Experimental Eye Research xxx (2013) 1–9

Contents lists available at SciVerse ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

TGF-β2 promotes RPE cell invasion into a collagen gel by mediating urokinase-type plasminogen activator (uPA) expression

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ARTICLE INFO

Article history:
Received 1 February 2013
Accepted in revised form 10 June 2013
Available online xxx

Keywords:
TGF-β
uPA
uPAR
EMT
cell invasion
retinal pigment epithelial cell

ABSTRACT

Transforming growth factor-beta (TGF-β) is one of the main epithelial–mesenchymal transition (EMT)-inducing factors. In general, TGF-β-induced EMT promotes cell migration and invasion. TGF-β also acts as a potent regulator of pericellular proteolysis by regulating the expression and secretion of plasminogen activators. Urokinase-type plasminogen activator (uPA) is a serine protease that binds to its cell surface receptor (uPAR) with high affinity. uPA binding to uPAR stimulates uPAR's interaction with transmembrane proteins, such as integrins, to regulate cytoskeletal reorganization and cell migration, differentiation and proliferation. However, the influence of TGF-β and the uPA/uPAR system on EMT in retinal pigment epithelial (RPE) cells is still unclear. The purpose of this study was to determine the effect of TGF-β2, which is the predominant isoform in the retina, and the uPA/uPAR system on RPE cells. In this study, we first examined the effect of TGF-β2 and/or the inhibitor of uPA (u-PA-STOP®) on the proliferation of a human retinal pigment epithelial cell line (ARPE-19 cells). Treatment with TGF-β2 or u-PA-STOP® suppressed cell proliferation. Combination treatment of TGF-β2 and u-PA-STOP® enhanced cell growth suppression. Furthermore, western blot analysis, fibrin zymography and real-time reverse transcription PCR showed that that TGF-β2 induced EMT in ARPE-19 cells and that the expression of uPA and uPAR expression was up-regulated during EMT. The TGF-β inhibitor SB431542 suppressed TGF-β2-stimulated uPA expression and secretion but did not suppress uPAR expression. Furthermore, we seeded ARPE-19 cells onto Transwell chambers and allowed them to invade the collagen matrix in the presence of TGF-β2 alone or with TGF-β2 and u-PA-STOP®. TGF-β2 treatment induced ARPE-19 cell invasion into the collagen gel. Treatment with a combination of TGF-β2 and the uPA inhibitor strongly inhibited ARPE-19 cell invasion compared with treatment with TGF-β2 alone. Furthermore, the interaction between uPA and ARPE-19 cells was analyzed using a surface plasmon biosensor system. The binding of uPA to ARPE-19 cells was observed. In addition, TGF-β2 significantly promoted the binding activity of uPA to ARPE-19 cells in a time-dependent or cell-number-dependent fashion. These results indicate that TGF-β-induced EMT-associated phenotype changes in ARPE-19 cells and the invasiveness of ARPE-19 cells into a collagen gel matrix are mediated, at least in part, by uPA.

1. Introduction

Retinal pigment epithelial (RPE) cells are believed to contribute to the development of proliferative retinal diseases such as proliferative vitreoretinopathy (PVR) (Hiscott et al., 1999). Epithelial–mesenchymal transition (EMT) is a cellular process in which epithelial cells lose cell–cell contacts and undergo dramatic remodeling of the cytoskeleton (Greenburg and Hay, 1982; Thiery, 2002). In general, the cells undergoing EMT acquire expression of mesenchymal components and manifest a migratory phenotype

Abbreviations: TGF-β, transforming growth factor-beta; EMT, epithelial–mesenchymal transition; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; RPE, retinal pigment epithelial; α-SMA, α-smooth muscle actin.

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0014-4835/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.exer.2013.06.020

(Kang and Massague, 2004). EMT is a crucial step in the acquisition of invasive capacity. In the case of ocular trauma or retinal detachment, RPE cells come off from Bruch's membrane and subsequently induce proliferative changes followed by EMT. These events are thought to be involved in the progression of PVR.

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine and is one of the main EMT-inducing factors in various cells through the activation of Smad and non-Smad signaling pathways (Heldin et al., 2012; Massague, 2008; Miyazono, 2009). In cancer cells, TGF-β-induced EMT promotes cell migration and invasion (Bakin et al., 2000). TGF-β also acts as a potent regulator of pericellular proteolysis by regulating the expression and secretion of plasminogen activators (Santibanez et al., 1999).

Urokinase-type plasminogen activator (uPA) is a serine protease that is synthesized by vascular endothelial cells, smooth muscle cells, monocytes, macrophages, fibroblasts, epithelial cells and malignant tumor cells of different origins (Tkachuk et al., 2009). uPA mediates a variety of functions such as regulation of cell recruitment, cell migration (Romer et al., 1991), cell adhesion (Madsen et al., 2007), chemotaxis (Kwak et al., 2005), metastasis (McMahon and Kwaan, 2008) and angiogenesis (Oh et al., 2003). uPA facilitates cell invasion by degrading the extracellular matrix (ECM) and activating other protease zymogens or growth factors (Rabbani and Xing, 1998). The efficiency of uPA activity is regulated by the number of the cell surface receptors (uPAR). uPAR possesses a glycosyl-phosphatidylinositol plasma membrane anchor instead of the conventional membrane-spanning polypeptide (Ploug et al., 1991). uPAR enhances proteolysis of uPA by binding to uPA (Blasi and Sidenius, 2010).

Several studies have shown the expression of uPA or uPAR and function of the uPA/uPAR system in RPE cells (Alfano et al., 2006; Elner et al., 2003; Siren and Immonen, 2003; Siren et al., 1999). However, it is not fully understood how TGF-β and the uPA/uPAR system are implicated in the proliferative changes and EMT of RPE cells in PVR.

In this study, we analyzed whether TGF-β-induced EMT-associated phenotype changes in RPE cells and the invasiveness of RPE cells into a collagen gel matrix were mediated by uPA. In addition, we investigated the effects of TGF-β antagonism on uPA and uPAR expression and invasion of RPE cells and examined the influence of TGF-β on binding activity between uPA and RPE cells.

2. Materials and methods

2.1. Reagents and antibodies

Human recombinant TGF-β2 was purchased from R&D Systems (Minneapolis, MN). The specific inhibitor of the TGF-β type I receptor (SB431542) was purchased from Sigma–Aldrich (St. Louis, MO). SB431542 was dissolved in dimethylsulfoxide. u-PA-STOP®, a selective uPA inhibitor, was purchased from American Diagnostica (Stamford, CT).

u-PA-STOP® [N-a-(2,4,6-triisopropyl-phenylsulfonyl)-3-amidino-(L)-phenylalanine-4-ethoxycarbonyl-piperazide hydrochloride hemi-hydrate] is a reversible competitive inhibitor of trypsin-like serine proteases that has a relatively high specificity for uPA (Sturzebecher et al., 1999).

2.2. Cell culture

The human retinal pigment epithelial cell line (ARPE-19 cell) was obtained from American Type Cell Culture (ATCC) and maintained in DMEM/F12 medium supplemented with 10% fetal calf serum (Gibco-BRL, Invitrogen, Grand Island, NY). For western blot analysis and fibrin zymographic analysis, ARPE-19 cells were plated at a density of 3×10^5 cells per 35 mm^2 plate. For real-time reverse-transcription PCR, ARPE-19 cells were plated at 3×10^4 cells per well in 12-well transwell plates. All experiments were performed after starvation of cell cultures for 24 h and in serum free DMEM/F12 medium.

2.3. Cell proliferation assay

ARPE-19 cells (1×10^3 cells) were seeded onto 96-well plates in 180 μl of culture medium alone or with u-PA-STOP® (1 μg/ml), TGF-β2 (10 ng/ml) or TGF-β2 (10 ng/ml) + u-PA-STOP® (1 μg/ml) at 37 °C in 5% CO2. After 24 h, 48 h and 72 h of incubation, 20 μl of MTT [3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenylterazolium bromide] (Sigma–Aldrich) was added and the cultures were incubated for 4 h at 37 °C. Formazan was dissolved in DMSO (100 μl/well), and absorbance of the cultures at 562 nm was measured using VERSAmax (Japan Molecular Devices, Tokyo, Japan). The experiments were performed in triplicate.

2.4. Western blot analysis

ARPE-19 cells were washed three times with ice-cold phosphate-buffered saline (PBS). Cell lysates were prepared by scraping the cells into 120 μl of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 200 μM Na_3CO_4, 10 mM NaF, 1% NP-40, and 1% protease inhibitor cocktail). The lysates were centrifuged at 13,000 × *g* for 15 min at 4 °C and the supernatants (equal amounts of protein) from different conditions were subjected to SDS-polyacrylamide gel electrophoresis on 8–16% gels. The separated proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in PBS containing 0.1% Tween 20 (PBST) for 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4 °C followed by three washes in PBST and incubation with peroxidase-linked secondary antibodies. The immunoblotted membrane was developed using an enzyme-linked chemiluminescence kit (GE Healthcare Bio-Sciences) according to the manufacturer's instructions.

2.5. Fibrin zymographic analysis

The activity of uPA was determined as described previously (Matsuo et al., 1986). Briefly, protein samples were loaded onto a 10% polyacrylamide gel containing 0.55 mg/ml of bovine fibrinogen and 0.056 NIH U/ml of thrombin (Sigma–Aldrich). After electrophoresis, the gels were soaked in 2.5% Triton X-100 solution for 60 min, and then incubated in reaction buffer (0.5 M glycine–HCl, pH 8.4) at 37 °C for 36 h. The gels were stained with Coomassie Blue R-250 for 1 h and destained with destaining solution (30% methanol, 10% acetic acid). Fibrin zymography was used to determine the total pro (single-chain) and active (two-chain, disulfide linked) levels of uPA. Previous papers showed that these two forms of uPA are both active on the zymograms because, under non-reducing conditions, both forms co-migrate and the single-chain forms are activated during of the zymogram gel processing (Corti et al., 1986; Warejcka et al., 2011). Although pro uPA is a single-chain form precursor of uPA, pro uPA possesses very low intrinsic activity. In general, mammalian uPA is secreted as single-chain pro uPA (Kasai et al., 1985) and is subsequently converted to active (two-chain) uPA by proteolytic enzymes. In this study, we determined the increase in total uPA expression (pro and active levels of uPA) activated by TGF-β. The intensity of the lytic band corresponding to uPA was measured with an LAS-1000 system (Fuji Film, Tokyo, Japan), which was calibrated using human standard uPA (0.1 IU).

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2.6. Total RNA extraction, reverse transcription (RT) and quantitative real-time PCR

Total RNA was isolated with an RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Single-strand complementary DNA (cDNA) was prepared from total RNA using random primers under standard conditions and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). cDNA was subjected to quantitative real-time PCR using SYBER Premix Ex Taq (Takara Bio, Shiga, Japan) and the ABI 7900HT Sequence Detection System (Applied Biosystems) in a 96-well plate according to the manufacturer's instructions. The PCR condition was 94 °C for 2 min, followed by 40 cycles of 94 °C for 0.5 min, 50 °C for 0.5 min, and 72 °C for 0.5 min. The primers were as follows:

uPA forward, 5′-ATCTGCCTGCCCTCGATGTATAA-3′;
uPA reverse, 5′-TTTCAGCTGCTCCGGATAGAGATAG-3′;
uPAR forward, 5′-GAGCTATCGGACTGGCTTGAAGA-3′;
uPAR reverse, 5′-AGGTAACGGCTTCGGGAATAGG-3′;
E-cadherin forward, 5′-TTAAACTCCTGGCCTCAAGCAATC-3′;
E-cadherin reverse, 5′-TCCTATCTTGGGCAAAGCAACTG-3′;
N-cadherin forward, 5′-CGAATGGATGAAAGACCCATCC-3′;
N-cadherin reverse, 5′-GGAGCCACTGCCTTCATAGTCAA-3′;
α-SMA forward, 5′-AGCAGGCCAAGGGGCTATATAA-3′;
α-SMA reverse, 5′-CGTAGCTGTCTTTTTGTCCCATT-3′.

The quantity of expression for each gene was calculated by normalizing the cycle threshold (Ct) of each gene to the Ct of the β-actin in the same sample according to the ΔΔCt method. Three independent experiments were performed.

2.7. Binding assay of ARPE-19 cells to uPA

The ability of uPA to bind ARPE-19 cells was measured with a real-time cell–molecular interaction assay system, IAsys resonant mirror biosensor (Affinity Sensors; Cambridge, UK) at a temperature of 20 °C using a three-dimensional carboxylate cuvette. In this cuvette-based resonant mirror instrument, binding of the molecule to the sensor surface causes a change in the refractive index; i.e., the resonant angle shifts. The change in the angle detected by the instrument is displayed in arc seconds with higher arc seconds revealing greater binding. uPA was immobilized on the surface of an IAsys carboxylate cuvette (Affinity Sensors) (Okada et al., 2008). Briefly, after equilibration with PBS to obtain a stable baseline, carboxylate on the cuvette surface was activated with 200 mM *N*-hydroxysuccinimide (NHS) and 50 mM 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDC) for 7 min. The activation solution was removed and washed out with PBS, and then uPA (final concentration, 10 μg/ml) was treated in 10 mM sodium acetate (pH 5.0) for 5 min for immobilization. After the removal of the uPA solution and washing out with PBS, the unbound surface of the cuvette was blocked with 1 M ethanolamine. The immobilization of uPA on the cuvette surface was confirmed by an increase in arc seconds. The binding ability of ARPE-19 cells (2.5×10^4 to 2×10^5 cells/ml) to immobilized uPA was measured. For the competition study, ARPE-19 cells were preincubated with or without TGF-β2 (10 ng/ml) for 24 h and then subjected to the binding assay. The sensor surface was regenerated after each experiment by washing with 0.1 M glycine–HCl buffer (pH 3.0) for 2 min followed by washing with PBS.

2.8. Invasion assay

The three-dimensional invasion assay in the collagen matrix was performed as described previously (Yamaguchi et al., 2011), with some modification. We used a Transwell chamber (Chemotaxicell; Kurabo, Osaka, Japan) that had a polycarbonate membrane with an 8 μm pore size. The hydrated collagen gel was prepared as previously described (Nishida et al., 1988). Briefly, type 1 collagen solution (Nitta Gelatine, Osaka, Japan) was mixed with 10 fold concentration of MEM and neutralized with 0.2 N NaOH. Three hundred microliters of collagen solution was placed into each well of a 12-well tissue culture plate. Collagen was allowed to gel in an incubator for 30 min at 37 °C, and then a Transwell chamber was placed onto the gel. 3×10^5 ARPE-19 cells were seeded onto the Transwell chambers and allowed to invade the collagen matrix in the presence of TGF-β2 (1 ng/ml and 10 ng/ml) alone, TGF-β2 (1 ng/ml and 10 ng/ml) + u-PA-STOP® (1 μg/ml) or in the absence of TGF-β2. At 3 days after incubation, the Transwell chambers were removed from the 12-well tissue culture plates. The collagen gel in each 12-well tissue culture plate was dissolved by collagenase L (1 mg/ml; Nitta Gelatine) and centrifuged. The number of cells in each collagen gel was determined by direct counting under a phase-contrast microscope.

2.9. Statistical analysis

Statistical comparisons between two groups were performed by an unpaired Student's *t*-test. An ANOVA was used to compare three or more conditions with post hoc comparisons using the Tukey–Kramer procedure; $p < 0.05$ was considered to be significant. Significant differences between groups are noted by * and †.

3. Results

3.1. Effect of TGF-β2, u-PA-STOP® or TGF-β2 + u-PA-STOP® on cell proliferation of ARPE-19 cells

The morphology of ARPE-19 cells treated with TGF-β2, u-PA-STOP® or TGF-β2 + u-PA-STOP® was examined after 48 h of the treatment. ARPE-19 cells without treatment or those that were treated with u-PA-STOP® showed a cobblestone-like epithelial morphology. By contrast, cells treated with TGF-β2 demonstrated more elongated morphology. The morphological change of the ARPE-19 cells treated with TGF-β2 + u-PA-STOP® was partially inhibited compared with that of the cells treated with TGF-β2 alone (Fig. 1A). TGF-β2 or u-PA-STOP® alone significantly inhibited cell proliferation in ARPE-19 cells. Moreover, TGF-β2 + u-PA-STOP® showed a significantly increased growth inhibitory effect in ARPE-19 cells compared with that of TGF-β2 alone or u-PA-STOP® alone (Fig. 1B).

3.2. Epithelial mesenchymal transition in ARPE-19 cells by TGF-β2

We determined the expression of the epithelial marker E-cadherin, mesenchymal markers N-cadherin and α-smooth muscle actin (α-SMA) to confirm the EMT of ARPE-19 cells after stimulation with TGF-β2. Western blot analysis of E-cadherin, N-cadherin and α-SMA protein levels after 48 h showed that TGF-β2 reduced E-cadherin and increased N-cadherin and α-SMA in a dose-dependent manner (Fig. 2A). The mRNA and protein levels of N-cadherin and α-SMA increased whereas those of E-cadherin decreased after stimulation of TGF-β2 in a time-dependent manner (Fig. 2B and C). These results suggested that EMT was induced by TGF-β2.

3.3. Enhancement of uPA expression, secretion and uPAR expression in ARPE-19 cells by TGF-β2

To investigate whether uPA and uPAR expression were up-regulated during EMT induced by TGF-β2, the protein and mRNA levels of uPA and uPAR were determined. The protein levels of uPA were determined by fibrin zymography, whereas those of uPAR

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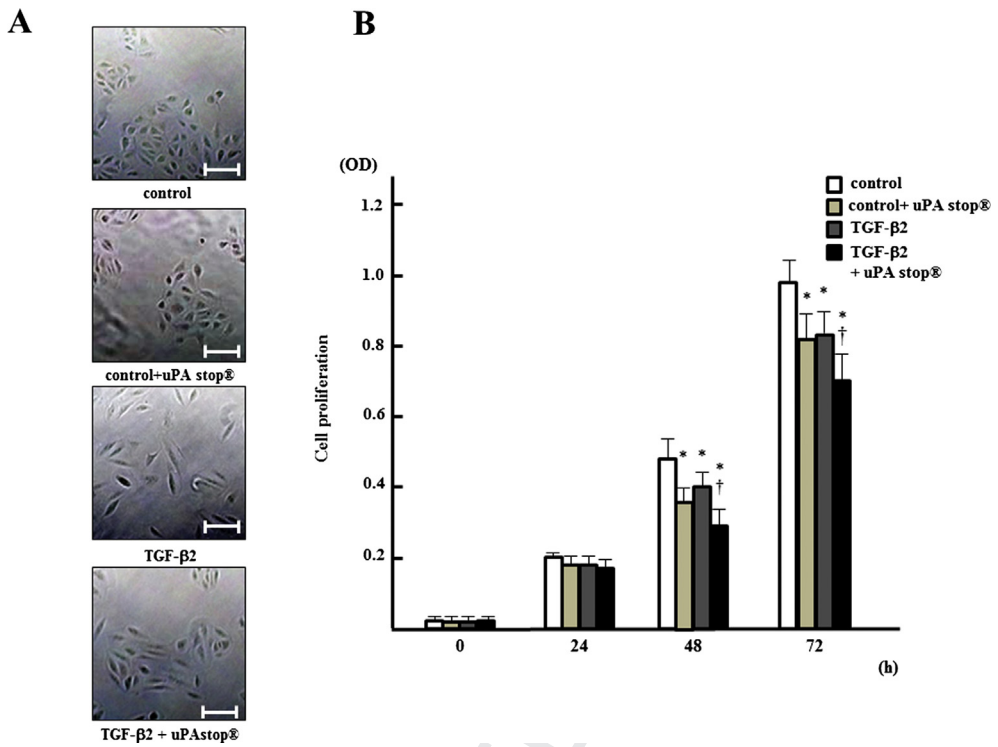

Fig. 1. (A) Morphological changes of the ARPE-19 cells treated with TGF-β2 (10 ng/ml), u-PA-STOP® (1 μg/ml) or TGF-β2 (10 ng/ml) + u-PA-STOP® (1 μg/ml). TGF-β2, not u-PA-STOP®, mediates EMT-like morphologic changes in ARPE-19 cells. u-PA-STOP® suppresses TGF-β2-induced morphologic changes in ARPE-19 cells. Scale bar, 100 μm. (B) Growth inhibitory effects of TGF-β2 (10 ng/ml) and u-PA-STOP® (1 μg/ml) in ARPE-19 cells evaluated using an MTT assay after incubation for 0 h, 24 h, 48 h and 72 h. The experiments were performed in triplicate. The error bars represent the SD. Significant difference compared with *the control, †TGF-β2. $P < 0.05$.

were determined by western blotting. The mRNA levels of uPA and uPAR were determined by real time RT PCR. Fig. 3A shows that TGF-β2 enhanced both cell-associated uPA expression and secretion in a dose-dependent manner.

The relative amount of uPA expression was determined using a LAS-1000 system with a gel for fibrin zymography (Table 1). Secreted uPA expression was significantly increased at a TGF-β2 concentration of 1 ng/ml and 10 ng/ml. An approximately 10 fold

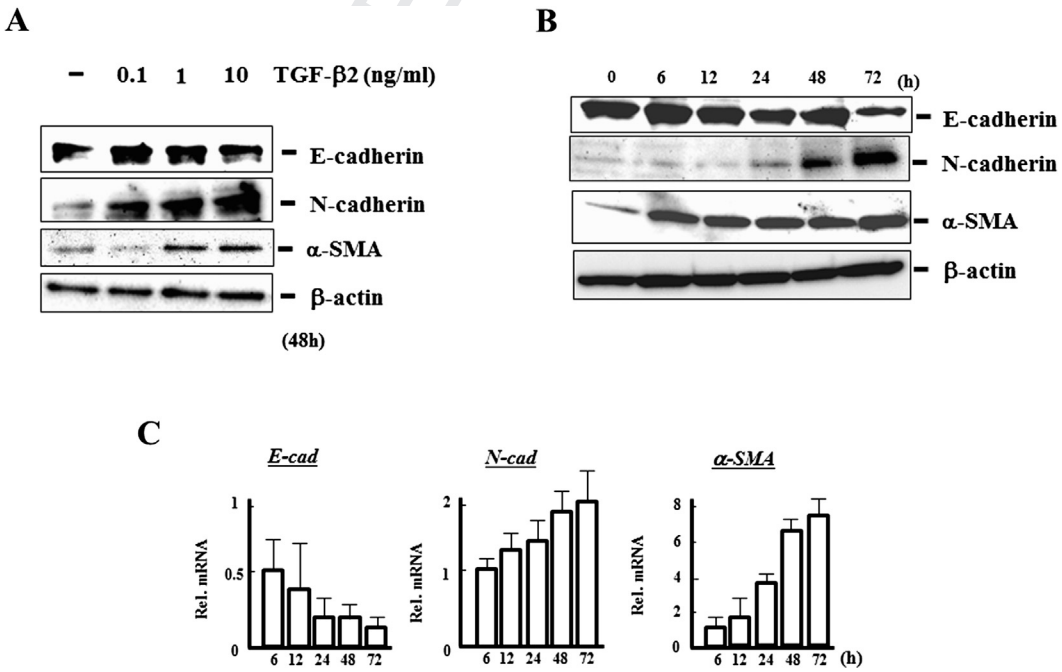

Fig. 2. Downregulation of E-cadherin and upregulation of mesenchymal markers N-cadherin and α-SMA by TGF-β2 in ARPE-19 cells. (A) Western blot analysis of E-cadherin, N-cadherin and α-SMA in ARPE-19 cells treated with TGF-β2 (10 ng/ml) for 48 h. β-actin was used as an internal control. (B) Western blot analysis of E-cadherin, N-cadherin and α-SMA in time-dependent responses to TGF-β2 (10 ng/ml) treatment in ARPE-19 cells. β-actin was used as an internal control. (C) Real time reverse transcription PCR analysis for mRNA levels of E-cadherin, N-cadherin and α-SMA in time-dependent responses to TGF-β2 (10 ng/ml) treatment in ARPE-19 cells. The experiments were performed in triplicate. The error bars represent the SD. *$P < 0.05$.

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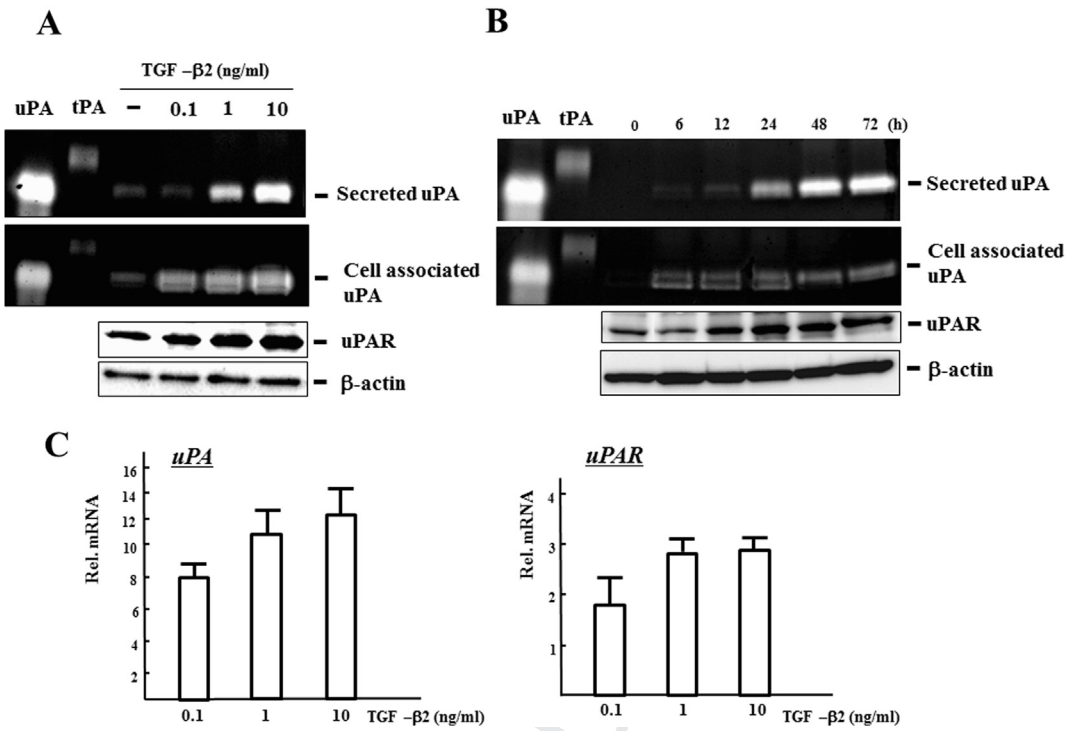

Fig. 3. (A) Fibrin zymographic analysis of uPA protein expression, uPA secretion and western blot analysis of uPAR expression in dose-dependent responses to TGF-β2 treatment in ARPE-19 cells. β-actin was used as an internal control. (B) Fibrin zymographic analysis of uPA protein expression and uPA secretion and western blot analysis of uPAR expression in time-dependent responses to TGF-β2 (10 ng/ml) treatment in ARPE-19 cells. β-actin was used as an internal control. (C) Time course study of mRNA expression of uPA and uPAR after TGF-β2 (10 ng/ml) treatment in ARPE-19 cells. The experiments were performed in triplicate. The error bars represent the SD.

increase was observed at the concentration of 10 ng/ml compared with the control. Cell-associated uPA expression was significantly increased at a TGF-β2 concentration of 0.1 ng/ml, 1 ng/ml and 10 ng/ml. An approximately 3 fold increase was observed at a concentration of 10 ng/ml compared with the control.

TGF-β2-stimulated uPA secretion gradually increased in a time-dependent manner (Fig. 3B). Cell-associated uPA expression increased at 6 h after the addition of TGF-β2 and was sustained up to 72 h (Fig. 3B). As expected, quantitative real-time PCR analysis revealed that mRNA levels of uPA rapidly peaked at 6 h with a 20-fold increase (Fig. 3C). This increased mRNA expression of uPA was maintained up to 72 h, however, its expression levels gradually decreased from after 12 h TGF-β2 stimulation. The protein expression of uPAR was up-regulated by TGF-β2 (Fig. 3B), which is consistent with the quantitative real-time PCR results. TGF-β2-stimulated mRNA expression of uPAR sustained an approximately 3-fold increase during the time course (Fig. 3C).

3.4. Influence of TGF-β I receptor blocker (SB431542) on TGF-β2-stimulated uPA expression, secretion and uPAR expression in ARPE-19 cells

SB431542 is a potent and specific inhibitor of activin receptor-like kinase (ALK) 4, 5, and 7 (Inman et al., 2002). The protein uPA was determined by fibrin zymography and uPAR expression was determined by western blotting. SB431542 effectively inhibited both TGF-β2-stimulated cell-associated uPA expression and uPA secretion in ARPE-19 cells (Fig. 4A). Interestingly, in contrast, uPAR expression was not inhibited by SB431542 (Fig. 4A). Quantitative real-time PCR results revealed that TGF-β2 stimulation increased mRNA expression of uPA, but that of uPAR was not suppressed by SB431542 (Fig. 4B).

Table 1
The total uPA expression of ARPE-19 cells stimulated by TGF-β2.

TGF-β2 (ng/ml)	0	0.1	1	10
Secreted uPA	5.3 ± 2.5	8.3 ± 4.7	29.6 ± 7.4*	53.1 ± 16.7*
Cell-associated uPA	32.1 ± 11.2	72.4 ± 18.5*	85.9 ± 9.4*	98.5 ± 11.4*

The relative amounts of total uPA expression were estimated as described in Material and Methods. Data shown as mean ± SEM ($n = 3$). *significant difference compared with the control at $P < 0.05$.

3.5. Binding assay of uPA to ARPE-19 cells

To investigate whether TGF-β2 enhances the binding ability of uPA to the surface of ARPE-19 cells, ARPE-19 cells were pre-incubated with TGF-β2 and subjected to an IAsys-binding assay. As shown in Fig. 5A, both ARPE-19 cells pretreated with and without TGF-β2 bound to uPA in a time-dependent or cell-number-dependent fashion. The binding ability of uPA to ARPE-19 cells was enhanced by pretreatment with TGF-β2 (Fig. 5B).

3.6. Influence of u-PA-STOP® on ARPE-19 cell invasion

TGF-β2 was observed to cause a significant enhancement of invasive capacity 3 days after TGF-β2 (1 ng/ml and 10 ng/ml) stimulation compared with TGF-β2 (0.1 ng/ml) stimulation or in the absence of TGF-β2. TGF-β2 effects on cell invasion were strongly inhibited by u-PA-STOP® (Fig. 6A). The invasive capacity of ARPE-19 cells caused by TGF-β2 (1 ng/ml and 10 ng/ml) stimulation for 3 days was significantly increased compared with that with or without TGF-β2 (0.1 ng/ml; Fig. 6B).

4. Discussion

RPE cell invasion to the vitreous cavity is the initial step in the development of PVR. In this process, EMT is believed to be essential

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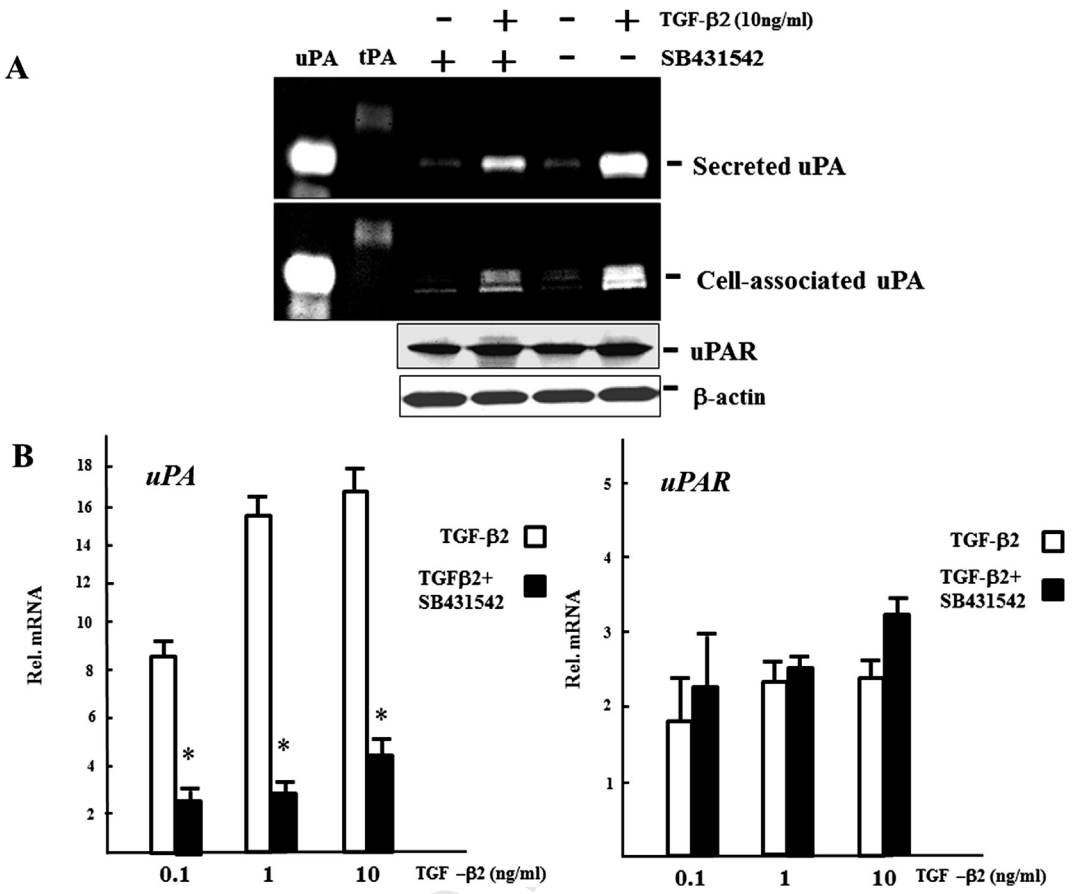

Fig. 4. The small molecule inhibitor of TGF-β (SB431542) inhibited TGF-β2-induced uPA secretion and expression, but not uPAR expression. (A) ARPE-19 cells were treated with TGF-β2 (10 ng/ml) in combination with SB431542 (10 μM) for 48 h. uPA protein secretion and expression were examined by fibrin zymography. uPAR expression was examined by western blot. (B) ARPE-19 cells were treated with TGF-β2 (10 ng/ml) in combination with SB431542 (10 μM) for 24 h. mRNAs of uPA and uPAR were examined by quantitative real-time PCR. The experiments were performed in triplicate. The error bars represent the SD. $^{*}P < 0.05$.

for RPE cells to acquire an invasive phenotype. However, the molecular mechanisms of invasive RPE cells undergoing EMT are poorly understood.

Our results show that uPA alters the response of RPE cells to TGF-β, particularly the ability to regulate cell proliferation and cause morphological change and invasion into a collagen gel matrix. Furthermore, we showed for the first time that uPA binds to ARPE-19 cells and the binding activity was enhanced by TGF-β2.

It is known that RPE cells show morphological trans-differentiation during an EMT (Casaroli-Marano et al., 1999; Lee et al., 2001). TGF-β was identified as the first factor that induced an EMT directly (Miettinen et al., 1994). The three forms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) have overlapping functions and mediate their effects through the TGF-β receptors and intracellular SMAD pathway (Shi and Massague, 2003). All three isoforms of TGF-β are expressed in the retina and the TGF-β2 isoform exists most abundantly in the tissue of the retina (Jobling et al., 2009; Pfeffer et al., 1994).

uPA was originally described as a matrix degrading enzyme. uPA converts plasminogen to plasmin. Plasmin activation of latent metalloproteinases (MMPs) leads to collagen degradation (Mazzieri et al., 1997).

In addition to the effects mediated by proteolysis on the cell surface, uPA binding to uPAR activates a number of cell signal proteins, including the Ras-extracellular signal-regulated kinase (ERK), the phosphatidylinositol 3-kinase (PI3K) and Rac 1, which regulate cell migration (Chandrasekar et al., 2003; Ma et al., 2002; Nguyen et al., 1998).

As uPAR is glycosyl-phosphatidylinositol (GPI)-anchored, uPAR mediated cell signaling requires co-receptors including integrins and growth factor receptors, such as the epidermal growth factor receptor (EGFR) (Jo et al., 2007; Wei et al., 1996).

In cancer cells, uPAR expression strongly correlates with their migratory and invasive phenotype (Ossowski et al., 1991; Wang, 2001). Moreover, there is a considerable amount of data on uPA/uPAR in EMTs in the field of cancer (Jo et al., 2009; Lester et al., 2007; Zhang et al., 2003).

Meanwhile, uPA/uPAR systems on EMT in RPE cells have been rarely studied. Few data are available on the TGF-β-induced expression of uPA and uPAR in RPE cells (Siren et al., 1999). Siren et al. (1999) reported that TGF-β1 upregulated uPAR expression in RPE cells. This is consistent with our results. Moreover, our results indicate that TGF-β enhances expression of uPA and uPAR, which could be involved in increased cell invasion and EMT.

The results presented in this study suggest that signal transduction of TGF-β on ARPE-19 cells could up-regulate uPA and uPAR expression. However, the signaling pathway of TGF-β, which induces up-regulation of uPA and uPAR, might be different. Since SB431542 blocks the EMT process observed in the fibroblastic phenotype, we employed SB431542 to clarify the relationship between TGF-β2 and uPA/uPAR expression during the EMT process. In the present study, as shown in Fig. 4A, TGF-β induced uPA and uPAR expression, while SB-431542 inhibited uPA expression but not uPAR expression. These results indicated that TGF-β-induced uPA expression is directly mediated by the TGF-β type 1 receptors ALK 4,

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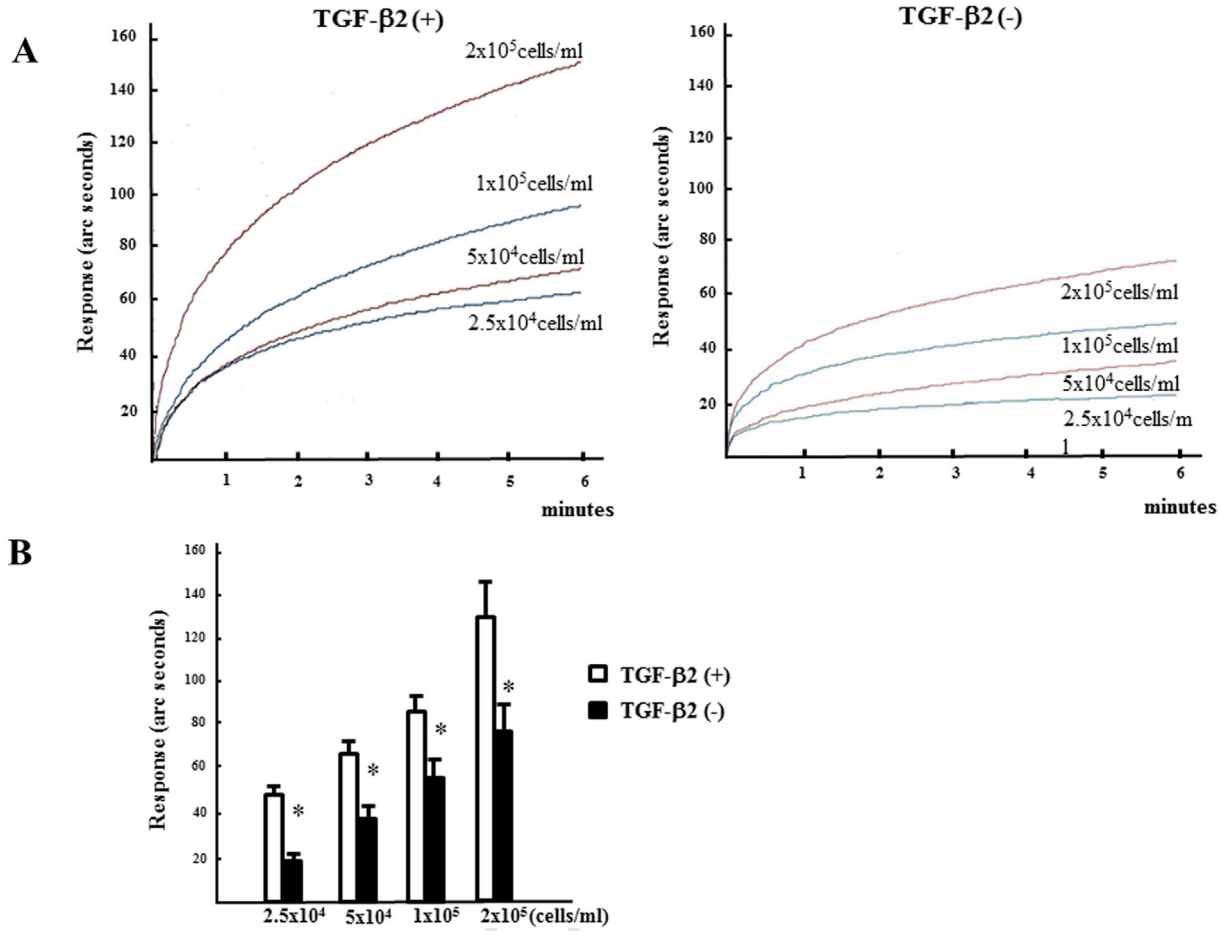

Fig. 5. Binding ability of ARPE-19 cells to uPA (A) Binding abilities of ARPE-19 cells to uPA were analyzed by the real-time biomolecular interaction assay system, IAsys, as described in Materials and methods. ARPE-19 cells were preincubated with or without TGF-β2 (10 ng/ml) for 24 h and they (2.5×10^4 to 2×10^5 cells) were added to a cuvette coated with uPA. The axis of ordinate shows the strength of binding between ARPE-19 cells and uPA. The axis of abscission shows the time-course of binding reaction. (B) Binding reaction between ARPE-19 cells pretreated with or without TGF-β2 and uPA were measured at 6 min. The experiments were performed in triplicate. The error bars represent the SD. $^*P < 0.05$.

5 and 7 but uPAR expression may not be directly mediated. TGF-β induced uPA expression is mediated by activating JNK- and ILK-signaling pathways (Lin et al., 2007; Santibanez, 2006). However, the precise mechanism by which TGF-β induces uPAR expression is still unclear. Yue et al. (2004) demonstrated that the TGF-β type 2 receptor is required for TGF-β-mediated JNK activation and up-regulation of uPAR. Wu et al. (2012) reported that Smad3 siRNA effectively inhibited TGF-β induced uPAR expression, but SB431542 did not effectively inhibit uPAR expression in human mononuclear phagocytes, suggesting that this may be due to the inefficiency of SB431542. Our results were similar to their results.

Further investigation is needed to elucidate the mechanism of uPAR up-regulation by TGF-β-dependent cell signaling in RPE cells. In this work, the interaction between uPA and ARPE-19 cells was examined by a surface plasmon biosensor. The analysis showed that the binding activity of uPA to ARPE-19 cells was in a time-dependent or cell-number-dependent manner and that TGF-β2 enhanced the binding activity of uPA to ARPE-19 cells. The present results suggest that TGF-β2 facilitates binding between uPA and uPAR. uPAR not only promoted uPA activity on the cell surface, but also formed a functional unit with integrin (Wei et al., 1996). uPAR participates in cell adhesion, migration and proliferation through the integrin-mediated pathway (Bernstein et al., 2004; Wei et al., 2007). Overexpression of uPAR in kidney epithelial cells promotes EMT responses via the interaction of uPAR with integrin α3β1 (Zhang et al., 2003).

In addition, TGF-β signaling alters integrin expression, which correlates with the process of cancer invasion (Galliher and Schiemann, 2006; Hood and Cheresh, 2002). The mechanism by which TGF-β2 enhances the affinity of uPA to uPAR is not clear at present. One interpretation of this effect is that TGF-β2 changes integrin expression, which subsequently forms a preferable structure for uPA-uPAR binding.

Finally, our results from the invasion assay suggested that uPA is necessary for TGF-β to affect RPE cell invasion given that there are reductions when u-PA-STOP® is add to media containing TGF-β. These data support the hypothesis that TGF-β induced enhancement of ARPE-19 cell invasion is mediated by uPA-dependent pericellular proteolytic activity. In addition, uPA/uPAR expression occurring in a manner that coincides with EMT may promote TGF-β induced ARPE-cell invasion. At present, the precise mechanism by which the uPA inhibitor abolished TGF-β stimulation of ARPE-19 cell invasion remains to be elucidated.

Integrins are a family of cell adhesion receptors that interact with many ECMs and are involved in cell attachment, migration and invasion. Integrins have also been considered to be important factors influencing the cell shape and intracellular signaling by interacting with molecules that regulate cytoskeletal organization and signal transduction (Hood and Cheresh, 2002).

Although our current results do not address the roles of uPA/uPAR and integrins, there is a possibility that integrin might be one

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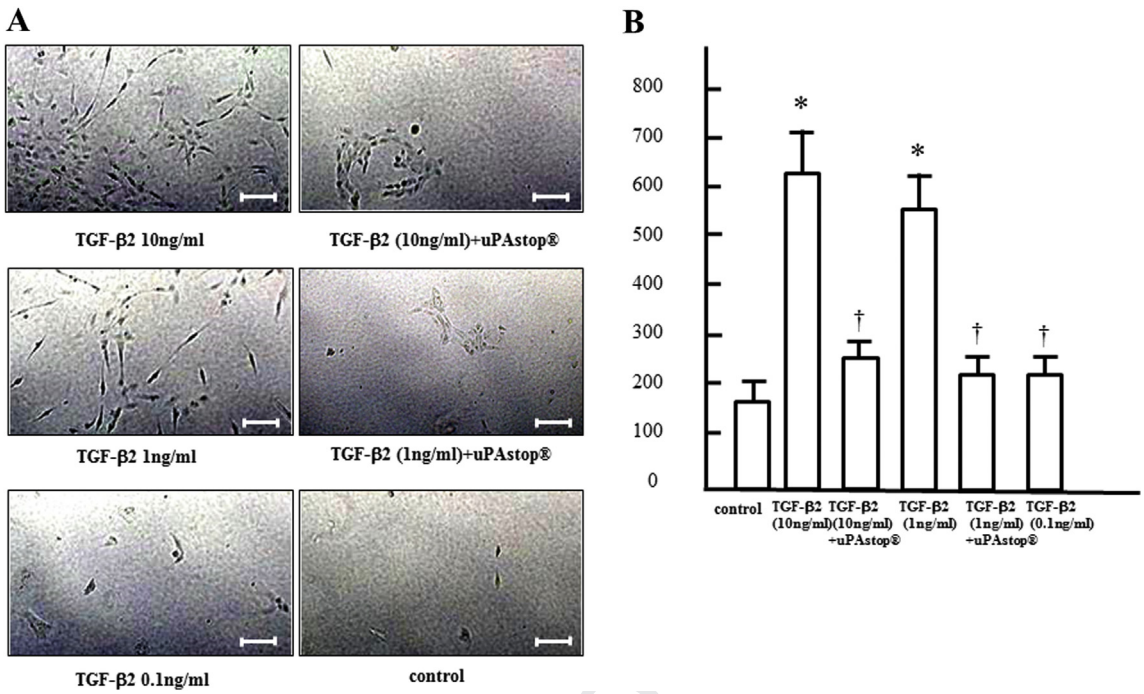

Fig. 6. TGF-β2-induced ARPE-19 cell invasion was inhibited by u-PA-STOP®. (A) ARPE-19 cells (3×10^5) seeded in the upper compartment of Transwell chambers were incubated in the absence and in the presence of TGF-β2 (0.1 ng/ml, 1 ng/ml and 10 ng/ml) for 3 days. u-PA-STOP® was added at 1 μg/ml together with TGF-β2 (1 ng/ml and 10 ng/ml). Scale bar, 100 μm. (B) The number of invaded cells was counted as indicated in Materials and methods. Each bar represents the mean (±SD) of triplicates. *$P < 0.05$. Similar results were obtained in two independent experiments. Significant difference compared with *the control, †TGF-β2 (10 ng/ml). $P < 0.05$.

of the key factors for the uPA/uPAR dependent invasiveness of ARPE-19 cells into a collagen gel matrix. Further study is required to clarify the mechanism of uPAR/integrin interaction.

5. Conclusion

The present study provides evidence that TGF-β-induced EMT-associated phenotype changes in ARPE-19 cells and the invasiveness of ARPE-19 cells into a collagen gel matrix are at least in part mediated by uPA. An antagonist of the type I receptor of TGF-β inhibited uPA expression and secretion but did not affect uPAR expression. Inhibition of uPA activity suppressed TGF-β-induced ARPE-19 cell invasiveness, suggesting that TGF-β-induced ARPE-19 cell invasiveness occurs via its regulation of uPA production. TGF-β2 also significantly promoted the binding activity of uPA to ARPE-19 cells. Results of the present study regarding the roles of the uPA/uPAR system may lead not only to better understanding of its biological functions but also to clinical applications of those molecules.

Acknowledgments

This work was partly supported by a Grant-in-Aid for Scientific Research (C) (23592596) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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