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Research article

Synergistic effect of olfactory ensheathing cells and alpha-crystallin on restoration of adult rat optic nerve injury

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HIGHLIGHTS

- We study the synergistic effect of OECs and α-crystallin on the optic nerve regeneration.
- OECs transplantation could promoted RGCs survival and axonal regeneration.
- α-Crystallin vitreous injection could promoted RGCs survival and axonal regeneration.
- Compared with OECs or α-crystallin, the synergistic effect of OECs and α-crystallin on the optic nerve regeneration was much more striking.

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ABSTRACT

Olfactory enshesathing cells (OECs) and α-crystallin all can promote axonal regeneration after optic nerve injury. However, their mechanisms were different. Here, we study the synergistic effect of OECs and α-crystallin on the optic nerve regeneration. α-Crystallin was injected into vitreous cavity, and OECs were transplanted to the optic nerve injury area. The regeneration length of optic nerve were measured by anterograde tracing using cholera toxin subunit B (CTB). The survival of RGCs were assessed by counting the numbers of βIII-tubulin-labeled RGCs in a retinal whole mount. The results that OECs and α-crystallin all could promoted RGCs survival and axonal regeneration ($P < 0.01$). Especially in the combination group, the length of axonal regeneration was 5.6 mm after optic nerve injury for 3 months. These findings indicated that compared to OECs and α-crystallin alone, the combination of OECs and α-crystallin could promote axonal regeneration more effectively.

1. Introduction

Retinal ganglion cells (RGCs) fail to spontaneously regenerate their axons after optic nerve injury. Apoptosis of RGCs, deficiency of neurotrofic factors, glial scar, and inhibitory microenviroment are the major reasons of inhibiting axonal regeneration after optic nerve injury [4,26,31]. Olfactory enshesathing cells (OECs) can secrete variety neurotrophic factors including nerve growth factor(NGF), neurotrophin-3, brain derived neurotrophic factor(BDNF), glial-derived neurotrophic factor(GDNF), ciliary neurotrophic factor(CNTF), and fibroblast growth factor(FGF) [6]. OECs also produce a range of extracellular matrix proteins, such as laminin and fibronectin, which also facilitate axon outgrowth [21]. Recent research have demonstrated that transplantation of OECs and their accompanying olfactory nerve fibroblasts (ONFs) into lesions in the adult spinal cord can promote axonal regeneration across the lesion, remyelination of axons, and partially restoration of functions when only 1% of axons are present [5,16,17]. OECs could also inhibit the activation of astrocytes and reconstruct glial scar by moderating nuclear factor kappaB translocation [3]. In optic nerve, the RGCs axons could regenerate and elongate for 10 mm together with the transplanted OECs/ONFs at 6 months after optic nerve was transected [10]. However, the regenerating RGC axons terminate in large expansions and do not continue further distally in the optic nerve stump, Whereas, the regenerating axons are able to leave the transplanted OECs/ONFs and re-enter the host tracts in the spinal cord [10]. It suggested that only OECs/ONFs transplantation can not achieve the target of long-distance regeneration of optic nerve.

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As the small heat shock protein (sHSP), α-crystallin and play a beneficial role in preventing stress-induced cell death, including retinal pigment epithelial cells(RPE) [20], photoreceptors [12], lens epithelial cells [1] and dopaminergic olfactory bulb neurons [14]. In the optic nerve, α-crystallins could promote directly the survival of RGCs after optic nerve axotomy, and the survival rate was 95% at 14 days [13]. α-Crystallins could also protect RGCs indirectly by inhibiting microglia activation via downregulating the expression of TNF-α and iNOS [28]. Moreover, the axonal density of RGCs distal to the crush site was significantly higher than in untreated controls up to 4 weeks after a single intravitreal administration of α-crystallin [30]. Presently, our studies showed that α-crystallin could counteract the effect of myelin inhibitory factors through the regulation of RhoA/Rock signaling pathway, and promoted the axonal growth after optic nerve injury [24]. However, the effect of promoting axonal regeneration declined by 4 weeks after α-crystallin injection [30]. It suggested that only α-crystallin injection can not promote the effective axonal regeneration of optic nerve.

OECs/ONFs can provide neurotrofic factors, inhibit astrocyte activation, reconstruct glial scar, and form a bridge to protect axonal regrowth. α-Crystallin can promote RGCs survival, decrease the RhoA activation, and protect axonal regeneration in inhibitory microenvirament. The combination of OECs and α-crystallin might overcome the four major inhibitory factors of axonal regrowth at the same time, and promote the effective axonal regeneration. In this work, we studied the synergistic effect of OECs and α-crystallin on the restoration of adult rat optic nerve after crushed injury.

2. Materials and methods

2.1. Animals

Adult (200–250 g) Long Evans rats were used for the experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Third Military Medical University. And all studies were conducted according to the Declaration of the NIH Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Cell cultures and identification

Primary cultures of OECs/ONFs were prepared as previously described [10]. Briefly, adult rats were decapitated. The outer nerve and glomerular layers of olfactory bulbs were dissected out, and dissociated in 0.125% trypsin for 15 min at 37 °C. Activity of exogenous proteases was eliminated by washing the tissue with DMEM/F-12 containing 10% fetal bovine serum (FBS).Then, cells were mechanically dissociated using a pipette and plated on 35 mm dishes coated with poly-L-lysine and cultured in DMEM/F-12 containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO2. At day 9, the cultrued cells were transfected with a lentivirus harboring enhanced GFP, and continued to culture for 14–17d, at which time each dish yielded a density of 1.5×10^6. As previously described [9,23], two primary antibodies were used to identified cultured cells. Rabbit monoclonal anti-S-100 (1:200, Sigma, USA) labels OECs. Mouse monoclonal anti-fibronectin labels ONFs (1:200, Santa Cruz Biotechnology, USA). Goat anti-mouse IgG-FITC and goat anti-rabbit IgG-cy3 (Boster, China) were separatedly used as second antibodies.

2.3. Surgical procedures

The right optic nerves were crushed (ONC) intraorbitally using a standard protocol [24]. In brief, adult animals were anesthetized by intraperitoneal injections of chloral hydrate (3.5 mg/kg). A 1-cm incision was made in the conjunctiva at the temporal side of each eye. The optic nerve was exposed under an operating microscope, and its dura was opened longitudinally. Using the blood vessel forceps, the optic nerve was crushed 2 mm behind the eye for 10s, avoiding injury to the ophthalmic artery. Immediately after ONC, 1 μL GFP- OECs/ONFs suspension (1×10^7/ml) or the same volume PBS (0.01 M) were injected dilatorily into the proximale side to the cruched optic nerve using a 35G-microinjector (World Precision Instruments, USA).Nerve injury was verified by the appearance of a clearing at the crush site, and the vascular integrity of the retina was verified by funduscopic examination. As our previously described [24,25], α-Crystallin (10^{-4}g/L;5 μL) or PBS (0.01 M;5 μL) were injected into vitreous space by posterior approach in the right eyes, taking care not to damage the lens. Treatment groups were: (1) OECs and α-crystallin combination group: injecting OECs/ONFs into the proximale side to the cruched optic nerve and α-crystallin into vitreous. (2) OECs group: injecting OECs/ONFs into the proximale side to the cruched optic nerve and PBS into vitreous. (3) α-crystallin group: injecting PBS into the proximale side to the cruched optic nerve and α-crystallin into vitreous. (4) PBS control groups: injecting PBS into the proximale side to the cruched optic nerve and into vitreous. And 18 animals were taken per group. In addition, a sham control group (only PBS injection without ONC) was observed. We found that only PBS injection did not cause significant damage to RGCs and axons(data not shown).

2.4. RGCs survival assay

After optic nerve injury, RGCs survive for 5d and then die abruptly in large numbers, reducing to approximately 50% of normal by day 7 and to less than 10% on day 14 [2]. This study, RGCs survival assay was performed at 1, 2 weeks and 1 month after ONC. As Luo J.M.and Sargoy A described [11,18],a eyecup was made by cutting off the cornea, removing lens and vitreous from the inner retinal surface. Then the retinal wholemounts were removed from the eyecup,and fixed in 4% paraformaldehyde for 1 h, washed in PBS,and blocked for 6 h with 1% bovine serum albumin. The primary antibodies was mice monoclonal anti-βIII tubulin (1:200, Cell Signaling, USA), which is a marker of RGCs [11]. Retinal wholemounts were incubated with monoclonal anti-βIII tubulin overnight at 4 °C for 24 h, washed in PBS, incubated at 37 °C for 6 h in the secondary antibody. After washed in PBS, retinal wholemounts were laid flat on a microscope slide with the RGCs layer up.The RGCs numbers were counted in 8 fields of the whole retina. The field (about 0.36 mm^2) was in a pattern of grid intersections, at a fixed distance of 5/6,1/2 radius from optic disc, which was at superior,inferior, nasal and lateral region respectively of retina. And 18 retinas erery groups were measured.

2.5. Evaluating axonal regeneration

In order to anterogradely label regenerating axons, cholera toxin B subunit (CTB; 1 g/L, Molecular Probes, USA) was intravitreally injected 2 days before sacrificing the animals as previously described [24]. Apoptosis of RGCs is associated with the loss of axons. To study axon regeneration over longer times and distances,regenerating axons were visualized in longitudinal sections of the optic nerve at 2 weeks, 1 and 3 months after surgery. As Takuji Kurimoto described [7], Optic nerves were dissected and postfixed in 4% paraformaldehyde, impregnated with 10% and then 30% sucrose, embedded in OCT Tissue,frozen, cut in the longitudinal plane and mounted on coated slides. The thickness of every frozen section was 7 μm. Five sections of one optic nerve(1 section every 6 sections) were used to evaluate axonal regeneration. And 18 optic nerves erery groups were measured. The longest length of regener-