Enhanced Apoptosis in Choroidal Tissues in Lens-Induced Myopia Guinea Pigs by Activating the RASA1 Signaling Pathway

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Purpose. This study aimed to explore the role of the RAS p21 protein activator 1 (RASA1) signaling pathway in apoptosis in choroid tissues from guinea pigs with negative lens-induced myopia (LIM).

Methods. Biometric measurements were performed to examine refractive status, ocular parameters, and choroidal thickness (ChT) after myopia induction. The choroidal morphology was observed by hematoxylin and eosin (H&E) staining and TUNEL assay. The expression of the RASA1 signaling pathway at the mRNA and protein levels in choroidal tissues was measured by real-time quantitative PCR (qPCR) and western blot assays.

Results. Compared with the normal control (NC) group, the ocular length of the guinea pigs in LIM increased remarkably, as did the myopic refraction. ChT decreased after myopia induction. H&E staining showed that the thickness and laxity of the choroidal tissues in LIM were strikingly reduced. The number of apoptotic cells in the LIM eyes was increased. Moreover, qPCR and western blot assays showed that the expression levels of both RASA1 and BCL-2-associated agonist of cell death (BCL-2) were higher in the LIM group than in the NC group, whereas the expression level of B-cell lymphoma 2 (BCL-2) was decreased after 2 weeks of experimental myopia. However, the trend of RASA1, BAD, and BCL-2 expression was reversed after 4 weeks of experimental myopia compared with levels after 2 weeks of experimental myopia.

Conclusions. Results showed that the RASA1 signaling pathway is activated in choroidal tissues in myopic guinea pigs. Activated RASA1 signaling induces high BAD expression and low BCL-2 expression, which in turn promotes apoptosis and ultimately causes ChT thinning in myopic guinea pigs.

Keywords: RASA1 signaling pathway, lens-induced myopia, choroidal thickness, BCL-2, apoptosis

The complications of high myopia can lead to serious visual impairment, and the most significant and common complications are atrophic myopic macular degeneration/retinopathy and even blindness. However, the pathogenesis of myopia remains unclear. The choroid is a subretinal layer of the retina that can provide metabolic support for the retinal pigment epithelium, provide blood supply for the outer retina, and participate in lymphatic drainage. Currently, we recognize that the choroid is a five-layered structure consisting (from the outside to the inside) of the basement membrane of the choriocapillaris, outer collagenous zone, elastic layer, inner collagenous zone, and basement membrane of the retinal pigment epithelium. The choroid is composed of blood vessels, melanocytes, fibroblasts, resident immune cells, and supporting collagen and elastic connective tissue. Furthermore, in addition to...
the five layers of structure described above, the choroidal interstitium contains collagen and elastic fibers, fibroblasts, nonvascular smooth muscle cells, and numerous very large melanocytes that are closely apposed to the blood vessels. As in other types of connective tissue, there are numerous mast cells, macrophages, and lymphocytes. As the most important supply tissue, the choroid supplies blood to the prelaminar portion of the optic nerve and absorbs excessive light penetrating the retina. The central retinal artery supplies the inner part of the retina, and the choroidal venous network supplies the remaining third of the outer part. Abnormal choroidal blood flow leads to retinal photoreceptor dysfunction and photoreceptor death; thus, the choroid plays an important role in the pathophysiology of myopia. In addition, it has been shown that choroidal blood flow plays an important role in the development of myopia, not only by providing abundant blood flow to the fundus but also by acting as a signal transduction pathway between the retina and the sclera, thereby mediating the development of myopia.

**Materials and Methods**

**Animals**

The present study was approved by the Ethics Committee of Shandong University of Traditional Chinese Medicine and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Prior to enrollment, guinea pigs with various ocular conditions, including cataracts and corneal disease, were excluded. In the present study, 42 healthy 2-week-old guinea pigs (*Cavia porcellus*, English short-hair stock, tricolor strain; Danyang Changyi Experimental Animal Company, Jiangsu, China) with a mean weight of 120 g were chosen for the rele-

**Preparation of the LIM Guinea Pig Model**

Refractive and other ocular measurements were measured prior to myopia induction. In the present study, we used −6.0 D spherical resin lenses to establish the LIM model.

The right eyes of guinea pigs in the LIM group were covered with a −6.0 D lens to induce myopia for 2 and 4 weeks, whereas both eyes of the animals in the NC group were left untreated. During myopia induction, when any lenses fell off they were promptly reattached. To ensure the validity of the LIM model in guinea pigs, all lenses were cleaned and wiped every morning and evening and replaced promptly if there were obvious scratches.

**Biometric Measurements**

After 2 and 4 weeks of myopia induction, refractive error was measured for animals from both the NC and LIM groups. Prior to the examination, the lenses covered on the eyes of guinea pigs were gently removed with scissors, and 10 mg/mL of cyclopentolate hydrochloride eye drops (Alcon, Geneva, Switzerland) was dropped into the conjunctival capsule of the guinea pigs three times, one drop each time, at an interval of 5 minutes. Refractive examinations were performed 30 to ~45 minutes after the last eye drop application. Each eye was measured at least six times, and the average value was taken as the experimental result. Ophthalmic A-type ultrasonography (Cinescan; Quantel Medical, Cournon-d’Auvergne, France) was used for the measurement of ocular axis length. Then, two drops of oxybuprocaine hydrochloride (Santen Pharmaceutical, Osaka, Japan) were administered, with one drop each time before measurement. Topical anesthetic (Santen Pharmaceutical) was administered before measuring ocular length. The anterior chamber propagation velocity was 1557 m/s, the lens propagation velocity was 1725 m/s, and the vitreous propagation velocity was 1540 m/s. A total of 10 readings were averaged to serve as the final one, as described previously.

**Histopathological Staining and TUNEL Assay**

Three guinea pigs in each group were randomly selected at 4 weeks of myopia induction for histopathological staining. Guinea pigs were euthanized with an intraperitoneal injection of 4% pentobarbital followed by enucleation of the eye and removal of periocular tissues. The eyeball was immediately fixed in 4% paraformaldehyde, followed by conventional dehydration, paraffin embedding, and sectioning into 5-μm sections for hematoxylin and eosin (H&E) staining.

Briefly, paraffin sections of the eye tissues were dewaxed, treated with proteinase K, and then incubated at 37°C for 20 minutes. Subsequently, the sections were quenched in 3% hydrogen peroxide solution, incubated with terminal deoxynucleotidyl transferase, and labeled with biotin–dUTP labeling mix for the apoptotic DNA fragments. Furthermore, the sections were subjected to chromogenic detection and hematoxylin staining using diaminobenzidine (DAB) chromogenic solution. Finally, the sections were observed using light microscopy (Eclipse 55i; Nikon, Tokyo, Japan) and analyzed using NIS-Elements D 3.2 software (Nikon).

**Measurement of ChT**

We used spectral-domain OCT (Heidelberg Engineering, Heidelberg, Germany) and OCT to scan the optic disc center of the guinea pigs to obtain the relevant parameter of ChT. As illustrated in Figure 1, the upper boundary of the choroid was defined as the outer surface of the retinal pigment...
epithelium, and the lower boundary was the inner surface of the sclera. The optic disc was the center referred to by Zhang et al., who made two concentric circles with radii of 600 μm and 1500 μm. We also measured the ChT by choosing the area around the intersection of the two concentric circles with the yellow line, and the averaged ChT was calculated. We then performed correlation analysis between the axis length and ChT.

### Real-Time Fluorescent Quantitative PCR

Choroidal tissues from 2- and 4-week-old NC and LIM guinea pigs were extracted and frozen in liquid nitrogen. Then, equal amounts of choroidal tissues were taken and ground separately using a modified tissue/cellular RNA rapid extraction kit (SparkJade Science Co., Ltd., Jinan, China) to extract total RNA. The RNA purity and concentration were measured by an ultraviolet spectrophotometer (SparkJade Science Co., Ltd., Jinan, China) to extract total RNA. The RNA purity and concentration were measured by an ultraviolet spectrophotometer (SparkJade Science Co., Ltd., Jinan, China)

The primer sequences for target genes are listed in the Table. The qPCR conditions were as follows: 94°C for 5 seconds, 1 cycle; 94°C for 5 seconds, 54°C for 15 seconds, and 72°C for 10 seconds for 45 cycles. The expression level of the target gene in each sample was normalized to the internal reference level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the result was analyzed using the 2^−ΔΔCT method.

### Western Blot Analysis

To investigate the protein levels of the molecules related to the RASA1 signaling pathway, we used western blot assays to determine the RASA1, RAS, BAD, and BCL-2 protein levels in LIM and NC guinea pigs. After 2 and 4 weeks of myopia induction, eight guinea pigs were randomly selected in each group, and the choroidal tissues were isolated. Phenyln-methylsulfonyl fluoride–containing radioimmunoprecipitation assay buffer lysate was then added at a mass volume ratio of 10 mg:100 μL. Furthermore, the tissues were fully ground by electric homogenization at 4°C for 120 seconds and centrifuged at 5000 rpm for 5 minutes (NEST Biotechnology, China), and the supernatants were then collected. In the present study, 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the target proteins, and a polyvinylidene difluoride (PVDF) membrane was used for membrane transfer. RASA1 (dilution 1:1000; ABclonal Biotechnology, Wuhan, China), RAS (dilution 1:1000; ABclonal Biotechnology, Wuhan, China), BAD (dilution 1:500; ABclonal Biotechnology, Wuhan, China), and BCL-2 (dilution 1:1000; BIOSS) primary antibodies were incubated with the membranes overnight at 4°C, and then the PVDF membrane-loaded transferred target proteins were incubated with secondary antibodies against RASA1 (dilution 1:1000), RAS (dilution 1:1000), BAD (dilution 1:500), and BCL-2 (dilution 1:1000) for 1 hour at 4°C. Finally, we used the FUSION-FX7 imaging system (Vilber Lourmat, Marne-la-Vallée, France) for development using DAB (Sigma-Aldrich, St. Louis, MO, USA) and quantified results using fusion CIFT software (Vilber Lourmat).

### Statistical Analysis

SPSS Statistics 23.0 (IBM, Chicago, IL, USA) was used for statistical analysis. In the current study, a paired t-test was performed for each refractive parameter between eyes at different treatment time points, and an independent sample t-test was performed for the right eye of the experimental (LIM) group and the ipsilateral eye of the NC group. An
FIGURE 2. The mean values of refraction (A) and axial length (B) in the left and right eyes of guinea pigs at 0, 2, and 4 weeks were compared between the LIM and NC groups (mean ± SD). LIM represents the negative lens-induced group, and NC represents the normal control group. At 0, 2, and 4 weeks, the LIM group was compared with the NC group (*P < 0.05, **P < 0.01).

independent sample t-test was performed for the NC and LIM groups of gene expression. Correlation analysis was used for choroidal thickness and ocular length, and one-way ANOVA was used to analyze the choroidal thickness in the NC and LIM groups. P < 0.05 was considered statistically significant.

RESULTS
Changes in Refraction and Axial Length
We measured the refraction and axial length of both eyes of all guinea pigs prior to myopia induction and found no significant differences between the groups (all P > 0.05). However, after myopia induction for 2 and 4 weeks, we noted that, compared with the self-control (left) eyes in the LIM group and the ipsilateral eyes in the NC group, the refraction of modeled myopic eyes in the LIM group significantly increased (*P < 0.05, **P < 0.01 in Fig. 2A), and the ocular length was also increased (*P < 0.05, ***P < 0.01 in Fig. 2B).

Pathological Analysis and TUNEL Assay
To investigate the effect of LIM on choroid morphology, we performed H&E staining at 4 weeks after myopia induction. The results indicated that the structural arrangement of the choroidal tissue in the NC group was denser and the capillary vascular structure was tighter than that in the LIM group, whereas the structural arrangement within the choroidal stroma of the LIM group was looser, with more obvious fracture dissolution and more sparse vascularity. Magnification, 400×.

FIGURE 3. H&E staining of ocular tissue sections showing histopathological features of the choroid of 4-week-old guinea pigs. The results indicate that the structural arrangement of the choroidal tissue in the NC group was denser and the capillary vascular structure was tighter than that in the LIM group, whereas the structural arrangement within the choroidal stroma of the LIM group was looser, with more obvious fracture dissolution and more sparse vascularity. Magnification, 400×.

FIGURE 4. TUNEL staining of ocular tissue sections. The TUNEL assay was performed for the LIM guinea pigs after modeling for 4 weeks. The choroidal tissue in the LIM group had more dark brown areas than the NC group and an increased number of TUNEL-positive cells compared to the NC subjects. Magnification, 400×.

Changes in ChT
Considering that LIM could lead to changes in choroidal morphology, we further measured ChT in guinea pigs before and after myopia induction. As shown in Figure 5, the results indicated that there was no significant difference between the two groups prior to myopia induction (NC, 64.9432 ± 4.6822 μm; LIM, 64.9205 ± 3.01987 μm). However, after myopia induction for 2 and 4 weeks, we found that the ChT...
was significantly reduced in the LIM group (2 weeks: NC, 72.55 ± 2.406 μm; LIM, 70.69 ± 1.590 μm; 4 weeks: NC, 86.29 ± 2.510 μm; LIM, 68.94 ± 4.345 μm; ***P < 0.001), demonstrating that the ChT of the LIM guinea pigs showed a negative correlation with axial length (Fig. 5).

**RASA1, BAD, RAS, and BCL-2 Expression**

As shown in Figure 6, qPCR showed that, compared with that in the NC group, RASA1 gene expression in the LIM group was elevated at 2 weeks after myopia induction and was statistically significant (P < 0.05) (Fig. 6A), whereas it decreased at 4 weeks after myopia induction (P < 0.01) (Fig. 6A). Similarly, the BCL-2 level was elevated at 2 weeks and decreased at 4 weeks after myopia induction compared to the NC group (Fig. 6B). In contrast, the BAD gene levels were reduced at 2 weeks and increased at 4 weeks after myopia induction compared with the NC group (Fig. 6C).

Consistent with RASA1, BAD, RAS, and BCL-2 gene expression, Figure 7 indicates that RASA1, RAS, and BCL-2 protein expression levels were upregulated at 2 weeks after myopia induction and downregulated at 4 weeks after myopia induction compared with the NC group. In contrast, the BAD protein level was reduced at 2 weeks and increased at 4 weeks after myopia induction compared with the NC group; therefore, we may infer that the aberrant expression of BCL-2 and BAD will activate apoptotic signaling and induce the apoptosis of choroid-related cells, and the increment of apoptotic cells is related to choroidal thinning.

**DISCUSSION**

In this study, we found that RASA1 regulates the expression of RAS, BAD, and BCL-2 in the downstream molecules of the RASA1 signaling pathway and that RASA1-regulated downstream molecules showed different expression trends in the choroid tissues of myopic guinea pigs at 2 weeks and 4 weeks. Meanwhile, the OCT angiography results showed that the ChT of guinea pigs in the LIM group showed an attenuated trend. We speculate that the varying levels of RASA1 at different time points could be attributed to the state of cellular growth and the stress levels in guinea pigs.
induced by LIM. This would decrease cell viability in the choroid, influence the normal physiological function, and affect the growth and development of choroidal vessels, which would impact the ChT of guinea pigs and aggravate myopia.

Current research on RASA1 signaling is mostly limited to cancer and other diseases,27 and little research has concentrated on myopia. It has been shown that microRNAs can regulate expression of the RASA1 gene and thus play a role in myocardial fibrosis and vascular lymphatic vessel development, in addition to driving the cellular output of type IV collagen to control the development of lymphatic veins and venous valves in mice;28,29 therefore, we propose that there is a close relationship between RASA1 signaling and ChT development. The choroid is mainly composed of blood vessels and lymphatic tissue, and studies have shown that RASA1 and its downstream molecules affect blood vessel growth and development.27 Thus, in the present study, we performed relevant experiments to explore the relationship between the expression levels of RASA1 signaling-related molecules and ChT in LIM guinea pigs. We demonstrated that RASA1 showed high expression at 2 weeks after myopia induction and low expression at 4 weeks after myopia induction. RASA1 is a regulator of GTP and RAS GTP, which are involved in multiple physiological processes, including angiogenesis, cell proliferation, and apoptosis.18 It has been reported that overexpression of RASA1 can suppress cell growth and promote apoptosis.30 Moreover, RASA1 signaling governs multiple downstream molecules, including BCL-2, BAD, and RAS, which are also associated with the apoptotic signaling pathway. In this study, we noted that BCL-2 was highly expressed at 2 weeks after myopia induction and was expressed at low levels at 4 weeks after myopia induction.

Cellular stress is sensed by a few molecules and pathways that can stimulate either cell survival or apoptosis, and the stress response depends on the degree of stimulation or molecule-mediated differential posttranslational modifications.32 RASA1, also known as p120 RasGAP, is a regulator of RAS guanosine diphosphate and GTP. It is regarded as a signaling scaffold protein, playing a vital role in anti-apoptotic and cell death-inducing effects and relying on activation of the fragment C and fragment N split from RasGAP.32 Fragment N can activate the anti-apoptotic response and inhibit apoptosis when the organism suffers from mild stress. However, if the organism is suffering from strong stress, fragment C would be activated to induce apoptosis, ultimately leading to cell death,33 suggesting that the organism can regulate cellular function, the microenvironment, and organism homeostasis through stress responses.34,35 Nevertheless, stress responses may diminish over time when external stimuli persist; hence, cells will eventually die under constant intense stress due to the limitation of the stress response.

In the present study, we found that RASA1 expression was significantly elevated at 2 weeks of myopia induction to promote survival. In contrast, RASA1 expression decreased significantly at 4 weeks after myopia induction, accompanied by elevated apoptosis of choroidal cells and significant thinning of choroidal thickness. Based on the experimental results, we hypothesize that, during the early phase of myopia induction (in the first 2 weeks), external stimulation serves as a mild stressor and will activate the anti-apoptotic response, thereby inhibiting apoptosis. As myopia induction continues (after myopia induction for 4 weeks), the external stimulus turns into a strong stressor, which activates the apoptotic response, leading to apoptosis (Fig. 8).

BCL-2 plays an anti-apoptotic role that normally induces the formation of mitochondrial pores by inhibiting apoptosis,36,37 thereby preventing the release of cytochromes from mitochondria.38 It has been reported that mitochondria are closely associated with protein synthesis; meanwhile, as power-generated organelles, they can also generate chemical energy (adenosine triphosphate). In addition, mitochondrial dysfunction is also correlated with neurodegenerative diseases and the aging process,39 and these
Figure 8. The RASA1 signaling pathway mediates apoptosis. RASA1 signaling has anti-apoptotic and cell death-inducing effects. The specific mechanism of action leads to the partial cleavage of RasGAP into fragment C and fragment N. When an organism suffers from mild stress, fragment N activates BCL-2, which generates an anti-apoptotic response and inhibits the further response to apoptosis. However, when an organism suffers from stronger stress, fragment C will be activated, leading to an apoptotic response in the downstream BAD and cell death.

In this study, we found that, after myopia induction for 2 weeks, sparse vascularization of the choroidal tissue in guinea pigs occurred due to the presence of negative lens induction, resulting in sustained high expression of RASA1 (Fig. 3), leading to low expression of the downstream signaling molecule BCL-2 as well as elevated BAD and thus mediating apoptosis. When myopia induction proceeded (4 weeks after myopia induction), the expression levels of RASA1 decreased, affecting BCL-2 expression and thereby promoting apoptosis.

In this study, although we found no significant difference in the choroidal thickness between the NC and LIM groups at 2 weeks, there was a statistically significant difference between the two groups after myopia induction for 4 weeks, and this result is also consistent with the gene expression profile. We further confirmed a negative correlation between ChT and ocular length in the LIM group. According to the literature, there is a strong correlation between ChT and choroidal blood perfusion in guinea pigs. A relevant study done in humans confirmed a significant decrease in ChT in highly myopic patients. There is a significant correlation between ChT and ocular length, and ChT also appears to decrease significantly in children in the early stages of myopia. In our study, the ChT of guinea pigs in the LIM group was consistent with the results of the literature. We also found that there was a negative correlation between ChT and vessel density of the choriocapillaris with the development of myopia.

The initiation of the RASA1 signaling pathway and the low expression of the downstream BCL-2 result in choroidal cell apoptosis, thus leading to a decrease in ChT. With the decrease in ChT, the rapid change in the choroid makes the retina move back, allowing objects to fall out of the retina. In this case, myopia progression reduced the ChT in LIM guinea pigs, thus resulting in an increased ocular length.

Nevertheless, the current study still has limitations. First, pharmacological manipulations are required at the basic level to establish a relationship between RASA1 and apoptosis, which is missing in this study. In addition, whether the choroid influences the function of the retina and sclera and thus regulates the development of myopia is still unclear. Although we could not define the specific type of apoptosis, we propose new avenues to explore the pathogenesis of myopia.

Conclusions

In summary, we confirmed that choroidal thickness gradually decreased in guinea pigs with negative lens-induced myopia, which was accompanied by a negative correlation with ocular length. We also noted that negative lens-induced myopia can cause activation of the RASA1 signaling pathway and apparently lead to low expression of BCL-2 and elevated expression of BAD, initiating an apoptotic pathway and thereby leading to apoptosis of choroidal tissue-related cells and a decrease in choroidal thickness. In conclusion, the increased number of apoptotic cells in choroidal tissues mediated by myopia drives the decrease in choroidal thickness. Our findings indicate that the activation of RASA1 signaling can induce the apoptosis of choroidal cells that reduce choroidal thickness and may be a new target in the treatment of myopia.

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