

Differentiation of mesenchymal stem cell in the microenvironment of retinitis pigmentosa

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Abstract

• **AIM:** To assess the differentiation of rat mesenchymal stem cell (MSC) in the microenvironment of retinitis pigmentosa (RP) induced by the administration of sodium iodate.

• **METHODS:** *In vitro* cultured Lewis rat MSC were injected into the subretinal space of NaIO₃ induced RP rat eyes (30g/L NaIO₃ 100mg/kg). To observe the trace and differentiation of MSC by immuno-fluorescent method successively in 5 weeks after the surgery.

• **RESULTS:** The majority of the transplanted cells stay in retinal pigment epithelium (RPE) layer and cones and rods layer. From the 2nd week after transplantation, the engrafted MSC expressed PCK and rhodopsin under fluorescent microscope.

• **CONCLUSION:** MSC can survive mainly in the outer layer of retina in the microenvironment of RP and differentiate forward the RPE cell and photoreceptor.

• **KEYWORDS:** mesenchymal stem cell; NaIO₃; transplantation in subretinal space; differentiation; retinitis pigmentosa

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INTRODUCTION

In the ongoing discussion of using stem cells for cell replacement strategies, the most promising group of stem cells are those isolated from the adult organism. Among these adult stem cells are the mesenchymal stem cells (MSC) from the bone marrow stroma, which have recently experienced a high level of attention as they can be easily isolated and obtained in almost unlimited numbers. Bone marrow stromal cell (BMSC) can be expanded through as many as 20 to 50 population doublings in approximately 10 weeks^[1]. Recently, it has been reported that bone marrow cells differentiate into

various cell types, including hepatocytes, endothelial cells of the blood vessels, cardiac muscle, and skeletal muscle. It has also been reported that MSC differentiate into neural cells and astrocytes *in vitro*. Furthermore, they differentiate into astrocyte-like cells *in vitro* when transplanted into the normal or ischemic brain. In addition, in the case of the rat eye, it has been published that MSC can differentiate into photoreceptor-like cells^[2]. This study is to observe the growth and differentiation of MSC that are transplanted into the sub-retinal space of rats with the destruction of RPE cells.

MATERIALS AND METHODS

Materials DMEM/F12(1:1) culture medium and fetal calf serum (Hyclone), NaIO₃, Dulbecco fluid (Gibco), Bromodeoxyuridine (BrdU, Sigma), mouse monoclonal anti-BrdU antibody (Boster Biotechnology Co. Ltd.), PE conjugated anti-rat CD45 monoclonal antibody and FITC conjugated anti-rat CD90 monoclonal antibody (Caltag Laboratory), anti-rat rhodopsin (Opsin) monoclonal antibody (NeoMarkers), anti-rat PCK monoclonal antibody (NeoMarkers), Cy3 conjugated anti-mouse secondary antibody, Sheep blood serum (Boster Biotechnology Co. Ltd.). Lewis rats, deparaffinized, 4-5 weeks old, weighing from 100-120g.

Anesthetize the Lewis rat in overdose, take the tibia and fibula and immerse them in 750mL/L alcohol for 10 minutes. Wash the medullary cavity of bones with the PBS fluid repeatedly. Centrifuge the washed PBS fluid at the speed of 1000r/min for 5 minutes. Collect the cells and put them in the culture of DMEM/F12 including 100mL/L fetal calf serum. Finally put them in the petri dish and culture them at the condition of 37°C, 50mL/L CO₂ and saturated humidity. Change the culture media on alternate days. 12-14 days later, transfer the passage when the cells confluence mostly. Digest the MSC that confluence mostly with the 2.5g/L trypsin. Put PE-conjugated CD45 and FITC-conjugated CD90 monoclonal antibody after centrifugation. Place them in the dark at 4°C for 30 minutes and then wash them by PBS fluid, suspend them again and check them by flow cytometry. 2-3 days later, there are some sporadic spindle cells adherent to the culture dish. 5-7 days later, the sporadic cells change into colonies. 9-10 days later, the colonies increase and coalesce. 12-14 days later, there are whirlpools in some place. We can transfer the passage when cells confluence. 6-7 days later after transferring, the cells can confluence again. The result of flow cytometry indicates that more than 70% of the cells are CD90(+) and CD45(-) which are from passage 1 to 2. And

more than 93% of the cells are CD90(+) and CD45(-) which are from passage 3 to passage 6(Figure 1). MSC cells labeled by BrdU monoclonal antibody can be stained into brownish red. The cytoplasm is not stained. Un-labelled cells are not stained at all. We can see the damage of RPE cells and photoreceptor cells and the inner nuclear layer being in direct contact with Bruch's membrane (Figure 2). Put DMEM culture with 10mg/L BrdU, 100mL/L fetal cow blood serum in the MSC of passage 3, then culture at the condition of 37°C, 50mL/L CO₂ and saturated humidity, change the culture media on alternate days. Observe the label effect by immunohistochemistry. When MSC of passage 3 labelled by BrdU confluence mostly, digest with 2.5g/L trypsin and centrifugate them. Dilute with PBS fluid to the concentration of 3×10^{10} /L and put in the sterile canoula for use.

Twenty-five Lewis rats, 4 weeks old, weighing from 100-120g, intra-peritoneally administered 100mg/kg NaIO₃ dissolved just before use for one week. We observed the pathological sections of RPE cells and photoreceptor cells.

Methods We anesthetized the Lewis rat, dilated the pupil, stabbed the sclera and pars plana of ciliary body under the operating microscope. Then we put the slender glass needle made by ourselves and the micro-amount syringe in the vitreous through the pars plana of ciliary body stabbed just now, and injected 10μL fluid into the sub-retinal space. We repeated the procedures and completed the surgeries to 25 rats. The 25 right eyes were considered as experimental eyes and 25 left eyes as controls. The fluid injected in the right eye was 10μL MSC suspension while in the left eye was PBS fluid in equal amount. All the fundus of the rat eyes were observed 1 day, 7, 14, 21 and 28 days after operation. Twenty-five Lewis rats were divided into 5 groups randomly. One group contained 5 rats. We anesthetized the Lewis rat in overdose, enucleated the eyes of the rats 7, 14, 21 and 28 days after operation. The eyes were frozen immediately and cut in sections labelled by mouse monoclonal Anti-BrdU antibody, FITC-conjugated anti-mouse secondary antibody; Anti-rat rhodopsin monoclonal antibody, Cy3-conjugated anti-mouse secondary antibody; Anti-rat PCK monoclonal antibody, Cy3-conjugated anti-mouse secondary antibody; Fluorescence microscope was used to observe the result.

RESULTS

One day after operation, the conjunctivas were congestive in all eyes. All the corneas were transparent. Seven lenses became slightly opaque including 4 right eyes and 3 left eyes. No hemorrhage was seen in vitreous and retina of all eyes checked by indirect ophthalmoscope. Local retinal detachment could be seen in all eyes. 7 days after operation, the congestion in conjunctiva regressed. Seven lenses became opaque including 4 right eyes and 3 left eyes. Their fundus could not be seen accordingly. The detached retina became reattachment. 2-4 weeks after operation, seven eyes including 4 right eyes and 3 left eyes changed into cataract. The retinas remained no change. One week later after injection of MSC into sub-retinal space, the slices of the retina of Lewis rats indicate that MSC labelled by BrdU show green conjugated

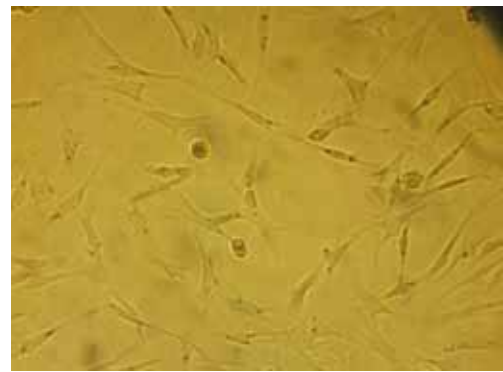


Figure 1 MSC original passage in the fourteen days.

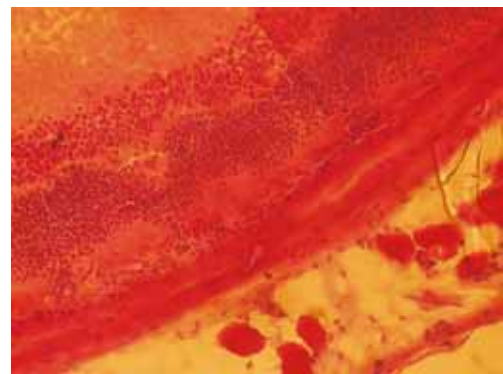


Figure 2 One week after NaIO₃ injection.

with FITC. The cells that express PCK or rhodopsin showed red conjugated with Cy3. If one scene is overlapped to another, we can see that some cells express PCK and the other express rhodopsin(Figure 3,4). This indicates the MSC can differentiate into the way of RPE and photoreceptor cells.

DISCUSSION

There are a number of animal models of retinal degeneration. They contain the inherited animal models and chemical induced retinotoxicity models. In the inherited animal models, arrested outer segment development followed by photoreceptor cells loss is the primary event in the rd3 and rds mouse. By contrast, defects of the phagocytic activity in the RPE cells is the primary site of the genetic mutation in the RCS rat, with the rdy mutation in which photoreceptor cells are affected occurring as a secondary event. Among the chemicals, N-methyl-N-nitrosourea (MNU) and urethane cause photoreceptor cell degeneration without initial damage to the RPE cells. NaIO₃ possesses selective toxicity for the RPE cells. The damage of photoreceptor is secondary to the damage of RPE cells. In the literature, the RPE cell nuclei were hyperchromatic at 6 hours after the NaIO₃ treatment, and the RPE cell number decreased at 12 hours. At this time point, photoreceptor cell nuclei as well as photoreceptor segments remained normal. At 24 hours, the number of RPE cells was decreased and the arrangement of the photoreceptor segments was disorganized. But by day 3, destruction of the photoreceptor segments was more advanced, macrophage migration was seen, and the number of photoreceptor cell nuclei was decreased in some areas. Finally, at day 7 and day 28, an abrupt transition from a relatively normal retina to an area with loss of RPE cells and photoreceptor cells resulting in

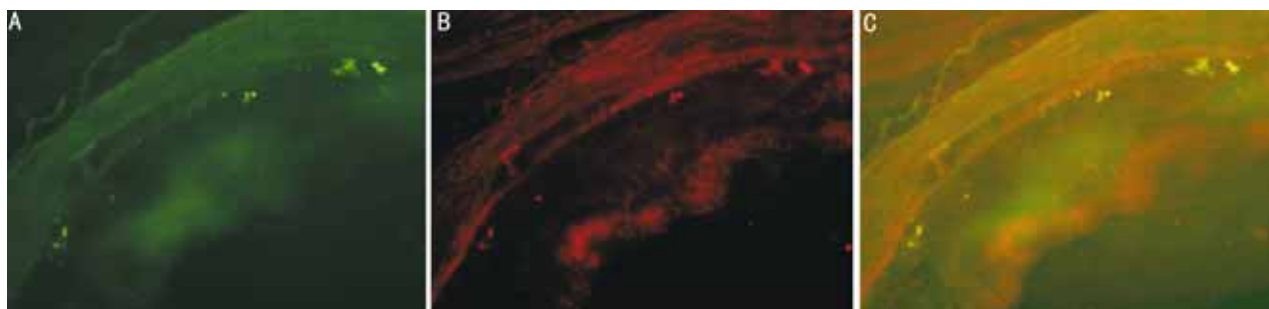


Figure 3 The fifth week after sub-retinal transplantation A; Labeled by BrdU(+); B; Labeled by rhodopsin(+); C; Two overlay (+).

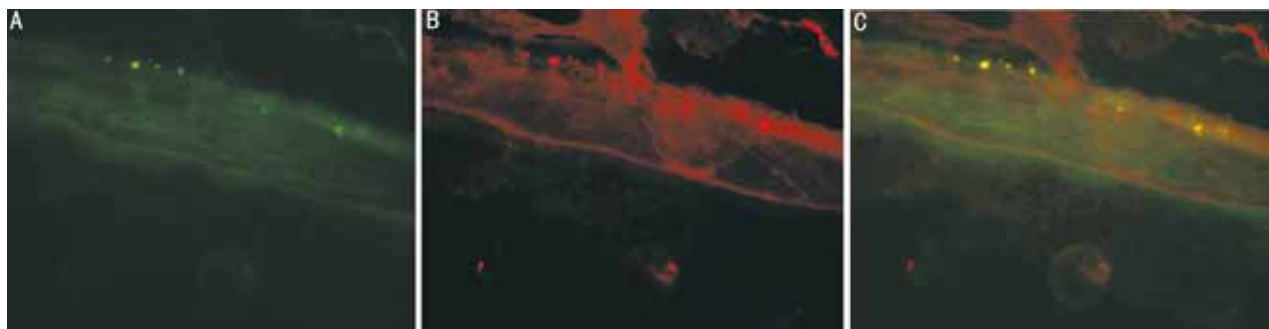


Figure 4 The fourth week after sub-retinal transplantation A; Labeled by BrdU(+); B; Labeled by PCK(+); C; Two overlay (+).

the inner nuclear layer being in direct contact with Bruch's membrane was seen. In this study, we select NaIO_3 to induce our animal model. As a result, we can see that the damage of RPE cells and photoreceptor cells and the inner nuclear layer being in direct contact with Bruch's membrane.

MSC are the precursor of the bone marrow stroma cells. There are several sight-threatening retinal disorders for which treatment is not yet available or produces poor results. In some of these diseases, retinal degeneration occurs early in life and might be quite rapid, whereas in other disorders, retinal degeneration begins later and progresses very slowly. There are some possible sources for the repair of retinal degeneration. A current therapeutic approach to neovascular eye diseases involves the application of angiostatic or antiproliferative agents to the eye. A cell-based therapy, however, should be explored, because, in contrast to inhibiting angiogenesis with small molecules or recombinant factors, a cell-based approach might enable the cell to adapt and respond to a changing environment. Cell-based therapy likely involves numerous factors produced by the cell that can be appropriately modulated in response to changing conditions^[3]. It was demonstrated that MSC are able to secrete neurotrophic factors that promote neural cell survival^[4]. The multipotentiality of BM-MSC as well as their easy isolation and culture properties and their high expansion potential makes these cells an ideal source for autologous transplantation aimed at a cell-based therapy for retinal degeneration repair^[5]. We select the marker of CD90 and CD45 to identify the MSC by flow cytometry. We have achieved a lot in the transplantation of NSC. NSC could incorporate into the retina and express the Cytokeratin and CD68 which are the markers of RPE cells when transplanted

into sub-retinal space^[6,7]. MSC can incorporate into the retina and express the GFAP which is the marker of astrocyte and rhodopsin which is the marker of photoreceptor cells. Kicic *et al*^[8] transplanted the MSC into the sub-retinal space of RCS rat and discovered that most of the MSC distributed in the layer of cones & rods and express rhodopsin which is the marker of photoreceptor cells. Pan *et al*^[9] discovered there are about 0.174% \pm 0.082% MSC can survive and incorporate into all the layers of the retina 2 weeks after transplantation. Inoue *et al*^[5] discovered transplanted MSC can delay the retina degeneration of RCS rat. Also, a recent study compared MSC and retinal progenitor cells to evaluate their potential as a source for retinal transplantation. Both cell types expressed neuronal markers *in vitro* but some MSC differentiated into cells that resembled microglia rather than neural cells. These findings suggested that retinal progenitor cells are the best choice for retinal transplantation studies, but MSC remain an attractive candidate as a therapeutic tool for retinal repair in autologous transplantation.

The purpose of the study is to observe if the MSC can differentiate into RPE cells which are damaged. RPE cells are damaged by celiac injection of NaIO_3 . In this case, the MSC can differentiate into the way of RPE cells. Because the photoreceptor cells are damaged at the same time, we can also observe that the MSC differentiate into the way of photoreceptor cells. As for the differentiated cells, we don't know if they have the function of the RPE and photoreceptor cells and need further study. But it is difficult to detect the retinal function of the animals because it needs to keep the most retina intact to examine the electrophysiology or action. At least, we can deduce two principles from this study: (1) the transplanted MSC can survive in the sub-retinal space,

without exclusion and proliferation; (2) the MSC can differentiate into the way of RPE and photoreceptor cells.

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骨髓间充质干细胞在视网膜色素变性大鼠视网膜下的分化

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摘要

目的:研究骨髓间充质干细胞(MSC)在视网膜色素变性(RP)大鼠体内的分化。

方法:Lewis 大鼠腹腔注射 30g/L NaIO₃ 100mg/kg,建立大鼠 RP 模型,将体外培养的 MSC 植入视网膜下腔,用免疫荧光标记的方法对 MSC 进行追踪,并观察术后第 1,2,3,4,5wk MSC 在该微环境中的分化。

结果:术后第 1wk 即可见 MSC 位于视网膜色素上皮(RPE)层与光感受器细胞层,但全角蛋白(PCK)及 rhodopsin 标记阴性,第 3wk 开始可见 MSC 在体内表达 PCK 及 rhodopsin。

结论:MSC 植入 RP 模型大鼠视网膜下腔后可存活,主要分布于 RPE 层和视锥、视杆细胞层,并表达 RPE 细胞和光感受器细胞的表面标志。

关键词:骨髓间充质干细胞;NaIO₃;视网膜下腔移植;分化;视网膜变性