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Endothelin-1 Mediated Regulation of Extracellular Matrix Collagens in Cells of Human Lamina Cribrosa

Vidhya R Rao^a, Raghu R Krishnamoorthy^a, and Thomas Yorio^{a,*}

a Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, Fort Worth, TX 76107, USA

Abstract

Endothelin-1(ET-1), a potent vaso-active peptide, mediates extracellular matrix regulation resulting in an increase in collagen deposition in various cell types and tissues and has been proposed to play a key role in glaucoma pathology. The role of ET-1 in the regulation of extracellular matrix collagens at the level of optic nerve head is not known. In this study we have examined the role of ET-1 in extracellular matrix collagen regulation in primary cultures of human lamina cribrosa cells. Our hypothesis is that ET-1 increases remodeling of the ECM of cells of the lamina cribrosa. Such actions could contribute to the development of optic neuropathy. QPCR analysis revealed that ET-1 mediated an increase in mRNA levels of collagen type I alpha 1 and collagen type VI alpha 1 chains at all doses of ET-1 with a significant increase at 1nM and 10nM concentration in LC cells. A dose dependent increase in collagen type I and type VI protein deposition and secretion was also observed by Western blot in response to ET-1 and was significant at 10nM and 100nM concentrations of ET-1. ET-1 increased the [³H] proline uptake in LC cells suggesting that ET-1 contributed to an increase in total collagen synthesis in LC cells. ET-1 mediated increase in collagen type I, type VI and total collagen synthesis was significantly blocked by the ET_A receptor antagonist, BQ610, as well as with the ET_B receptor antagonist, BQ788, suggesting the involvement of both receptor subtypes in ET-1 mediated collagen synthesis in LC cells. These results suggest that ET-1 regulates extra cellular matrix–collagen synthesis in LC cells and may contribute to ECM remodeling at the level of LC of POAG subjects who have elevated plasma and aqueous humor levels of endothelin-1.

Keywords

Endothelin; Lamina Cribrosa; Extacellular matrix; Collagens; Glaucoma

1. Introduction

Primary Open Angle Glaucoma (POAG), a leading cause of irreversible blindness worldwide, is a progressive optic neuropathy characterized by loss of retinal ganglion cells (RGC), optic nerve degeneration and excavation of the optic disc (Quigley 2005; Quigley and Broman 2006). Elevated intra-ocular pressure (IOP) and age are important risk factors (Leibowitz et al., 1980; Klein et al., 1992). Various mechanisms including elevated IOP, ischemia and glutamate mediated excitotoxicity have been implicated in retinal ganglion cell death (Kuehn

*Corresponding author. Dr Thomas Yorio, Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, Fort Worth, TX 76107, USA., E-mail address: yoriot@hsc.unt.edu (T. Yorio)., Ph. No. 817-735-2490, Fax. No. 817-735-2043.

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et al., 2005). The primary site of injury however appears to be at the level of lamina cribrosa (LC), a distinct perforated connective tissue region of the ONH through which the RGC axons exit the eye. (Anderson 1969 ; Birch et al., 1997; Hernandez, 2000; Quigley, 2005.). Marked disruption in the architecture of the LC is observed in POAG subjects which includes, backward displacement, distortion, collapse of connective tissue plates and extensive extracellular matrix (ECM) remodeling (Miller and Quigley 1988; Hernandez et al., 2000). These changes in LC have been associated with blockade of axonal transport, resulting in optic nerve degeneration and loss of RGCs by apoptosis (Quigley et al., 1983; Sakugawa and Chihara 1985; Martin et al., 2003). ECM remodeling with increase in ECM components including collagen type I, type IV, type VI, and elastin degeneration is observed in LC of POAG subjects and animal models of glaucoma (Hernandez et al., 1987; Miller and Quigley 1988; Morrison et al., 1989; Sawaguchi et. al, 1999; Hernandez et al., 2000). Excess accumulation of collagens, the principal components of ECM, results in fibrosis leading to loss in normal structure and function of the tissue (Varga et al., 2005). The changes in collagens observed in POAG could therefore alter the biomechanical properties of LC and result in the loss of structural integrity (Tengroth and Ammitzboll 1984; Rehnberg et al., 1987). Pathophysiological changes in lamina cribrosa (LC) cells and optic nerve head astrocytes (ONA), two important cell types of LC including, ECM regulation, hypertrophy, migration, and proliferation in response to elevated IOP, and cytokines such as endothelin-1 (ET-1) and transforming growth factor beta (TGF- β) have been attributed to the changes observed in LC (Hernandez 2000; Prasanna et al., 2002; Kirwan et al., 2005; Morrison et al., 2005; He et al., 2007).

Endothelin-1 a 21-amino acid vasoactive peptide plays a key role in glaucoma pathology (Yorio et al., 2002). POAG subjects have significantly higher levels of ET-1 in plasma and aqueous humor compared to their age matched controls (Sugiyama et al., 1995 and Noske et al., 1997). Animal models of glaucoma with elevated IOP also demonstrate significant increase in ET-1 levels (Kallberg et al., 2002; Prasanna et al., 2005). Intravitreal administration of ET-1 in various animal models results in loss of retinal ganglion cells by apoptosis, blockade of axonal transport, activation of optic nerve head astrocytes contributing to optic neuropathy similar to that observed in glaucoma (Stokely et al., 2002; Chauhan et al., 2004; Lau et al., 2006). ET-1 is also recognized as an important pro- fibrotic factor in initiating and maintaining fibrosis of various tissues, by enhancing collagen synthesis and deposition in several cell types including fibroblasts, cardiac myocytes, and smooth muscle cells (Eng and Friedman 2000; Eddy 2000; Wakatsuki et al., 2004; Clozel and Salloukh 2005; Tsukada et al., 2006; Khan ZA 2006). The role of ET-1 in the regulation of ECM collagens at the level of optic nerve head remains to be studied. In the present study we have examined ET-1 mediated extra cellular matrix collagen changes in human lamina cribrosa cells.

2. Materials and methods

2.1. Cell culture

Primary cultures of human Lamina cribrosa (LC) cells derived from four normal donors (ages 58, 84, 85, 87 yrs) were characterized previously as an LC population and were a generous gift from Dr Robert Wordinger (UNTHSC, Fort worth, TX) and Dr Abe Clark (Alcon labs, Fort worth, TX) (Lambert et al., 2004; 2001). The cells were maintained at 37°C and 5% CO₂ in Dulbecco's modified eagle medium (DMEM; Invitrogen-Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin/streptomycin/glutamine (Invitrogen-Gibco, Grand Island, NY).

2.2. QPCR

LC cells were grown to confluence in 100mm dishes. Following treatment with ET-1 (1nM, 10nM and 100nM), total cellular RNA was isolated using the Trizol B reagent (Life

Technologies, Rockville, MD, USA). cDNA was synthesized from 5 µg of total RNA using random primers and AMV Reverse Transcriptase (Promega, Madison, WI, USA). Reactions without reverse transcriptase were also performed and used as negative controls for experiments. For quantification of mRNA transcripts by QPCR, amplification was performed as previously described with modifications (Zhang et al., 2003). Briefly 2.5 µl cDNA samples were amplified with specific primers for COL I alpha 1, COL VI alpha 1 and β -actin was used as internal control were amplified using SYBR Green PCR core reagents (PE Applied Biosystems, Foster City, CA, USA). QPCR amplifications were performed for 50 cycles of denaturation at 95°C for 60sec, annealing 60°C for 60 sec, extension 72°C for 120 sec (for ET_A and β -actin) or 58°C annealing for 60sec and extension at 72°C for 30 sec (for ET_B) in Cepheid Smart Cycler (Cepheid, Sunnyvale, CA, USA). The melting curves were generated to detect the melting temperatures of the specific products immediately after the PCR run. The relative mRNA levels were determined by the comparative C_T method (as described in PE Biosystems User Bulletin #2: <http://docs.appliedbiosystems.com/pebi/docs/04303859.pdf>). The relative mRNA levels in treated versus control are represented as mean percentage $\pm$ SEM of four individual experiments. Amplified PCR products were run on 0.75% agarose gel stained with ethidium bromide in parallel with 100-bp DNA markers. Control RT-PCR reactions without reverse transcriptase or cDNA served as negative controls and did not result in amplification products suggesting that the reactions were not contaminated with genomic DNA. PCR primers for COL I alpha 1, COL VI alpha 1 and β -actin used in this study were designed from their respective cDNA sequence using Gene Jockey II program (BioSOFT, Ferguson, MO, USA) or Primer 3 program (provided in the public domain at <http://www.basic.nwu.edu/biotools/Primer3.html> by the Massachusetts Institute of Technology, Cambridge, MA). The authenticity of QPCR products was confirmed by DNA sequencing and a BLAST search of the sequence through National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). The PCR primers and their expected amplified product size are listed in Table 1.

2.3. Immunocytochemistry

LC cultured on glass coverslips were treated with or without ET-1 (100nM) in serum free medium for 48 hrs. Following treatment the cells were fixed with 4% paraformaldehyde. Nonspecific binding was blocked with 5% Bovine serum albumin (BSA). Cells were then incubated with 1:200 primary mouse monoclonal anti-collagen type I antibody (Calbiochem, Fremont, CA) or 1:200 primary rabbit polyclonal anti-collagen type VI antibody diluted in 1% BSA. Cells incubated with 1% BSA alone served as a negative control. Following washes cells were incubated with 1:400 dilution secondary anti mouse antibody, Alexa fluor 488 or 1:400 dilution secondary anti rabbit antibody, Alexa fluor 633 (Molecular probes, Eugene, OR). Following washes cells were incubated with 300nM DAPI to stain the nuclei. The coverslips were mounted on Fluorsave reagent (Calbiochem, San Diego, CA) and fluorescent images were taken using confocal microscopy (Carl Zeiss Meditec, Inc., Thornwood, NY).

2.4. Western Blot

LC cells were grown to confluency in 60mm dishes. Confluent wells were serum starved over night and subjected to various treatment conditions and included control with no treatment and cells treated with ET-1 (1nM, 10nM, 100nM). In some experiments cells were pre-incubated with the ET_A receptor antagonist (BQ788 -1 µM) or the ET_B receptor antagonist (BQ610 -1µM) for 30 min and subsequently treated with ET-1 (100nM) in the presence of respective antagonists, and the antagonists BQ610 or BQ788 alone. Following various treatments the media was collected and concentrated by Microcon centrifugal filter device (10-kDa cutoff; Amicon; Millipore, Bedford, MA). Cell lysates were obtained by directly lysing the cells in RIPA lysis buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in 1X PBS]. Protein content was determined by bicinchonic acid (BCA) protein assay (Pierce

Biotechnology, Inc, Rockford, IL). Equal amounts of proteins were supplemented with SDS sample buffer, separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were probed with primary anti collagen type I antibody [1:200 mouse monoclonal (Calbiochem, San Diego, CA) or 1:200 goat polyclonal (Santa Cruz Biotechnology, Inc, Santa Cruz, CA)] or primary anti collagen type VI antibody [1:200 rabbit polyclonal (Chemicon Temecula, CA)]. Following incubation with anti mouse/rabbit HRP conjugated secondary antibody (1:10000; GE Health Care, Piscataway, NJ) or anti goat HRP conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) the blots were developed with Super Signal West Femto Maximum Sensitivity Substrate kit (Pierce Biotechnology, Inc, Rockford, IL). The blots were stripped and reprobed with primary anti beta-tubulin antibody [1:200 rabbit polyclonal (Santa Cruz Biotechnology, Inc, Santa Cruz, CA)] followed by anti rabbit HRP conjugated secondary antibody (1:10000; GE Health Care, Piscataway, NJ) for normalizing the protein loading. Densitometric analysis of the bands was performed using the image-analysis software (Scion image; National institutes of health, Bethesda, MD). The relative band intensities in treated versus control is represented as mean percentage $\pm$ SEM of four individual experiments.

2.5. Measurement of total collagen synthesis by [^3H] proline incorporation assay

Collagen synthesis was assessed by measuring the uptake of [^3H] proline as previously described with slight modifications (Ku et al., 2006). Briefly cells were seeded into 24-well plates. Confluent wells were serum starved over night and subjected to various treatment conditions and included control with no treatment, ET-1 100nM, cells pre-incubated with the ET_A receptor antagonist (BQ788 -1 μM) or the ET_B receptor antagonist (BQ610 -1 μM) for 30 min and subsequently treated with ET-1 (100nM) in the presence of respective antagonists, and the antagonists BQ610 or BQ788 alone. [^3H] Proline (Perkin Elmer Waltham, Massachusetts) was added to each well at a final concentration of 1 $\mu\text{Ci/ml}$, and remained in the medium for the rest of the incubation period along with various treatments. After 48 hrs of treatment the media was removed from the wells. Proteins in the media were precipitated by adding trichloroacetic acid (TCA) to give a final concentration of 10%, and left on ice for one hour. Precipitated protein was collected by centrifugation at 14000 g for 30 min, washed with 4 ml ice-cold 10% TCA to remove any unincorporated labeled proline and centrifuged again. The supernatant was carefully removed and the pellet suspended in 0.3 ml of 0.3 M NaOH-0.1% SDS and warmed to 37 $^{\circ}\text{C}$ for 1hr. The cell layer was washed twice with PBS and precipitated with 1 ml of ice-cold 10% TCA for 30 min at 4 $^{\circ}\text{C}$. Following washes with 10 % ice cold TCA, the proteins were solubilised by incubating with 0.3 ml of 0.3 M NaOH-0.1% SDS at 37 $^{\circ}\text{C}$ for 1 hr. An equal portion (0.2 mL) of the solubilised proteins obtained from the cell layers or media were added to 3ml scintillation cocktail and utilized to count the radioactivity using beta counter, Packard Tricarb 1600 TR liquid scintillation analyzer (Packard, UK) while an equal portion of the solubilised proteins obtained from the cell layers or media (0.1 mL) were utilized to determine the total protein content using the bicinchoninic (BCA) protein assay (Pierce Biotechnology, Inc, Rockford, IL). The total radiocactivity counted in each sample were normalized to the respective total protein content. Data are expressed as the mean percentage $\pm$ SEM of [^3H] proline incorporated in cells or proteins in media of 8 individual wells of similar treatment groups.

2.6. Statistical Analysis

Data are represented as Mean $\pm$ SEM. Comparisons between multiple groups was analyzed by analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparison test. Statistical analysis with values of $p < 0.05$ was considered significant.

3. Results

3.1. ET-1 mediated regulation of COL I $\alpha 1$ and COL VI $\alpha 1$ mRNA expression in LC cells

QPCR analysis of total RNA isolated from LC cell lines treated with 1nM, 10nM and 100nM, ET-1 for 24hrs was performed. ET-1 at all doses increased the COL I $\alpha 1$ mRNA levels (Fig. 1(A) and Fig. 1(B)). The most significant increase was observed at 1nM concentration followed by ET-1 10nM concentration. A similar trend was observed for the expression COL VI $\alpha 1$ mRNA levels where in, ET-1 increased the COL VI $\alpha 1$ mRNA levels at all doses with a most significant increase at 1nM concentration followed by 10nM concentration (Fig. 1(C) and Fig. 1(D)). These results suggested that ET-1 regulated the expression of COL I $\alpha 1$ and COL VI $\alpha 1$ at the level of transcription and increased the steady state levels of COL I $\alpha 1$ and COL VI $\alpha 1$ mRNA.

3.2. ET-1 mediated regulation of collagen type I and type VI expression in LC cells

An increase in immunoreactivity for collagen type I protein and type VI protein was observed following ET-1 100nM treatment for 48 hrs in LC cell lines, suggesting that ET-1 mediated an increase in collagen type I and type VI deposition (Fig. 2(A) and Fig. 3(A)). Cells incubated with secondary antibody alone in the absence of primary antibody showed no immunoreactivity and served as control (Fig. 2(A) and Fig. 3(A)). Western blot analysis of LC cell lysates treated with 1nM, 10nM and 100nM, ET-1 for 48hrs, revealed that ET-1 increased the collagen type I and type VI expression in a dose-dependent manner with a significant increase at 10nM and 100nM concentrations (Fig. 2(B), Fig. 2(C), Fig. 3(B) and Fig. 3(C)). Western blot analysis of LC cell culture media also revealed a dose dependent increase in collagen type I and type VI secretion (Fig. 2(D) and Fig. 2(E)). These results suggested that ET-1 increased both the deposition and secretion of collagen type I and type VI in a dose-dependent manner in LC cells.

3.3. ET-1 mediated regulation of collagen type I & type VI expression in LC cells and Role of endothelin receptors ET_A and ET_B

In order to determine the role of ET-1 receptors in ET-1 mediated collagen regulation, the cells were preincubated with either a specific ET_A receptor antagonist, BQ610 (1 μ M), or a specific ET_B receptor antagonist, BQ788 (1 μ M) and subsequently treated with ET-1 for 48hrs. Western blot analysis of LC cell lysates revealed that 100nM ET-1 significantly increased the expression of collagen type I and type VI protein and the increase was blocked significantly with both the ET_A receptor antagonist BQ610 and as well as ET_B receptor antagonist BQ788. The antagonists BQ610 and BQ788 alone had no effects on collagen type I and type VI expression (Fig. 4(A), Fig. 4(B), Fig. 4(C) and Fig. 4(D)). These results suggested that both ET_A and ET_B receptors are involved in ET-1 mediated regulation of collagen type I and type VI in LC cells.

3.5. ET-1 mediated regulation of collagen synthesis in LC cells as determined by [³H] proline incorporation assay

Based on the previous data that ET-1 appears to increase both collagen type I and type VI we decided to measure increases in collagen synthesis. The major biosynthetic destination of proline is collagen and therefore the amounts of radioactive [³H] proline incorporated into insoluble and soluble protein fractions provides a reliable index of total collagen synthesis (Mukherjee and Sen 1990; Ku et al., 2006). In order to determine the receptors involved in ET-1 mediated increase in collagen synthesis, the cells were preincubated with either an ET_A receptor antagonist, BQ610 (1 μ M) or an ET_B receptor antagonist, BQ788 (1 μ M) and subsequently treated with ET-1 for 48hrs in the presence of [³H] proline. ET-1 at 100nM significantly increased the [³H] proline incorporation in cell layer (collagen deposited and collagen within cells) and as well as in cultured media. ET-1 mediated increase in [³H] proline was blocked with the ET_A receptor antagonist BQ610 and as well as ET_B receptor antagonist,

BQ788. The antagonists BQ610 and BQ788 alone did not have any effect on [^3H] proline incorporation (Fig. 5(A) and Fig. 5(C)).

4. Discussion

The connective tissue of LC comprises of various extra cellular matrix molecules including collagens, elastin, fibronectin and proteoglycans (Rehnberg et al., 1986; Goldbaum et al., 1989; Morrison et al., 1989). The composition of ECM renders resiliency and compliance to LC and therefore its ability to sustain changes in intraocular pressure (IOP) without the loss of structural integrity (Burgoyne et al., 2005; Morrison et al., 2005). Increase in collagen type I and collagen type VI is a characteristic feature of ECM remodeling in LC of glaucomatous subjects and animal models of glaucoma (Hernandez 2000; Morrison et al., 2005). Increase in collagen type I has been associated with marked reduction of compliance of several tissues resulting in fibrosis and loss of normal structure and function of the tissue (Varga et al., 2005). Associated with fibrotic tissues is the increase in collagen type VI and is considered as an early marker for tissue fibrosis (Specks et.al, 1995; Hatamochi et.al., 1996; Gerling et.al, 1997; Groma 1998; Zeichen et.al., 1999). Increase in collagen VI has also been proposed to increase the rate of collagen type I fibril formation (Harumiya et al., 2002; Minamitani et al., 2004;), result in loss of elastic properties (Hatamochi et al., 1996) and contribute to migration of glioblastoma cells (Han and Daniel 1995; Han et al., 1995). Elevated IOP models of glaucoma have demonstrated, the deposition of collagen I and VI at the optic nerve head to be an early event and are correlated linearly to degree of IOP- induced injury (Johnson et al., 2000; Guo et al., 2005; Morrison et al., 2005; Johnson et al., 2007). The ability of LC cells to respond to profibrotic triggers such as mechanical stress and transforming growth factor- β (TGF- β) resulting in enhanced ECM synthesis, implicates LC as an important pro-fibrotic tissue that could lead to loss of structural integrity resulting in collapse of LC and associated neuronal loss in glaucoma (Hernandez 2000 and Kirwan et al., 2005). Endothelin-1 has been increasingly recognized for its role as a pro-fibrotic factor resulting in enhanced ECM synthesis and has been widely implicated in the pathology of various connective tissue disorders (Eng and Friedman 2000; Eddy 2000; Wakatsuki et al., 2004; Clozel and Salloukh 2005; Tsukada et al., 2006; Khan ZA 2006). We were therefore interested in studying the role ET-1 in regulation of collagens type I and VI in LC cells. The dose and time point selected for ET-1 in the present study are consistent with previous studies (Shi-wen et al., 2001; Hafizi et al., 2004; Horstmeyer et al., 2005; He et al., 2007).

ET-1 treatment in LC cells resulted in an increase in mRNA levels of both collagen type I α I and collagen type VI α I chains at all doses of ET-1, suggesting a transcriptional regulation of collagen genes by ET-1. Western blot analysis following ET-1 treatment also demonstrated an increase in deposition and secretion of both collagen type I and type VI proteins in LC cells. The increase in protein expression of collagen type I and VI was dose-dependent with a significant increase at 10 and 100nM concentrations of ET-1 in contrary to the increase in mRNA levels which were significantly increased at 1nM and 10nM concentrations of ET-1. Besides increase in transcription the overall increase in collagen synthesis has been attributed to increase in half life and mRNA stability, or decreased collagen degradation (Stefanovic et al., 1997; Stefanovic et al., 1999; Friedman et al. 2000; Bedossa and Paradis 2003). Endothelin-1 not only enhances collagen synthesis in various tissues and cell types but also limits degradation by increasing the activity of tissue inhibitors of matrix metalloproteases (TIMPS), key enzymes that inhibit matrix metallo proteases (MMP's) responsible for collagen degradation (Thirunavukkarasu et al., 2004; Koyama et al., 2007;). Our lab has recently shown that ET-1 at 100nM significantly increases the levels of TIMP1 and TIMP2 in optic nerve head astrocytes (He et al., 2007). ET-1 mediated increases in collagen levels in LC cells could therefore involve both an increase in expression and decrease in collagen degradation. ET-1 mediated increase in collagen type I is well characterized in various tissues, however to the

best of our knowledge, current study presents for the first time that ET-1 can directly regulate collagen type VI expression.

Endothelin-1 (ET-1) mediates its effects through seven transmembrane G-protein coupled receptors, endothelin receptor A (ET_A) and endothelin B (ET_B) that are coupled to different G proteins and down stream targets (Yanagisawa 1994; Takagi et al., 1995). In cardiovascular and pulmonary tissues ET-1 mediated collagen synthesis and ECM deposition have been primarily attributed to stimulation of ET_A receptors (Hafizi et al., 2004; Rodriguez-Vita et al., 2005). In dermal and hepatic tissues however, ET_B receptors, contribute to ET-1 mediated collagen synthesis and ECM deposition (Gandhi et al., 2000; Shi-wen et al., 2001). ET_A receptor serve as regulators of collagen homeostasis by inducing both its synthesis and degradation while it has been shown that ET_B receptor mediates only collagen synthesis (Guarda et al., 1993; Shi-wen et al., 2001; Tostes et al., 2002). Chronic exposure of ET-1 in cultured fibroblasts also resulted in a switch of receptor subtype from ET_A to ET_B, resulting in an ET_B mediated increase in collagen synthesis (Shi-wen et al., 2001; Horstmeyer et al., 2005). An upregulation of ET_B receptors results in autoinduction of ET-1 synthesis and further enhances ET-1 effects through ET_B receptors (Iwasaki et al., 1995). Prevention of ET_B upregulation indeed inhibits hepatic stellate cell activation and collagen synthesis (Chi et al., 2003). An upregulation of ET_B receptors has also been observed in human glaucomatous optic nerve head as well as elevated IOP models of glaucoma (Prasanna et al., 2005 and Wang et al., 2006). In our previous study, we have shown that ET-1 treatment in LC cells resulted in an upregulation of ET_B receptors that was significant at 100 nM concentrations of ET-1 (Rao et al., 2007). The shift in receptor subtype from ET_A to ET_B could in part explain the increase in collagen deposition that is observed at 100nM concentrations of ET-1 in the present study. Both BQ610 an ET_A receptor antagonist and BQ788 an ET_B receptor antagonist were able to each partially inhibit collagen type I and collagen type VI deposition suggesting the involvement of both receptor subtypes in ET-1 mediated collagen deposition in LC cells. Similar results have been reported previously where dual ET_A and ET_B receptor antagonists effectively inhibit ET-1 mediated collagen synthesis (Shi-wen et al., 2001; Morgera et al., 2003; Clozel and Salloukh 2005).

The primary structure of collagen chains is made for the most part of repeating Gly-Xaa-Yaa triplets with a high content of Pro in the Xaa position and OH-Pro in the Yaa position (Van der Rest and Garrone 1991). ET-1 mediated enhanced [³H] proline incorporation has been used for determining the effects of ET-1 on total collagen synthesis in several tissues and cell types (Guo et al., 2004; Hafizi et al., 2004). ET-1 also mediated enhanced [³H] proline incorporation in cell layer and cultured media of LC cells. ET-1 mediated [³H] proline incorporation was blocked by both the ET_B receptor antagonist, BQ788, and the ET_A receptor antagonist, BQ610. These results suggest that ET-1 increases total collagen synthesis in LC cells is mediated by both ET_B and ET_A receptors.

In conclusion, we have demonstrated that ET-1 contributes to enhanced collagen synthesis and secretion in LC cells. ET-1 mediated increase in collagen synthesis by LC cells could therefore contribute to ECM remodeling observed at the level of LC in glaucomatous subjects and further contribute to the pathology of primary open angle glaucoma.

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Fig. 1. Effects of ET-1 on COL I α 1 and COL VI α 1 mRNA determined QPCR analysis

QPCR products separated on ethidium bromide stained 0.75 % agarose gel of COL I α 1, COL VI α 1 and internal control β -actin, following the treatment with ET-1 (1, 10 & 100nM) for 24 hrs (A, C). QPCR data is presented as the mean percentage $\pm$ SEM of mRNA levels of COL I α 1, COL VI α 1 expression compared with the respective control (B, D). ET-1 increased the mRNA expression of COL I α 1, COL VI α 1 message at all doses of ET-1. A significant increase was observed at 10nM and 100nM concentrations of ET-1. *Statistical significance of ET-1 treatment versus control ($p < 0.05$). Experiments were repeated 4 times, two times each in LC cell lines from two different donors.

Fig. 2. Effect of ET-1 on collagen type I

Immunofluorescent staining for collagen type I in LC cells not treated (control) or treated with ET-1 (100nM) for 48hrs (A). Negative control, treated with secondary antibody alone showed no staining for collagen type I (A). Nuclei are DAPI stained (blue). Scale bar in L=50 μ m.

Representative Western blot of collagen type I and internal control beta-tubulin protein expression in LC cell lysates (B), representative Western blot of collagen type I in LC cell culture media (D) following the treatment with ET-1, (1, 10 & 100nM) for 48 hrs. Coomassie stained gel suggested a uniform loading (D). The quantification of band intensities of collagen type I in LC cell lysates and LC culture media is represented as mean percentage $\pm$ SEM compared with the corresponding control band (C, E). *Statistical significance of ET-1 treatment versus control ($p < 0.05$). Experiments were repeated 4 times, two times each in LC cell lines from two different donors.

Fig. 3. Effect of ET-1 on collagen type VI

Immunofluorescent staining for collagen type VI in LC cells not treated (control) or treated with ET-1 (100nM) for 48hrs (A). Negative control, treated with secondary antibody alone showed no staining for collagen type VI (A). Nuclei are DAPI stained (blue). Scale bar in L=50 μ m. Representative Western blot of collagen type VI and internal control beta-tubulin protein expression in LC cell lysates (B), representative Western blot of collagen type VI in LC cell culture media (D) following the treatment with ET-1, (1, 10 & 100nM) for 48 hrs. Coomassie stained gel suggested a uniform loading (D). The quantification of band intensities of collagen type VI in LC cell lysates and LC culture media is represented as mean percentage $\pm$ SEM compared with the corresponding control band (C, E). *Statistical significance of ET-1

treatment versus control ($p < 0.05$). Experiments were repeated 4 times, two times each in LC cell lines from two different donors.

Fig. 4. Effects of ET-1 on collagen type I and type VI is mediated by ET_A and ET_B receptors
Representative western blot of collagen type I, collagen type VI and internal control beta-tubulin protein expression in LC cell lysates, with or without the treatment with ET-1, 100nM, ET-1 100nM treated in the presence of ET_A antagonist BQ610 or ET_B antagonist BQ788 or BQ610/BQ788 alone for 48 hrs (A, C). The quantification of band intensities of collagen type I and collagen type VI is represented as mean percentage $\pm$ SEM compared with the corresponding control band (B, D). * Statistical significance of ET-1 versus control; **statistical significance of ET-1 +BQ610 versus ET-1 alone; ***statistical significance of ET-1 +BQ788 versus ET-1 alone ($p < 0.05$). Experiments were repeated four times, two times each on LC cell lines obtained from two different donors.

Fig. 5. Effect of ET-1 on collagen synthesis as determined by [³H] proline incorporation assay [³H] proline uptake in cell layer (collagen deposited and collagen within cells), (A) and proteins secreted into the media (B) following various treatment conditions for 48 hrs including, control with no treatment, ET-1 100nM, cells pre-incubated with ETA receptor antagonist (BQ788 – 1μM) or ^{ET}B receptor antagonist (BQ610 – 1μM) for 30 min and subsequently treated with ET-1 (100nM) in the presence of respective antagonists, and the antagonists BQ610 or BQ788 alone. Data are expressed as the mean percentage ± SEM of [³H] proline incorporated. * Statistical significance of ET-1 versus control; **statistical significance of ET-1 +BQ610 versus ET-1 alone; ***statistical significance of ET-1 +BQ788 versus ET-1 alone (p<0.05).

Experiments were repeated eight times, four times each on LC cell lines obtained from two different donors.

Table 1

PCR primer sequences and expected product sizes

Gene	Primer Sequence	Product size (bp)
COLI alpha 1	(S) GATGGACTCAACGGTCTCC (A) CCTTGGGGTTCTTGCTGATG	458
COL V1 alpha 1	(S) CTGGGCGTCAAAGTCTTCTC (A) ATTCGAAGGAGCAGCAGCACA	211
Beta-Actin	(S) TGTGATGGTGGGAATGGGTCAG (A) TTTGATGTCACGATTCC	514