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Insulin-like Growth Factor-I Gene Cloning and Protein Expression in Bovine Trabecular Meshwork Tissue and Cells*

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Summary: Whether cultured bovine trabecular meshwork cells and trabecular tissue ex vivo express insulin-like growth factor-I (IGF-I) messenger RNA (mRNA) and protein was investigated. Total RNA of cultured bovine trabecular meshwork cells as well as trabecular meshwork tissue freshly excised from bovine eyes was extracted, and reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect IGF-I mRNA. RT-PCR product was verified by sequencing. Immunohistochemical stain was used to detect IGF-I protein. The results showed that a single PCR amplified product was obtained, and the sequence was homologous to the known sequence.. IGF-I immunostain was positive in the cytoplasm of trabecular meshwork cells. It was concluded that trabecular meshwork cells produce IGF-I and contribute to the presence of IGF-I in trabecular meshwork microenvironment as well as aqueous humor. Trabecular meshwork cells were affected by IGF-I not only through paracrine, but also autocrine action. Whether abnormal down-regulations in IGF-I production may contribute to the pathogenesis of primary open-angle glaucoma and the possibility of promoting the autocrine action of IGF-I by trabecular meshwork cells to treat the diesease is worth further investigation.

Key words: trabecular meshwork; insulin-like growth factor-I; reverse transcription-polymerase chain reaction; immunohistochemistry; primary open-angle glaucoma

Primary open-angle glaucoma (POAG) is a leading cause of blindness, which involves optic neuropathy accompanied by characteristic visual field defects and is often associated with elevated intraocular pressure due to disturbance of aqueous humor outflow through the trabecular meshwork (TM)[1]. The pathophysiology of the TM in POAG has been characterized by an increase in extracellular matrix components and a decrease in the number of TM cells[2]. Polypeptide growth factors are critical modulators that control normal cell functions such as proliferation, motility, differentioation, phagocytosis and extracellular matrix synthesis and degradation. Studies of secretion of growth factors which function on TM cells, especially on proliferation and extracellular matrix metabolism, are critical to our understanding of POAG and the development of new antiglaucoma therapy[3].

Insulin-like growth factor-I (IGF-I) has been reported to be present in human and bovine aqueous humor[4,5]. Serum and vitreous humor are considered to be the exogenous and partially endogenous source of IGF-I in the aqueous humor respectively[5-7]. High-affinity receptors for IGF-I was identified on cultured porcine TM cells[3]. Also, cultured human TM cells and ex vivo human TM tissues were found to express mRNA for IGF-I receptors[8], and administration of exogenous IGF-I stimulated TM cells proliferation significantly and induced a moderate increase in production of two kinds of metalloproteinases, stromelysin and gelatinase B[8,9]. So can we suggest that IGF-I may affect TM cells through paracrine and/or autocrine mechanism. But there is still no direct evidence about whether IGF-I could be produced by TM cells and acts locally through autocrine signalling in the TM. The purpose of this study was to use reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry to determine whether IGF-I is expressed by TM cells.

1 MATERIALS AND METHODS

1.1 Cell Culture and Tissue Preparation

Cultures of TM cells were initiated from fresh bovine eyes that were obtained within 2 h after decapitation, as described previously[10-12]. Briefly, the eyes were placed in sterile petri dish and transected 5 mm posterior to the limbus. Under a dissecting microscope, the lens, the iris, the majority of ciliary body and the pectinate ligaments were carefully removed away with microsissors. The TM were gently lifted with jeweler's forceps and cut into pieces, and then placed on the surface of the tissue culture flask, incubated at 37 ℃ in RPMI-1640 (BRL, USA) culture medium containing 20 % fetal bovine serum (BRL, USA), 10 mmol/L HEPES buffer (Sigma,

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USA), 100 U/ml pennicillin (Hebei Pharmaceutical Factory, China), 100 μg/ml streptomycin (Hebei Pharmaceutical Factory, China) and 2.5 μg/ml fungizone (Shanghai Pharmaceutical Factory, China). The growth medium was changed twice a week and the cells were treated with 0.125 % trypsin (Sigma, USA) and 0.01 % EDTA (Sigma, USA) and passaged after confluence. The third passage cultures were used to performe experiments and the whole TM tissues for analysis were frozen and stored at −70 ℃.

1.2 Extraction of Total RNA

Total RNA was extracted from 1×10^6 cultured cells or whole tissues by using TRIZOL Reagent (BRL, USA). Briefly, 1 ml TRIZOL was added to homogenize 100 mg TM samples for 5 min at 20 ℃. Then 0.2 ml of chloroform was added per 1 ml TRIZOL and tubes shaken vigorously for 15 s and incubated for 3 min at 20 ℃. After the samples were centrifuged at 12 000 r/min for 15 min at 4 ℃, the aqueous phase was transferred to a fresh tube. The RNA from the aqueous phase was precipitated by mixing with isopropyl alcohol and centrifuged at 12 000 r/min for 10 min at 4 ℃. The supernatant was removed, the RNA pellet was washed with 75 % ethanol and centrifuged at 7500 r/min for 5 min at 4 ℃. At the end of the procedure, the RNA pellet was briefly air-dried, RNA dissolved in DEPC (Sigma, USA)-treated Rnase-free water and the amount of RNA and $A_{260/280}$ ratio determined by using a spectrophotometer (Milton Roy Company, USA).

1.3 RT-PCR and Sequence Analysis

The sequences of the primers specific for IGF-I were as follows (BRL, USA): forward primer, 5'-TCG CAT CTC TTC TAT CTG GCC CTG T-3' and reverse primer, 5'-GCA GTA CAT CTC CAG CCT CCT CAG A-3', with an expected size of the amplified sequence of 240 bp[13].

250 ng RNA, RT buffer (50 mmol/L Tris, pH 8.3, 75 mmol/L KCl, 3 mmol/L $MgCl_2$), 10 mmol/L DTT and 150 μmol/L dNTPs (each of dATP, dGTP, dCTP, and dTTP) with three drops of Liquid Wax were denatured for 5 min at 65 ℃. The subsequent RT was done at 42 ℃ for 60 min by adding 10 pmol of reverse primer P1b, 10 U of AMV reverse transcriptase (Promega, USA), 20 U of Rnasin Rnase inhibitor. The samples were then heated for 1 min at 95 ℃ to terminate RT. The following PCR was performed as a hot start PCR with 2.5 U of thermostable DNA polymerase (Sangon, Canada) in an automatic DNA Thermal Cycler (Biometra, USA) by adding 30 μl of a PCR master mixture containing 1×PCR buffer, $MgCl_2$ (to final concentration of 1.45 mmol/L) and 10 pmol of forward primer P1a to the cDNA samples. 30 cycles (30 s at 94 ℃, 30 s at 60 ℃, 90 s at 72 ℃) followed by additional 5 min at 72 ℃ for complete amplification of all PCR products were performed. In each experiment water was used as a negative control.

Aliquots of the PCR products were analyzed by electrophoresis through 1.5 % agarose in 1 TAE (50 TAE: 2 mol/L Tris, 1 mol/L acetic acid, 90 mmol/L EDTA pH 8.5) and visualized by ethidium bromide staining. To verify each PCR product, double-strand sequencing was performed (TaKaRa Biotech, China).

1.4 Immunohistochemistry

Suspension of 6×10^5 TM cells was distributed in each well of a 6-well disk, with coverlips placed at the bottom of the well and incubated at 37 ℃ in a humind, 5 % CO_2 plus 95 % air atmosphere. When the cell growth reached confluence on the coverslips, the coverlips were taken out, stuck onto a slide, washed twice with phosphate buffered saline (PBS), fixed in petri dishes for 30 min in 4 % paraformaldehyde, then washed three times in PBS for 2 min each wash, dried with air. After treatment with 0.5 % H_2O_2 for 30 min to block endogenous peroxidase and with 1 % bovine albumin serum for 10 min to block free binding sites, 10 μg/ml monoclonal rabbit anti-human IGF-I (Sigma, USA) and samples were incubated overnight at 4 ℃ in a humidifying box. After rinsed three times in PBS, sections were incubated with 6 μg/ml biotinylated goat anti-rabbit IgG at 37 ℃ for 30 min, then washed three times, added 1 % streptavidin-biotin-horseradish peroxidase complex (Vector Lab, USA), incubated at 37 ℃ for 30 min. Rinsed four times before the slides were incubated with chromogenic horseradish peroxide substrate 500 mg/l DAB (Vector Lab, USA) over a period of 30 min. Finally, the slides were counterstained in 0.7 % hematoxylin, examined and photographed on a photomicroscope (Olympus, Japan). PBS instead of primary antibody was used as negative control.

2 RESULTS

2.1 Extraction of Total RNA

Approximately 18 μg of RNA was recovered from 1×10^6 cultured bovine TM cells, 14 μg from 100 mg ex vivo bovine TM tissue. All of the RNA was substantially free of DNA and contaminating protein, exhibiting $A_{260/280}$ ratio about 1.91.

2.2 RT-PCR and Sequence Analysis

By using primers specific for IGF-I, we detected a PCR-amplified product that corresponded to 240 bp from TM cells and ex vivo TM tissue, as well as from the positive control IGF-I DNA templete (fig. 1). The size of amplified fragment was determined by regression analysis with PCR Markers (SABC Biotech, China) and by comparison with the product from the positive control template. Double-strand sequencing of the PCR product showed that the sequence were 100 % homologous to the known IGF-I sequence[14].

2.3 Immunohistochemistry

To confirm the expression of IGF-I in the cultured bovine TM cells, slides were probed with these antibodies and PBS instead of the primary antibody respectively. IGF-I was significantly expressed in

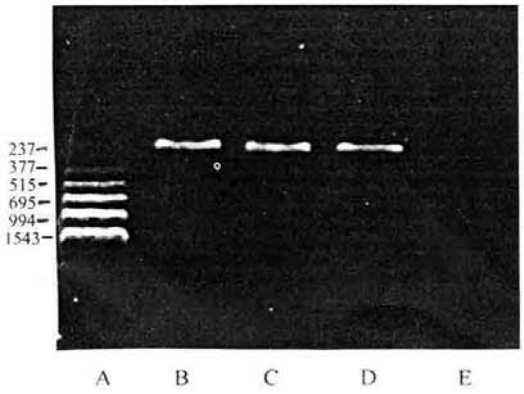

Fig. 1 PCR products of IGF-1 amplified from positive control DNA template (lane B), cultured bovine trabecular meshwork cells (lane C), tissue of trabecular meshwork (lane D), and negative control (lane E). PCR Markers are shown in lane A, and their lengths in base pairs are indicated on the left

the cytoplasm of TM cells (fig. 2). No immunostaining was seen in the negative control (fig. 3).

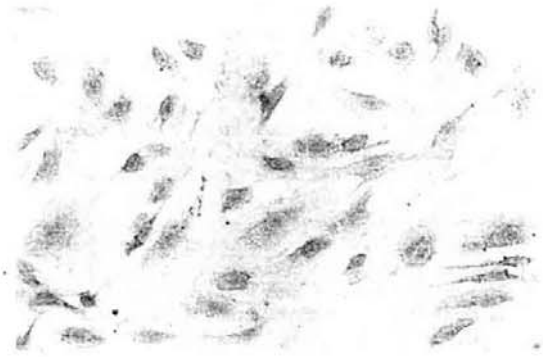

Fig. 2 IGF-1 staining (brown) localized immunohistochemically in the cytoplasm of cultured bovine trabecular meshwork cells (immunohistochemistry ×200)

3 DISCUSSION

It is generally accepted that, in patients with POAG, the TM offers increased resistance to the outflow of aqueous humor, which leads to a raised intraocular pressure and impairs the normal structure and function of the eye. Glaucomatous changes in the TM of the human eye include an abnormal accumulation of extracellular matrix components and decreased cellularity[15,16]. Growth factors present within the microenvironment of the TM may play a significant role in maintaining the normal physiology of the tissue, and it has been proposed that growth factors play a role in the pathogenesis of POAG[17].

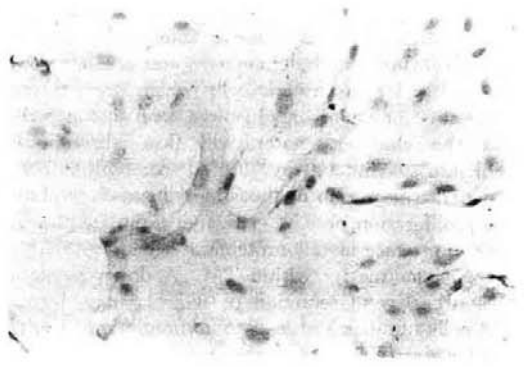

Fig. 3 No immunostaining can be seen in the negative control (immunohistochemistry ×100)

IGF-1 is homologous structurally and functionally to insulin. The biologic actions of IGF-1 are diverse, it affects the mitogenesis, differentiation and regeneration of many kinds of cells, including endothelial cells in health and after tissue injury[18]. These biologic responses were mediated by type I receptor which is related to the insulin receptor and has cytoplasmic tyrosine kinase activity[19].

Measurable quantities of IGF-1 has been reported in human and bovine aqueous humor, and the concentration is about 1.36±0.38 ng/ml of human and 0.62 nmol/L of bovine[4,5]. However, the origins and cellular source of synthesis of IGF-1 in the aqueous humor are not completely understood. Serum is considered to be the most likely exogenous source[6], and vitreous humor may partly contribute to the presence of IGF-1 in the aqueous humor as endogenous source[5,7]. By radioligand binding experiments followed by Scatchard analysis of the binding data, a single class of high-affinity receptors for IGF-1 was identified on cultured porcine TM cells with K_d=0.37 nM and 17 000 receptors per cell[3]. Also, cultured human TM cells and ex vivo human TM tissues were found to express mRNA for IGF-1 receptors by RT-PCR, and administration of exogenous IGF-1 within the physiologic range stimulated TM cells proliferation significantly[8]. In a recent study, Wirtz found that treatment of cultured porcine TM cells with IGF-1 induced a moderate increase in production of stromelysin and gelatinase B[9], which belong to metalloproteinases and catalyze the degradation of proteoglycan, fibronectin and type Ⅲ, Ⅳ, Ⅴ, Ⅸ collagens. So can we know that IGF-1 may function through paracrine mechanism and/or acts locally through autocrine signalling within the TM. But there is still no report about whether IGF-1 could be produced by TM cells in the TM.

In the current study, we demonstrated for the first time that cultured bovine TM cells and TM tissues expressed mRNA for IGF-1 by using RT-PCR and confirmed it by sequence analysis and immunohistochemistry. The results suggest that IGF-1 pre-

sent in the aqueous humor and TM microenvironment may be in part derived locally from the cells of the TM, although it is relative impossible that the growth factors for the chamber water are secreted in the trabecular meshwork cells which are upstream from anterior chamber aqueous, because the factors and the chamber water will flow through the Schlemm's channel away. IGF-Ⅰ has both autocrine and paracrine action in the TM system. It promotes the proliferation of TM cells and stimulate the TM cells to produce metalloproteinases which can degrade extracellular matrix within TM. A down-regulation of synthesis and secretion of IGF-Ⅰ may decrease TM cells number and lead to accumulation of extracellular matrix protein, associated with the pathogenesis of POAG.

Because of the multifunctional activities of IGF-Ⅰ and its possible involvement in the physiologic and pathologic processes of the TM, there may be a potential for the therapeutic approach through up-regulation IGF-Ⅰ production of TM cells to improve the function of TM and reverse the pathogenesis of POAG.

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