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Effect of Limbal Mesenchymal Stem Cell in Form Deprivation Myopia Animal Model

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Abstract

This study investigates the capability of limbal mesenchymal stem cell (LMSC) in reducing myopia progression in a form-deprivation myopia animal model. A total of 24 rabbits (*Oryctolagus cuniculus*) divided into three equal groups (n=8) as follows: form-deprivation myopia group (FDM), FDM group with treatment $1x10^5$ in 0,1 ml LMSCs injection (FDM+LMSC), and control group. Refraction status was evaluated by streak retinoscopy, and axial length was measured using a-scan biometry. The migration of LMSCs into the posterior sclera was evaluated with red-fluorescent cell dye (PKH26). α-smooth muscle actin (α-SMA) expression and posterior scleral thickness were evaluated in a 6-week follow-up. This study shows significant differences in α-SMA expression and scleral thickness between the three groups ($p<0,05$). Besides, there were significant differences in scleral thickness and α-SMA expression between the control and the FDM with the LMSCs group. LMSC has a potential role for myopia control in form-deprivation eye animal models by increasing α-SMA and sclera thickness.

Keywords: Form deprivation myopia, limbal mesenchymal stem cell, α- SMA, myofibroblast, scleral thickness

Introduction

Myopia is one of the global eye health issues with a high prevalence of sight-threatening complications, with 8 million people resulting from blindness due to myopia complication, with 153 million people over 5 years of age suffer from visual defects caused by uncorrected myopia [1,2].

Myopia development is mainly due to the postnatal accelerated growth of scleral tissue, which dominated the sclera's posterior part. A study showed that photoreceptors and retinal pigment epithelium in monkeys produce signals inducing scleral tissue remodeling[3,4]. The axial lengthening process was due to the remodeling of the extracellular matrix on the sclera. Another study showed that the posterior sclera on rabbits had the capability for induction and inhibition of myopia. There were similar changes of sclera changes in low myopia compared with other mammals with high myopia[5]. In animal models of myopia, the change of excessive ocular elongation is accompanied by thinning of the sclera, the structural framework that maintains ocular shape and integrity. In a previous study, myopia was induced with monocular deprivation of pattern vision for short-term or long-term periods. High myopia is associated with scleral thinning and changes in the diameter of scleral collagen fibrils in human[6]. Fibroblasts in the sclera play a significant role in cell communication and matrix degradation. Another study has shown that myopia-related

remodeling of the scleral extracellular matrix (ECM) is linked to the decelerated synthesis and accelerated degradation of ECM components[7].

One strategy for myopia therapy is mesenchymal stem cell (MSC) implantation. The use of MSC is initially designed for recovering strategy of the wounded tissue. The use of MSC in the myopia animal model is presumed to give anabolic effect on the sclera, reduce the thinning process of the sclera, and prevent axial lengthening, resulting in the therapy of myopia[8]. This study investigates the capability of limbal MSC (LMSC) in reducing myopia progression in the form-deprivation myopia animal model.

Literature Review

Myopia Progression

Myopia is a common ocular disorder, which is a global problem due to economic and social costs. It typically affects school-age children and seems to progress from 8 until 15 years of life, caused by the continuous growth of the eye during childhood. The pathophysiology of myopia is multifactorial and is not yet completely understood. There is proof that multiple genetic variations and environmental and lifestyle factors play an important role in the etiology of this disease. Family linkage analysis, genome-wide association studies, and next-generation sequencing studies, and a high correlation among monozygotic twins compared to dizygotic twins, show that myopia has a genetic component. Previous studies have already shown the relationship between myopia and environmental factors such as near work, light exposure, lack of physical activity, and a higher level of education, revealing their major involvement. New hypotheses suggest that myopia might also have an inflammatory component. The study showed an increased prevalence of refraction error in children with inflammatory diseases such as diabetes mellitus, juvenile chronic arthritis, uveitis, and systemic lupus erythematosus[31].

Scleral remodeling in myopia

The prominent feature of the sclera in a highly myopic eye is that it is thinned significantly, with the thickness sometimes approaching half that of the sclera in an emmetropic eye. Excessive degrees of scleral mainly thinning correlate with the posterior staphyloma. In animal models of myopia study, general thinning of the posterior sclera is more likely to feature all myopia development. The gross morphological change in the sclera at an ultrastructural level is associated with a preponderance of smaller diameter collagen fibrils in the scleral matrix, which leads to a reduction in the gradient in average fibril diameter between the inner and outer sclera. There are also slightly fewer collagen fiber bundles across the scleral tissue in myopic eyes, with those bundles also being thinner. As a result, the defined area of scleral tissue from a myopic eye has a lesser dry weight than a similar tissue area from an emmetropic eye. However, animal models of myopia have also shown that scleral thinning and tissue loss is not simply local phenomenon. Still, there is also a reduction in overall scleral dry weight, demonstrating that active tissue loss occurs as myopia develops, rather than just a simple redistribution of the tissue as the eye enlarges[10].

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Myofibroblast on sclera

Although the changes occurring in the extracellular matrix of the sclera in myopia have been massively studied, changes that may arise in the sclera's cellular components (fibroblasts and myofibroblasts) have been massively studied received little attention. Myofibroblasts are a specialized, differentiated form of fibroblast that express α -smooth muscle actin (α -SMA), which provides the contractile ability. The character of the myofibroblast population in mammalian sclera has thus far been very limited, and little knowledge of the changes to the myofibroblast population in myopia. These cells may be responsible for the ocular shortening seen over time in an animal model with increased intraocular pressure and the reduction in axial length sometimes observed in tree shrews recovering from induced myopia[31].

Stem cells for myopia

The common feature of a myopic eye is a weak, less rigid, and thinned sclera characterized by increased elasticity and reduced collagen content. In this context, developing strategies to improve scleral biomechanics and prevent myopia progression is attractive because this would address the common underlying causative factor. This concept has already been used with posterior scleral reinforcement surgery. While effective, this surgery can be complex and is justified only for specific cases of severe myopia. However, with a recently developed, minimally invasive, safe technique, it is now possible to deposit payloads of stem cells to the back of the eye, specifically to the space between the choroid and the sclera. This route is effective in the treatment of acute posterior uveitis in an animal model. It has also been used for the administration of biomaterials and tumor cells. Thus, while scleral reinforcement by mesenchymal stem cells is an attractive concept, alternative or supplementary stem cell-based therapies could also be used to prevent the progression of myopia. There are dynamic relations between the retina and the sclera, and one of the proposed mechanisms of myopia development is a disruption in that signaling. Dopaminergic signaling is central to these relations. There are extensive studies of evidence that dopamine also plays an important role in the growth of the eye and regulation and myopia control[8].

Methods

Animals

A total of 24 New Zealand rabbits (*Oryctolagus cuniculus*) aged 3-4 months and weighing 2-4 kg were used in the study. The animals were housed under standard laboratory conditions in a temperature-controlled room with 25° C with 12-hour light and dark cycles. Water and food were freely available. All animal care and treatment complied with the guidelines of ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental protocols were approved by the Animal Care and Ethics Committee of Veterinary Medicine.

Experimental protocols

All rabbits were randomly assigned to one of three groups: control group, form-deprivation

myopia group (FDM), and FDM group with treatment 1x10 ^{5} in 0,1 ml LMSCs injection (FDM+LMSC) (*n* =8 for each group). In the FDM group, the right eyes were induced by covering the right eye with a handmade opaque diffuser for 6 weeks, starting from form deprivation, leaving the left eye, nose, mouth, and ears exposed. The diffuser was attached carefully to the fur around the eye and did not contact the cornea. These diffusers were checked each day to ensure that they were in place. In the FDM-LMSC group, the right eye received an intrasclera injection of 0,1 mL LMSC ($1x10^5$/0,1 mL) at the fourth week of occlusion. LMSCs were injected intrasclera at 10 o'clock with 2,5 mm from the posterior limbus. Immunofluorescence examination was done to evaluate LMSCs migration into the posterior sclera using PKH26 labeling.

Isolation and culture of LMSCs

LMSCs were taken from the corneal limbus of rabbits. Rabbits underwent anesthesia with ketamine and xylazine (ketamine 40 mg/ml, xylazine 20 mg/ml) intramuscular. Full-thickness of corneal tissue was taken 1 mm at the peripheral part of the cornea and 3 mm part of the limbus. Tissues were then washed with phosphate buffer saline (PBS) 3 times and maintained at Dulbecco's modified eagle's medium (DMEM) +200U/ml penicillin – 200U/ml streptomycin transport media. LMSC was prepared with a semi-enzymatic isolation method and culture using a modification method by a previous study [9]. Primary media culture was alpha minimum essential eagle's medium (MEM) – Penicillin 200 U/ml – streptomycin 200 U/ml – 1 % amphotericin B – Non-essential amino acid (NEAA) 100nM – PBS 10%. Tissues were then incubated for 24 hours. Primary media culture was then replaced every 3 days until confluence cells reached 80-90%. Cells were continued with the trypsinization process with 2 ml 0,25% EDTA trypsin. After the fifth passage, cells were then placed in a culture dish for cell expansion. LMSCs was added with CD73, CD90, CD105 antibody for surface markers expression.

Refraction status and biometric measurement

All animals underwent biometric and refraction status measurements at baseline, fourth week, and sixth week after the initiation of form deprivation. Streak retinoscopy (NEITZ, Tokyo, Japan) and A-scan ultrasonography (11 MHz, AxisNano, Quantel Medical) with a resolution of 0.01 mm was used for axial length (AL) measurement. Refractions were measured using hand-held streak retinoscopy under cycloplegia. In this procedure, 1% Tropicamide Eye Drops (Cendo Mydriatil) was topically applied to the eye every 5 minutes for a total of four times; then, measurements were taken 30 minutes after the last drop was applied. The average refractive error along the horizontal and vertical meridians was taken as the spherical measurement. Topical anesthesia 0,5% proparacaine hydrochloride (Pantocain, Cendo) was used before measuring AL with A-scan ultrasonography. The measurements were repeated ten times for each eye, with the mean value calculated. The entire measurement procedure was performed without general anesthesia because the rabbits were compliant.

Histopathological examination

At the end of the experiment, animals were administered a lethal dose of pentobarbital sodium (120 mg kg^{-1}). Eyes were enucleated after six weeks of observation. The posterior sclera was excised, and the head of the optic nerve head was discarded. The eyeballs were fixed in 4% paraformaldehyde solution for 48 hours, embedded in paraffin, and cut into 5-6 μm sections. Haematoxylin and eosin (HE) staining was done to evaluate scleral thickness under a light microscope examination. The slides were deparaffinized, rehydrated, and blocked with 3% H_2O_2 and 10% normal goat serum. Immunohistochemistry to evaluate α-smooth muscle actin (α- SMA) expression was done with α- SMA antibody (ACTA2 / Smooth Muscle Actin Rabbit anti-Human Polyclonal, N-Terminus LS-C354532) incubated overnight at 4° C. α- SMA expression measured with Immunoreactive Score (IRS) with the percentage of positive cells staining intensity (score range 0-12). Scleral thickness measurement was taken using Olympus cellSens Software version 1.12 (Olympus, Japan).

Statistical analysis

Data were reported as the mean ± standard deviation (S.D.). All the statistical calculations were performed using SPSS software Version 25.0. Kruskal-Wallis was used to measured significant differences and Mann-Whitney for comparison between each group. $P < 0.05$ was considered statistically significant.

Results

LMSCs migration

The immunofluorescence examination showed migrating LMSCs to the posterior sclera, as shown in Fig.1. LMSCs were positive for surface markers with CD105, CD90, and CD73 expression, as shown in Fig.2.

Refraction and axial length status changes

There were changes of refraction status and axial length towards to be more myopic at the FDM group. Table 1 and Table 2 represent the refraction status and axial length data of each subject. There were significant differences in streak retinoscopy at all groups in the fourth week (p=0,013) and sixth week (p=0,001). There was a significant difference in periodic evaluation of refraction status in the FDM group (p=0,001). There was also a significant difference in periodic evaluation of refraction status in the FDM+LMSC group (p=0,001). Biometry results showed significant difference of axial length in control group (p=0,021), FDM group (p=0,001), and FDM+LMSC group (p=0,001). The sixth-week evaluation showed a significant difference at axial length (p=0,049).

α- SMA expression

α- SMA expression measured at posterior sclera after six weeks at all groups. Table 3 represents data of α- SMA expression in each group. Immunohistochemistry staining was

done with α- SMA antibody and measured with IRS. Results of α- SMA expression are 8,00 ± 1,50, 12,00 ± 1,50, and 12,00 ± 0,00. The study showed the increased intensity of α- SMA at the FDM group and FDM-LMSCs group. There was significant difference between control group and FDM-LMSC group at α- SMA expression (8,00 ± 1,50 and 12,00 ± 0,00, p=0,038; p<0,05). Statistics analysis showed significant differences in α- SMA expression between three groups (p=0,047, p<0,05).

Posterior scleral thickness

Table 4 shows posterior scleral thickness measurement of all groups. The study showed increased scleral thickness at myopia model group (FDM). Posterior scleral thickness evaluation showed significant difference in all groups (p=0,025). Posterior scleral thickness evaluation showed significant difference between control group and FDM-LMSC group (229,27 μm ± 84,60 and 293,37 μm ± 50,47; p=0,010, p<0,05). There were no differences between control and FDM groups (229,27 ± 84,60 and 245,82 ± 55,61; p = 0,279, p>0,05) nor between FDM and FDM-LMSC groups (245,82 ± 55,61 and 293,37 ± 50,47; p=0,083, p>0,05). Figure 3 shows as representative example of scleral thickness measurement of each group.

Discussion

Our study reports the use of LMSCs for preventing myopia progression. This study proved that there were effects of LMSCs in scleral thickness and α- SMA expression in the FDM animal model. This study showed changes in scleral thickness and α- SMA expression in myopia animal models with FDM. There was an increased thickness of the posterior sclera in both FDM groups with or without LMSC treatment. This could be explained by the fact that myofibroblasts had been working as the scaffolding in both groups. The previous study[10] described a high proportion of the scleral cell population were myofibroblasts with contractile potential. Scleral thinning in the myopia process results from local stress and collagen degradation. Still, autoregulation involving secondary effects increased collagen synthesis with fibroblast differentiation into myofibroblasts marked with α- SMA. However, the number of myofibroblasts in the previous study did not alter by inducing form-deprivation myopia, suggesting other factors than several cells affect axial length. Myofibroblasts are responsible for the normal turnover of collagen to prevent rapid expansion of the tissue on degrading the matrix with matrix metalloproteinases, so the myofibroblasts likely act as a scaffolding, taking the role of collagen until the new matrix is synthesized [11,12,13.] Other study[14] showed that myopia was related to remodeling of the scleral ECM, thus linked to decelerated synthesis and accelerated degradation of ECM components that weakens the scleral framework and thereby increases ocular elongation. However, in our study, the FDM group increased ocular elongation by axial length measurement without thinning of posterior sclera thickness. The previous study[15] showed that other factors affected the ocular elongation, such as anterior scleral thickness resulting in increased ocular elongation. Type 1 collagen degradation increases during myopia development resulting in scleral and axial changes[16,17]. Myofibroblasts are a product of differentiation of fibroblasts with α-SMA as a specific biomarker[18]. Another factor resulting

in scleral remodeling is the role of hypoxia[19]. Injection of LMSC plays a role in increasing fibroblast, which is presumed to differentiate into myofibroblasts and increase collagen synthesis, resulting in increases in scleral thickness and α- SMA expression. This showed that LMSC needs to be considered as an alternative potential therapy for myopia. The transplanted cells proposed to differ into fibroblasts that produce extracellular matrix, reinforcing sclera and preventing further elongation. The sclera also contains LMSC that would strengthen the sclera [20]. Form deprivation will reduce dopamine levels, whereas a local dopamine agonist, apomorphine, produces an antimyopic effect [21,22]. Application of dopamine using intravitreal injection had been proved to slow myopia progression [23]. Studies were focused on dopamine as a potential therapy for myopia for the last few decades, and dopaminergic cells were found in sources such as embryonic stem cells.[8] Inflammation plays an important role in the development of myopia. Previous study[24] reported an increase of interleukin-6 (IL-6) and matrix metalloproteinase-2 (MMP-2) in aqueous humor and axial lengths of the eye. However, the effects of stem cells on the inflammation process to prevent the progression of myopia had not yet been established. The antimyopic effect of an inflammatory cytokine such as basic fibroblast growth factor (bFGF), and cotransplantation of cells producing bFGF could also be considered[25] to help the survival and function of dopaminergic cells. These cells do not activate transforming growth factor-beta signaling, a possible susceptibility pathway for severe myopia[26]. Our study uses subscleral space injection due to its advantage of penetrating the signaling loop and safe application without causing bleeding or retinal hemorrhage. Another consideration for a route is the sclera itself. Still, the sclera had limited capability to expand, so it results in more technically challenging due to the need for multiple injections or a scleral tunnel [27]. The retrobulbar injection did not participate in sclera-retina signaling loop and targeting sub-tenon's space, which is commonly used for chemotherapeutic drugs or local anesthesia, could obstruct lymph circulation [28,29,30].

Conclusion

Our study reports the use of LMSCs for preventing myopia progression. This study proved that there were effects of LMSCs in scleral thickness and α- SMA expression in the FDM animal model. This study also showed changes in scleral thickness and α- SMA expression in myopia animal models with FDM. LMSC has a potential role in myopia control in form-deprivation eye animal models by increasing α-SMA and sclera thickness.

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