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Abstract

Retinal cells from chick embryos aged 7.5 days of gestation were cultured for two months in a non-adherent suspension culture dish to study the effects of growth factors and co-culture with retinal pigment epithelial cells on their differentiation. Dissociated retinal cells became cellular aggregates (multicellular spheroids) within a day, and rosettes were formed in the spheroids after 2 days. Ultrastructurally, neurons of the rosettes developed connecting cilia, ellipsoids (accumulation of mitochondria), and external limiting membrane, indicative of their differentiation into photoreceptor cells. Epidermal growth factor enhanced the expression of rhodopsin by rosette-forming neurons, while basic fibroblast growth factor induced the growth of Mueller cells at 4 weeks, and their transdifferentiation into lens-epithelial-like cells at 8 weeks. Co-culture of retinal cells with retinal pigment epithelial cells enhanced the formation of rosettes in spheroids. Multicellular spheroids formed in a dish for suspension culture would provide a convenient in vitro system to examine differentiation and transdifferentiation of the retina.

KEYWORDS: multicellular spheroids, differentiation, transdifferentiation, retina, retinal pigment epithelium, growth factors

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The Effects of Growth Factors on Multicellular Spheroids Formed by Chick Embryonic Retinal Cells

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Retinal cells from chick embryos aged 7.5 days of gestation were cultured for two months in a non-adherent suspension culture dish to study the effects of growth factors and co-culture with retinal pigment epithelial cells on their differentiation. Dissociated retinal cells became cellular aggregates (multicellular spheroids) within a day, and rosettes were formed in the spheroids after 2 days. Ultrastructurally, neurons of the rosettes developed connecting cilia, ellipsoids (accumulation of mitochondria), and external limiting membrane, indicative of their differentiation into photoreceptor cells. Epidermal growth factor enhanced the expression of rhodopsin by rosette-forming neurons, while basic fibroblast growth factor induced the growth of Mueller cells at 4 weeks, and their transdifferentiation into lens-epithelial-like cells at 8 weeks. Co-culture of retinal cells with retinal pigment epithelial cells enhanced the formation of rosettes in spheroids. Multicellular spheroids formed in a dish for suspension culture would provide a convenient *in vitro* system to examine differentiation and transdifferentiation of the retina.

Key words: multicellular spheroids, differentiation, transdifferentiation, retina, retinal pigment epithelium, growth factors

Reaggregation of dissociated retinal cells in a non-adherent culture environment has been used as an *in vitro* model to study differentiation of the retina since its introduction by Moscona (1-8). Later, Layer and Willbold reported that whole layers of retina could be formed by embryonic retinal cells in multicellular spheroids under certain conditions (6-8). A non-adherent environment was usually achieved by agitation of the media by rotation in the previous studies. Recently, the electric charge of the surface of culture dishes has been shown to be one of crucial factors for achieving a non-adherent environment (9), and plates for suspension culture have become commercially available. We cultured chick embryonic retinal cells in a dish for suspension culture to form retinal multicellular spheroids and studied the effects of growth factors and co-culture with retinal pigment epithelial cells upon their differentiation.

Materials and Methods

Isolation and culture of retinal cells. Egg shells were disinfected with 70% alcohol and 7.5-day-old chick embryos (10) were taken out from a small opening. Eyes were enucleated and cut at the midperiphery of the globe, and the anterior halves were removed together with the vitreous. The retina at this embryonic stage could be pealed off easily from the eye cup, and was incubated in 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (HBSS: Gibco BRL, Gaithersburg, MD, USA) to disperse retinal cells. Retinal cells were washed with Dulbecco's modified Eagle's medium (DMEM) after centrifugation and plated at a concentration of 5×10^6 cells/ml in wells of a 24-well multidish for suspension culture (Sumilon, Osaka, Japan) containing DMEM supplemented with 10% fetal calf serum (FCS), 100 mg/L streptomycin, and 100 mg/L ampicillin. Half of medium was changed twice weekly. Trypan blue dye exclusion test confirmed that 95% or more of retinal cells were viable at the beginning of culture.

Growth factors and co-culture. Epidermal growth factor (EGF: Human EGF, R & D Systems,

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Minneapolis, MN, USA), or nerve growth factor (NGF: mouse beta nerve growth factor, Austral Biologicals, San Ramon, CA, USA) at a concentration of 100 ng/ml, or basic fibroblast growth factor [bFGF: Human FGF basic (157 aa), R & D Systems] at a concentration of 200 ng/ml, was added to the media of retinal cells. Co-culture was done with a mixture of retinal pigment epithelial cells at a concentration of 3×10^6 cells/ml and retinal cells at a concentration of 5×10^6 cells/ml.

Light and electron microscopy. For light microscopy, multicellular spheroids were fixed with 3.7 % formaldehyde in phosphate buffered saline (PBS, pH 7.4) overnight, dehydraded with a graded series of alcohol and xylene, and then embedded in paraffin. Paraffin sections were cut and stained with hematoxylin-eosin. For electron microscopy, multicellular spheroids were fixed with 2.5 % glutaraldehyde in PBS overnight, fixed further with 1 % osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 3 h at room temperature, dehydrated with a graded series of alcohol and propylene oxide, and embedded in eposy resin (Epon 812 resin, Taab Laboratories Equipments, Berk, UK). Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed with an electron microscope (Hitachi, HS-9, Katsuta, Japan).

Immunohistochemistry. Paraffin sections were deparaffinized with xylene and washed with PBS after a graded series of alcohol hydrations. Sections were incubated with goat normal serum for 30 min to block non-specific binding, and incubated with anti-rhodopsin antibody (rabbit anti-bovine rhodopsin: LSL, Tokyo, × 2,000 dilution), anti-crystallin antibody (rabbit antibody to alpha B crystallin: Sero Tec, Oxford, England, × 2,000 dilution), or anti-bFGF receptor antibody (anti-chicken-FGF receptor, rabbit antiserum: Upstate Biotechnology, Lake Placid, NY, USA, × 1,000 dilution) for 90 min at 37 °C. After being washed 3 times each for 10 min in PBS containing 0.05 % Tween-20 (Bio-Rad Laboratories, Hercules, CA, USA), sections were incubated with biotinylated goat antibody against rabbit IgG for 60 min at 37 °C and washed again. Sections were then incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (ABC: Vector Stain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature and washed. Sections were color-developed by 0.1 % diaminobenzidine tetrahydrochloride and 0.02 % hydrogen peroxide in 0.1 M Tris buffer (pH 7.4) for 30 min and counterstained with 1 % methyl green for 30 min.

Results

Multicellular spheroids of retinal cells. Dissociated retinal cells aggregated with one another to form a multicellular spheroid within 12 h (Fig. 1a, 1b). Multicellular spheroids then fused with one another to become large membranous aggregates after 24 to 48 h (Fig. 1c). Rosettes were observed within multicellular spheroids after 3 days by phase-contrast microscopy (Fig. 1c, 1d, 1e), and a number of dead cells shedded off from the periphery of multicellular spheroids, resulting in spheroids in smaller sizes (Fig. 1d, 1e). Shedding of dead cells continued until 4 weeks when multicellular spheroids began to grow gradually in size once again until 8 weeks as observed (Fig. 1f).

Histologically, rosettes began to form in multicellular spheroids after 2 days (Fig. 2a) and appeared in a full scale at 5 days (Fig. 2b). Multicellular spheroids consisted of two parts, one part with a high density of nuclei and another with reticular structure at 1 week, and then the spheroids developed layered structures of nuclei at 2 weeks (Fig. 2c). From this point, until 8 weeks, cells located in the periphery of the multicellular spheroids shedded away, and the number of nuclei in spheroids decreased, leading to an increased area of eosinophilic reticular structure with an ambiguous layered structure of nuclei (Fig. 2d). Ultrastructurally, the reticular structures at 2 weeks consisted of numerous cellular processes such as neurites and processes of Mueller cells (Fig. 3a), among which observed desmosome-like structures and synapses (Fig. 3b). Condensed intercellular junctions developed among cells in rosettes in their apical region, indicating the formation of an external limiting membrane (Fig. 3c). Some cells in rosettes also contained oil droplets and accumulations of mitochondria known as an ellipsoid, connecting cilia, and basal bodies, indicating differentiation into photoreceptor cells (Fig. 3c). Immunohistochemically (Table), cells positive for rhodopsin were dispersed in spheroids at the earlier stages (Fig. 4a) and then accumulated in rosettes. In contrast, cells positive for crystallin were absent in multicellular spheroids of retinal cells at 2 weeks (Fig. 4c).

The area occupied by Mueller cells (11, 12) in the spheroids increased with the passage of time, occupying half of the whole area of the spheroids at 4 weeks (Fig. 3d) and three quarters at 8 weeks of culture. In contrast, neuronal cells decreased in number, although they re-

Table Immunohistochemical positivity for rhodopsin, crystallin and bFGF receptor in multicellular spheroids formed in the presence and absence of growth factors. First column for retinal cells only and second column for retinal cells co-cultured with RPE cells.

Factor	Retinal cells				Retinal cells + RPE cells		
	None	bFGF	EGF	NGF	None	bFGF	EGF
Anti-rhodopsin							
2 weeks	+	+	+	+	+	+	+
4 weeks	+	+	2+	+	+	+	−
8 weeks	+	n.d.	n.d.	−	−	−	n.d.
Anti-crystallin							
2 weeks	+	+	+	+	+	+	+
4 weeks	+	+	+	−	2+	2+	−
8 weeks	+	2+	n.d.	+	2+	2+	n.d.
Anti-bFGF receptor							
2 weeks	−	−	+	−	+	2+	+

The number of positive cells is graded into 3 stages: from none (−); a few to several (+); and many (2+). Abbreviations used are: n.d., not determined; bFGF, basic fibroblast growth factor (at 200 ng/ml); EGF, epidermal growth factor (at 100 ng/ml); NGF, nerve growth factor (at 100 ng/ml); and RPE, retinal pigment epithelial cells.

mained viable with many neurites forming synapses.

Multicellular spheroids of retinal cells in the presence of growth factors. The presence of growth factors did not influence the formation of multicellular spheroids and also did not protect cells in spheroids from shedding off. The presence of bFGF made multicellular spheroids grow in size and fuse with one another (Fig. 5a), and induced the formation of lentoid structures at 8 weeks (Fig. 6b). In contrast, NGF

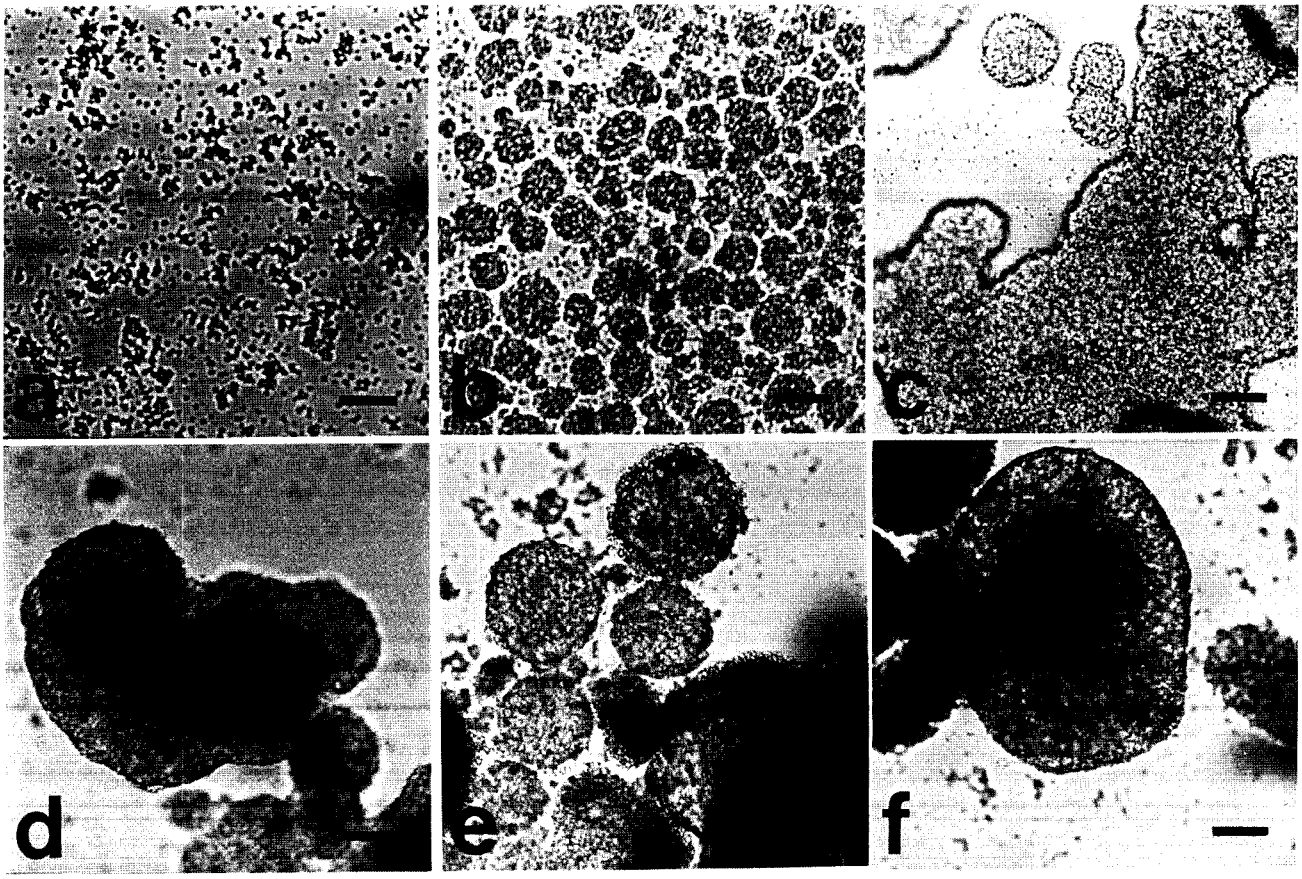


Fig. 1 Formation of muticellular spheroids by chick embryonic retinal cells observed by phase-contrast microscopy. At 6h (**a**), 12h (**b**), 2 days (**c**), 3 days (**d**), 4 weeks (**e**), and 8 weeks (**f**) of culture. Multicellular spheroids form at 12h (**b**), fuse with one another to become larger spheroids (**c**), and then decrease in size (**d**, **e**), and grow again (**f**). Bar = 100 μm.

and EGF did not influence the growth of spheroids or the formation of a layered structure of nuclei in spheroids (Fig. 6a). Ultrastructurally, bFGF enhanced the growth of Mueller cells which, as a result, occupied about three quarters of the spheroids at 4 weeks (Fig. 7a), and induced the growth of cells with homogeneous medium-electron-dense cytoplasms, characteristic of lens cells, at 8 weeks (Fig. 7b). Cells positive for crystallin increased in number dramatically after 4 weeks in the presence of bFGF (Table). EGF enhanced the formation of ellipsoids and increased the number of cells positive for rhodopsin, while the presence of NGF allowed the survival of well-differentiated rosettes until 8 weeks.

Multicellular spheroids formed by co-culture of retinal cells with retinal pigment epithelial cells. Retinal cells aggregated (Fig. 5b) and formed a layered structure (Fig. 6c) around a core of retinal pigment epithelial cells in their co-culture. The co-culture with retinal pigment epithelial cells did not prevent a number of dead retinal cells from shedding off the spheroids, but did enhance the formation of rosettes in spheroids, especially in the presence of bFGF (Fig. 5c) or EGF (Fig. 5d). Spheroids formed by the co-culture in the presence of bFGF grew in size after 4 weeks to twice as large a diameter (Fig. 5f) as those in the absence of bFGF (Fig. 5e), and large spherical lentoid structures, consisting of spindle-shaped cells with ovoid nuclei, developed at 8 weeks (Fig. 6d). Ultrastructurally, the number of cells with numerous rough endoplasmic reticuli, characteristic of Mueller cells, increased in the co-culture. Immunohistochemically (Table), expression of crystallin was enhanced in spheroids in co-culture after 4 weeks (Fig. 4d), as compared with those formed only from retinal cells. Many cells with small mitochondria and cytoplasmic

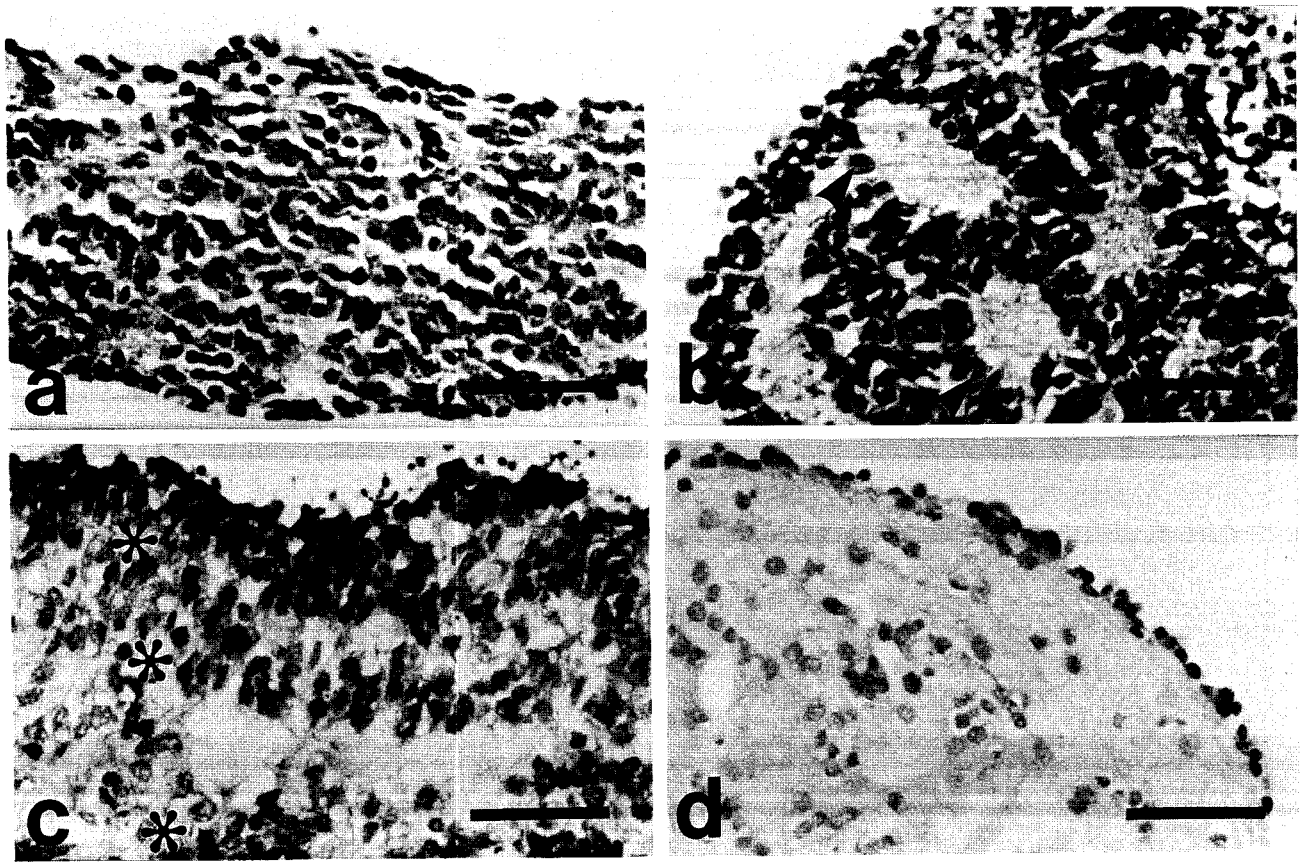


Fig. 2 Histological structure of multicellular spheroids formed by chick embryonic retinal cells. Rosettes (arrowheads) begin to form at 2 days of culture (**a**), and are fully formed at 5 days (**b**). A layered structure of neurons (asterisks) is formed at 2 weeks (**c**). The number of cells in a spheroid decreased at 8 weeks (**d**). Hematoxylin-eosin stain. Bar = 50 μm.

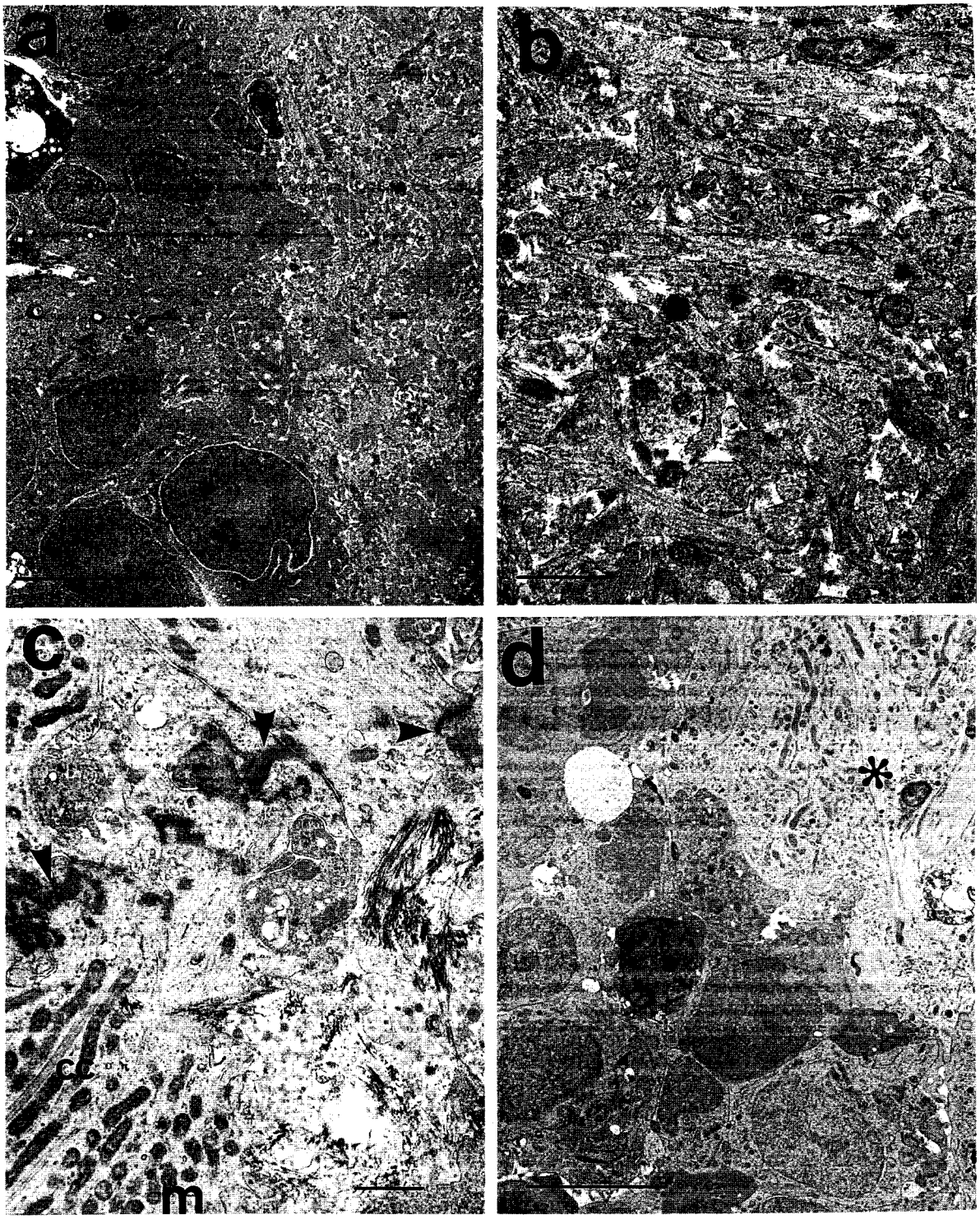


Fig. 3 Electron micrographs of multicellular spheroids formed by chick embryonic retinal cells. Neurons and their cellular processes in the outer layer of the spheroid at 2 weeks (**a**), and growth of Mueller cells (asterisk) replacing the neural processes at 4 weeks (**d**). Note many synaptic vesicles and desmosome-like junctions among cellular processes at 2 weeks (**b**). Note also accumulation of mitochondria (m in **c**) known as ellipsoids, formation of external limiting membrane (arrowheads in **c**), and connecting cilium (cc in **c**) at 2 weeks, characteristic of retinal neurons. Uranyl acetate and lead citrate stain. Bar = 5 μm in **a**, **d**. Bar = 1 μm in **b**, **c**.

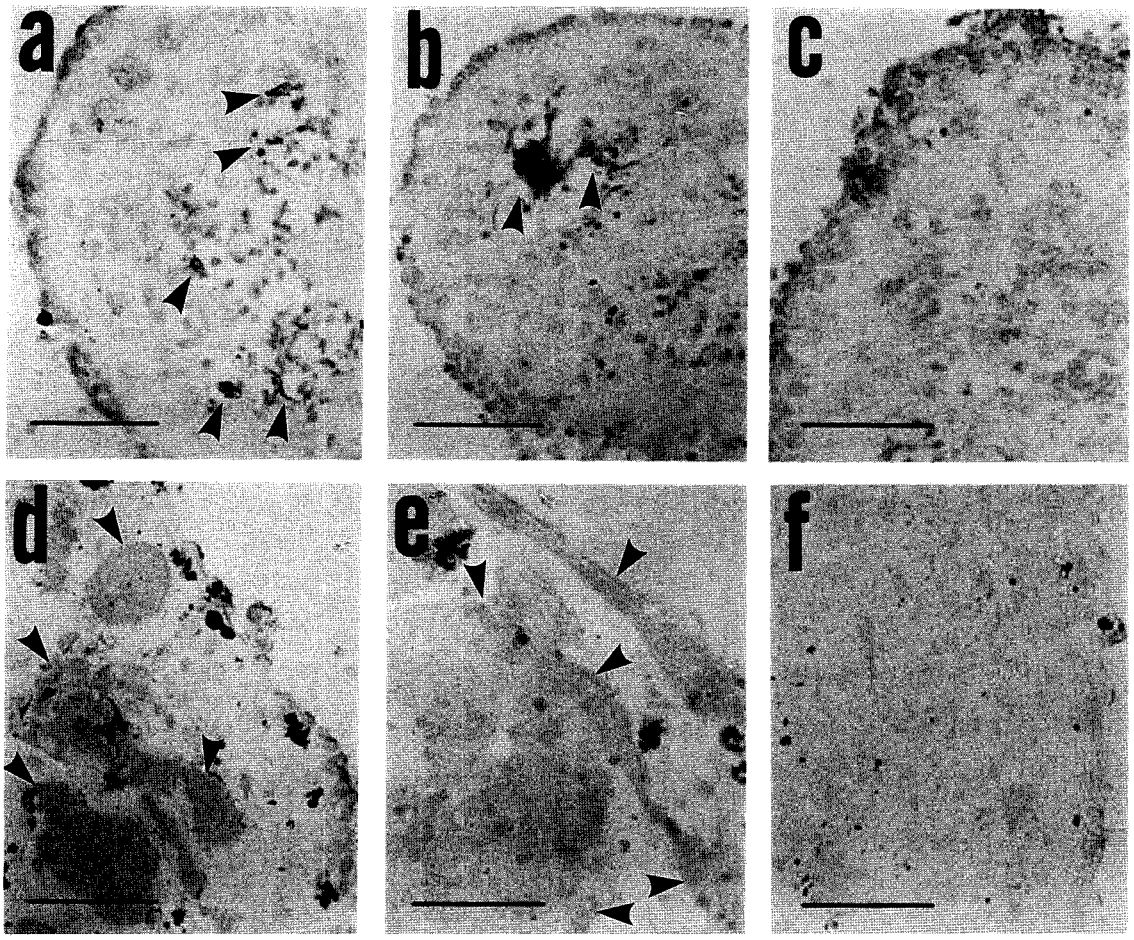
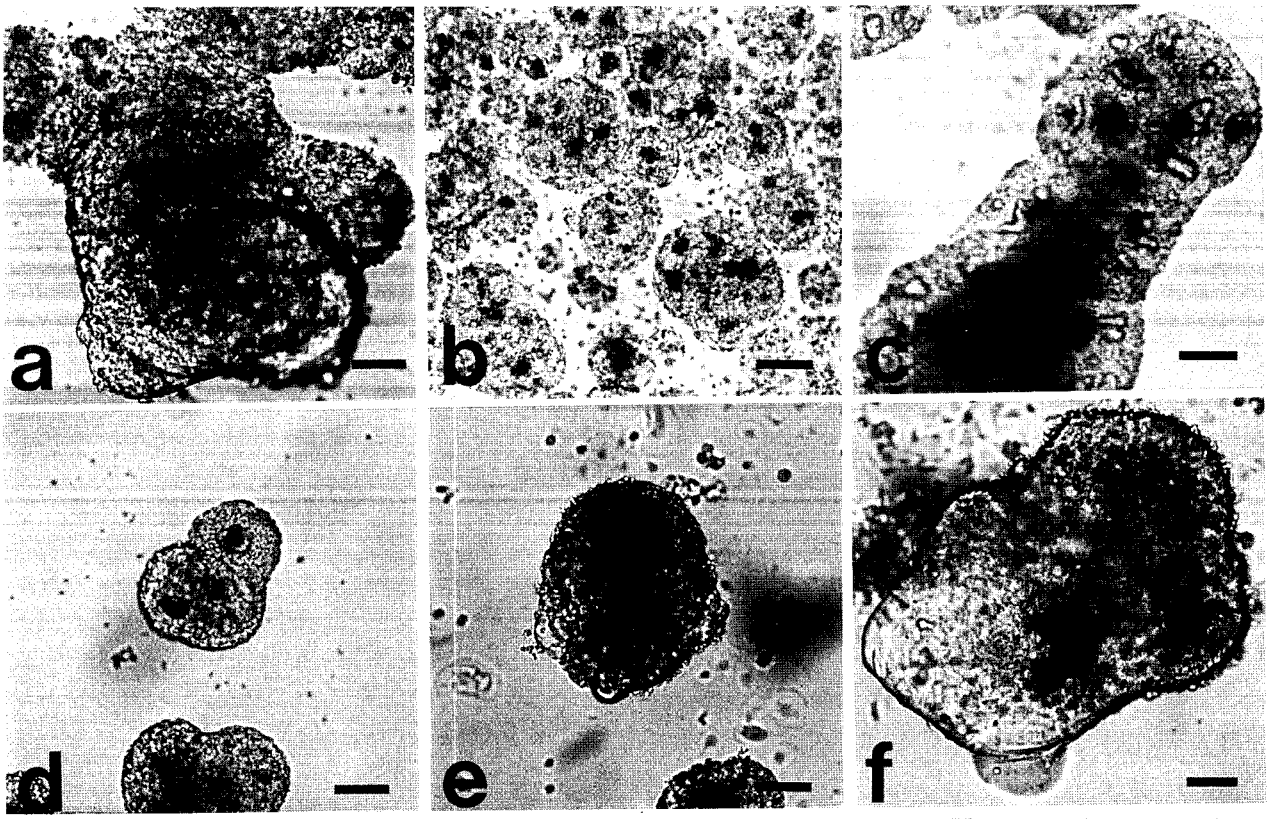


Fig. 4 Immunohistochemistry of multicellular spheroids formed by chick embryonic retinal cells. Positive cells for rhodopsin (arrowheads) are located separately in a control spheroid at 2 weeks (**a**), while accumulated in a rosette at 2 weeks by co-culture with retinal pigment epithelial cells in the presence of epidermal qrowth factor (EGF) (**b**). Positive cells for crystallin (arrowheads) are negligible in a control spheroid at 2 weeks (**c**), while accumulated in a spheroid by co-culture with retinal pigment epithelial cells at 8 weeks (**d**) and more markedly so in a spheroid by co-culture in the presence of basic fibroblast growth factor (bFGF) at 8 weeks (**e**). (**f**) is a control for (**e**). Methyl green counterstain. Bar = 50 μm.

Fig. 5 (Next page, top) Multicellular spheroids of retinal cells formed in the presence of growth factors and/or co-culture with retinal pigment epithelial cells. A large spheroid in the presence of bFGF at 8 weeks (**a**). Co-culture with retinal pigment epithelial cells at 24 h resulting in spheroids with pigmented cells at their core (**b**). A large spheroid in co-culture with retinal pigment epithelial cells and in the presence of bFGF at 7 days (**c**). Note many follicles in the spheroid, corresponding histologically to rosettes. A small spheroid in co-culture with retinal pigment epithelial cells and in the presence of EGF at 7 days (**d**). A small spheroid in co-culture with retinal pigment epithelial cells at 8 weeks (**e**), in contrast with a large spheroid in co-culture with retinal pigment epithelial cells in the presence of bFGF at 8 weeks (**f**). Bar = 100 μm. bFGF, EGF: See legend to Fig. 4. ➡

Fig. 6 (Next page, bottom) Histological structure of multicellular spheroids formed by chick embryonic retinal cells in co-culture with retinal pigment epithelial cells and/or in the presence of growth factors. Rosettes are formed at 4 weeks in the presence of EGF (**a**). A spheroid becomes a lentoid structure in the presence of bFGF at 8 weeks (**b**). A layered structure of retinal cells is formed around a core of retinal pigment epithelial cells in co-culture with retinal pigment epithelial cells at 4 weeks (**c**). Lens fiber-like cells fill a spheroid in co-culture with retinal pigment epithelial cells in the presence of bFGF at 8 weeks (**d**). Hematoxylin-eosin stain. Bar = 50 μm. EGF, bFGF: See legend to Fig. 4. ➡


a
b
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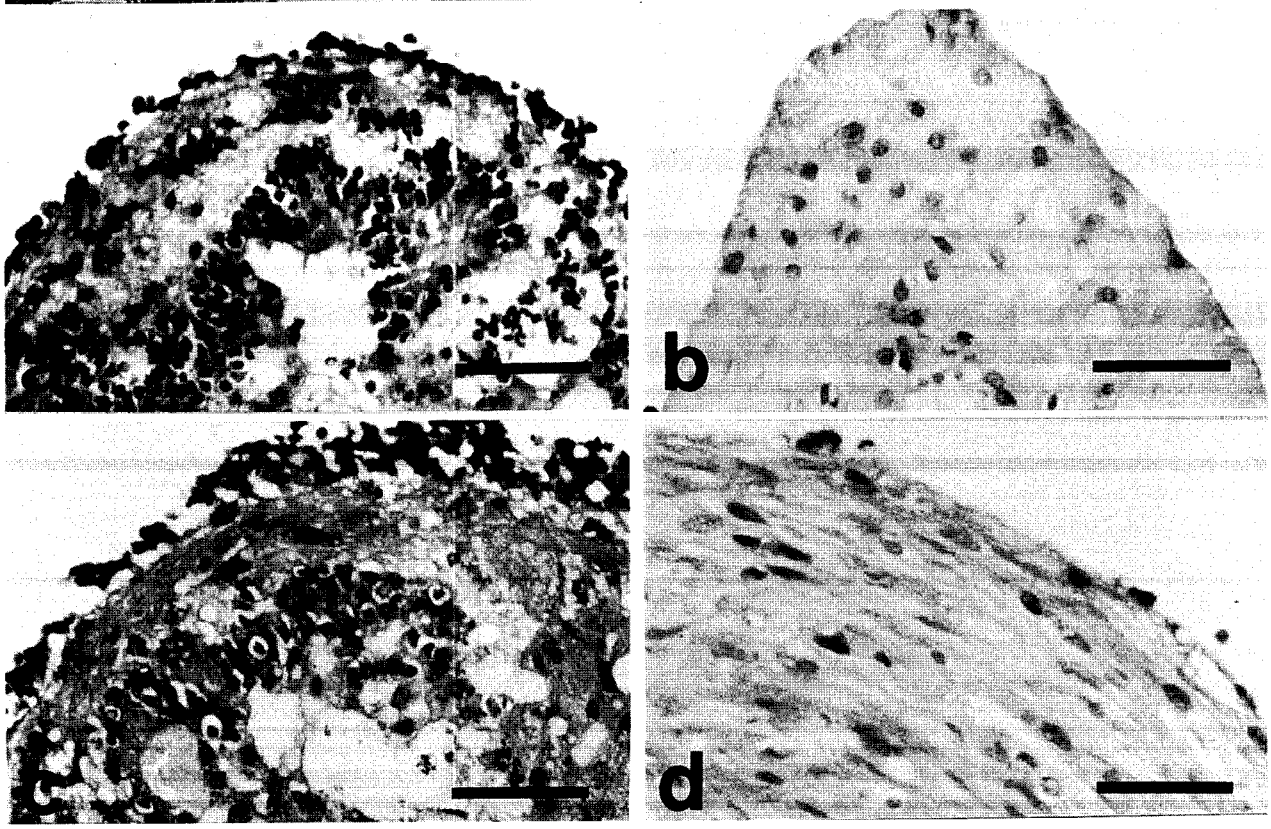
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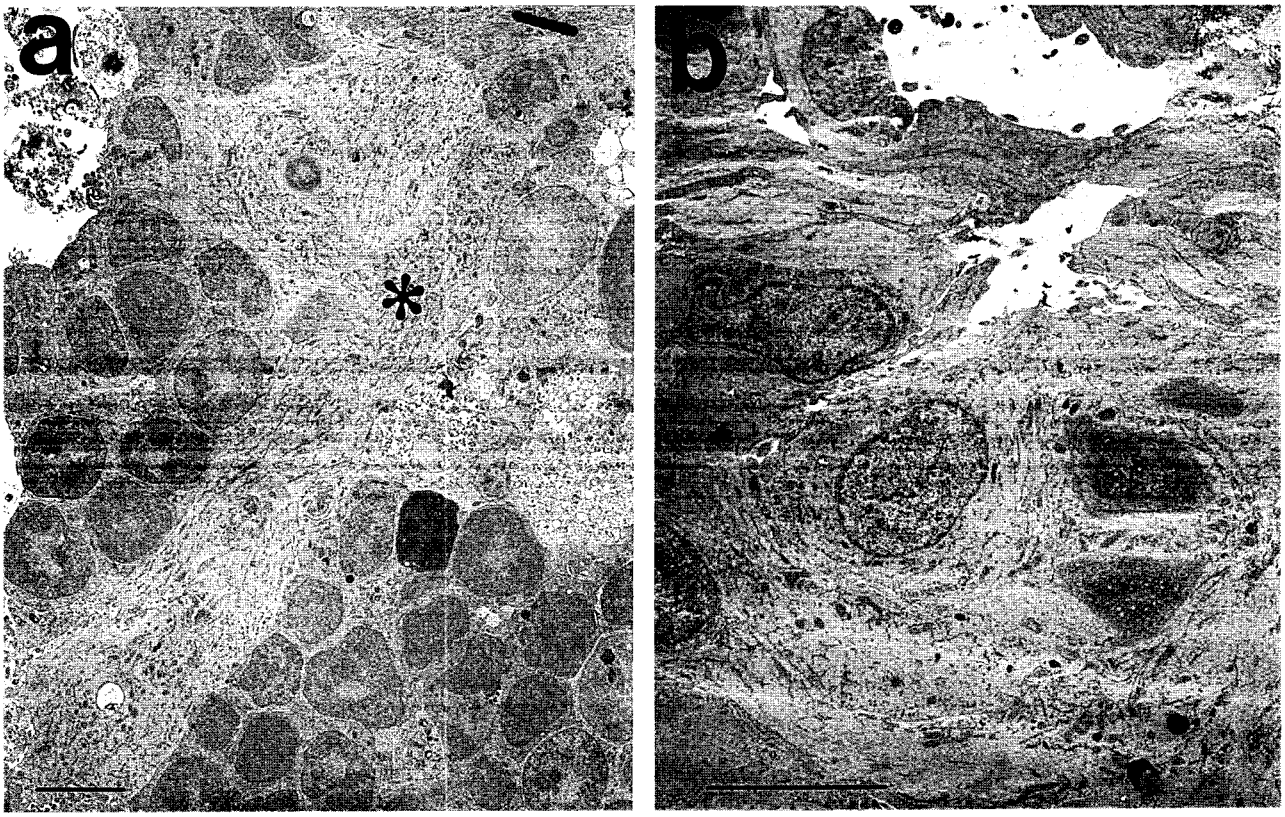

Fig. 7 Electron micrographs of multicellular spheroids of chick embryonic retinal cells in the presence of bFGF. Note growth of Mueller cells (asterisk) at 4 weeks (**a**) and lens-epithelial-like cells at 8 weeks (**b**). Uranyl acetate and lead citrate stain. Bar = 5 μm in **a**, **b**. bFGF: See Fig. 4.

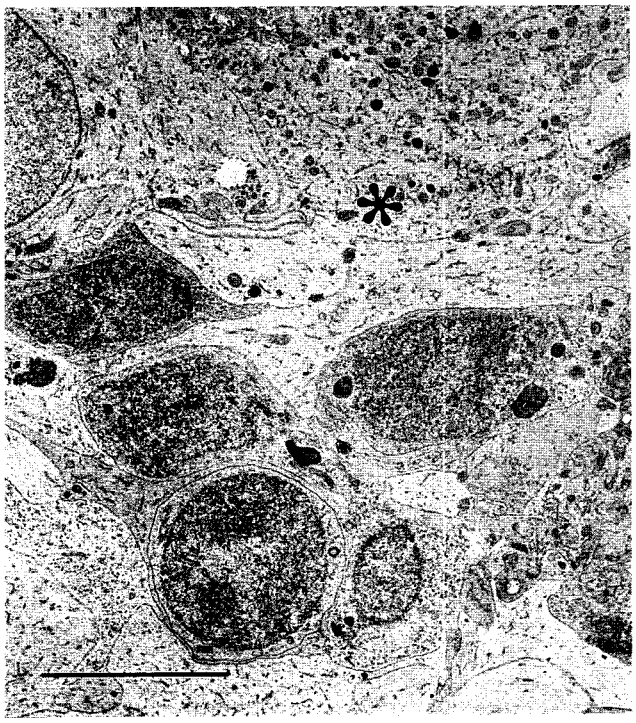

Fig. 8 Electron micrograph of a multicellular spheroid of chick embryonic retinal cells in co-culture with retinal pigment epithelial cells and in the presence of bFGF at 4 weeks. Note predominant Mueller cells some of which (asterisk) appear like lens epithelial cells. Uranyl acetate and lead citrate stain. Bar = 5 μm. bFGF: See Fig. 4.

fine granular materials, characteristic of lens-fiber cells, grew in the co-culture with bFGF (Fig. 8). Lentoid structures formed in co-culture in the presence of bFGF at 8 weeks were markedly positive for crystallin (Fig. 4e) and bFGF receptor. EGF in the co-culture enhanced accumulation of mitochondria and expression of rhodopsin (Fig. 4b) at the apical region of the cells in rosettes, indicating the formation of ellipsoids.

Discussion

In the present study, multicellular spheroids formed by chick embryonic retinal cells could be incubated in

suspension culture for up to 2 months with good viability. Retinal neuronal cells as well as Mueller cells could be maintained in a differentiated state in the spheroids. The spheroids showed the formation of rosettes at 5 days followed by the development of a layered structure of neurons at 2 weeks. Cells of the rosettes in the spheroids showed several characteristics present in the inner segments of photoreceptor cells, including ellipsoids composed of accumulations of mitochondria, myoids composed of numerous endoplasmic reticuli, connecting cilia, and external limiting membrane. However, retinal differentiation stopped at this stage and did not lead to the formation of outer segments in the spheroids from here on until termination of culture at 2 months. Nonetheless, this retinal differentiation at 2 weeks was advanced over that present at the time of harvest of the 7.5-day-old embryos, in which development of photoreceptor cells had not yet begun (13–16). The development of outer segments could not be induced by the addition of such growth factors as bFGF, EGF, and NGF, or co-culture with retinal pigment epithelial cells, indicating that some other factors or conditions for inducing further differentiation of photoreceptor cells were missing in the multicellular spheroids.

We used chick embryos aged 7.5 days old of gestation, corresponding to the Hamburger-Hamilton stage 32 (10), since this was the earliest stage at which the retina could be separated completely from the retinal pigment epithelium outside the optic fissure. We also tried to use younger embryos, but it was impossible to isolate the retina without contamination with retinal pigment epithelium because these two tissues were firmly adherent to each other at earlier stages. Previous studies used chick embryos at 6 days of gestation and described such advanced differentiation of retinal multicellular spheroids as the development of whole layers (3, 5–8). One reason for the limited retinal differentiation in the present study might be the older age of the embryos used. Retinal cells in these older embryos might lose the potential for giving rise to various types of cells.

Numerous retinal cells shedded off during the process of formation of multicellular spheroids from dissociated retinal cells through a sorting-out phenomenon (17), meaning exclusion of unwanted cells during their reaggregation. Pyknotic nuclei, considered the products of apoptosis, were also found in the spheroids with rosettes and layered structure of neuronal cells. These processes in the spheroids would correspond to selection of cells via apoptosis, as observed during the normal development of the retina (18–20). Growth factors used in the present study, such as bFGF, EGF, and NGF, did not rescue dying cells during the sorting-out process.

The presence of bFGF induced a marked expression of crystallin in multicellular spheroids of retinal cells and led to the formation of a lentoid structure filled with lens fiber-like cells at 8 weeks, indicating transdifferentiation of retinal cells into lens epithelial cells as described previously (21–29). The growth of Mueller cells (11, 12) was enhanced by bFGF at 4 weeks, supporting the previous finding that Mueller cells transdifferentiated into lens epithelial cells (27). Co-culture of retinal cells with retinal pigment epithelial cells, together with bFGF, induced further growth of Mueller cells and resulted in an earlier formation of a large lentoid structure, suggesting that retinal pigment epithelial cells might enhance transdifferentiation. However, a more plausible explanation for this enhanced transdifferentiation is that retinal pigment epithelial cells themselves in co-culture might transdifferentiate into lens cells, since retinal pigment epithelial cells have been known to transdifferentiate into lens cells in the presence of bFGF (30).

Co-culture of retinal cells with retinal pigment epithelial cells did not result in the development of outer segments as described previously (6, 7). These previous studies used retinal pigment epithelial cells near the ciliary epithelium (6, 7), in contrast with more posteriorly located retinal pigment epithelial cells used in this study. Another factor underlying the present results could be a smaller number of retinal pigment epithelial cells mixed with retinal cells. However, the co-culture in the present study did enhance differentiation of Mueller cells and neurons to some extent, suggesting that direct contact of retinal cells with retinal pigment epithelial cells as well as diffusable factors from retinal pigment epithelial cells (31, 32) plays a role in retinal differentiation in the spheroids.

Our floating culture system allowed embryonic retinal cells to survive as multicellular spheroids for 2 months and induced retinal cells at an undifferentiated stage to differentiate further. Multicellular spheroids formed in a dish for suspension culture would be a convenient *in vitro* system to study the effect of various factors on the differentiation of the retina. In addition, differentiated or transdifferentiated multicellular spheroids might be used as transplants for the retina or lens in the future (33, 34).

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