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Methods in eye research

Simple explant culture of the embryonic chicken retina with long-term preservation of photoreceptors

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ABSTRACT

Structurally stable *in vitro*-model systems are indispensible to analyse neural development during embryogenesis, follow cellular differentiation and evaluate neurotoxicological or growth factor effects. Here we describe a three-dimensional, long-term *in vitro*-culture system of the embryonic chick retina which supports photoreceptor development. Retinal tissue was isolated from E6 chick eye, and cultured as explants by continuous orbital rotation to allow free floatation without any supporting materials. Young stage (E6) immature retinas were cultured for various time periods in order to follow the differentiation of cell types and plexiform layers by immunocytochemical methods. These explants could be cultured for at least 2–3 weeks with remarkable retention of retinal architecture. Interestingly, photoreceptors developed in the absence of pigment epithelium. Electron microscopic studies revealed formation of structures resembling photoreceptor outer segments, a feature not reported previously. Thus, the verification of photoreceptors, Müller cells, inner retinal cells and the inner plexiform layer described in our study establishes this explant culture as a valuable *in vivo*-like model system.

1. Introduction

The need for well-organized *in vitro*-culture systems, which can mimic an *in vivo* situation, is still a high priority, in spite of remarkable advances made since the last century. The central nervous system (CNS), especially brain or retinal tissues from rat (LaVail and Hild, 1971; Rzeczinski et al., 2006), mouse (Caffé et al., 1989), and chicken (Kato et al., 1983; Hoff et al., 1999) were highly exploited in the development of *in vivo*-like culture systems that could be employed in different areas of study including embryonic development, cellular differentiation, pharmacology, transplantation (Johnson and Martin, 2008; Johnson et al., 2010) and other medical aspects.

Several methods have been developed for culturing retinal explants from different species. Earlier techniques involved the usage of different substrata on which retinal tissues were placed for support, including plasma clots (Barr-Nea and Barishak, 1970), and chicken gizzard extracts (Kato et al., 1983). Histotypic differentiation of mouse retina was obtained when tissues were explanted flat on a piece of nitrocellulose membrane (Caffé et al., 1989). Successful preservation of the cytoarchitecture over a period of several weeks was observed in slice culture techniques from rodent CNS (Gähwiler et al., 1997). Organotypic organization in chick retinal explants was demonstrated when the tissues were embedded in a fibrin clot on glass cover slips and rotated in roller tubes (Hoff et al., 1999). Recent developments have paved ways to culture retinal slices from adult rat retina by free floating them in roller drums under constant rotation (Rzeczinski et al., 2006).

Though complete retino-architecture was obtained and maintained in retinal explants for long culture periods, in either standard or roller cultures, no system has been established up to now that could preserve the development of photoreceptor outer segments. Studies show that photoreceptor outer segments developed to a certain extent when explants were cultured in the presence of retinal pigment epithelium but totally failed in the absence of the latter (Caffé et al., 1989). Electron microscopic studies also suggested the absence of photoreceptor outer segments in chick retinal explants (Araki et al., 1987).

Our aim was to establish an *in vivo*-like explant culture system from chick that differed significantly from other systems developed so far. The main difference compared to other explant systems was the absence of any substratum used as a support for the tissues. Instead of slicing the retinal tissues, we used whole retinal tissues

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; DACs, displaced amacrine cells; DAPI, 4′,6-diamidine-2-phenylindole-dihydrochloride; div, days in vitro; E, embryonic day; GCL, ganglion cell layer; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segments; OD, oil droplet; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment; SE, standard error.

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as explants which were subjected to continuous and constant orbital rotation. The explants were cultured in a spacious dish rather than using narrow roller tubes, in which they were allowed to float freely in order to obtain a 3D culture environment. We report here a simple but effective culture technique that allowed us to maintain the retinal architecture for at least 2–3 weeks. During the culture periods, the explants reached a near *in vivo*-like tissue organization. Since we used embryonic chick retina, we were able to follow complete differentiation of all retinal layers, starting from an early neuroblastic stage until the development of photoreceptor outer segments.

2. Materials and methods

Fertilized eggs of White leghorn chicken were purchased from a local hatchery and incubated at 37 °C in a humidified chamber until 6, 10 and 15 days of embryonic development for explant cultures. Different stages starting from embryonic day 6–17 were used to perform parallel *in vivo* retinal development studies. For explant cultures, eggs were opened at embryonic day 6 (E6) and the eyes collected in calcium free Hank's Basal Salt Solution (HBSS). Using a fine scissors the lens was removed by dissection along the ora serrata in such a way that most of the peripheral retina was lost. The vitreous body was next removed with sharp forceps. The central retina which was associated with the pigment epithelium was removed carefully with a spatula without any contamination of the latter. The whole retinal cup was flattened with the ganglion cell layer facing upwards without any damage and cut into two equal parts with a micro scissors (see Fig. 1). For *in vivo* studies, whole eyes of different stages were collected.

2.1. Establishment of explant culture

The equal retinal halves were transferred to 35 mm culture dishes containing DMEM (Dulbecco's Modified Eagle's Media) with a supplement of 10% fetal calf serum, 2% chicken serum, 1% L-glutamine, and 0.15% penicillin/streptomycin (all from Gibco, Berlin, Germany). No substrata such as Millipore filters or poly-cationic substances were used in order to plant the retinal tissues, but, instead, the dishes with explants were placed on a self-made rotatory shaker installed in a Heraeus incubator and allowed to rotate continuously in an orbital manner with a constant speed of 72 rpm. The explant culture was supplied with 95% air, 5% CO_2 and maintained at 37 °C throughout a culture period of 2–3 weeks. Fresh medium was changed every second day. Due to continuous rotation and avoidance of substrata, the tissues were not allowed to adhere to any surface, and thereby kept free-floating and preserving their 3D architecture.

2.2. Fixation and cryosectioning

The explants from different culture days beginning from div 2 till 20 were collected in Eppendorf tubes with 1 ml tips which were cut at the tip to have a wide mouth. The tissues were washed thrice with PBS (Phosphate Buffered Saline) and fixed in 4% para-formaldehyde (Merck, Darmstadt, Germany) for an hour and immersed in 25% sucrose (Roth, Karlsruhe, Germany) overnight at 4 °C. The fixed explants were transferred to tissue-tec (Richard-Allan Scientific) solution and sectioned on a cryostat (Microm, Heidelberg, Germany) at 10–12 μm and mounted on frost-free or gelatine-coated glass slides. The sections were stored at −20 °C until further use. Whole eyes for *in vivo* studies were also fixed in 4% paraformaldehyde overnight at 4 °C, immersed in 25% sucrose and sectioned as whole mount eyes as mentioned above.

2.3. Immunocytochemistry

Sections of explants and *in vivo* whole mount eyes were subjected to immunostaining in order to follow and compare the development and differentiation of different types of retinal cells in both the systems. Prior to staining procedures, the frozen sections were dried on a heating plate at 37 °C. The area around the sections was marked with a greasy liquid blocker (Roth, Karlstuhe, Germany) and the slides were pre-incubated in blocking solution which contains PBS, 3% BSA (Bovine Serum Albumin) and 0.1% Triton-X-100 for at least 30 min. 100 μl of primary antibodies which were diluted in the blocking solution were applied on the sections inside the area which was surrounded by the liquid blocker and then incubated for 90 min at room temperature or overnight at 4 °C. In case of double staining, one polyclonal and one monoclonal

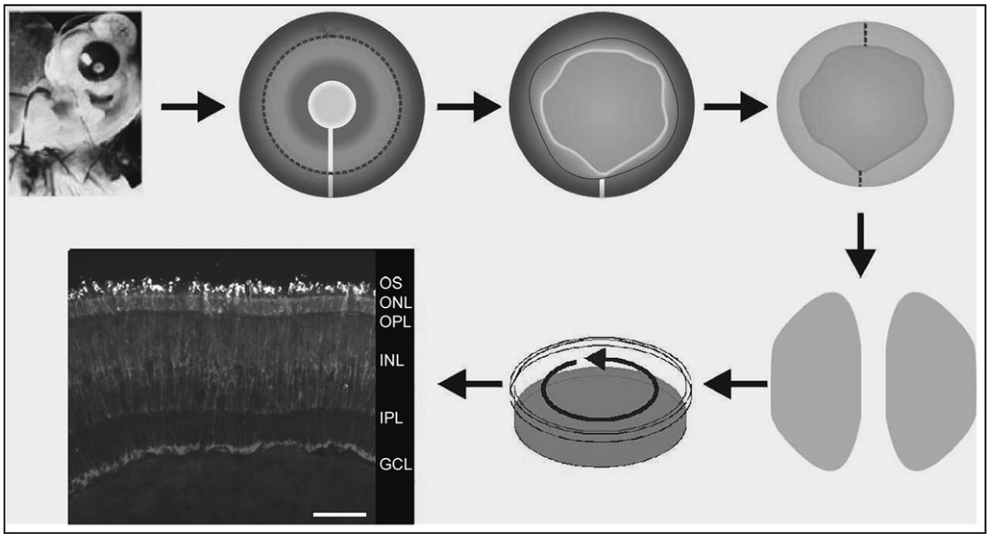

Fig. 1. Schematic representation of the method of preparing explants from an E6 chick embryo. Eyes from E6 embryos were removed and whole retinal cups were isolated without pigment epithelium. The tissues were cut into two equal parts and seeded in 35 mm dishes with medium in the absence of substratum. Constant orbital rotation (72 rpm) results in the growth of retinal explants in a 3D-like environment.