cells such as PECs and lens cells. The key agents directing pineal cells to differentiate into ocular cells are dimethyl sulfoxide (DMSO), basic fibroblast growth factor (bFGF) and hexamethylene bisacetamide (HMBA). The results show that the pineal cells of embryonic quail are multipotent, and that they can be directed to one differentiation pathway, shown typically in the case of differentiation of PECs. The differentiation system of pineal cells may be suitable to search for regulatory genes acting in the determination of pigmented epithelial cells and lens cells.

Abbreviations used in this paper: PECs, pigmented epithelial cells; DMSO, dimethyl sulfoxide; bFGF, basic fibroblast growth factor; HMBA, hexamethylene bisacetamide; MM-CK, skeletal muscle type creatine kinase; TPBS, PBS containing Triton X-100.

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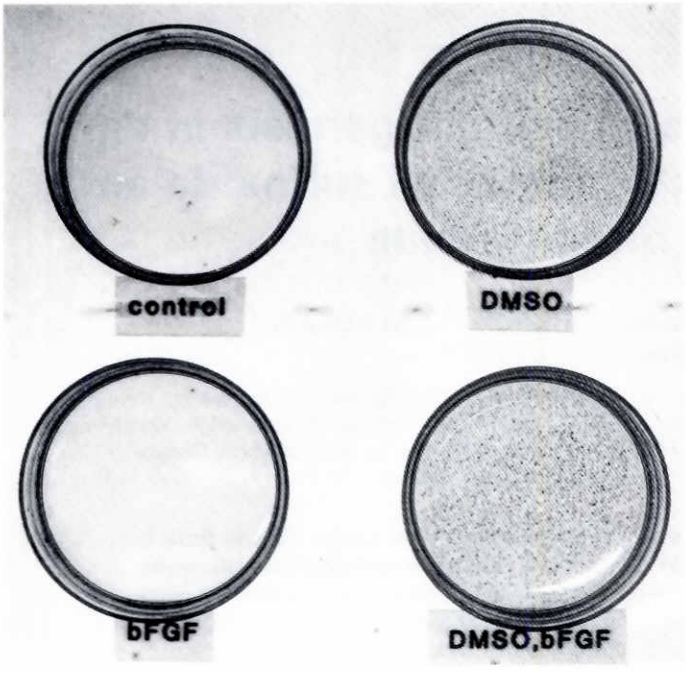
0214-6282/94/$03.00



Results

Predominant expression of pigmented cell feature under the effect of DMSO

Cultured pineal cells for a few days are distinguished morphologically into flat epithelial cells and small round cells (Watanabe *et al.*, 1988). In the basal medium, small round cells disappeared gradually and most of the epithelial cells became fibroblastic in about one week (Figs.1, 2A). Thereafter, skeletal muscle fibers and pigmented epithelial cells were sometimes differentiated.

Administration of 2% (280 μM) DMSO in the culture completely inhibited the differentiation of fibroblastic cells, whereas epithelial cells became predominant in the culture. At a concentration of 1% DMSO, some fibroblastic cells were differentiated. Two types of epithelial cells could be observed in 2% DMSO-medium around

Fig. 1. Dishes culturing pineal cells for 2 weeks in 4 different media. *Control dish was supplemented with the basal medium. DMSO, bFGF and DMSO+bFGF dishes were supplemented with the basal medium administered with 2% DMSO, 10 ng/ml bFGF and both, respectively. In control and bFGF cultures, only a few pigment foci were observed. In DMSO and DMSO+bFGF cultures, numerous foci were readily detectable.*

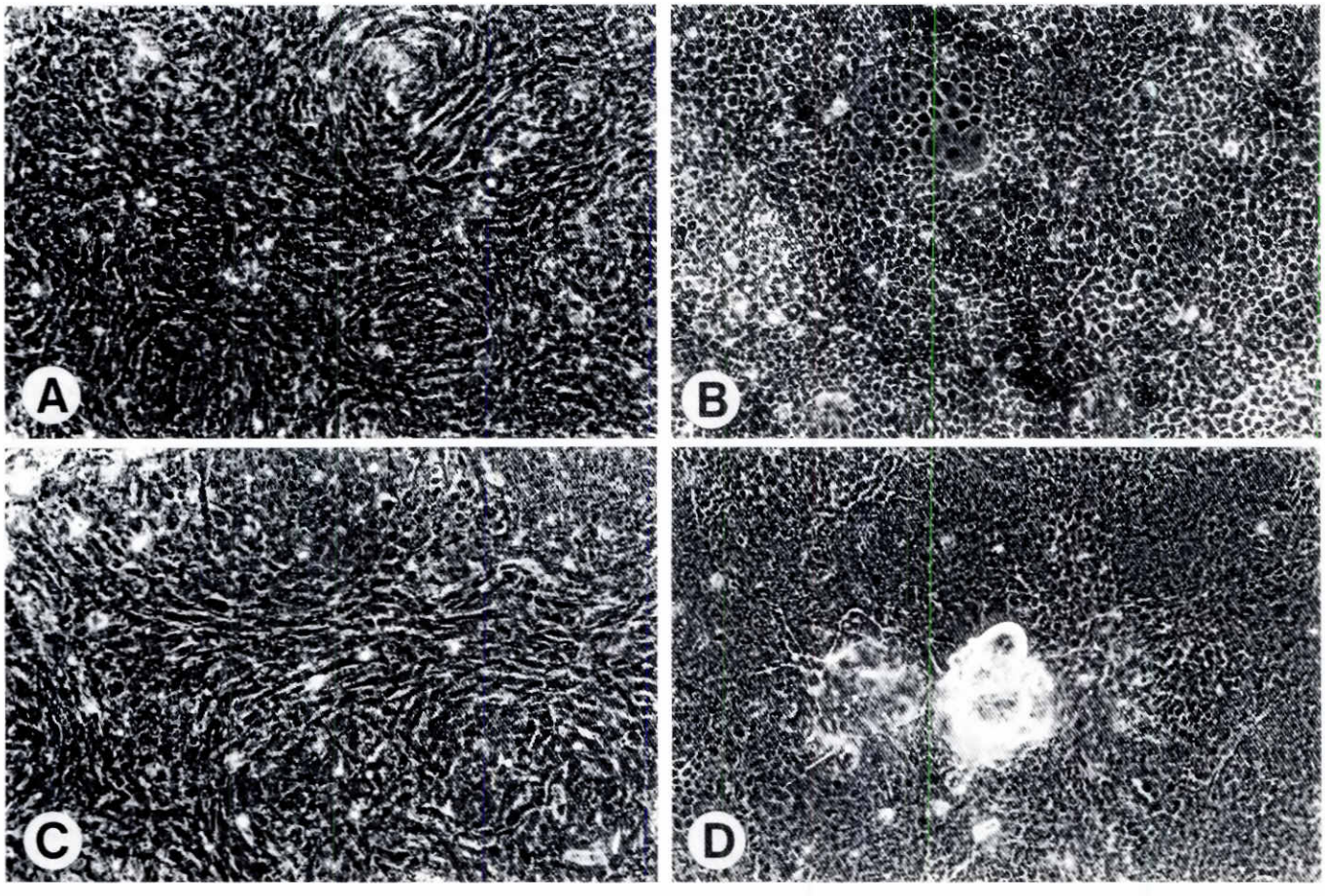


Fig. 2. Phase contrast micrographs of the same dishes in Fig. 1. *Fibroblastic cells were dominant in control culture* **(A)** *and bFGF administered culture* **(C)**. *Pigmented epithelial cells were exclusively differentiated in DMSO-medium* **(B)**. *Lentoid bodies were frequently observed in DMSO+bFGF medium* **(D)**. *x67.*

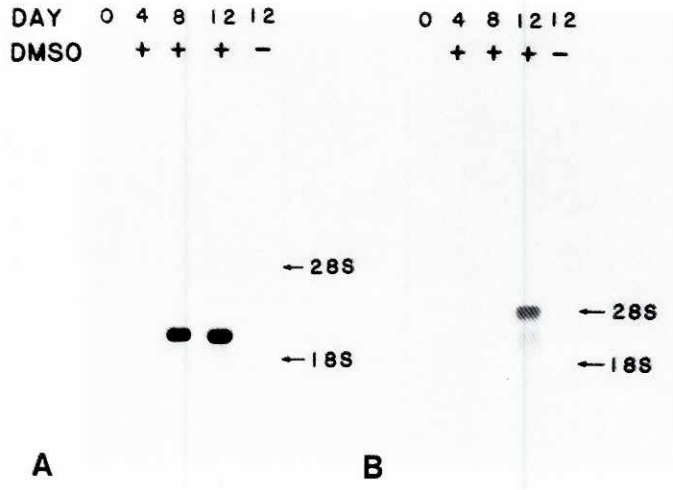


Fig. 3. Northern blot analysis for MMP115 (A) and pP344 (B) genes in pineal cells cultured in DMSO-medium (+) and basal medium (-).

one week culturing. One type of cells were small packed epithelial cells, whose boundaries were difficult to distinguish. The other ones were polygonal epithelial cells with or without pigmented granules and they were similar to retinal pigmented epithelial cells in shape. Continuing culture, polygonal cells increased and small epithelial cells decreased (Figs.1, 2B). Lentoid bodies were hardly observed in these conditions.

Lentoidogenesis of pineal cells under the coeffect of DMSO and bFGF

Basic fibroblastic growth factor (bFGF) is a ubiquitous peptide with profound biological effects on various types of cells (Rifkin and Moscatelli, 1989). In the case of pineal cells, we have already observed that bFGF promotes neuronal differentiation from the pineal cells suppressed in their DNA synthesis (Araki *et al.*, 1993).

Under the effect of 2% DMSO and 10 ng/ml bFGF, pineal cells became mostly small round epithelial cells and the differentiation of PECs was markedly delayed. Lentoid bodies were constantly differentiated around two weeks of the culture (Figs.1, 2D) and increased gradually in number. Decreased DMSO concentration (1%) in combination with bFGF was more effective on lentoidogenesis, whereas, bFGF alone was not effective on the lentoidogenesis of pineal cells (Figs.1, 2C).

Expression of cell type specific genes under the effect of DMSO and/or bFGF

Expression of genes specific to pigmented epithelial cells (MMP115 and pP344) and lens cells (δ-crystallin) were examined in the pineal cell culture under the effect of DMSO and bFGF. In a pineal body, MMP115 gene was expressed at a very low level corresponding with the pigment feature of *in situ* pineal cells (see discussion). However, pP344 and δ-crystallin genes were not expressed (Fig. 3, day 0). In 2% DMSO-medium, MMP115 and pP344 genes were expressed in 8 days and 12 days, respectively. It should be emphasized that pP344 is a specific marker of PECs and is not expressed in melanocytes of the skin (Agata *et al.*, 1993). δ-crystallin was not expressed in the basal medium or in 1% and 2% DMSO-medium. The δ-crystallin gene was expressed only in DMSO-medium containing bFGF. The expression of these genes was paralleled with the morphological change of cultured pineal cells.

Lentoidogenesis of pineal cells by HMBA

HMBA as well as DMSO is an agent which induces erythroleukemia cells to differentiate into red blood cells (Marks and Rifkind, 1989). Administration of HMBA into the culture of pineal cells promoted the differentiation of epithelial cells and inhibited fibroblastic differentiation (Fig. 5). The epithelial cells became polygonal cells which resembled pigmented epithelial cells, although they had no pigment at the light microscopical level. They became gradually smaller in size. Lentoid bodies were differentiated from smaller packed epithelial cells in the overall dish.

Expression of δ-crystallin gene was detected from 12 day culturing and then increased (Fig. 6). MMP115 gene, which is a marker of pigmented cells, was also expressed in pineal cells cultured in HMBA-medium for 8 days. The results were beyond our expectations, because pineal cells in HMBA-medium were never pigmented. The other marker of pigmented cells, tyrosinase gene, was not expressed in the cultured cells in HMBA-medium (data not shown).

Irreversible direction of pineal cells into ocular cells by DMSO treatment

It has been shown in the present study that pineal cells are differentiated into PECs by the DMSO treatment. On the other hand, the pineal cells respond to hypertonicity, and differentiate into skeletal muscle fibers (Watanabe *et al.*, 1988). It was examined whether differentiation of PECs conducted by DMSO is accompanied by the loss of myogenicity of pineal cells or not.

Pineal cells were treated with DMSO for various periods and then with the hypertonic medium for 3 days. The cells were fixed and immunostained with anti-MM-CK, to examine the differentiation of skeletal muscle fibers. With DMSO treatment for 3 days, the number of MM-CK positive cells was only 2, whereas it was 678 without DMSO treatment (Fig. 7). The results have suggested that a few days' treatment of DMSO directs the pineal cells to the

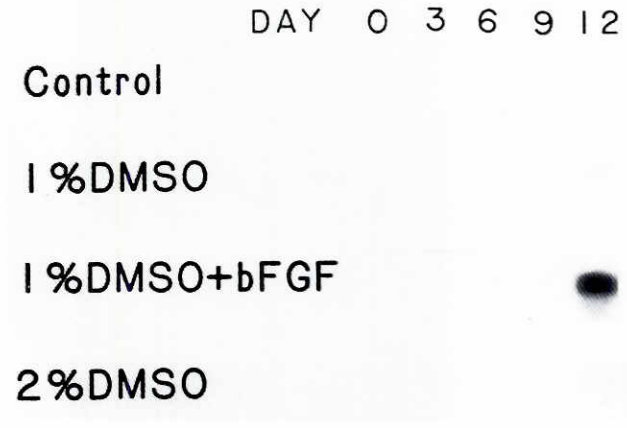


Fig. 4. Northern blot analysis for δ-crystallin gene under the effect of DMSO and bFGF. *Culture condition is described in the text.*

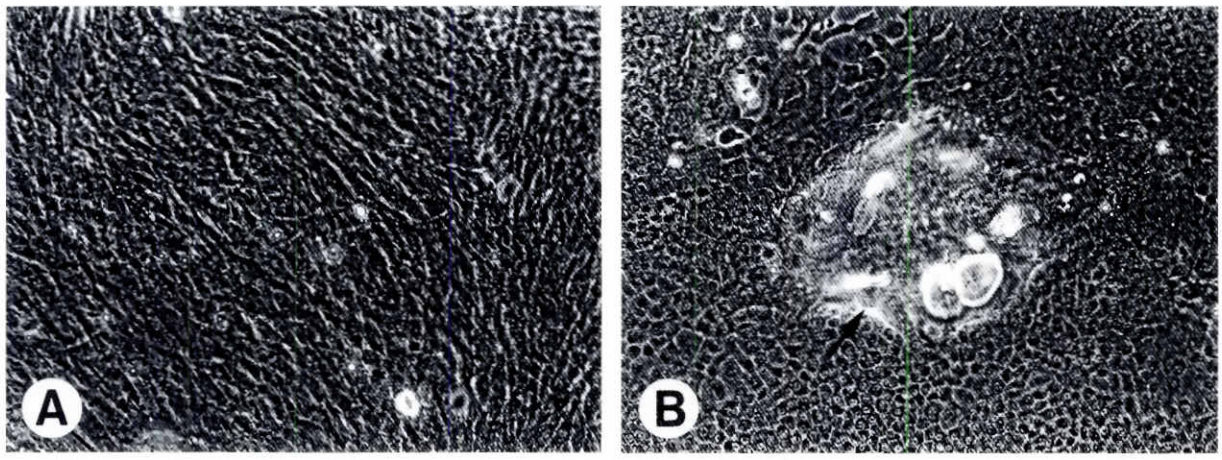


Fig. 5. Phase contrast micrographs of pineal cells cultured for 16 days in basal (A) and HMBA (B) medium. *Lentoid body is indicated by an arrow in* **B**. *x141.*

differentiation pathway of PECs, accompanied by the loss of myogenic potency.

Clonal analysis of differentiation pathways of pineal cells

To study ocular differentiation pathways of pineal cells, clonal analysis was performed. The medium for clone culture was 1% DMSO-medium containing bFGF and 25% conditioned medium, which might be adequate for differentiation of both pigmented epithelial cells and lens cells in mass culture. After 4 weeks culturing, 52 growing colonies were obtained out of 4000 inoculated cells, giving a clonal efficiency of 1.3%. The colonies containing lentoids numbered 10, of which one colony contained both pigmented epithelial cells and lentoids (Fig. 8). The other 42 colonies were fibroblastic or undefined cell types.

The results suggest probable pathways of lentoidogenesis from pineal cells. The first is the direct course from pineal cells into lens

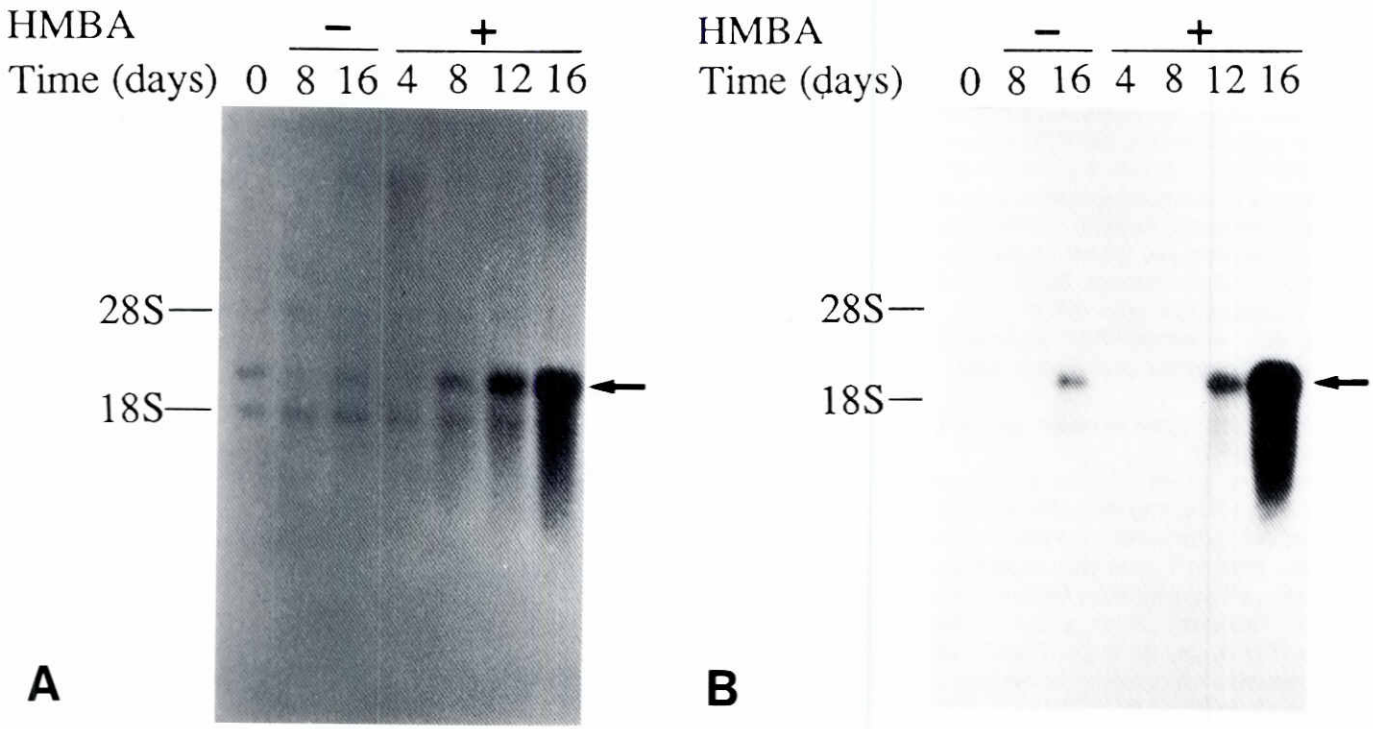


Fig. 6. Northern blot analysis for MMP115 (A) and δ-crystallin (B) genes in pineal cells cultured in HMBA-medium (+) and basal medium (-). *Signals are indicated by arrows.*

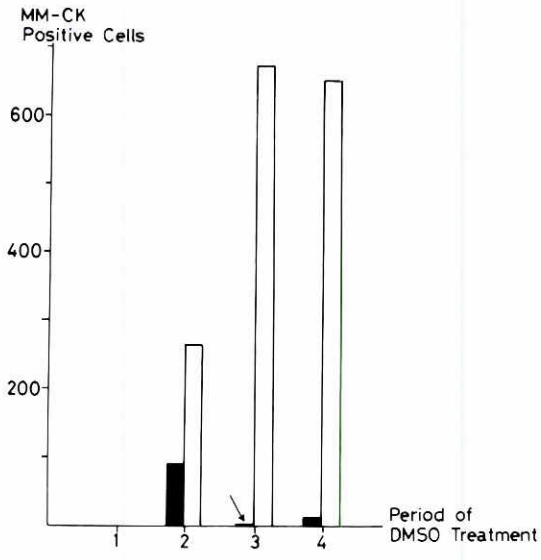


Fig. 7. Inhibition of myogenicity from pineal cells by DMSO treatment. *DMSO was given on the next day of inoculation. After treatment with DMSO-medium (closed bar) and basal medium (open bar) for 2, 3 and 4 days, DMSO-medium was changed to hypertonic medium. After another 3 day culturing, skeletal muscle cells were immunostained with anti-MM-CK, and the number of nuclei in positive cells was counted. Muscle differentiation was clearly inhibited by 3 day treatment of DMSO.*

cells. The second is the indirect course in which lentoidogenesis of pineal cells is followed via bipotent ocular cell state. The third is lentoidogenesis by transdifferentiation of PECs, derived from pineal cells (see below).

Transdifferentiation of pineal-PECs into lentoids

Transdifferentiation of retinal pigmented epithelial cells into lentoids has been well characterized (Eguchi, 1992). PECs differentiated from pineal cells have the possibility to transdifferentiate into lentoids. When pineal cells were cultured in the DMSO-medium containing bFGF and transferred every week, PECs and lentoids were constantly differentiated after about 7 days in the culture of second passages onwards. We were able to pick the area of PECs on the culture and transferred the cells onto the other dishes. In this case, we constantly found lentoidogenesis from PECs (data not shown). However, when we cultured primary pineal cells in DMSO-medium without bFGF for a longer period of 2 weeks, and then transferred every week, PECs were hardly transdifferentiated into lentoids. The results may suggest that there is a critical stage of cell differentiation of pineal PECs in respect to lentoidogenesis. Before that stage, pineal PECs are ready to transdifferentiate into lentoids. After that, they rarely do and may need a prolonged culture period or other differentiation agents such as phenyl-thiourea (Itoh and Eguchi, 1986).

Effect of substrate on the differentiation of pineal cells

The cell-substrate interaction is one of the environmental cues to decide the differentiation states of cells. The effects of substrates were examined for differentiation of pineal cells using plastic dish for culture dish coated with the type I collagen as used in the former sections and non-treated polystyrene dish (Corning 15000). The former two were not significantly different. Noteworthy findings came from the use of a non-treated dish which is not usually suitable for the adhesion, growth and differentiation of the cells. On that plate, pineal cells were first aggregated and then gradually emigrated and grew, if they were nourished with DMSO-medium containing bFGF. Some of them were epithelial and others were fibroblastic. Lentoid bodies were differentiated gradually, and pigmentation was completely suppressed up to a month. Furthermore, aggregates of small neuronal cells were maintained and developed. Cell islands consisting of only lentoids or neurons appeared in a patchy fashion. DMSO is responsible for the maintenance and growth of the pineal cells on the non-treated dish (data not shown). bFGF may be related to the neurite extension (Araki *et al.*, 1993) and lentoidogenesis.

Discussion

Cultured pineal cells of the 8-day embryonic quail are distinguished in two cell types. The small round cells observed only in early culture period express neuronal phenotypes (Araki *et al.*, 1993). Epithelial cells are multipotent cells which have potency to differentiate into both pigmented epithelial cells and skeletal mus-

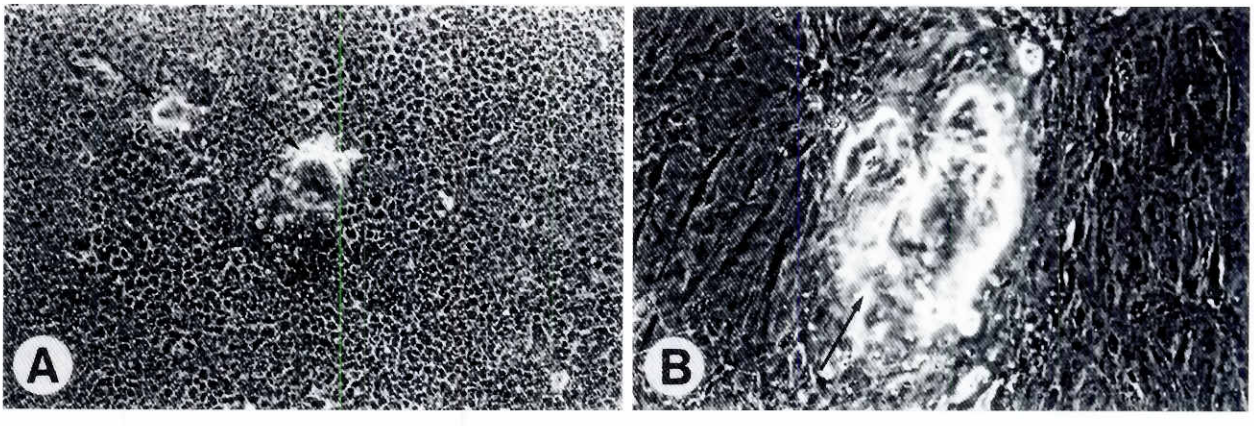


Fig. 8. Colonies differentiated in the clone culture. *A colony mixed with pigmented epithelial cells and lentoids (arrows)* **(A).** *One of the colonies with lentoids (arrow)* **(B).** *(A) x60, (B) x90.*

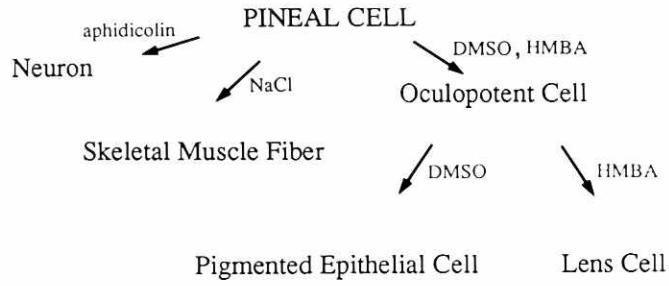


Fig. 9. A scheme showing differentiation pathways from multipotent pineal cells and relevant agents.

cle fibers (Watanabe *et al.*, 1988) and also into neurons (Araki *et al.*, 1993). Multipotent epithelial cells of the pineal body or single pineal cells have now been shown to be directed exclusively into the ocular differentiation pathway by DMSO and HMBA.

Suppression of myogenicity of pineal cells

It is not probable that fibroblastic or myoblastic precursor cells other than multipotent epithelial cells in the pineal body are suppressed in their growth by DMSO or HMBA, because myoblasts and fibroblasts from muscle tissue grow well in DMSO-medium (data not shown). Furthermore, clonal analysis has shown the presence of bipotent cells to differentiate into both PECs and skeletal muscle fibers (Watanabe *et al.*, 1988). Multipotent pineal cells are probably directed into the ocular differentiation pathways by these agents, accompanied by the loss of myogenic or fibroblastic potency.

Differentiation states of in situ pineal cells

Melanogenesis is the earliest differentiation property known in the pineal body. When the pineal body is formed during the three days of ontogenic development, it already contains tyrosinase which synthesizes melanin from tyrosin, revealed by DOPA-histochemistry (Watanabe *et al.*, 1992), and also MEBL-1 antigen, which is specifically expressed in melanocytes and their precursors, and retinal pigment epithelium (Takiguchi-Hayashi and Kitamura, 1993). In the 8-day pineal body, there are three differentiation states in respect to melanogenesis (Watanabe *et al.*, 1992). The first state is pigmented and, of course, tyrosinase positive. The second is tyrosinase positive but not pigmented. And the third is tyrosinase negative. Three different states observed in pineal cells *in situ*, from at least 8-day embryos, have now been shown to be arranged into similar states (PECs) by DMSO treatment *in vitro*.

Pineal cells and retinal PECs

Some pineal cells *in situ* possess melanogenic properties common to retinal PECs. However, the two cell types are different in morphology and differentiation potency. The pineal body of 8-day quail forms follicles, constituting pseudostratified epithelium. The pigmented retina forms single cuboidal epithelium. Pineal cells have the potency to differentiate into skeletal muscle fibers, but retinal PECs do not. Pineal cells do not respond to phenyl-thiourea (data not shown), which is an effective agent to promote transdifferentiation of retinal PECs (Itoh and Eguchi, 1986). However, retinal PECs cultured in the DMSO-medium containing bFGF were not transdifferentiated up to 10 passages during two months (data not shown). It is another unsettled problem whether or not pineal PECs *in vitro* and retinal PECs are similar in their differentiation potency.

Cell-substrate and cell-cell interaction in the differentiation of pineal cells

Cells themselves synthesize substrate such as collagen, fibronectin and laminin, and stabilize their own differentiation states. On the other hand, cells respond to given substrates and change their differentiation states. For example, retinal PECs *in vitro* respond to laminin and transdifferentiate into neurons (Reh *et al.*, 1987). Transdifferentiation of retinal PECs is enhanced on the plastic substrate rather than collagen coated dish (Yasuda, 1979).

A developed system of transdifferentiation of retinal PECs into lentoids includes phenyl-thiourea and crude testicular hyaluronidase (Itoh and Eguchi, 1986), whose effective component has recently been found to be bFGF (Hyuga *et al.*, 1993). Pineal cells also respond to bFGF in their differentiation. Basic FGF displays the activities on many types of cells, including increased cell growth and migration, and induction of plasminogen activator and collagenase (Rifkin and Moscatelli, 1989). As a consequence of bFGF activity, extracellular matrices are decreased, and their composition may be changed. Modulation of differentiation of pineal cells may also be explained from the interaction of bFGF with extracellular matrix. On this point, it might be noteworthy that DMSO also remarkably decreased the amount of substrate synthesized by the pineal cells (data not shown). On the non-treated dish, cell-substrate interaction seems to be limited in the cell aggregates, and contribution of cell-cell interaction may increase in determination of differentiation states. The patchy appearance of lentoid bodies and neurons might be related to this situation.

Signal transduction and cell differentiation

DMSO and HMBA are known to promote differentiation of mouse erythroleukemia cells into red blood cells, and they also affect differentiation and growth of other several types of cells (Marks and Rifkind, 1989). They act multifunctionally on membrane permeability, protein kinase C activity, and cellular oncogenes. A brief survey was done to find out other agents which are related to signal transduction and to affect differentiation of pineal cells. However, no clear effects have been found yet from the administration of retinoic acid, okadaic acid, forskolin, genistein, transforming growth factor β (TGF-β and phorbol 12-myristate 13-acetate (TPA).

Multipotency of pineal cells

The probable differentiation pathways from multipotent pineal cells are summarized in Fig. 9. Embryonic pineal cells *in situ* have pigment phenotype, but are different from retinal PECs and melanocytes of the skin. They will differentiate into endocrine cells in the *in situ* conditions and may lose multipotency. When pineal cells are dissociated and cultured, they become highly sensitive to environmental conditions in their choice of differentiation pathways. Dissociated pineal cells are probably more akin to neural cells, as they are ready to differentiate into neural cells by the cessation of DNA synthesis using aphidicolin. The differentiated neural cell types are similar to the neural retinal cell (Araki, *et al.*, 1993). In this case, bFGF works in the maintenance of neurons and in the extension of neuronal processes. Several cell divisions may be needed for the expression of other differentiation phenotypes.

Muscle differentiation from pineal cells occurs in a few days culturing in hypertonic conditions. The only known molecular event accompanied by muscle differentiation is the new appearance of 56 kDa protein in the pineal cells cultured in hypertonic medium (Watanabe *et al.*, 1988). The pathway to muscle differentiation from pineal cells is closed by administration of DMSO and HMBA. PECs differentiate around the 7th day of culturing and lens cells do a little later. We tentatively assume the bipotent cells (oculopotent cells) which can differentiate into both PECs and lens cells. Both DMSO and HMBA put forward the pineal cells into the oculopotent cells. A further effect of DMSO on oculopotent cells is to promote PEC differentiation and to inhibit lens cell differentiation. Basic FGF modulates this pathway and opens the lens cell differentiation in some oculopotent cells, whereas, HMBA inhibits the oculopotent cells to differentiate into PECs and promote them into lens cell differentiation. Although fibroblasts are the possible precursors of muscle cells and fat cells (Tailor and Jones, 1982), the fibroblastic cells in pineal cell culture merely mean unidentified cells. Much must be settled to understand how cellular differentiation is stabilized and unstabilized, and our present system may be useful to search for cellular determinants in the differentiation of ocular cells.

Materials and Methods

Materials

Fertilized quail eggs were purchased from a local quail yard and incubated at 38°C. Eight-day embryos were used for materials.

Cells

Pineal bodies of 8-day embryos were dissociated into cells and cultured by a procedure similar to the one described by Watanabe *et al.* (1985, 1988). Approximately $2x10^5$ cells were inoculated on 3 cm dish (Corning) in 5% CO_2 atmosphere. Culture dishes were coated with type I collagen from rat tail tendon.

The basal culture medium was Eagles' MEM (Nissui, Tokyo) supplemented with 10% dialyzed fetal bovine serum (Itoh and Eguchi, 1986), 1% chicken serum, 250 μg/ml sodium pyruvate, 50 μg/ml ascorbic acid and 0.1 i.u./ml insulin. Other reagents were dimethyl sulfoxide (DMSO), basic fibroblast growth factor (recombinant bFGF, Collaborative Research Biochemicals Ltd.) and hexamethylene bisacetamide (HMBA, Sigma). Hypertonic medium for muscle differentiation from pineal cells contained additional 75 mM NaCl in the basic medium (Watanabe *et al.*, 1988). DMSO, HMBA or additional NaCl were administered on the next day of cell inoculation to avoid cell damage. For clone culture, conditioned medium of pineal cells in mass culture was stocked at -20°C, and used at 25% concentration.

Immunocytochemistry

To detect skeletal muscle type creatine kinase (MM-CK), cultures were fixed overnight with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) at room temperature and immunostained using anti-MM-CK, second antibody and peroxidase-anti-peroxidase complex in accordance with the procedure of Watanabe *et al.* (1988). All antibodies were diluted with phosphate-buffered saline containing 0.1% Triton X-100 (TPBS). TPBS was also used for the rinse after the antibody treatment. Coloring was done using 3-amino-9-ethylcarbazol after a brief wash with PBS.

Northern blot analysis

Total RNA was extracted from cultured cells by Chirgwin *et al.* (1979). Five or 10 μg of total RNA was glyoxylated, separated on 1% agarose and transferred to nylon membrane (Maniatis *et al.*, 1982). Hybridization using ^{32}P-labeled probe was performed as described (Mochii *et al.*, 1988a). The probes were isolated from chicken cDNA library previously. MMP115 is a melanosomal matrix protein, expressed in pigmented epithelial cells of retina and melanocytes of skin (Mochii et al., 1988b and 1991). pP344 is expressed specifically in the pigmented epithelial cells, not in the melanocytes (Agata *et al.*, 1993). Tyrosinase is a key enzyme in melanin synthesis, expressed in pigmented cells (Mochii *et al.*, 1992). δ-crystallin is a major structure protein in lens cells (Yasuda *et al.*, 1984).

Acknowledgments

This work was done at the Division of Morphogenesis, Department of Developmental Biology, National Institute for Basic Biology.

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