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RESEARCH ARTICLE

Comparing the Adhesion and Survival of Adult Rod and Cone Photoreceptor Neurons on 'Poly-D-Lysine' and 'Concanavalin-A' Substrate

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Rod and cone photoreceptors (PR) are the primary sensory neurons of the visual system. Currently there are very few *in vitro* cell culture models available for adult rod and cone neurons that can be used for high throughput drug screening for retinal photoreceptor disorders, toxicology research, cell patterning and regenerative medicine. A robust culture model for adult photoreceptor neurons thus, would be of utility to these research areas. We intend to develop such a model and as a first step, we compare here the adhesion, growth and survival of adult rod and cone neurons obtained from fish (*Cyprinus carpio*) retina on the 'poly d lysine' and 'concanavalin A' substrate. The two substrates were characterized by contact angle measurements and atomic force microscopy. Rod and cone neurons cells adhere and survive on 'Concanavalin A' substrate for 12 ± 3 days but failed to adhere to 'poly-d-lysine' substrate. The cell survival was monitored using cell-viability assay. Photoreceptor cells surviving on the 'Concanavalin A' were characterized morphologically and by Immuno-cytochemical assay; the binding of photoreceptor specific antibodies. Further we have discussed the surface features of these two substrates, which promote adhesion and survival of these cells exclusively on 'Concanavalin A' substrate.

Keywords: Rod, Cone, Photoreceptor, Adult Neuron, Poly D Lysine, Concanavalin A, Defined Medium.

1. INTRODUCTION

In vitro animal cell culture models are powerful tools for studying cellular physiology, initial screening of wide range of drugs, evaluating short and long term effects of different environmental toxins and for basic studies on regenerative medicine.[1–12] Currently there are very limited *in vitro* culture model for adult retinal rod and cone photoreceptor neurons;[13–17] the primary sensory neurons of the visual system involved in translating the light intensity and color information to electrical impulses before transmitting it to the brain for further processing.[18, 19]

Rod and cone photoreceptor neurons are extremely specialized cells with a very unique morphology. They have an inner segment (IS), which constitutes the cell body and nucleus; and an outer segment (OS) housing the light sensitive pigments in saucer-like disc structures stacked in a pile. In the intact retina, these cells grow on top of the retinal pigment epithelial (RPE) cells and the surrounding *in vivo* extracellular matrix is extremely complex.[18, 19] The complex cyto-architecture of the retina is shown in Figure 1. It is an exceptionally challenging task to isolate these cells from the complex cyto-architecture of intact adult retina. Further they fail to adhere on different synthetic and natural substrates because of the complex extracellular matrix in which they are adapted to grow.[18–23]

Thus the challenge to develop an *in vitro* model for rod and cone neurons is three fold viz.

a. Isolating intact photoreceptor cells,

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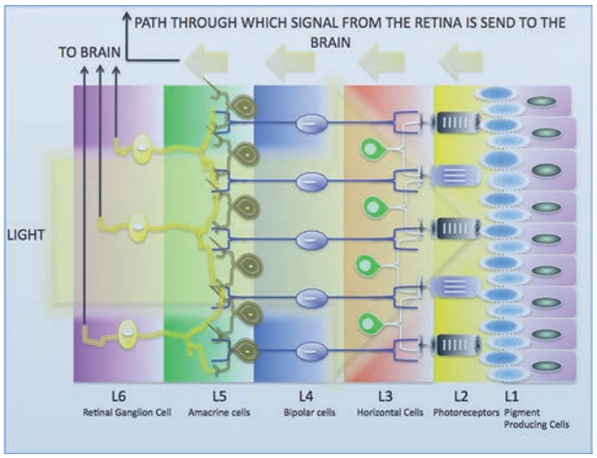

Fig. 1. Complex cyto-architecture of the retina showing the different layers of cell stacked over one another (L1–L6). The L2 layer showing the photoreceptor cell sitting on top of the retinal pigment epithelial (RPE) cell layer (L1). The photoreceptor cells derive necessary nutrients from these RPE cells and are surrounded by a complex extracellular matrix.

b. Finding a suitable substrate which promotes adhesion and survival,
c. Developing a defined medium that will allow them to survive.

In this study, we addressed the above mention challenges in culturing adult rod and cone photoreceptor cells. Firstly we developed a simple protocol to isolate the intact rod and cone photoreceptor cells from adult fish retina followed by evaluation of adhesion and survival of adult rod and cone photoreceptor neurons on the 'poly d lysine' and 'concanavalin A' substrates in the presence of a chemically defined medium.

'Poly-d-lysine' is a synthetic polymeric substrate and is one of the most widely used substrate in neural cell culture.[1–3, 24–27] This synthetic polymer is believed to alter the surface charge of glass or plastic base upon being adsorbed thus promoting adhesion and growth of almost all kinds of neurons.[1–3, 24–27] 'Concanavalin A' commonly known as jack bean lectin, is a plant lectin which binds to carbohydrate moieties present on the cell surface.[28–31] Till date very few cell types have been successfully grown on 'Concanavalin A' substrate.[1–3, 32, 33] We have characterized the glass surfaces coated with these substrates using contact angle measurement and atomic force microscopy (AFM). Cell attachment and survival is evaluated using live dead assay. Rod and cone cells were characterized morphologically and immuno-cytochemically using photoreceptor specific antibodies. Here we are reporting that photoreceptor cells failed to adhere to 'poly d lysine' substrate but adhere and survive on 'concanavalin A' substrate, thus highlighting the complex interaction between the photoreceptor cells and their surrounding extracellular matrix in intact retina.

2. MATERIALS AND METHODS

2.1. Maintaining Common Carp Population

The common carp population is maintained in the laboratory aquariums. The adult carp used for the study were 18 months old. In Indian subcontinent the carp population matures by 6–8 months.[34–37] The temperature of the water was set at 30 °C. Optimal aeration is maintained in the aquariums.

2.2. Isolation of Rod and Cone PR from Fish Retina and Culturing Them

Earlier Professor Satoru Kawamura developed a simple, reliable method to isolate rod and cone PR cells from the retina of adult *Cyprinus carpio*.[18] We have followed a similar protocol to isolate these cells. We used one adult fish (2 retina) for culturing the rod and cone cells for each experiment. Before the dissection, we removed the fish from the tank and placed it in an ice bucket covered with ice flakes. After 4–5 minutes the fish was decapitated and retina was dissected. Anatomically the rods and cones are faced to the pigment epithelium cell layer of the retina that is attached to the sclera (Fig. 2(a)).[18, 19]

The first step is to isolate the retina from the eyeball. After removal of the eyeball from the fish, a sharp razor blade was used to make a small cut along the equator of the eyeball (Fig. 2(b)). A fine scissor was inserted along the incision and the eyeball was cut into two halves, a front

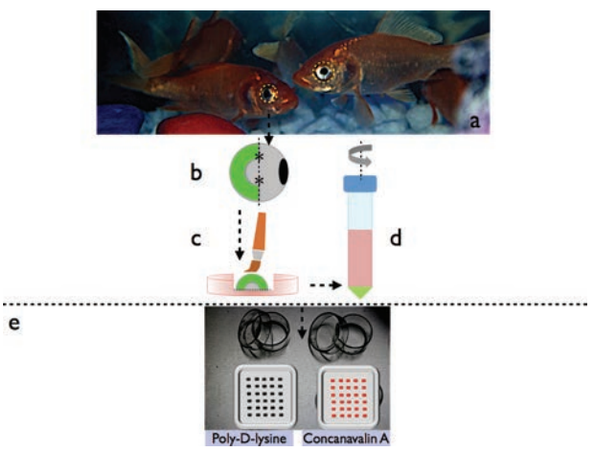

Fig. 2. Overall outline of the experiment. (a) Adult carp (*Cyprinus carpio*) fish in the laboratory fish tank (© Photograph taken by Professor Mainak Das). (b) Picture of the eyeball, showing the retina (green) on the back of the eye. The lens is shown in black in the front. The dotted line shows the incision line for cutting and isolating the retina from the eyeball. (c) The isolated retina is placed on a dish with small medium and using a brush the rod and cone cells are mechanically detached from the retinal tissue. (d) The mechanically dissociated retinal photoreceptor cells are collected in a sterile falcon tube along with the medium. The curved arrow indicating that the cells are centrifuged and further and the pellet is re-suspended in the defined medium used for culture. (e) The schematic diagram showing the poly-D-lysine and concanavalin A substrates used for this study. Inset showing in house fabricated glass culture dishes used for the culture.

and a back half. The edge of the retina was also cut during this surgical manipulation. The cut-end of the back half was placed on a dish and a small amount of cold 'defined medium' (Table I) was added (Fig. 2(c)). The sclera was lifted up gently with a pair of forceps to remove the retina from the pigment epithelium. The retina is firmly bound to the sclera through the optic nerve at the optic disc. Thus one needs to cut the nerve to isolate the retina. This step helps to lift the sclera off the retina completely and make the photoreceptor side is up. We further added more of the medium on the dish. The retina was tapped using a brush to mechanically dissociate the cells. This resulted in a suspension containing rods and cone PR cells. This cell suspension was centrifuged for 4 minutes at 500 rpm (Fig. 2(d)). The cell pellet was further re-suspended in the defined medium (Table I) and further used for culture.

2.3. Defined Medium

The composition of the defined medium is provided in Table I.

2.4. Coating of Glass Substrates With Cell Adhesion Molecules (Poly D lysine, Concanavalin A) for Rod and Cone PR Growth

(Fig. 2(e)).

2.4.1. Cleaning the Glass Coverslips

The coverslips are initially washed with soap, followed by acid cleaning and rinsing with deionized water and finally dried in a laminar flow hood under UV lighting for further use. The acid treatment protocols were described earlier.[1–3]

2.4.2. Polylysine

Poly-D-lysine hydrobromide (average mol wt 30,000–70,000) was used a substrate. It was purchased from Sigma-Aldrich (Catalogue number P7280). We used 0.5 mL of a 0.1 mg/ml solution of poly-D-lysine to coat the glass substrates. The technique is well documented in previously published works.[1–3, 24–27, 38–40]

Table I. Composition of the defined medium developed for rod and cone culture.

Number	Component name and source	Quantity
1	Leibovitz's L15, Himedia laboratories, India, Catalogue number AT011	500 ml
2	B27 Supplement, Gibco®, Life technologies™, Catalog number 17504-044	10 ml
3	GlutaMAX™ Supplement, Gibco®, Life technologies™, Catalog number 35050-061	5 ml
4	100 X Antibiotic-antimycotic, Gibco®, Life technologies™, Catalog number 15240-062	5 ml
	Total volume	520 ml

2.4.3. Lectin (Concanavalin A)

Concanavalin A (Catalogue number TC220-100MG) was purchased from HiMedia® laboratories private limited, Mumbai, India. It is dissolved in sterile water to make a solution of 2 mg/ml and is used further for coating the glass wells. The glass wells are coated with concanavalin A solution and the protein is allowed to settle on the surface for 2–4 hours. Soon after that the glass culture wells are ready for plating the cells. We do not store the 'concanavalin A' coated coverslip for more than 24 hours, since this deteriorates the cell attachment.

2.5. Contact Angle Measurement

The static contact angle measurements were performed using a Cam 200 contact angle goniometer (KSV).

2.6. Atomic Force Microscopy

AFM images for the different substrates is obtained using Park XE 70 AFM instrument.

2.7. Cell Plating and Growth Analysis

In every experiment, in order to maintain the uniformity, we used one adult fish (1 pair of retina) to isolate the PRs. The cell suspension that was obtained from one pair of retina was directly plated on the glass cell culture dishes (as mentioned earlier in Section 2.4.1). After initial plating of the cells, we waited for 1 h in order to allow the cells to settle on the underlying substrate; thereafter we filled the culture well with the defined medium (Table I). Since in the adult neuron culture, it is really difficult to do a cell count initially, due to the presence of lot of debris,[41–44] so we allowed the cells to adhere and grow on the culture dish for 24 hours and then physically counted the number of live PR cells which have adhered to the substrate. Such a counting methodology ensured that the cells failed to adhere to the substrate were not counted. Through this process we got a more accurate estimate of the cells surviving following dissection and plating. We monitored the growth of the PRs for 3 weeks and performed a statistical analysis of the number of rod and cone cells surviving in the culture at day 1 and day 10. Half of the medium of the culture wells was changed after every 5th day.

2.8. Live Dead Assay

Life technologies,' live/dead viability/cytotoxicity assay kit (L-3224) was used for quantifying the viability the rod and cone cells in the culture.[27, 41–44] The guideline for using the kit is provided by the manufacturer. The live cells showed green fluorescence when incubated with the dye. The green fluorescently labeled live cells are counted for analysis.

2.9. Immunocytochemistry

We used mouse anti-rhodopsin monoclonal antibody, MAB 5316 (Millipore, Temecula California) to label the rhodopsin protein present ubiquitously in the rod PR cells.

We performed the immune labeling of the cells, which were 10 days old in the culture. In order to ensure the labeling of the primary antibody (MAB 5316), the cells were fixed in two steps. In the first step, we added a solution of 80 μl of paraformaldehyde+ 1920 μl of 1X phosphate buffer saline (calcium, magnesium free PBS) for 5 minutes. The cell fixing reaction was carried out by placing the culture wells on the ice. After 5 min, the coverslips we rinsed free of medium with phosphate-buffered saline (PBS) and we proceeded to the second step. In the second step, cells were fixed for 20 min at room temperature with a cold fixative (11.1 ml of formalin+89.9 ml of PBS+200 μl of glutaraldehyde+4 g of glucose). After 20 min, cells were permeabilized for 5 min using a permeabilizing solution (50 mM lysine+ 0.5% Triton X-100+100 ml of PBS). After rinsing with PBS, the nonspecific sites were blocked using 5% normal donkey serum and 0.5% Triton X-100 in PBS. The cells were blocked for 2 h, and then the cells were incubated with the primary antibody (MAB5316) for 12 h at 4 °C. After 12 h, the cells were rinsed free of the blocking solution using PBS and then further incubated with the fluorescently conjugated secondary antibodies (Alexafluor R 488-cojugated affinipure goat anti mouse lgG (H+L), 1:10, Jackson Immuno Research). After rinsing four times in PBS, the cover slips were mounted with Vectashield mounting medium (H1000, Vector Laboratories, Burlingame, CA). Control cultures without primary antibody were found to be negative.[27,41–44]

3. RESULTS AND DISCUSSION

3.1. Characterization of Poly-D Lysine and Concanavalin A Substrate Using Contact Angle Measurements and Atomic Force Microscopy (AFM)

3.1.1. Contact Angle Measurement

The concanavalin A substrate is significantly more hydrophilic as compared to the poly D lysine substrate (Figs. 3(a), (b)). The observed average contact angle of poly-d-lysine coated glass coverslip is around 45°. On the other hand, the average contact angle of concanavalin A is around 13°.

3.1.2. AFM

The AFM image showed that poly-d-lysine substrate is rougher as could be seen from the image. On the other hand, the concanavalin A substrate is smooth (Figs. 3(c), (d).

3.2. Choice of Fish Species

Common carp or *Cyprinus carpio* belongs to the family Cyprinidae. In natural habitat, they generally feed on decayed plant matter and different benthic organisms. These fishes have the ability to withstand and wide range of environmental conditions. They can withstand a wide variation of temperature. We chose 18 months old adult common carp (Cyprinus carpio) as the model system to isolate the rod and cone PR cells from the dissected retina

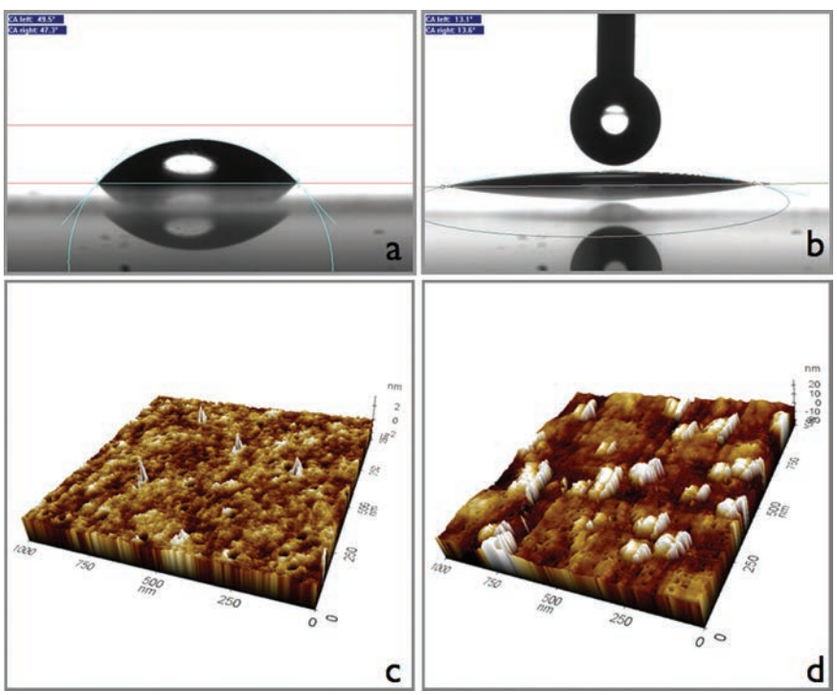

Fig. 3. Representative pictures of the contact angle measurement and AFM analysis of the 'poly d lysine' and 'concanavalin A' coated glass substrate. (a) Contact angle of poly D lysine substrate. (b) Contact angle of concanavalin A substrate. (c) AFM image of poly-d lysine substrate. (d) AFM image of concanavalin A substrate.

(Fig. 1(a)). The reason to choose this system is the easy accessibility to tissue and ease of maintenance of these fishes.[34–37]

3.3. Isolation of Rod and Cone

During a culture, we normally isolate 2 retina of one adult fish. The total PR cells isolated from the two retinas are used for the cell culture. We used a simple protocol to isolate the PR cells as described earlier in the methods section. It is very essential that the cells are isolated with minimum mechanical damage otherwise the outer segment of the PR cells gets damaged during isolation from the retina. Special care is to be taken to minimize the contamination form the retinal pigment epithelial cells. There will be very few retinal pigment epithelial (RPE) cells which could be observed as dark stained in appearance will be present in the culture. But with proper care during dissection such contaminating RPE cells could be avoided.[18]

3.4. Defined Medium

To grow these cells, we chose to develop a medium, which is free from bicarbonate buffering system. We empirically developed a chemically defined medium to culture the PR. We used L15 as the base medium, which is already optimized for promoting cell growth in the absence of exogenous carbon dioxide. We supplemented the medium with B27 supplement which has already shown to support survival and growth of adult and embryonic CNS and PNS neurons. Further we added 5 ml of glutamax and 5 ml of antibiotic/antimycotic to 500 ml of base medium (L15). All throughout the dissection and culture, we use this same medium. We have empirically derived a serum-free defined medium (Table I). The medium is suitable for growing the rod and cone PR neurons in the ambient condition without any exogenous carbondioxide.[45] The base medium is Leibovitz (L15) supplemented with B27. This medium supported the growth and survival of the neurons for 4 weeks. B27 is a specialized supplement formulation for growing different types of neurons.[2, 27, 38–44]

3.5. Comparing the Growth of PR on Poly-Lysine and Concanavalin A Substrates

We did not observe any form of cell attachment on poly-lysine substrate. The cells remain floating for 48 hours and after that starts to die out. In contrast, we observed that PR neurons attached on the lectin (concanavalin A) substrate

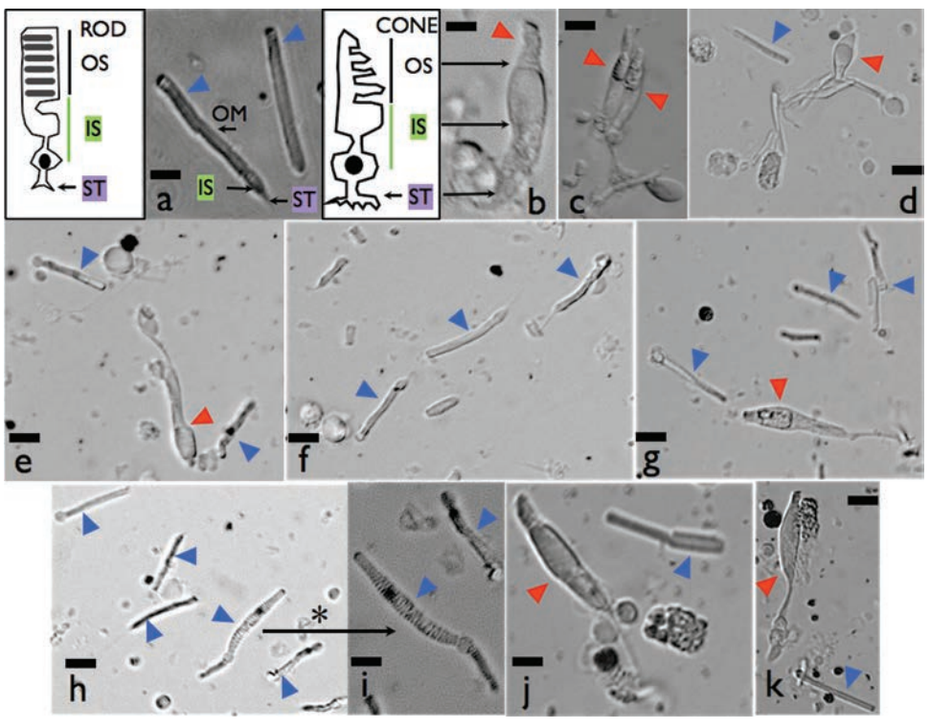

Fig. 4. The representative picture of the rod and cone cells at day 2 in the culture. The red and blue arrows indicate the cone and rod cells respectively. The scale bar is 5 μ. (a) The sketch of a characteristic rod cell showing the outer segment (OS), inner segment (IS) and the synaptic terminal (ST). The OS houses the large amount of rhodopsin pigments. The IS contains the nucleus and other cell organelle. The photograph shows two characteristic rod cells in the culture. (b) The sketch of a characteristic cone cell showing the outer membrane (OS), inner membrane (IS) and the synaptic terminal (ST). The OS houses the large amount of conopsin pigments. The IS contains the nucleus and other cell organelle. The photograph shows a characteristic cone cell in the culture. (c) Characteristic twin-cone cell. Such twin cones are documented in the retina of the bluegill fishes. (d, e, g, j, k) The representative rod and cone cells of varied morphologies seen in the culture at day 1. (f, h, i) The representative pictures of the rod cells at different resolutions.

soon after plating. The cells take 1 hour to firmly attach on the concanavalin A substrate and thereafter grow for three weeks. The representative pictures of the different morphologies of rod and cone cells at 2 different days in the culture are shown in Figures 4, 5 at day 2 and ay 5 in the culture respectively. Thus we observed attachment and growth of the rod and cone neurons on the concanavalin A substrate.

3.6. Estimating the Viable PR Cells in the Culture

At day 1 in the culture, we estimated that from one pair of carp retina, we could get approximately on an average 2000 and 8000 cones and rod cells respectively which adhere on the concanavalin A substrate (Cone: 2109 ± 460 cells, $n = 10$ cultures; Rod: 8013 ± 460 cells, $n = 10$ cultures; The data is expressed as 'average number of cells surviving in the culture $\pm$ standard deviation,' $n =$ total number of cell culture experiments from which data has been obtained). By day 10, half of the initially plated cone cells are lost and there is also reduction in the number of rod cells, which were initially plated on the culture dish (Cone: 1057 ± 542 cells, $n = 10$ cultures; Rod: 5867 ± 183 cells, $n = 10$ cultures). The data is graphically represented in Figure 6; fluorescent labeling shows the representative pictures of the viable photoreceptor cells.

3.7. Immuno-Cytochemical Identification of the Rhodopsin Molecule in the Rod PR Cells

We verified the presence of rhodopsin molecules in the rod cells by using anti-rhodopsin antibody. This antibody (MAB5316) reacts with the 39 kDa protein rhodopsin (opsin). The antibody specifically labels the synaptic pedicles and the axons of the rod PR cells (Fig. 7).

3.8. Overall Result Summary

In this work, we have developed a cell culture model to grow the adult PR cells obtained from the carp retina. Here we have documented the following:

i. A simple cell isolation protocol for dissecting out the rod and cone cells from the carp retina (Fig. 2).

ii. Developed a chemically defined medium (Table I), which supports the growth of these cells in carbon dioxide free system.

iii. Utilized a chemically defined substrate of plant origin, the jack bean lectin 'Concanavalin A' to successfully grow the adult PR cells (Figs. 3–7).

3.9. Why the Photoreceptor Prefers Concanavalin A Substrate?

The most intriguing aspect of this study is '*why the adult PR cells prefer to attach and survive only on 'concanavalin A' substrate as against a well known synthetic substrates which is known to support the attachment and growth of all kind of neurons*.' Four decades back, the cell biologist observed that the outer membrane of rod and cone PR cells have high concentration of carbohydrates on their outer surface of the membrane.[20–23] The presence of these carbohydrate molecules on the outer surface of the outer membrane of the PR may have roles in orienting and organizing the rhodopsin molecules.[21] Since the outer

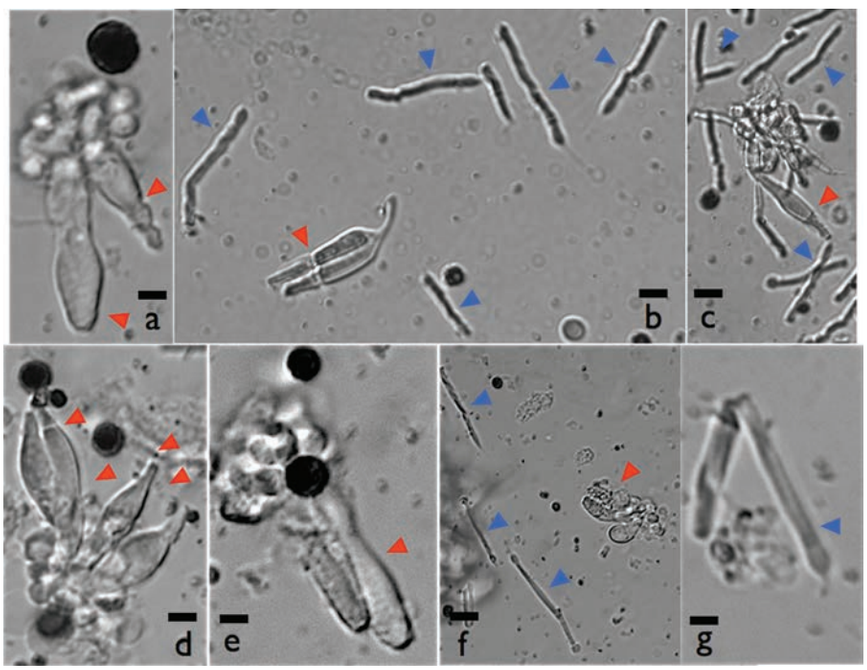

Fig. 5. (a)–(g) Representative picture of the rod and cone cells at day 5 in the culture. The red and blue arrows indicate the cone and rod cells respectively. The scale bar is 5 μ. The black circular cells are the contaminating retinal pigment epithelial cells in the culture.

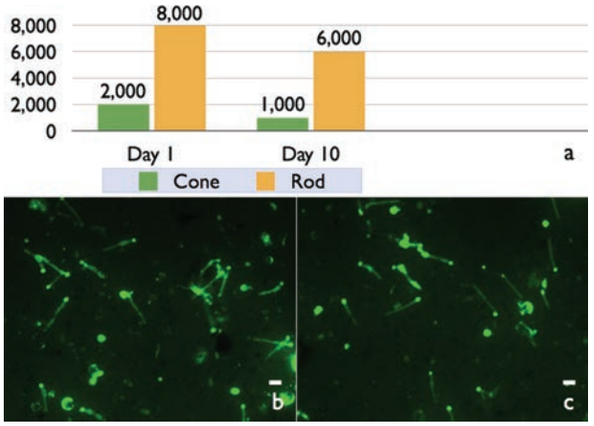

Fig. 6. Cell viability assay (a) Graph showing the statistical summary of the rod and cone cells surviving in the culture at day 1 and day 10. (b) The green fluorescent cells showing the live rod and cone cells at day 1. (c) The green fluorescent cells showing the live rod and cone cells at day 10. The scale bar is 25 μ.

segment (OS) of the PR cells has a comparatively larger surface area as compared to the inner segment (IS), so the attachment of these cells depends on how one can couple the outer segment (OS) to the substrate. The simplest way could be to use certain carbohydrate binding molecules as a substrate. So we follow this strategy. We coated the substrate with jack bean lectin 'Concanavalin A,' a protein that binds to carbohydrate. We observed that the PR cells upon plating successfully attach and grow on such a substrate. More than two decades back, it was documented that adult salamander PR cells also prefers to grow on lectin substrates.[14] But the immediate question is 'Does these adult PR cells lack integrin receptors, which could sense the ligands like laminin, collagen, fibronectin and ECM?' Our conservative guess will be 'No.' It is probably the large density of the carbohydrate molecules on the surface of the PR cells, which outnumber the attachment of other possible ligands like collagen, laminin, fibronectin or ECM. Another aspect, which is worth mentioning here is, the synthetic substrates like polylysine, which depends on surface charge manipulation to promote attachment of the neural cells too failed in the case of adult PR cells. Our study and a previous study dating back to 1993,[14] is indicating that the adult PR cells needs certain very special surface chemical cues to attach and regenerate in an *in vitro* system. We believe that these substrate chemical cues may come very handy in designing smart substrates for differentiating stem cells to form rod and cone PR cells.

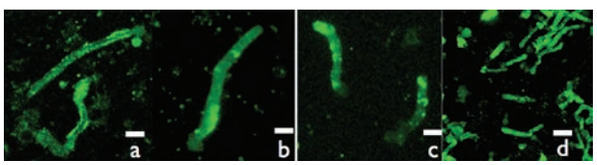

Fig. 7. Representative picture of the immune-stained rod PR cells. (a)–(d) The 10-day-old cultures were stained with anti-rhodopsin antibody. (a)–(c) The scale bar is 3 μ. (d) The scale bar is 10 μ.

4. CONCLUSIONS

Based on our experimental results, we are concluding that 'tethering to a lectin (concanavalin A) substrate is an absolute requirement for adult PR to grow.' Understanding the molecular details of this unique substrate specificity of the PR cells could be useful in designing smart materials for retinal regenerative medicine in future.

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