

The effect of ET1-CTGF mediated pathway on the accumulation of extracellular matrix in the trabecular meshwork and its contribution to the increase in IOP

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Abstract:

Purpose: Excessive accumulation of extracellular matrix (ECM) in the trabecular meshwork (TM) increases resistance to the outflow of aqueous humor, which inevitably contributes to the elevation of intraocular pressure (IOP) in primary open-angle glaucoma (POAG). Endothelin 1 (ET-1), a potent vasoconstrictor peptide, is reported to enhance profibrotic processes in multiple organs like liver, lung, and kidney tissues, which also significantly increases aqueous humor in POAG patients. Here we tested the hypothesis that ET-1 plays a key role in excessive accumulation in the trabecular meshwork and increase in IOP.

Methods: To test the effect of ET-1 on expression of fibronectin (FN), collagen type IV (COL-IV) and its receptor pathway, cultured human TM cells (HTMCs) were treated with ET-1, ET-1+ ETAR antagonist (BQ123) or ETBR antagonist (BQ788). The protein expression levels of FN, and COL-IV were evaluated by western-blot. A time course effect of ET-1 on the transcription level of the connective tissue growth factor (CTGF) was investigated by qRT-PCR. Next, we down regulated the transcription level of CTGF by its antisense oligodeoxynucleotide sequence and then tested the expression levels of FN, and COL-IV. Lastly, we observed the effect of ET-1 on IOP changes, expression of FN, and COL-IV in an ex-vivo model of cultured anterior eye segment.

Results: In cultured HTMCs, the expression of FN and COL-IV was significantly increased after ET-1 treatment, and this could be blocked by ETAR antagonist (BQ123) but not ETBR antagonist (BQ788). Further, the CTGF mRNA level also increased significantly and reached the top level at 48h after ET-1 treatment. However, the induced ET-1 increased the expression of FN and COL-IV in HTMCs and could be reversed by the downregulation of CTGF. In an ex-vivo model, incensement of FN and COL-IV expression were observed too after ET-1 perfusion. Furthermore, IOP significantly increased after ET-1 administration. Nevertheless, this could be decreased by the ETAR antagonist (BQ123) but not the ETBR antagonist (BQ788).

Conclusions: These results indicate that ET-1 in aqueous humor could lead to the abnormal synthesis of FN and COL-IV via the ETA-CTGF pathway which could result in the accumulation of ECM in trabecular meshwork and incensement of IOP.

Key words: glaucoma, endothelin-1, trabecular meshwork, fibronectin, collagen type IV

Introduction

Glaucoma is a leading cause of blindness worldwide and is characterized by optic nerve degeneration and visual field defect [1]. The most common form is primary open angle glaucoma (POAG) which affects about 2.2% people all over the world[2]. One study predicted that the number of people aged 40-80 years who have glaucoma are estimated to reach 111.8 million by 2040[3]. Pathologically elevated intraocular pressure (IOP) is the major risk factor. It is widely acknowledged that obstruction to aqueous humor outflow results in elevated aqueous outflow resistance, which subsequently contributes to the pathogenesis of elevated IOP.

The trabecular meshwork (TM) comprises the main pathway responsible for draining the aqueous humor and affects IOP primarily by regulating fluid resistance from the anterior chamber into the Schlemm's canal (SC) via aqueous vein collector channels into the venous system [4]. The TM cells express a variety of extracellular matrix (ECM) proteins such as fibronectin, collagens, hyaluronate and proteoglycans [5]. The accumulation, remodeling and metabolic imbalance of ECM can directly affect the outflow resistance of aqueous humor [6]. Therefore, pathologic changes in ECM could be involved in the pathogenesis of POAG. Thus, targeting TM ECM could possibly be a potential therapeutic way of lowering IOP.

Endothelin-1 (ET-1), originally isolated from endothelial cells, is a potent vasoconstrictor peptide [7]. It is reported that ET-1 has effects on promoting fibrosis, proliferation, differentiation, and extracellular matrix (ECM) synthesis. ET-1 and its receptor are abundant in TM and SC. Accumulative evidence implies that ET-1 is involved in the pathogenesis of glaucoma [8]. For example, the concentration of ET-1 in aqueous humor is 2 to 3 times higher than that in plasma [9]. ET-1 levels in aqueous humor are elevated in eyes with POAG compared to those in normal subjects [10]. Most studies have focused on the role of ET-1 in mediating the contraction of TM cells [11-15]. In this study, we hypothesized that ET-1 can regulate the ECM in TM, contributing further to the incensement to the outflow resistance of aqueous humour and IOP.

Materials and methods

Human TM cells isolation, culture, and treatment

Human cadaver eyes were acquired from the Department of Ophthalmology, Wuhan Red Cross Medical Center (Hubei, China). This study was approved by the ethics committee of Tongji Hospital and adhered to the tenets of the Declaration of Helsinki. Human TM cells (HTMCs) were isolated from donor eyes as previously described in Polansky's work[16]. Briefly, the posterior segment, lens, iris, and ciliary body were carefully removed after decontamination in 1% povidone iodine solution for 5 min and three rinses with D-hanks equilibrium solution. The trabecular meshwork tissue between the Schwalbe line and scleral spur was separated with microscopic tweezers under a dissecting microscope and then rinsed in sterile D-Hanks equilibrium solution. TM tissue was cut into 2mm×1mm fragments and cultured in low glucose Dulbecco's Modified Eagle Medium, supplemented with 20% fetal bovine serum (Gibco, USA), penicillin (100U/ml)/ streptomycin (0.1mg/ml) and glutamine (0.29mg/ml). Tissue culture flasks were maintained in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C. The tissue explant was removed when HTMCs migrated onto plate about 1 week later; the medium was changed every three days. After reaching 70-80% confluence, cells were passaged using 0.25% trypsin and split at a ratio of 1:3. Cells passaged to the third and fourth generations were selected for subsequent experiments.

In vitro experiment, HTMCs were seeded into 6-well plates at a density of 10⁵

cells per well. To study the role of ET-1 and its receptor pathway, HTMCs were treated with ET-1 (10^{-7} mol/L, sigma, St Louis, MO, USA) ET-1+ BQ123 (10^{-7} mol/L, sigma, St Louis, MO, USA) ET-1+ BQ788 (10^{-7} mol/L, sigma, St Louis, and MO, USA) for 72 hours at 37°C. To assess the role of CTGF in promoting the expression of FN and Col IV, cells were treated with ET-1, ET-1 plus liposome-CTGF-antisense oligonucleotides (CTGF-ASODN, Lipofectamine 2000, Thermo Fisher Scientific, USA). The full sequence of human CTGF mRNA was obtained through GenBank (<http://www.ncbi.nlm.nih.gov/>) and imported into RNA structure software to design the CTGF antisense oligonucleotides (CTGF-ASODN). The Oligo 6.0 program was used to evaluate the GC content of the sequence and the Blast program (<http://www.ncbi.nlm.nih.gov/blast>) was used for genetic evaluation of each sequence. Four CTGF-ASODN sequences (shown in Table 1) were synthesized and tested by qRT-PCR. The most efficient sequence was selected.

Western blot analysis

Western blot analysis was performed as previously described [17]. Cells were scraped and lysed in lysis buffer (50mmol/L Tris, PH 7.2, 0.1% SDS, 150mmol/L NaCl, 1% NP-40 and 1mmol/L EDTA) supplemented with protease and phosphatase inhibitors for 30 min. The samples were centrifuged for 5 min at 12000 rpm, and protein was collected in the supernatant. After that, 1× SDS sample loading buffer was added to the required protein and boiled for 10 min. An equal amount of protein was loaded onto SDS-PAGE gels and transferred to nitrocellulose (NC) membranes. The membranes were blocked with 5% BSA diluted in PBS for 1 hour at room temperature and subsequently incubated at 4°C overnight with primary antibodies specific for FN (1:300 dilutions; Santa Cruz, USA) and COL-IV (1:500 dilutions; Bioss, Beijing, China). The following day, after three washes with Tris buffered saline with Tween 20 (TBST), the membranes were incubated with HRP-conjugated secondary antibody (1:2000, Boster, Wuhan, China) for 1 hour at room temperature. Afterwards, the proteins were visualized with an enhanced chemiluminescence (ECL) kit and recorded with the image lab imaging system. The blots were analyzed with Quantity One 4.62 software.

Immunofluorescence

After treatment, TM cells were fixed with 4% paraformaldehyde for 15 min followed by three washes with PBS for 15 min. Then, fixed cells were permeabilized with PBS containing 0.5% Triton X-100 for 10 min at room temperature. After that, cells were treated with 5% goat serum for 10 min at room temperature to block nonspecific bindings. Subsequently, cells were incubated overnight at 4°C with primary antibodies anti-FN (1:1000; Santa Cruz, USA) or anti-Col IV antibody (1:500; Bioss, Beijing, China). The second day, the cells were rinsed with PBS and then incubated with FITC-conjugated secondary antibodies (1:2000, Boster, Wuhan, China) for 2 hours at room temperature. For immunohistochemistry, the cultured anterior segments were dehydrated in gradient concentration of ethanol (75%, 85%, 95%, 5min; 100%, 1h, twice) which were then embedded in paraffin to enable TM to be cut perpendicular to its longitudinal axis. For immunofluorescence, the paraffin sections of eyes were dewaxed in dimethylbenzene twice and rehydrated in gradient concentration of ethanol (100%, 95%, 85%, 75%). The staining methods were carried out as mentioned above.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was harvested from cultured human TM cells using Trizol reagent (Aidlab, China), and quantified with a spectrophotometer (Eppendorf, Germany). Then reverse transcription was performed to synthesize cDNA. Quantitative real-time PCR protocol was performed on the ABI7900 real-time detection system (Illumina, USA).

SYBR Green Master Mix (TAKARA, Japan) was utilized to detect amplification. The sequences of the specific primers for qRT-PCR were designed based on the previous reported sequence of human genes (CTGF, GADPH) by Oligo 6.0 program. For standardization, the housekeeping gene GAPDH was used as an internal control. Relative changes in gene expression were calculated using the comparative ($2^{-\Delta\Delta CT}$) method. The conditions of PCR reaction were performed on the basis of the manufacturer's protocol: Incubated at 50°C for 2 minutes, at 95°C for 10 minutes and then followed by 40 cycles at 95°C for 30s and 60°C for 30s. The sequences of specific primers were designed and synthesized as summarized in supplementary Table 1.

Anterior segment perfusion culture and IOP recording

The anterior segment perfusion model was established as reported by Johnson [18]. In brief, the human eye was cut at the equator in order to separate the anterior and posterior segments. The vitreous, lens, iris and ciliary processes were removed from anterior segments. The remaining anterior segment, which consisted of the cornea, sclera, TM, was then moved into a sterile perfusion culture system, fixed with screws and O-ring. The inlet of the perfusion culture system was connected to a uniform speed micropump, which was used for medium infusion, and the outlet was connected to a tonometer to monitor the changes of IOP in real time. The perfusion culture medium containing DMEM supplementary with 100U/ml penicillin, 100U/ml streptomycin and 0.25ug/ml amphotericin was infused into a micropump at a constant infusion rate of 2.5ul/min for 24 hours. After 24 hours of perfusion culture equilibrated in the anterior segment of human eyes, the baseline of IOP of the eyes were recorded, respectively. In total 12 eyes with stable perfusion pressure were used for further study. They were divided into four groups (n=3), correspondently perfused with DMEM only, ET-1(10^{-7} mol/L) ET-1 plus BQ123(10^{-7} mol/L) or BQ788(10^{-7} mol/L) in DMEM. IOP were recorded every 6 hours. After 72 hours, the anterior segments were fixed with 4% paraformaldehyde for immunohistochemistry study.

Statistical analysis

All results were presented as mean \pm SEM. Western blot, qRT-PCR data were analyzed by One-way ANOVA followed by Bonferroni post hoc test. Repeat-measure ANOVA was used to analyze IOP after administration of ET-1 and its antagonist. SPSS 21.0 statistical software was used for all analyses and $p < 0.05$ was considered statistically significant in this study.

Results

1. ET-1-ETAR pathway mediated proliferation of ECM in HTMCs

We first tested the effect of ET-1 on the expression of FN and COL-IV in vitro. Cultured HTMCs were treated with 10^{-7} mol/L ET-1 only, and ET-1 with its antagonist BQ123 or BQ788. Western-blot results showed that, for those HTMCs treated with 10^{-7} mol/L ET-1 for 72h, a significant increase in FN and COL-IV was noted compared with the control group. The same result was found in those HTMCs co-treated with ET-1 and BQ788, but not in cells co-treated with ET-1 and BQ123(Figure1.1). The immunofluorescence study showed changes in morphology after treatments. FN was expressed around or within cells, whereas COL-IV was distributed in the cytoplasm or around the cell diffusely. After being treated with ET-1, the expression of FN and COL-IV was significantly increased, and the cells were elongated. The same occurred in those cells which were co-treated with ET-1 and ETAR antagonist BQ788, but not in those treated with ET-1+BQ123 (See Figure 1.2, Figure 1.3). Therefore, we concluded that ET-1 could up regulate the expression of FN and COL-IV in HTMCs, and mainly through the ETA receptor but not the ETB receptor.

2. The role of CTGF in regulating the expression of FN and Col IV by ET-1

To further clarify the exact mechanism of ET-1 in promoting the expression of FN and COL-IV in Human TM cells, we aimed to study the role of CTGF. The CTGF mRNA levels were tested by qRT-PCR after treatment with ET-1. Results indicate that the mRNA level of CTGF increased 24h after ET-1 administration, reached a peak at 48h and then decreased significantly at 72h after treatment (Figure 2A). Next, we designed four CTGF-ASODN sequences and tested using the qRT-PCR (sequence was listed in Table 1). The sequence with a down-regulation rate of more than 40% on average was used for further study (Figure2B). Next, we treated HTMCs with liposome encapsulated-ASODN4 when exposed to 10^{-7} mol/L ET-1. Western-blot results showed that the expression FN and COL-IV were significantly reversed in those cells treated with ET-1 and liposome encapsulated-ASODN4, compared with those treated with ET-1 only (Figure C&D). These results indicate that the downregulation expression of CTGF could reverse ET-1 -induced increase in FN and COL-IV expression in HTMCs.

3. Blocking ET-1+ETAR pathway prevented ET-1 induced IOP on an ex-vivo model

Then we tested the effect of ET-1 on IOP using an anterior segment cultured model. After 24 hours of perfusion culture equilibrated in the anterior segment of human eyes, the baseline of IOP was recorded, respectively. A total of 12 eyes with the right baseline were randomly divided into four groups (n=3). The control group was perfused with DMEM only. The ET-1 treated group was perfused with 10^{-7} mol/L ET-1 in the perfusate. The remaining two groups were correspondingly treated with ET-1 and ETAR BQ123 (10^{-7} mol/L) or BQ788 (10^{-7} mol/L). The IOP in the four groups were recorded respectively during the next 72 hours. After the anterior segment of human eyes was treated with ET-1, the IOP increased gradually and reached a plateau of about 24mmHg within 48 hours. The ETAR BQ123 prevented ET-1 induced increment of IOP but not BQ788 (Figure 3). These results indicate that ET-1 induced the increase in IOP mainly through the ETA pathway.

4. Blocking the ET-1+ETAR pathway prohibits ET-1 -induced increasing expression of ECM in an ex-vivo model

Next, we detected the expression of FN and COL-IV in the human anterior segment of the trabecular meshwork tissue after perfusion with tissue immunofluorescence staining. After 72 hours of perfusion with 10^{-7} mol/L ET-1, the expression and distribution of FN and COL-IV in the human anterior segment of the trabecular meshwork tissue increased significantly compared with the control group. BQ123 could significantly block ET-1 -induced increased expression of FN and COL-IV, but not BQ788 (Figure4.1& 4.2). This experiment further confirmed that ET-1 mediates the expression of FN and COL-IV through the ETA receptor.

Discussion

IOP is mainly produced in response to a resistance to aqueous humor flow in the trabecular outflow pathways. In humans, 75% of the resistance to aqueous humor outflow is localized within the TM. The TM extracellular matrix (ECM) has been suggested to be responsible for the increased outflow resistance at this specific site. Here, in this study we found that ET-1 could induce increase in IOP in an ex-vivo model. The possible mechanism could be that ET-1 up-regulates the expression of FN and COL-IV, which are key components of ECM. The ETA receptor and CTGF could be the potential pathway for this pathophysiological process.

The trabecular meshwork anatomically can be divided into three layers from inside

to outside: the uveal meshwork, the corneoscleral meshwork and the juxtacanalicular meshwork [19]. The JCT, where outflow resistance is thought to reside, is composed of a loosely arranged extracellular matrix (ECM) and 2 to 5 layers of TM cells dispersed within the ECM. TM cells have both contractile and fibroblastic properties [4]. The ECM is highly dynamic and is composed of many bioactive molecules that influence outflow resistance. FN and COL-IV regulate the outflow resistance of aqueous humor in TM by directly constituting mechanical resistance and participating in cellular signal communication. FN is a macromolecular glycoprotein and one of major structural components of the ECM, which also plays a key role in its maintenance. FN scatters along the basement membranes of the inner wall of SC and the trabecular beams, and is abundant in the periphery of the sheath material surrounding the elastin tendons in the JCT [20]. COL-IV is a basement membrane-related antigen, which is in the form of amorphous fibrous granules and is widely present in the basement membrane of cells in the aqueous humor outflow channel [21]. Abnormal synthesis or secretion of FN and COL-IV are critical pathological factors for the obstruction of aqueous humor outflow, thus resulting in the development of POAG. For example, the expressions of FN and COL-IV in patients with glaucoma increased by 5 times and 20.5 times, respectively [22]. With the progression of glaucoma, the expression of FN increased in JCT and TM, which was positively correlated with the degree of IOP. Besides, the mRNA level of COL-IV increased significantly in glaucoma resected tissues [23]. Another study indicated that FN could regulate the assembly of other ECM, such as COL-IV, fibrillin, and laminin within the TM [24].

ET-1 is a powerful vasoconstrictor peptide and has been shown to be involved in the regulation of intraocular pressure (IOP) by constricting the trabecular meshwork. ET-1 is reported to enhance profibrotic processes in some organs like liver, lung, and kidney tissues, and plays an important role in cell proliferation and differentiation [25]. It has also been acknowledged that ET-1 is a key factor for ECM remodeling. Along with the upregulation of ET-1, the expression of FN and collagen increased markedly in cardiac fibroblasts [26] and in human skin scleroderma fibroblasts [27]. Here in this study, we noted that ET-1 could increase FN and COL-IV expression in HTMCs. In eyes, ETA is expressed in human ciliary smooth muscle, ciliary nonpigmented epithelium, and trabecular meshwork [28]. ETB is mainly distributed in the retina, choroid, cornea, trabecular meshwork, and ciliary body [29]. Here we observed that the block ETA receptor could efficiently prohibit ET-1 induced up-regulation expression of FN and COL-IV, and similarly for IOP. These findings are in line with some other studies. It was previously reported that ETAR antagonist significantly blocked ET-1-induced increase in FN and COL-I in skin fibroblasts [30]. Other studies concluded that ET-1 also promoted the expression of ECM through ETAR in tissues such as myocardium, lung, kidney, and nipple [31-33].

Connective tissue growth factor (CTGF) is a secreted peptide rich in cysteine that can promote the production and aggregation of ECM. This study also determined that the levels of FN and COL-IV in TM cells increased later than that of CTGF after ET-1 intervention, indicating that the increase in FN and COL-IV using ET-1 was likely to be mediated by CTGF. After the application of CTGF-ASODN, the expression levels of FN and COL-IV were decreased significantly, demonstrating that CTGF was an important intermediate molecular protein. Xu et al. [34] showed that ET-1 response elements were present in the basic element (BCE-1) of the CTGF promoter. ET-1 could activate the CTGF promoter and induce expression of CTGF at both mRNA and protein levels, suggesting that CTGF might be involved in the process of ET-1 mediated ECM accumulation [35]. A study revealed that ET-1 induced the expression of CTGF through

ETAR/JNK/AP-1 signaling pathway in human lung fibroblasts [36]. Beyond that, other possible mechanisms could also be involved. A report concluded that ET-1 could promote the secretion of ECM (COL-I, FN) through Akt and Erk signaling pathways in RPE cells in vitro [37]. And other studies demonstrated that ET-1 could activate the TNF- β /Smad pathway [38] or initiate the MEK/ERK/MAPK pathway [34, 39] to promote ECM synthesis. In addition, ET-1 induced inflammation, oxidative stress by up-regulating a variety of cytokines (ICAM-1, MCP-1, IL-1, 6, 8, TNF- α), resulting in the accumulation of ECM [40].

There are several limitations of this study. For donated resources are precious and limited, the sample size of our study using the ex-vivo model was small. Another limitation was that the ex-vivo model may not totally represent the in vivo pathophysiological process. And the third is how CTGF works on FN and COL-IV needs further investigation.

In conclusion, ET-1 in aqueous humor could lead to abnormal synthesis of FN and COL-IV, inducing accumulation of ECM via the ETA-CTGF pathway in trabecular meshwork. As a result, IOP increased. These findings indicate that the ETA antagonist or inhibitors targeting CTGF could be potential therapeutic targets for POAG in the future.

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Table 1: The sequences for qRT-PCR and antisense oligonucleotides

Sequence name	Base Sequence
CTGF—sense	5'-GCCCAGACCCAACTATGA-3'
CTGF— Antisense	5'-CGTCGGTACATACTCCACA-3'
GAPDH—sense	5'-TCAAAGGTGGAGGAGTGG-3'
GAPDH—Antisense	5'-GGAGTCCACTGGCGTCTT-3'
ASODN1	5'-GCCCTTCTTAATGTTCTC-3'
ASODN2	5'- GCGGTTGTCATTGGTAAC -3'
ASODN3	5'- GACAGTTGTAATGGCAGG -3'
ASODN4	5'- AGGACCACGAAGGCGACG -3'

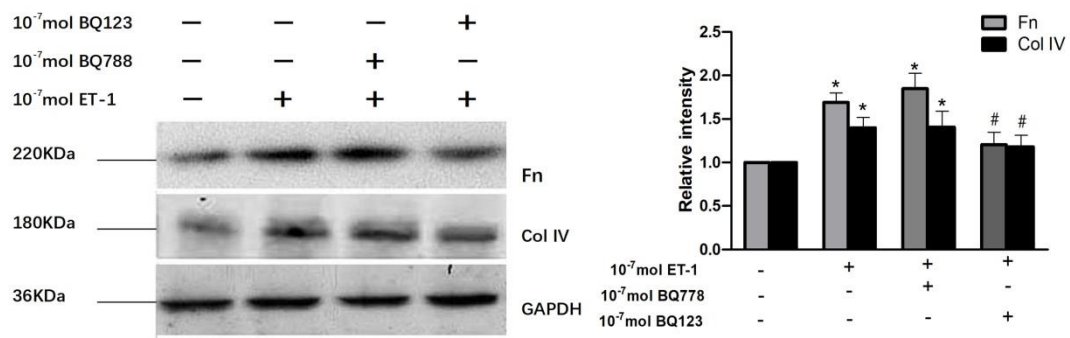


Figure1.1: Western-blot analysis of the effect of ETA antagonist (BQ123) and ETB antagonist (BQ788) on the expression of the FN and Col IV induced by ET-1 in human TMCs, the protein level of FN and Col IV are indicated as column diagram, (* , VS control group; # , VS ET-1 treated group $p < 0.05$) .

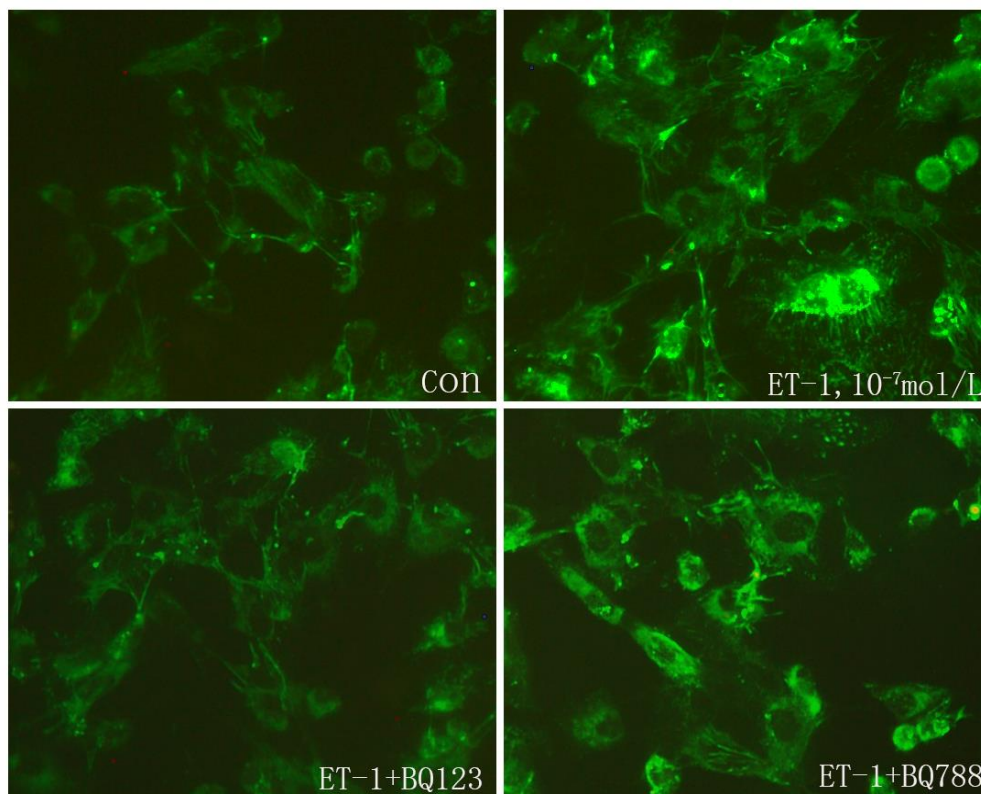


Figure 1.2: An Immunofluorescence analysis of the effect of ET-1, antagonist (BQ123) and ETB antagonist (BQ788) on the expression of the FN in human TMCs (scale bar =100 μ m).

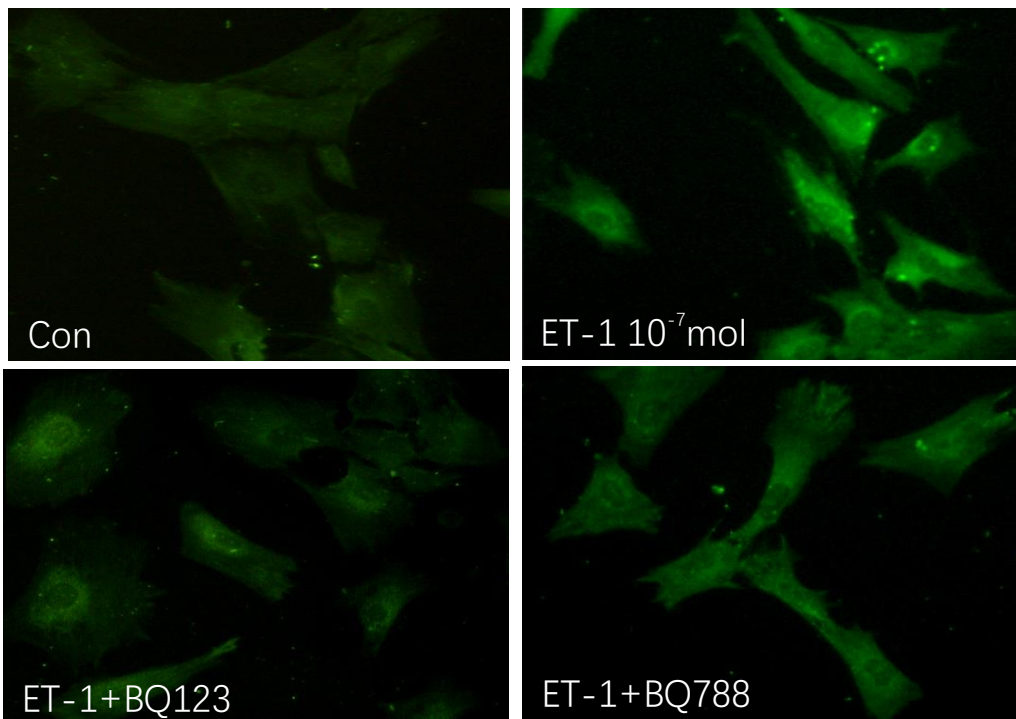


Figure 1.3: An Immunofluorescence analysis of the effect of ET-1, antagonist (BQ123) and ETB antagonist (BQ788) on the expression of the Col IV in human TMCs (scale bar =100 μ m).

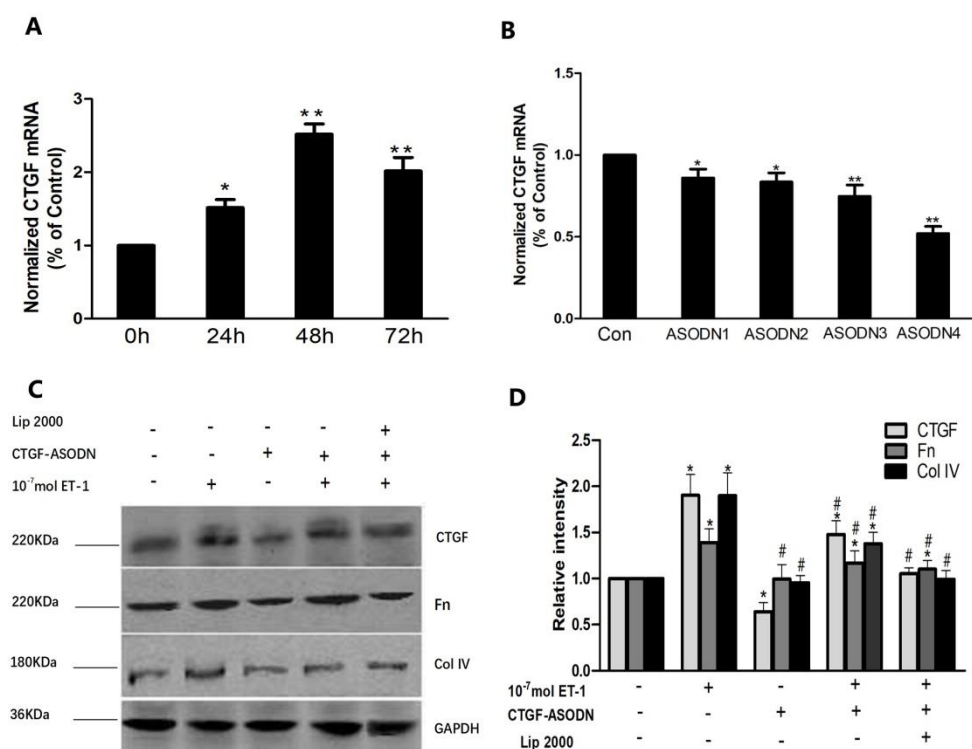


Figure 2: A: qRT-PCR analysis of the effect of ET-1 on CTGF expression; B: the effect of CTGF-ASODNs on the expression of the CTGF in human TMCs. C: Western-blot analysis of the effect of CTGF-ASODNs on the expression of the CTGF, FN and Col IV induced by ET-1 in human TMCs. D: statistical graph of the protein level of CTGF, FN and Col IV. (#, *, VS control group; *, VS ET-1 group p<0.05)

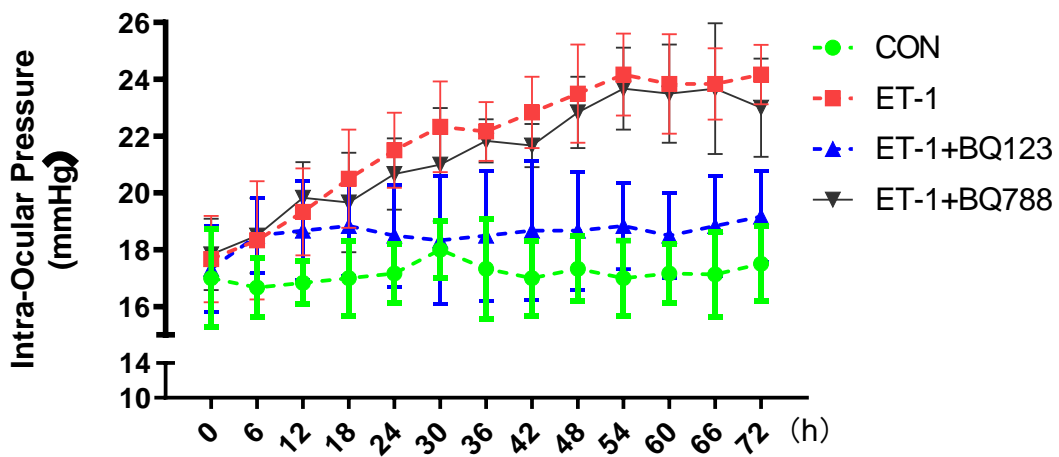


Figure 3: Effect of ET-1 on IOP and its receptor pathway in a human anterior segment perfusion culture model (*, VS control group; #, VS ET-1 group, P < 0.05);

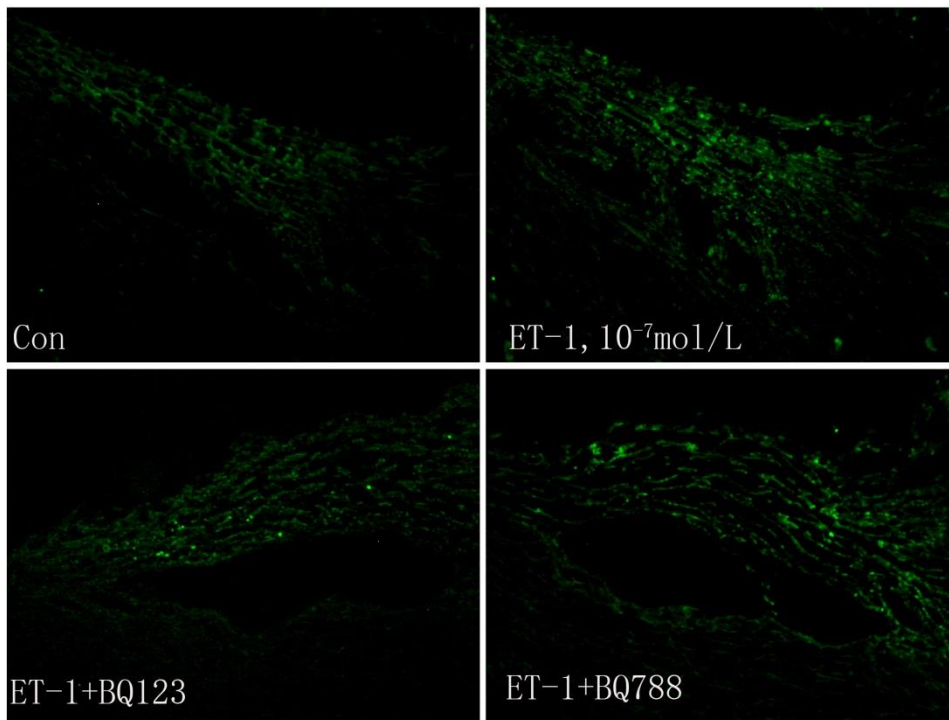


Figure 4.1: An Immunofluorescence analysis of the effect of ET-1 and its receptor pathway on the expression of the FN in trabecular meshwork in a human anterior segment perfusion model (scale bar = 100 μm)

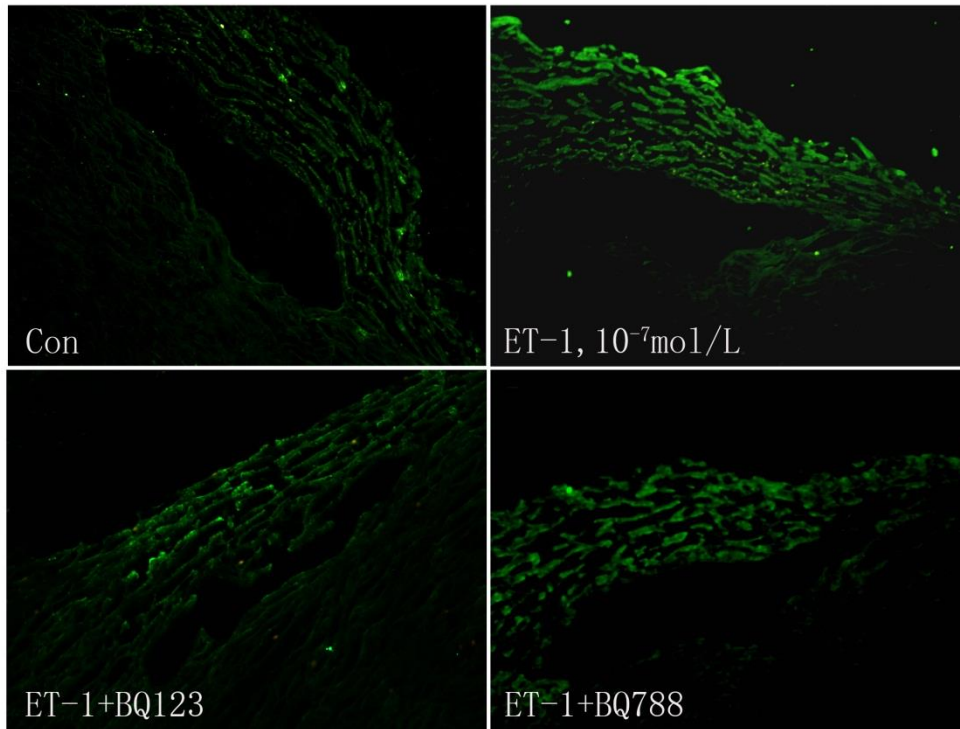


Figure4.2: An Immunofluorescence analysis of the effect of ET-1 and its receptor pathway on the expression of the COL-IV in trabecular meshwork in a human anterior segment perfusion model (scale bar =100μm)