90 年度計畫執行成果報告

生長因子在雞之實驗性近視所扮演角色之研究(2/2)

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Introduction

The prevention and treatment of myopia are important issue of public health in many countries, especially in Taiwan where the prevalence rate of myopia is extremely high. The most important complication of extreme myopia is retinal degeneration affecting the posterior pole that is associated with elongation of ocular axial length. Unfortunately, the actual mechanism of the development of myopia is still unknown

Studies of the sclera of chicks with form-deprivation myopia showed an increase in thickness and in mitotic activity in the cartilaginous sclera, caused primarily by increased amount of DNA synthesis and production of extracellular matrix within the posterior segment of the ocular globe. All these findings question a previous "passive scleral stretch" hypothesis and give support to the idea of "active scleral tissue remodeling". Active remodeling of connective tissues is a complex process. Cellular growth factors and matrix metalloproteinases may contribute to the delicate balance between degradation and synthesis of various extracellular matrix components. Growth factors probably release from the retina-retinal pigment epithelium (RPE)-choroid complex and modulate the tissue remodeling of sclera. Growth factors known to modulate fibroblast proliferation and the composition of the extracellular matrix, such as bFGF, and TGF-β, are the promising candidates. Rohrer and Stell suggested that bFGF acts as a stop signal and TGF-β acts as a go signal to modulate postnatal ocular growth in the chicken. However, experimental data from several studies are controversial. Rohrer and Stell reported that the intravitreal and subconjunctival injection of bFGF prevented excessive eye growth in chicken with form deprivation myopia. Seto et al. found that the bFGF content were significantly lower in the sclera in the posterior region of the myopic eyes than in the control eyes. The bFGF content were similar in the retina-RPE-choroid complex in myopic and control eyes. The TGF-β content was significantly higher in myopic eyes in both the retina-RPE-choroid complex and the sclera. However, Honda et al. found that axial elongation in form-deprivation myopia was correlated with the reduction of TGF-β in the retina-RPE-choroid complex. In addition, the roles of other growth factors or cell growth modulating factors, such as IGF and vasoactive

intestinal peptide, have not been investigated. The role of these cellular growth factors in the pathogenesis of myopia needs further investigation.

In the second year of experiment, we investigated myopia from protein level. Protein from retina-RPE-choroid and sclera of myopic and control eyes was extracted and analyzed with two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis will provide the highest resolution for protein analysis. More et al. used this method to find the proteome of the rhodopsin-bearing post-Golgi compartment of retinal photoreceptor cells. Nishizawa et al used this method to characterize bovine retina proteins. In our experiments the difference of protein expression between myopic and control eyes will be examined and the candidate proteins will be further analyzed with N-terminal sequence analysis and mass spectrometry. In this project, we used this powerful tool to study the pattern of protein expression in myopic eyes and to find the candidate genes responsible for the development of myopia. In addition, pharmacological agents, such as atropine, and pirenzepine haven been reported to modulate ocular growth in experimental myopia. We also examined the effect of these agents on the growth factors protein expression.

Materials and Methods

1. Induction of form deprivation myopia in chick.

Male white Leghorn chicks were used in the experiments. Form deprivation myopia was induced in the right of neonatal chicks since one day old. The right eye was covered with a semi-spherical translucent plastic goggle, and the left eye was uncovered for control. The chicks were maintained on 12 hours light-dark cycle for 2-21 days during the study, then they were sacrificed and both eyes enucleated at intervals of 3 days.

2. Pharmacological modulation of ocular growth.

Male white Leghorn chicks were used in the experiments. The chicks were anesthetized by intramuscular injection of ketamine and xylazine. Intravitreal injection of atropine, apomorphine and pirenzepine was performed on the temporal side of the right eye with a 30 gauge needle and repeated every 3 days for 6 times, the left eye was untreated for control. Both eyes were covered with a

semi-spherical translucent plastic goggle. The chicks will be maintained on 12 hours light-dark cycle for 2-21 days during the study, then they will be sacrificed and both eyes enucleated at intervals of 3 days. The enucleated eyes will be subjected to two- dimensional gel electrophoresis.

3. Sample preparation

The part of the sclera behind the scleral ossicles was dissected from each eye. Using a surgical trephine, tissue buttons with diameter of 8.5 mm was excised from part of each posterior hemisphere located temporal to the exit site of the optic nerve. Specimen of retina-RPE-choroid and sclera was separated from these tissue samples. Specimen from 5 eyes was pooled together for further experiments. The sample will be frozen with liquid nitrogen and ground into powder. Phosphate buffer (50 mM, pH 7.0) containing 1 mM of PMSF will be added into the sample with 1:1 volume ratio. The sample will be incubated in ice for 1 hour and then centrifuged at 14,000 rpm for 30 minutes. The supernatant will be precipitated by 5% trichloroacetic acid and kept at –20 ℃ overnight. Sample will be centrifuged at 14,000 rpm for 30 minutes and washed with cold acetone twice and finally stored at –20 ℃.

4. Two dimensional protein gel electrophoresis.

4.1. First, protein extracted from the myopic and control eyes was subjected to one-dimensional SDS-PAGE with 15% polyacrylamide gel. The gels were stained using a silver staining kit or 0.25 % coomassie brilliant blue R-250 to detect proteins.

4.2. Protein was dissolved in a sample buffer composed of 8 M urea, 2 % 2-mercaptoethanol, 0.8 % Pharmalyte, 0.5 % Nonidet P-40, and 0.01 % bromophenol blue. After centrifugation, the supernatant was applied to a sample cup placed on an immoblilized pH gradient gel (IPG) strip (Immobiline DryStrip pH4-7 or pH 3-10) which was rehydrated in a solution composed of 8 M urea, 10mM dithiothreitol, 0.5 % Nonidet P-40, and 2 mM acetic acid. Isoelectric focusing was performed using Multiphor II according to the manufacturer's instructions. After IEF, the IPG stripwas equilibrated in two stages with gentle

shaking in a 50 mM Tris/HCl buffer (pH 6.8) containing 6 M urea, 1% SDS, 16 mM dithiothreitol, 30 % glycerol. Iodoacetamide, 243mM, and bromophenol blue were added to the second stage in place of 16 mM dithioeritol. The strip was electrotransferred to ExcelGel SDS Homogeneous 12.5 ℃ at the maximal setting (600 V, 20mA, 30W) for about 20 min at 15 ℃. Electrophoresis proceeded for about 1 hour at the maximal setting (600V, 50mA, 30W). The gel was fixed and stained

4.3. Immunoblotting and sequencing of N-terminal amino acid residues

Two dimensional gels was removed from their backing and proteins was electrotransferred to a polyvinylidene difluoride membrane. The membrane was either immuonblotted as described below or stained with Coomassie brilliant blue for protein visualization. Membranes was incubated overnight with the pooled serum diluted 1:5 in NaCl/Pi containing 0.2 % bovine serum albumin and 0.5 % Tween 20, followed by 1:5000 diluted anti-growth factor antibodies for 30 minutes, and then by peroxidase-labeled anti-IgG antibodies for 30 min. Proteins were detected using the enhanced chemiluminescence ECL Western blotting system according to the manufacturer's instructions. Protein spots was analyzed by Edman degradation using an HP G10000 A protein sequencer. The sequences determined were searched for homology with known proteins using the SWISSPROT database.

4.4 In gel digestion and peptide mass fingerprinting

For proteolytic digestion 2D-slab gel was stained with 0.5% Coomassie brilliant blue for 5 min and destained. The protein spots was excised from the gels. The gel pieces was washed with 60% CH_3CN in 200 mM NH_4HCO_3 by shaking until the color of CBB will no longer visible. After washing, the gel pieces was air-dried briefly, then 2 μl of 0.1 mg/ml TPCK-trypsin was applied on each sides of the gel pieces. The gel pieces was incubated in 200 ml of 200 mM NH_4HCO_3 (pH 8.6) at 30 ℃ for 24 hr. The reaction was terminated by addition of 0.5 μl TCA. The peptides was extracted from the gel pieces by shaking with 200 μl 60% CH_3CN and 0.1% TCA three times. The extracts was combined and concentrated by lyophilization.

4.5 Peptide mass fingerprinting by matrix-assisted laser desorption ionization

time-of-flight (MALDI-TOF) mass spectrometry.

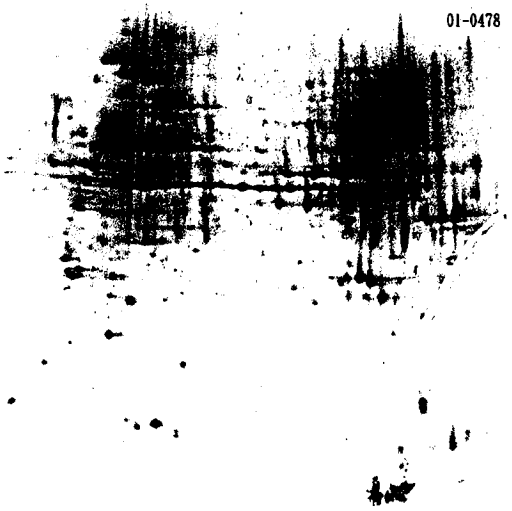
The tryptic peptides obtained from the 2D gel piece was dissolved in 10 μl of water following lyophilization. A half μl of the peptide mixture was mixed with 0.5 μl of a matrix solution. The mixture was examined by the MALDI-TOF mass spectrometry

Results

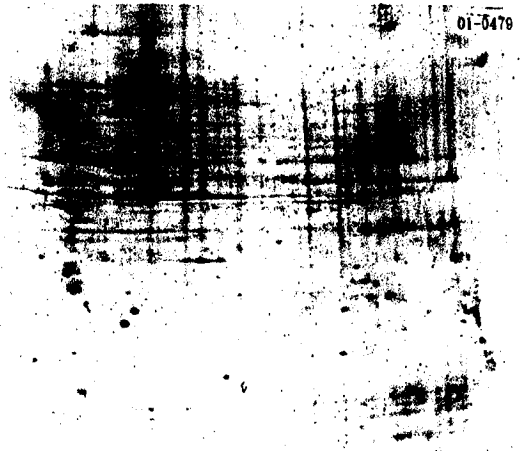
In our preliminary results, we had successfully separated the proteins in the retina and sclera from the normal and myopic chick eyes by two-dimensional gel electrophoresis. The expression pattern of proteins between normal eyes and myopic eyes were different. The significance of these differences needs further investigation.

Retina control-3

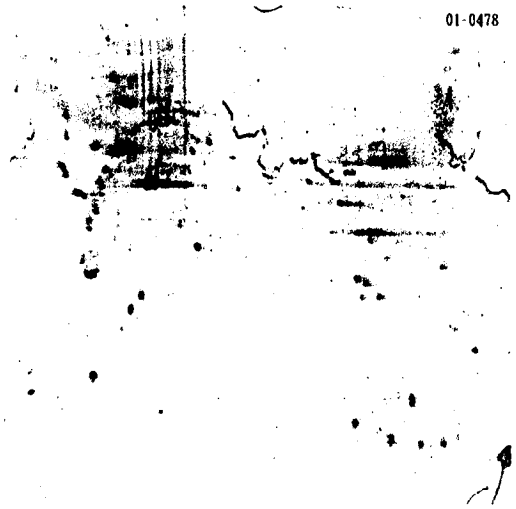
Sclera control-3



Retina myopia-3



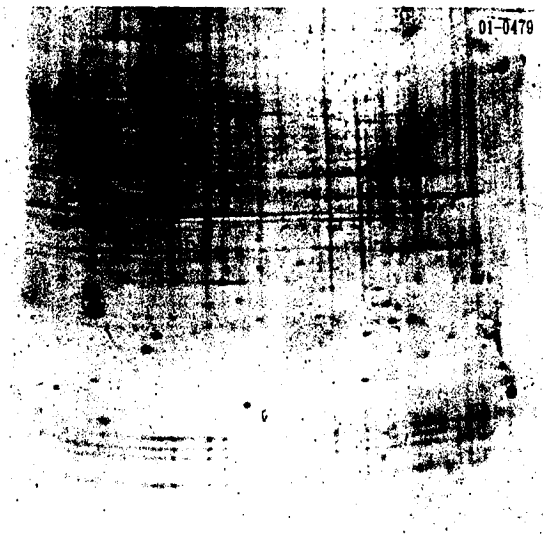
Sclera myopia-3



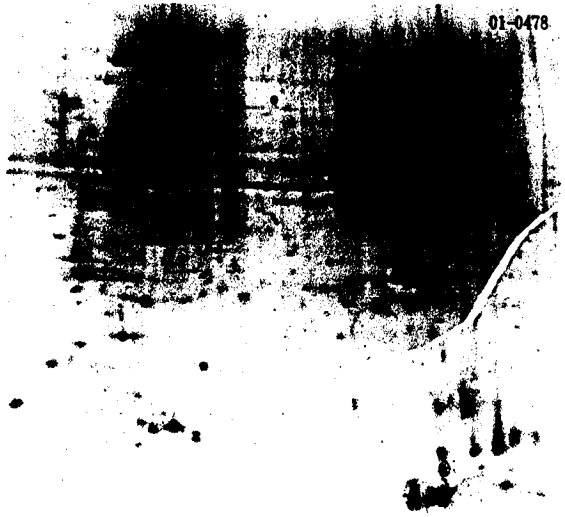
Sypro Ruby stain

Retina control-3

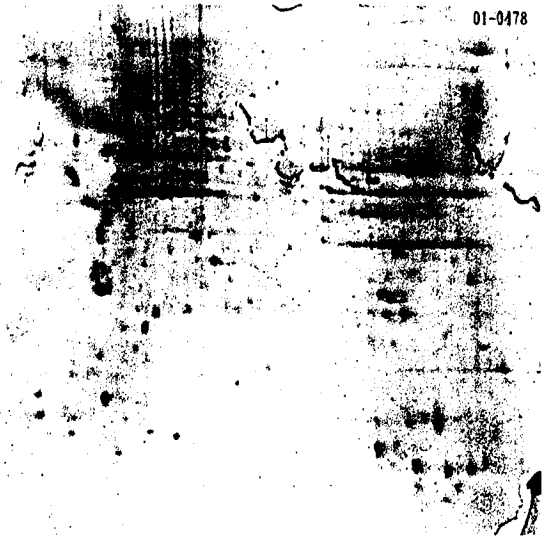
Retina myopia-3



Sclera control-3



Sclera myopia-3



Sypro Ruby stain