The role of induced pluripotent stem cell (IPs) in the transplantation of glaucoma

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ABSTRACT

Aim Glaucoma is a heterogeneous group of optic diseases that affect almost 1% to 2% of the population older than 40 years. There are many types of glaucoma but the most common type is primary open angle glaucoma. In this study we have investigated the role of muller cell lines in the transplantation of glaucoma model in rats.

Methods Intra ocular pressure was created with the help of laser treatment in rats. The induced pluripotent stem cells (IPs) were transplanted into the vitreous or sub-retinal space of glaucomatous or untreated eyes. Double therapy was used for the prevention of graft rejection. The rats were served with the mixture of two drugs in the drinking water. For this purpose cyclosporine (20mg/kg/day) and azathioprine (2mg/kg/day) were used. This drug therapy started three days before induction of glaucoma.

Results The transplanted cells were survived in vivo for 2 to 3 weeks and reduction in graft survival was also seen at the 4th week. Immunohistochemical analysis showed that a large number of oligodendrocyte precursor cells (OPCs), positive for the nuclear marker Olig2, survived in the vitreous, proximal to the inner surface of the retina, in glaucomatous eye for up to four weeks.

Conclusion Differentiating IPs cells within the glaucomatous eye produced cells that expressed glial cell markers.

Key words: diseases, muller cells, laser, eyes
INTRODUCTION

Glaucoma is a heterogeneous group of optic diseases that affect almost 1% to 2% of the population older than 40 years (1). It is estimated that 66 million people in the world are suffering from glaucoma, although fewer than half of those patients do not realize that they have the disease (2). There are many types of glaucoma but the most common type is primary open angle glaucoma (2-4). One of the major risk factors for the development of primary open-angle glaucoma (POAG) is elevated intraocular pressure (IOP) (3). Vision loss caused by glaucoma is irreversible, and glaucoma is the second leading cause of blindness in the world. This disease is the main cause of blindness in many countries.

The overall risk of developing glaucoma increases with the number and strength of risk factors. It increases substantially with the level of intraocular pressure elevation and with increasing age (3). Oligodendrocyte precursor cells (OPCs) hold great responsibility for the generation of oligodendrocytes in the developmental stage, whereas in adult individuals, they play a vital role in demyelinating pathologies and remyelinating of axons (4-6). Oligodendrocyte precursor cells appear to contain the majority of the stem cell characteristics (6) and have been shown to be neuroprotective in vitro (3).

The measurement of intraocular pressure is not an effective method for screening populations for glaucoma. Moreover, the most commonly used method for measurement, underestimates the true intraocular pressure (IOP) of patients with thin corneas and overestimates it in patients with thick ones. Almost half of all patients with primary open angle glaucoma have pressures below 23 mmHg at a single screening (7).

Recent findings prove the link between glucocorticoid and glaucoma. Actually, G1C1A is the first glaucoma gene which was mapped to chromosome 1q (8-10), and mutation in this gene is responsible for autosomal dominant juvenile glaucoma (ADJG) (11). Autosomal dominant juvenile glaucoma is a rare form of glaucoma. Stem cells have been proposed as a new approach for the regeneration of ganglion cells (12-13). On the other hand, the use of other stem cells, like embryonic stem cells and neural stem cells is greatly restricted due to some ethical issues and graft rejection. Muller cells have the characteristics of stem cells and it has been reported in a wide range of mammalian species, including the adult human retina (13).

A recent study explains that potential retinal stem cells and different types of stimuli have been shown in the muller cells of chicken and rats (14-15). Based on these facts this study was designed to investigate the role of induced pluripotent stem cell (iPS) in the transplantation of glaucoma.

MATERIAL AND METHODS

Ethical statement

The use of all the animals, as well as all animal experiments in this study was approved by the Ethical Committee of the Department of Ophthalmology, Shanghai First People’s Hospital Affiliated to Shanghai Jiaotong University. All the animals, albino rats (n=50) used for the experiment were kept at safe place and nourished with food, which is approved by food and drug administration (FDA).

Experimental design

Pilot experiment was done for checking graft rejection in cells but it showed that administration of cyclosporine alone was not enough for the prevention of total graft rejection. Double therapies were used for the prevention of graft rejection. The rats were served with the mixture of two drugs in the drinking water. For this purpose cyclosporine (20mg/kg/day) and azathioprine (2mg/kg/day) were used. This drug therapy started three days before induction of glaucoma. The serum levels of cyclosporine were measured in all samples at different intervals. The average concentration was 502.2 ± 79 ug/lit.

Preparation of animals

Animals in this study were anaesthetized with ketamine and xylene; later few drops of anesthetic agent were dropped into the eyes. Intraocular pressure was measured with the help of tonometer. Unilateral intracocular pressure was induced in the eyes with the help of laser (546 nm). Initial treatment consists of 60 to 65 spots of 45 μm diameter for 40 seconds. This laser treatment was repeated for 7 to 8 weeks after every one week. Then tissues of the eye were collected for further
processing and then washed with PBS and preserved at -80°C.

**Preparation of induced pluripotent stem cell (IPs) culture**

The cell lines were prepared from postmortem human neural retina and engineered to express purple fluorescent protein to facilitate tracking on transplantation. The cell lines were purified for purple fluorescent protein for cell culturing. Cells were used for transplantation at passages 46 to 48. The cells were maintained as an adherent cell lines in 70 cm² tissue culture flasks which contain D-MEM (containing 0.45 g/L glucose, sodium pyruvate and stabilized L-glutamine) and penicillin. Thnreafter, the cells were washed with phosphate buffer saline (PBS) then detached from the flask with the help of trypsin. Then, we washed the complete cell culture medium and then converted it into fresh clean flask.

**Histochemical analysis**

The cells were washed with PBS and then blocked with PBS-triton whose concentration was 0.5%. All the monoclonal antibodies were diluted with this blocked. Then this whole section was incubated at 4°C overnight in an incubator. After incubation cells were again washed with PBS and then added the purple fluorescent protein with primary antibodies. Then again slides were washed and cover slip was put on the slides. Mouse IgG1 antibodies were used as a primary antibody.

**RESULTS**

Figure 2 (A to I) shows the induced pluripotent stem cells which were not found to migrate into the uninjured adult retina after intra vitreal delivery.

![Figure 1. Glaucoma in the human eye: histological section of the optic disk in a glaucomatous eye (Luo D, 2013)](image1)

![Figure 2. Survival and migration of induced pluripotent stem cells after transplantation into the eye. A, B) The cells were transplanted; C) The cells were engrafted; D) Magnified Figure C; E, F) The eyes were stained with death associated protein 1(DAP1) (purple); G) intravitreal delivered EGFP positive IPs stem cells did not migrate into the retina; H) Magnified G; I) Engrafted EGFP positive IPs stem cells (Luo D, 2013)](image2)

![Figure 3. Facilitated IPs cells in the glaucomatous eye after transplantation of every 2 weeks in-vivo. A-D) Intraocular (IO) injection of EPO and then cells migrate to retina. D) Antibodies were used to detect DPA1 purple; E-F) Intraocular migration is done; G) Engrafted cells; H) Tissues were counterstained with DAP1 purple. Chondroitinase ABC activity was confirmed (Luo D, 2013)](image3)
After the grafting of all layers of retina no human nuclear cells were observed in the retinal layer (Figure 2C, Figure 2D). The transplantation of the cells after one week was shown in Figures 2A, and 2B. Cells were engrafted after two weeks (Figures 2C and 2D). All induced pluripotent stem cells did not retain their EGFP expression. Most of human nuclear antigens were negative as shown by histochemical labeling (Figure 2A, 2B, 2C and 2D).

For the encouragement of chondroitinase activity, it has combined with erythropoietin. This attempt modified the inhibitory retinal environment. It calculated 19 to 35 cells EGFP+-IPs cells/eye. These cells were not observed in the retina (Figure 2A-2I). It should be noted that this count is likely to be greatly underestimated, given that less than a half of the induced pluripotent stem cells were injected into the eye marked by EGFP (Figure 2).

The retina is exposed to EPO, either they were invitreous form or in subretinal space (Figure 3). EFGP-positive cells appeared in the left side of the retina. The presence of EPO (Figure 3A) was one of the surviving grafts of the retina. These cells appeared in the induced pluripotent stem cells and extended from ganglion to the outer nuclear layer (Figure 3B). Co injection of chondroitinase ABC with IPs cells into the glaucomatous eye greatly enhanced the ability of the transplanted cells to invade the retina (Figure 3E and 3G).

The differentiation of induced pluripotent stem cells after their incorporation into the glaucomatous retina was also examined in this experiment. Immunohistochemical analysis showed that a large number of OPCs, positive for the nuclear marker Olig2, survived in the vitreous, proximal to the inner surface of the retina, in glaucomatous eye for up to four weeks.

DISCUSSION

Muller glial cells are the radial glial cells of the retina and have been shown to share a common phyletic lineage with retinal neurons and to derive from a common multipotent progenitor cells (16-18). Retinal ganglion cell replacement (RGCR) is one of the best possible methods to restore the vision after glaucoma (19-21). Stem cell transplantation has been shown to neuronal loss and also replaces outer neuronal membrane (22-23). In the present study we have found that the IPs cells posses the stem cell like properties and a human muller cell lines which have the potential and ability to divide and be regenerated. These cells are well explained in the treatment of many diseases in many experiments (19). We have concluded that muller cells remain very easily in the eye and also respond to the environment.

As previously reported (24), the throat also been shown to increase expression of MBP by oligodendritic precursor cells (OPCs) in vivo and optionally oligodendritic precursor cells (OPC) mediated myelination of RGC axons normally unmyelinated retinal. This study has observed less oligodendritic precursor cells (OPC) differentiation into MBP - expressing cells in the retina than previously reported. It is not clear why this difference in myelin production was detected, but it may be due to the use of different breeds of rats which strain differences in the inflammatory response and protective autoimmune has been documented.

Oligodendritic precursor cells (OPCs) were injected into the vitreous of both injured and glaucomatous eye was found to survive well in all experiments. In addition to these, the grafted OPCs observed to spread across the inner membrane of retinal surface, which puts them for mediating the observed neuroprotection. The number of grafted cells was lower in chronic graft, which had been OPCs in vivo for 12 weeks, compared with acute graft in vivo for only four weeks.

In this study it was found that the retina of both normal and glaucomatous eyes did not permit the integration of induced pluripotent stem cells because they are without extra cellular matrix modification. Even in the direct contact of retina IPs cells are unable to penetrate in the membrane (25-28). It was demonstrated that induced pluripotent stem cells and the two other similar cell lines could integrate into the retina of neonatal and injured adult rats and then they differentiated into different cells type (19, 29, 30). The results of this study have shown the ability of muller cells to differentiate in the glaucomatous eye when cells were delivered in both cases either invitreally or subretinally. The intra-vitreous route is more reliable as compared to subretinal delivery because they fail in grafting. This experiment demonstrated that intra-vitreal injection was not without tribulations, as it was often complicated to place the cells precisely adjacent to the retina (31).
In summary, we have demonstrated that the human-derived IPs progenitor cell line induced pluripotent stem cells is capable of surviving within the glaucomatous eye and of acquiring neural morphology upon intravitreal transplantation. Additionally, it has been found that delivery of EPO and chondroitinase have the ability to migrate into the adult retina. With the particular environment retinal environment of chondroitinase facilitated the IPs cells into the mature retina.

The results explain that differentiating induced pluripotent stem (IPs) cells within the glaucomatous eye produced cells that expressed glial cell markers.

FUNDING
This research was supported by the Key sub Project of Chinese National Programs for Fundamental Research and Development973 sub project-2011CB707506, Project of Shanghai Natural Science Foundation (13ZR1433200) and Opening Project of Shanghai Key Laboratory of Fundus Diseases (07Z22911).

TRANSPARENCY DECLARATION
Competing interests: None to declare.

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