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Transforming growth factor-β regulates the expression of anosmin (KAL-1) in human retinal pigment epithelial cells

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Abstract

In a microarray analysis of human retinal pigment epithelial cells (HRPE) treated with TGF-β, in addition to the alteration of a number of known extracellular matrix (ECM)-related genes regulated by TGF-β, we found a significant increase in the expression of Kallmann Syndrome (KAL)-1 gene, that codes for the protein anosmin-1. Enhanced expression of KAL-1 by TGF-β was validated by real-time PCR analysis. In in vitro experiments, TGF-β receptor inhibitor abolished TGF-β-induced expression of KAL-1. Immunofluorescence staining showed increased presence of anosmin-1 in TGF-β treated HRPE cells, with distinct localization at the intercellular junctions. Treatment of HRPE cells with TGF-β enhanced secretion of anosmin-1 and the release of anosmin-1 was further augmented by heparin sulfate. Enhanced secretion of anosmin-1 in the presence of TGF-β and heparin was also observed in other ocular cells such as corneal epithelial and corneal fibroblast cultures. The role of anosmin-1, a protein with adhesion functions, in retinal structure, function and pathology has not been known and remains to be investigated.

Keywords

Anosmin; KAL-1; TGF-β; adhesion proteins; retinal pigment epithelium; retina; cornea

Introduction

Retinal pigment epithelium (RPE) consists of a single layer of epithelial cells strategically located between choroids and neurosensory retina. Extracellular matrix (ECM) components act as cementing substances and play critical roles in the maintenance of adhesion of neurosensory retina to RPE [1, 2]. ECM proteins also play an important role in the integrity of RPE basement membrane complex known as Bruch's membrane that is critical in preserving the barrier between choroid and retina. Aberrant production and secretion of ECM by RPE would lead to retinal disorders like retinal detachments, proliferative vitreoretinopathy and macular degeneration, the diseases that may lead to loss of visual function [1, 2].

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Transforming growth factor-beta (TGF-β) is a multipotent cytokine that regulates cell proliferation, differentiation and synthesis of ECM like collagens, fibronectin, heparin and chondroitin sulfate proteoglycans [3]. Using conditional gene knock-out mice, we have recently shown that retinal specific TGF-β signaling deficiency leads to retinal detachments [4]. Loss of TGF-β actions due to non-functional TGF-β receptor 1 resulted in significantly diminished chondroitin sulfate proteoglycan in the subretinal space causing retinal detachments [4]. In addition, elevated expression of TGF-β in the vitreous, retina and RPE has been reported in retinal fibrosis, retinal detachments and choroidal neovascularization [5, 6].

Our previous studies have showed that TGF-β is a potent inducer of vascular endothelial growth factor, platelet derived growth factors [7, 8, 9]. To further elucidate the genes altered in response to TGF-β in human RPE cells, we employed microarray analysis using Affymetrix GeneChip. One of the genes that demonstrated a significant upregulation following treatment with TGF-β was an extracellular adhesion protein, anosmin-1 (the gene is called Kallmann Syndrome-1 or KAL-1). In this study, we further investigated the expression of anosmin-1 in epithelial and fibroblast cells derived from human retina and cornea. Though we have previously shown that anosmin-1 is a cancer-regulated protein [10], the expression and role of anosmin-1 in the pathophysiology of retinal and other ocular disorders have not been known.

Materials & Methods

Cell Cultures

Human retinal pigment epithelial (HRPE) cell cultures were prepared from donor eyes as reported earlier [7, 8, 9]. Positive staining for cytokeratin confirmed the epithelial nature of HRPE. Human corneal epithelial cell line (HCE-T) was obtained from RIKEN Cell Bank. Human corneal fibroblasts (HCRF) were prepared from donor eyes or corneal buttons as reported earlier [11].

Anosmin polyclonal antibody

Polyclonal antibodies to anosmin-1 were developed in rabbits by using a peptide with the amino acid sequence “CSHLKHRHPHHYKPSPERY” conjugated to KLH [10]. The affinity purified antibody was prepared by coupling the peptide on to a SulfoLink affinity matrix (Pierce) and performing the affinity chromatography with the polyclonal antibody [10].

Analysis of global gene expression profile in HRPE cells by Microarray

Confluent cultures of HRPE were treated with TGF-β1(10 ng/ml) for 8 hr in serum free medium before preparing total RNA. Reverse transcription, cRNA synthesis, hybridization, washing, staining with streptavidin-phycoerythrin (SAPE, Molecular Probes) and amplification with biotinylated anti-streptavidin antibody were performed as per Affymetrix protocols. GeneChip Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA) was used for hybridization. Affymetrix GeneChip Operating software (GCOS) was used for absolute expression analysis. Normalization, filtering and cluster analysis of the data were performed with GeneSpring software (Silicon Genetica/Agilant, CA)

Real-Time RT-PCR analysis of KAL-1 mRNA expression

Total RNA prepared from cells was used for the relative quantitation of the levels of KAL-1 mRNA. To test the specificity of TGF-β, HRPE cells grown to confluence were treated with TGF-β R1 kinase inhibitor (Calbiochem, La Jolla, CA) 30 minutes prior to TGF-β treatment. Taqman master mix reagents and assays-on-demand gene expression products and FAM labeled KAL-1(Applied Biosystems, CA) were used according to the

manufacturer's instructions. Relative quantification method was used for the expression of KAL-1 gene in TGF-β treated samples in comparison to control samples. KAL-1 mRNA was quantified in relation to GAPDH.

Detection of Anosmin-1 by indirect immunofluorescence

HRPE cultures were grown to near confluence in 8 well chamber slides and treated with TGF-β for 24 hr. Cultures were fixed with acetone:methanol mixture (1:1) for 10 min and preincubated for 30 min in PBS containing 10% normal goat serum for blocking non-specific binding. Then cells were incubated with preimmune rabbit serum, polyclonal anti-anosmin-1 rabbit serum or affinity purified rabbit anosmin-1 antibody prepared in PBS containing 2% goat serum for 1 hr. Cells were washed and further incubated for 1 hr in the presence of goat anti-rabbit IgG conjugated to FITC prepared in PBS containing 2% goat serum. The slides were washed with PBS, mounted (Vectashield[AQ], Vector Labs, Burlingame, CA) and observed for fluorescence.

Western blot analysis of secreted Anosmin (KAL-1)

HRPE, HCE-T and HCRF cell cultures were grown to confluence in 100 mm dishes. Cultures were left in serum free medium overnight and then treated with TGF-β1(10 ng/ml) for 24 hr in serum free medium. Two hours before harvesting the culture supernatant fluids, heparin sulfate was added to a final concentration of 50 μg/ml. Culture supernatants were concentrated by 50 fold by using centrifugal filter devices (Centriprep, Millipore, Bedford, MA). Concentrated culture supernatants were separated by PAGE, transferred to nitrocellulose membranes and incubated with affinity purified anosmin antibodies. After incubating with HRP conjugated secondary antibodies, blots were developed by using chemiluminiscence substrate.

Results and Discussion

Microarray analysis of global gene expression profile of primary HRPE cell lines treated with TGF-β showed two fold up or down regulation of 2200 gene targets (**data not shown**). Several genes coding for proteins related to the ECM were upregulated following TGF-β treatment (Table 1). Since most of the ECM genes are known to be upregulated by TGF-β, we focussed further studies on KAL-1 gene. There was a 3.5 fold increase in the expression of KAL-1 gene in the cells treated with TGF-β (Table 1). The expression of KAL-1 in ocular tissues, its regulation by cytokines and growth factors, and its possible role in normal and pathological conditions in the ocular tissues are not known.

To validate the microarray results of TGF-β enhanced expression of anosmin-1, we performed RT-PCR analysis of total RNA isolated from HRPE cells treated with TGF-β and found that TGF-β treatment significantly enhanced the expression of KAL-1 (Fig.1a) while we did not observe any significant effects by cytokines such as IFN-γ, TNF-α, IL1-β and platelet derived growth factor (data not shown). We used TGF-β R1 kinase inhibitor, a small molecule inhibitor of TGF-β, to test TGF-β specificity in KAL-1 induction (Fig 1a). HRPE cultures were preincubated with the inhibitor for 30 m before adding TGF-β. Total RNA prepared after 8 h was used for the analysis of KAL-1 mRNA. The inhibitor completely abolished TGF-β induced expression of KAL-1 at both concentrations tested (Fig.1a).

As seen in Fig 1b, when HRPE cells were treated with TGF-β, anosmin-1 protein expression was also markedly elevated. In addition, TGF-β treatment resulted in enhanced the expression of anosmin-1 protein in human corneal epithelial (HCE), corneal fibroblast (HCRF) (Fig.1b) and in human choroidal fibroblast (**not shown**) cells. Intense bands were observed on the protein blots of TGF-β treated culture supernatant to which heparin sulfate

was added prior to termination of the culture (Fig.1b), demonstrating heparin-mediated disruption of the cell surface bound anosmin-1 [12].

In order to further confirm anosmin-1 expression, HRPE cultures grown to confluence were treated with TGF-β1 for 24 h in serum free medium and formalin-fixed cells were incubated with either preimmune serum or antibodies to anosmin as described in the methods section. Untreated and TGF-β treated HRPE cells treated with pre-immune serum showed only background fluorescence staining (Fig.1c, **panel A**). Untreated cells incubated with anosmin antibodies showed faint staining that is diffused and appears to be in the cytoplasm and cell surface (Fig.1c, **panel B**). In contrast, TGF-β treated cells exhibited intense staining both intracellular and cell surface. In some projections where cell-cell contacts were maintained after fixing, intercellular staining along the borders of the cells is clearly evident (Fig. 1c, **panel D**).

Anosmin gene (KAL-1) spans 210 kb with 14 exons and encodes a 680 amino acid extracellular matrix protein with a signal peptide and six potential N-glycosylation sites [12,13]. Anosmin has whey acidic protein (WAP) motif and four contiguous fibronectin type III repeats which are reported to be associated with serine protease activity and cell adhesion molecules. Loss of function due to mutations results in Kallmann syndrome, an X linked autosomal disease, characterized by hypogonadism and anosmia [14]. These symptoms are reported to be due to defects in the migration of neuronal precursors of both olfactory and gonadotrophin-releasing hormone cells during embryonic development [14]. Anosmin-1 may also play a role in cancer and inflammation [10,15]. Though the role of anosmin-1 in the olfactory tract extracellular matrix has been strongly implicated in facilitating migration of neurons in embryogenesis, its role in other tissues or in adult-hood is not known. Our finding indicates that TGF-β stimulates the production of anosmin-1; however the mechanism of TGF-b dependent expression of anosmin-1 remains to be investigated.

The results obtained in this study demonstrate that KAL-1 gene expression is enhanced by TGF-β in HRPE and corneal cell lines. The expression of KLA-1 gene in photoreceptors and other neuroretinal cells have not been evaluated in the present studies. However, further studies are needed to elucidate the specific function of anosmin-1 in ocular tissues and its role in diseases such as macular degeneration and diabetic retinopathy where extracellular matrix and TGF-β are known to play a profound role.

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Highlights

*Anosmin-1 is an extracellular matrix protein important in the migration of olfactory neurons in embryogenesis.

*Our experiments demonstrate that TGF-beta upregulates anosmin-1 in human ocular tissues.

*This finding has significance in diseases such as macular degeneration where TGF-beta plays a critical role.

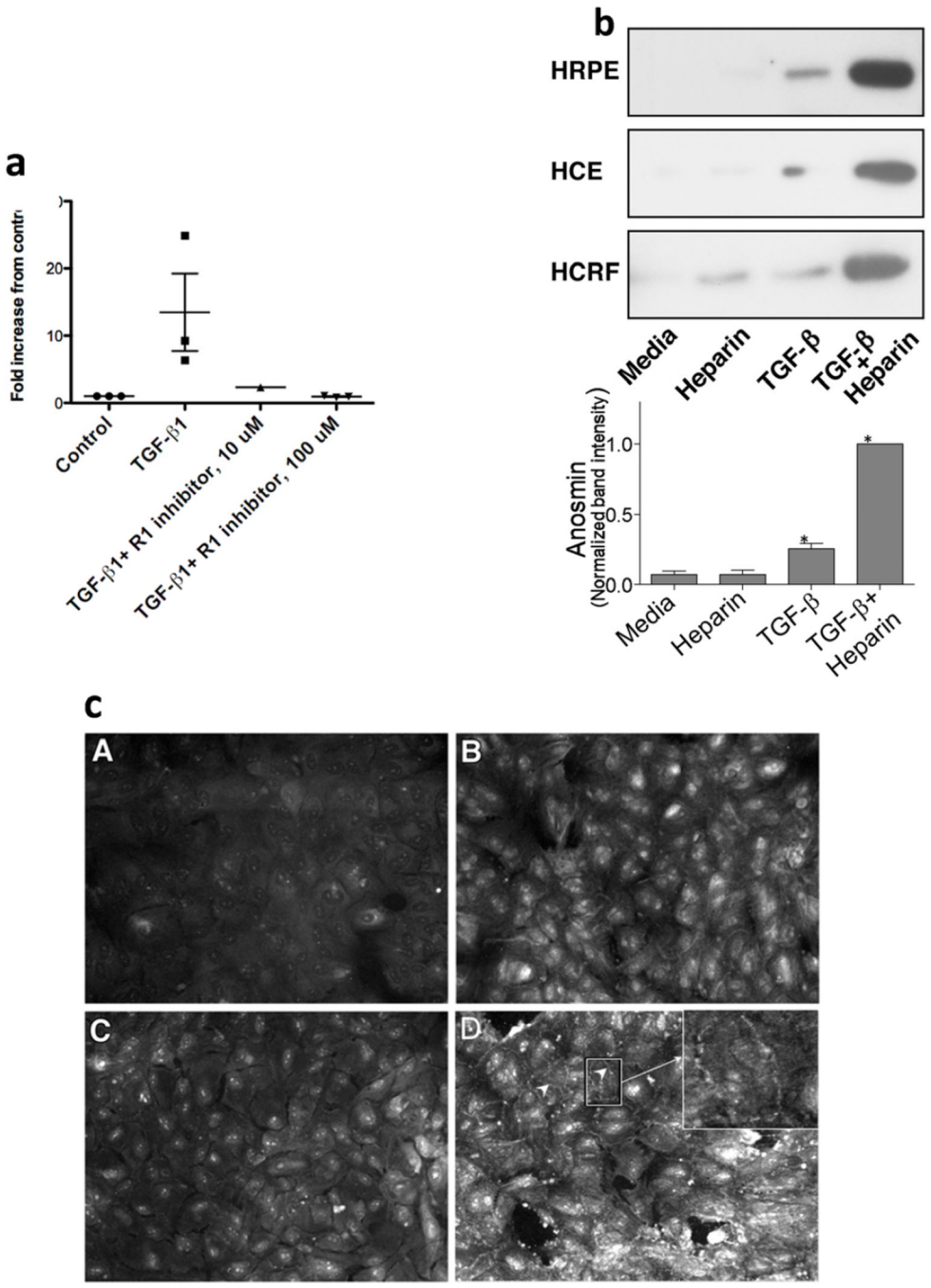

Fig. 1.

a. Real-time PCR analysis of KAL-1 gene expression in HRPE cells treated with TGF-β1. HRPE cultures were treated with TGF-β1 (10 ng/ml) in the absence or presence of TGF-β receptor 1 inhibitors for 8 h. Total RNA prepared was used for Real time PCR analysis as described in the methods.

b. Immunoblot analysis of anosmin (KAL-1) secreted by cell cultures. Cultures grown to confluence were treated without or with TGF-β (10 ng/ml) for 24 h in serum free media. Two hr before harvesting, heparin was added to give 50 ug/ml concentration. Culture supernatants collected were concentrated 50 fold and used for immunoblot analysis as

described in the methods section. **HRPE**, Human retinal pigment epithelial cells; **HCE**, Human corneal epithelial cells; **HCRF**, Human corneal fibroblast cells. In repeated experiments the molecular size of anosmin appeared around 75kD. **Lower panel**: Anosmin-1 level in the culture supernatant was quantified by Western blot, in four separate experiments using HRPE cells. Band intensities were normalized to the intensity of the sample with highest anosmin-1 level and expressed as mean+standard error. *=$p<0.05$ when compared to the controls.

c. Immunofluorescence detection of Anosmin (KAL-1) in HRPE cells. HRPE cultures grown to confluence in 8 well glass slides were treated with TGF-β for 24 hr, fixed, incubated with pre-immune serum or rabbit polyclonal antibody followed by FITC conjugated secondary antibody. **A.** Control cells incubated with pre-immune serum; **B.** Control cells incubated with anosmin-1 antibody; **C.** TGF-β treated cells incubated with pre-immune serum; **D.** TGF-β treated cells incubated with Anosmin antibody (arrow heads indicate intercellular staining)

Table 1
Gene expression changes in HRPE cells treated with TGF-β

HRPE cultures were grown to confluence were treated with TGF-β1(10 ng/ml) for 8h in serum free medium. Total RNA prepared was used for microarray analysis using Affymetrix GeneChip as described in Materials and Methods section.

Affymetrix ID	Fold Change	Gene name
215646_s_at	17.83	chondroitin sulfate proteoglycan 2 (versican)
202310_s_at	15.6	collagen, type I, alpha 1
204619_s_at	14.98	chondroitin sulfate proteoglycan 2 (versican)
202311_s_at	14.54	collagen, type I, alpha 1
213640_s_at	14.51	lysyl oxidase
214701_s_at	13.42	fibronectin 1
201107_s_at	13.07	thrombospondin 1
205828_at	12.6	matrix metalloproteinase 3 (stromelysin 1, progelatinase)
226535_at	12.27	integrin, beta 6
221731_x_at	11.68	chondroitin sulfate proteoglycan 2 (versican)
226847_at	11.54	follistatin
204298_s_at	10.46	lysyl oxidase
204620_s_at	10.32	chondroitin sulfate proteoglycan 2 (versican)
211571_s_at	9.588	chondroitin sulfate proteoglycan 2 (versican)
201645_at	9.249	tenascin C (hexabrachion)
215446_s_at	8.532	Human lysyl oxidase (LOX) gene, exon 7.
204475_at	7.224	matrix metalloproteinase 1 (interstitial collagenase)
214602_at	6.707	collagen, type IV, alpha 4
212865_s_at	6.676	collagen, type XIV, alpha 1 (undulin)
214702_at	6.637	fibronectin 1
201109_s_at	6.37	thrombospondin 1
201108_s_at	5.155	thrombospondin 1
216893_s_at	4.939	collagen, type IV, alpha 3 (Goodpasture antigen)
221729_at	4.462	collagen, type V, alpha 2
208083_s_at	4.368	integrin, beta 6
222073_at	4.327	collagen, type IV, alpha 3 (Goodpasture antigen)
211980_at	4.317	collagen, type IV, alpha 1
211981_at	3.998	collagen, type IV, alpha 1
214641_at	3.903	collagen, type IV, alpha 3 (Goodpasture antigen)
216898_s_at	3.778	collagen, type IV, alpha 3 (Goodpasture antigen)
219697_at	3.506	heparan sulfate (glucosamine) 3-O-sulfotransferase 2
205206_at	3.461	Kallmann syndrome 1 sequence
202620_s_at	3.087	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2
201069_at	2.845	matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
211966_at	2.299	collagen, type IV, alpha 2
212464_s_at	2.199	fibronectin 1

Affymetrix ID	Fold Change	Gene name
201656_at	0.485	integrin, alpha 6
219909_at	0.481	matrix metalloproteinase 28
239273_s_at	0.302	matrix metalloproteinase 28
239272_at	0.221	matrix metalloproteinase 28