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Ceramide inhibits connective tissue growth factor expression by human retinal pigment epithelial cells

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Abstract

Connective tissue growth factor (CTGF) is known to be involved in retinal fibrotic disorders. We used human retinal pigment epithelial cells (HRPE), which play critical roles in retinal fibrosis, to examine the expression of CTGF and its regulation by ceramide and TGF- β . Real-time PCR analysis showed downregulation of CTGF mRNA by C₂ ceramide and upregulation by TGF- β . C₂ Ceramide also inhibited constitutive and TGF- β -enhanced CTGF secretion by HRPE cells. Predominant secretion (>80% of total) of CTGF from the apical side was observed in highly polarized HRPE cells. Fumonisin, an inhibitor of ceramide synthesis, stimulated CTGF secretion while 4HPR, an activator of ceramide synthesis, downregulated CTGF secretion. Based on these results demonstrating ceramide regulation of CTGF secretion by HRPE, we suggest that ceramide may have therapeutic potential for the treatment of retinal fibrotic diseases by inhibiting CTGF production.

Keywords

Retinal pigment epithelium; Ceramide; TGF- β ; CTGF; retinal fibrosis

Introduction

Retinal pigment epithelium (RPE), is a uniquely specialized and polarized tissue consisting of a single layer of epithelial cells sandwiched between neuroretina on the apical side and highly vascularized choroid on the basal side [1]. RPE synthesizes and secretes a number of extracellular matrix (ECM) proteins [2, 3]. Regulated synthesis and degradation of ECM

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proteins are critical for the integrity of RPE basement membrane and adhesion of neural retina to RPE [3].

Connective tissue growth factor (CTGF), a secreted matricellular protein that is sequestered into the ECM, is expressed by a variety of cells including fibroblasts, epithelial cells and smooth muscle cells [4]. CTGF interacts with cell surface proteins such as integrins, growth factors, cytokines, proteases and other ECM components [4]. CTGF production is enhanced during wound healing and tissue repair, and CTGF is known to be overexpressed in fibrotic diseases [5,6]. We demonstrated CTGF as a major mediator of retinal fibrosis, a pathophysiological phenomenon associated with proliferative vitreoretinopathy and choroidal neovascularization complicating age-related macular degeneration [6,7].

Ceramide, a membrane sphingolipid, plays critical roles in apoptosis, cell proliferation and senescence [8,9]. Ceramide regulates gene expression by modulating a variety of protein kinases and phosphatases and activation of transcription factors [9]. Ceramide exposure of human RPE (HRPE) cells causes oxidative stress and mitochondrial dysfunction [10].

The effect of oxidative stimuli such as ceramide on CTGF production by RPE cells has not been hitherto investigated. Further, our previous research demonstrated that TGF- β induces CTGF expression in RPE [11]. The mechanisms of regulation of TGF- β induced CTGF secretion are not clearly understood. In this report, we show for the first time, suppression of constitutive and TGF- β enhanced CTGF by exogenous ceramide and further demonstrate apical secretion of CTGF by polarized HRPE cells. Our findings suggest a potential use for ceramide as a retinal anti-fibrotic therapeutic molecule.

Materials and methods

Materials

C₂ Ceramide, 4 HPR (fenretinide), fumonosin and TGF- β 1 were obtained from Enzo Life Sciences, Farmingdale, NY. CTGF ELISA kit was purchased from Antigenix America Inc. Huntington Station, NY. Fetal bovine serum and all other cell culture media were obtained from Laguna Scientific, Laguna Niguel, CA. SYBR Green PCR master mix, gene-specific primers for CTGF and GAPDH were purchased from Roche Inc.-and Valuegene Inc, San Diego.

Preparation of Human Retinal Pigment Epithelial cell cultures

This protocol adhered to the tenets of the Declaration of Helsinki. Primary human RPE cells were isolated from fetal eyes (gestational age 16–18 weeks; Advanced Bioscience Resources, Inc., Alameda, CA) and used at passages 3 to 4 [12]. For the preparation of polarized RPE cultures, passage 1 HRPE cells (1×10^5 cells/well) were seeded on fibronectin coated Transwell membranes (0.4 μ pore size), and cultured in RPE medium with 1% FBS for a month as previously described [12]. The formation of functional tight junctions among the cells was determined by measuring transepithelial resistance (TER). Polarized HRPE cells with a TER of >350 ohms.cm² were used in all experiments.

Real-time PCR analysis of CTGF gene expression

Confluent HRPE cells in 6 well plates were treated with C₂ ceramide or TGF- β for 12h. Total RNA was isolated and was used for gene expression analysis. PCR analyses were performed using SYBR Green PCR master mix and 0.2 μ M each of gene-specific primers for CTGF and GAPDH. The sequences of the primers for CTGF were 5'-CTCCTGCAGGCTAGAGAAGC-3' and 5'-GATGCACTTTTGGCCCTTCTT-3'. Gene expression in treated samples was presented as fold change in comparison to control samples using GAPDH as a reference standard.

Analysis of cellular and secreted CTGF by ELISA

HRPE cells grown to confluence in 6 well plates were incubated in DMEM containing 0.5% FBS overnight. This was followed by incubation of cultures in media, C₂ ceramide, TGF- β , and other combinations. Where indicated cultures were pre-treated with C₂ ceramide for 60 min before adding TGF- β . After 24h, culture supernatants were collected and used for the determination of CTGF by ELISA. CTGF was also determined in cell lysates.

Effect of 4HPR and Fumonisin on TGF- β induced CTGF secretion

Confluent HRPE cultures in 6 well plates were pretreated with 4HPR (5 μ M) or Fumonisin (25 μ M) for 60 min before adding TGF- β (4 ng/ml). After incubation for 24h in the presence of TGF- β and /or 4HPR or Fumonisin, culture supernatants were collected and CTGF levels in the medium were determined.

Polarized secretion of CTGF by HRPE cells

Polarized HRPE cultures were prepared as earlier described. Apical and basal compartments contained one ml culture media. Only inserts that showed TER of >350 ohms.cm² were used for determining the effect of polarity on secretion. Cells were treated with media (control), TGF- β or TGF- β + C₂ ceramide for 24h and culture supernatants were collected from both apical and basal sides of the transwell inserts and secreted CTGF was determined.

Results and discussion

Effects of C₂ ceramide and TGF- β on CTGF gene, protein expression and secretion

Based on preliminary experiments on cell viability and toxicity, we selected 25 μ M C₂ ceramide and 4 ng/ml TGF- β as optimal doses for all subsequent studies. The RPE viability remained unchanged after treatment at these doses. C₂ ceramide decreased CTGF mRNA levels while TGF- β treatment showed a significant increase ($p < 0.01$ vs untreated; Figure 1A). Cotreatment with C₂ ceramide suppressed TGF- β induced increase in CTGF mRNA ($p < 0.01$).

A significantly reduced CTGF secretion was found when RPE cells were treated with C₂ ceramide ($p < 0.01$) while TGF- β significantly increased secreted CTGF levels ($p < 0.05$) (Figure 1B). Cotreatment with ceramide suppressed CTGF secretion to levels lower than in untreated controls ($p < 0.01$). However, cellular CTGF levels were not significantly altered by treatment with ceramide alone or in combination with TGF- β (Figure 1C).

Effect of regulation of ceramide synthesis on CTGF secretion in HRPE cells

To further elucidate the role of ceramide on CTGF secretion, we determined the effects of treatment with 4HPR, a known stimulator of intracellular ceramide and fumonisin, an inhibitor of ceramide biosynthesis [10,13]. 4HPR significantly inhibited both constitutive CTGF secretion and also TGF- β induced secretion of CTGF ($p < 0.01$ vs. TGF- β) (Figure 2A). In contrast, fumonisin, enhanced TGF- β induced secretion of CTGF ($p < 0.05$ vs TGF- β) while having minimal effect when RPE were treated alone with fumonisin. These data suggest that TGF- β as well as exogenous C₂ ceramide treatment modulate intracellular ceramide thereby regulating CTGF secretion.

Polarized secretion of CTGF by hRPE cells

We investigated whether HRPE cells exhibit preferential secretion of CTGF towards apical or basal compartments. For these studies, we used highly polarized HRPE cells grown on Transwell membrane filters with a high TER (>350 ohms/cm²). A predominant portion of CTGF (amounting to $>80\%$) was released at the apical side i.e. the domain facing the photoreceptors of the neuroretina under normal unstimulated conditions (Figure 2B; $p < 0.01$, apical vs basal). TGF- β treatment caused a significant ($p < 0.01$ vs control) elevation in CTGF secretion at both apical and basal sides. The TGF- β induced increase in secretion was significantly inhibited by co-treatment with C₂ ceramide ($p < 0.01$ vs TGF- β treated).

Thus the important finding of this study is that exogenous ceramide causes downregulation of CTGF expression in RPE cells. Experimental evidence for the regulation of TGF- β -induced CTGF production by ceramide is also presented. We further show for the first time that CTGF is secreted predominantly from the apical domain of polarized RPE cells and ceramide significantly inhibited TGF- β -induced CTGF secretion at both the apical and basal sides. Further confirmation for the regulatory role played by ceramide was obtained from the use of an inhibitor of ceramide biosynthesis which caused downregulation of CTGF secretion and this phenomenon was also true for TGF- β stimulated CTGF secretion. CTGF secretion was significantly lower in control polarized RPE as compared to control nonpolarized RPE (*cf.* Figure 1B vs Figure 2B; $p < 0.01$). While the number of cells in the polarized cultures is 3-fold less than in the nonpolarized 6-well plate cultures, statistical comparisons for these control samples were made after correction to normalize the number of cells. It is possible that additional differences between the culture systems, such as the substrate, also play a role in determining the level of secretion. This lower level of secretion in polarized cells may reflect the amount of extracellular CTGF needed for normal maintenance and survival of photoreceptor [14]. The significant upregulation of apical secretion of CTGF with TGF- β in polarized RPE may represent a response to protect the photoreceptors from further injury. Thus, ceramide may prove to be a valuable agent in preventing increased CTGF production and may find use in profibrotic, proangiogenic and other ocular disorders.

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References

1. Strauss O. The retinal pigment epithelium in visual function. *Physiol Rev.* 2005; 85:845–881. [PubMed: 15987797]
2. Hoffmann S, He S, Ehren M, Ryan SJ, Wiedemann P, Hinton DR. MMP-2 and MMP-9 secretion by rpe is stimulated by angiogenic molecules found in choroidal neovascular membranes. *Retina.* 2006; 26:454–461. [PubMed: 16603966]
3. Thumann, G.; Dou, G.; Wang, Y.; Hinton, DR. Cell Biology of the Retinal Pigment Epithelium. In: Ryan, Stephen J., editor. *Retina*. Fifth Edition. Elsevier; 2013. p. 401-414. Editor-in-Chief
4. Cicha I, Goppelt-Struebe M. Connective tissue growth factor; Context dependent functions and mechanisms of regulation. *Biofactors.* 2009; 35:200–208. [PubMed: 19449449]
5. Kuiper EJ, de Smet MD, van Meurs JC, Tan HS, Tanck MWT, Oliver N, van Nieuwenhaven FA, Goldschmeding R, Schlingemann RO. Association of connective tissue growth factor with fibrosis in vitreoretinal disorders in the human eye. *Arch Ophthalmol.* 2006; 124:1437–1462.
6. Hinton DR, He S, Jin ML, Barron E, Ryan SJ. Novel growth factors involved in the pathogenesis of proliferative vitreoretinopathy. *Eye.* 2002; 16:422–428. [PubMed: 12101449]
7. He S, Jin ML, Worpel V, Hinton DR. A role for connective tissue growth factor in the pathogenesis of choroidal neovascularization. *Arch. Ophthalmol.* 2003; 121:1283–1288. [PubMed: 12963611]
8. Hannun YA. The sphingomyelin cycle and the second messenger function of ceramide. *J. Biol. Chem.* 1994; 269:3125–3128. [PubMed: 8106344]
9. Ruovolo PP. Intracellular signal transduction pathways activated by ceramide and its metabolites. *Pharm. Res.* 2003; 47:383–392.
10. Kannan R, Jin M, Gamulescu M-A, Hinton DR. Ceramide-induced apoptosis: role of catalase and hepatocyte growth factor. *Free Radic. Biol. Med.* 2004; 37:166–175. [PubMed: 15203188]
11. He S, Chen Y, Khankhan R, Barron E, Burton R, Zhu D, Ryan SJ, Hinton DR. Connective tissue growth factor as a mediator of intraocular fibrosis. *Invest Ophthalmol Vis Sci.* 2008; 49:4078–4088. [PubMed: 18450591]
12. Sonoda S, Spee C, Barron E, Ryan SJ, Kannan R, Hinton DR. A protocol for the culture and differentiation of highly polarized human retinal pigment epithelial cells. *Nature protocols.* 2009; 4:662–673.
13. Sreekumar PG, Zhou J, Sohn J, Spee C, Ryan SJ, Maurer BJ, Kannan R, Hinton DR. N-(4-hydroxyphenyl) retinamide augments laser-induced choroidal neovascularization by retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 2008; 49:1210–1220. [PubMed: 18326751]
14. Hauck SM, Gloeckner CJ, Harley ME, Schoefmann S, Boldt K, Ekstrom PAR, Ueffing M. Identification of a paracrine neuroprotective candidate proteins by a functional assaydriven proteomics approach. *Mol and Cellular Proteomics.* 2008; 7:1349–1361.

Highlights

1. TGF- β upregulates CTGF expression by human fetal retinal pigment epithelial cells (HRPE)
2. Exogenous C₂ ceramide inhibits constitutive and TGF- β -induced CTGF secretion by HRPE cells
3. CTGF secretion is predominantly from the apical side in polarized HRPE cells
4. Suppression of TGF- β -induced CTGF by ceramide may find application in therapy of retinal fibrotic disorders

FIG. 1.

A. Effects of TGF-β and C₂ ceramide treatment on the expression of CTGF mRNA in HRPE cells. Cells were treated with TGF-β (4 ng/ml) and C₂ ceramide (25 μM) for 12h and total RNA prepared was used for analysis of CTGF mRNA by real-time PCR. **B.** Effects of TGF-β and C₂ ceramide treatment on CTGF secretion. RPE cells were treated with TGF-β (4 ng/ml) and/or C₂ ceramide (25 μM) for 24 h and the culture supernatants collected were used for the determination of secreted CTGF by ELISA. **C.** Effects of TGF-β and C₂ ceramide treatment on cell associated CTGF levels, HRPE cells were incubated with TGF-β

(4 ng/ml) and or ceramide (25 μ M) for 24h. Culture supernatants were removed and CTGF in the cell extracts was determined by ELISA. Results are mean $\pm$ SEM (n=6–8). *p < 0.05; ** p<0.01; NS, not significant.

FIG 2.

A. Effects of TGF- β , 4HPR and fumonisin treatment on CTGF secretion by HRPE cells. Cells were treated with TGF- β (4 ng/ml), 4HPR (5 μ M) or fumonisin ((5 μ M) for 24h and culture supernatants collected were used for the analysis of secreted CTGF by ELISA.

B. Polarized secretion of CTGF by HRPE cells treated with TGF- β and C₂ ceramide. RPE cultures were treated with indicated reagents for 24h. Culture media from both apical and basal compartments were used for the determination of CTGF levels. Results are mean $\pm$ SEM (n=6).

*p < 0.05; ** p<0.01; NS, not significant.