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Lycopene inhibits PDGF-BB-induced retinal pigment epithelial cell migration by suppression of PI3K/Akt and MAPK pathways

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ABSTRACT

Retinal pigment epithelial (RPE) cells play a dominant role in the development of proliferative vitreoretinopathy (PVR), which is the leading cause of failure in retinal reattachment surgery. Several studies have shown that platelet-derived growth factor (PDGF) exhibits chemotaxis and proliferation effects on RPE cells in PVR. In this study, the inhibitory effect of lycopene on PDGF-BB-induced ARPE19 cell migration is examined. In electric cell–substrate impedance sensing (ECIS) and Transwell migration assays, significant suppression of PDGF-BB-induced ARPE19 cell migration by lycopene is observed. Cell viability assays show no cytotoxicity of lycopene on RPE cells. Lycopene shows no effect on ARPE19 cell adhesion and is found to inhibit PDGF-BB-induced tyrosine phosphorylation and the underlying signaling pathways of PI3K, Akt, ERK and p38 activation. However, PDGF-BB and lycopene show no effects on JNK activation. Taken together, our results demonstrate that lycopene inhibits PDGF-BB-induced ARPE19 cell migration through inhibition of PI3K/Akt, ERK and p38 activation.

Introduction

Proliferative vitreoretinopathy is a major complication in rhegmatogenous retinal detachment and serious ocular trauma. It is the leading cause of failure in retinal reattachment surgery [1,2]. The formation of PVR membranes is originated from the transient breakdown of blood–retinal barrier which promotes the proliferation and migration of retinal pigment epithelial (RPE) cells, fibroblasts, glial cells and macrophages into the vitreous and subretinal space. The contraction of PVR membranes may lead to retinal detachment and blindness [1,3,4]. Among these cells, there are strong indications that RPE cells play a dominant role in the development of PVR [1,5].

There are several growth factors, released through a break in the blood–retinal barrier, that are expressed in the PVR membranes [6–9]. Among these growth factors, PDGF exhibits a high-level of chemotaxis and proliferation effects on RPE cells [1]. These findings indicate that PDGF-induced RPE cell proliferation and migration are an important step in PVR development.

Lycopene, a member of carotenoids, is present in tomatoes and other fruits with red color. It is one of the most potent antioxidants among dietary carotenoids and possesses photoprotection, antioxidant effects, immunomodulation and anticancer activities [10,11]. Besides, lycopene has shown the capabilities to regulate gap-junction communication and gene function, and inhibit cancer cell migration [12,13]. Owing to their lipophilic nature, lycopene and related tomato carotenoids are found to concentrate in the adrenal gland, testes, liver and prostate gland [12,14,15]. In addition, they are present at biologically significant concentrations in human RPE and choroids [16].

We have demonstrated that lycopene can inhibit PDGF-BB-induced signaling and migration in human dermal fibroblasts through direct binding with PDGF-BB [17,18]. Thus, we propose that lycopene can also inhibit PDGF-BB-induced RPE cell proliferation and migration through the same mechanism. In this study, we investigated the inhibitory effect of lycopene on PDGF-BB-induced ARPE19 cell migration and the possible mechanisms involved. These mechanisms include the influence of lycopene on ARPE19 cell adhesion, migration, and the expression of anti-phosphotyrosine antibodies (4G10), phosphatidylinositol-3 kinase

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(PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathway activation.

Materials and methods

Materials. Bovine serum albumin (BSA), aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium fluoride (NaF), and sodium orthovanadate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human plasma fibronectin was from Invitrogen Life Technologies (Carlsbad, CA). Antibody (Ab) raised against phospho-extracellular signal-regulated kinase 1/2 (ERK1/2) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ab raised against phosphotyrosine (4G10) was from Upstate Biotech, Inc. (Lake Placid, NY, USA). Abs raised against total ERK1/2 and phospho-p38 were from R&D systems, Inc. (Minneapolis, MN, USA). Abs raised against phospho-c-Jun N-terminal kinase (JNK), JNK and p38 were from New England Biolabs, Inc. (Beverly, MA, USA). Lycopene was purchased from Extrasynthese (Genay, France). Tetrahydrofuran (THF) containing 0.025% butylated hydroxytoluene as an antioxidant was purchased from Sigma Chemical Co., and used as a vehicle to dissolve lycopene.

Cell cultures. Adult human retinal pigment epithelial cells (ARPE19) were purchased from Food Industry Research and Development Institute (Hsinchu, Taiwan). They were maintained in DMEM/F12 supplemented with 10% fetal calf serum (GibcoBRL, Invitrogen Life Technologies, Carlsbad, CA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma Chemical Co., St. Louis, MO). The cells were cultured in a humidified incubator at 37 °C and 5% CO_2. For most of the experiments, cells reaching a 90–95% of confluence were starved and synchronized in serum-free DMEM/F12 for 24 h before they were subjected to further analysis.

Lycopene treatment and PDGF-BB incorporation. Lycopene was dissolved in tetrahydrofuran (THF) to a concentration of 15 mmol/L. This stock solution was prepared with minimal exposure to air and light and stored at −70 °C. Immediately before the experiment, THF–lycopene aliquots from the stock solution were added to the serum-free cell culture medium to a final concentration of 1, 3, 10 μmol/L. The final concentration of THF in the serum-free cell culture medium was 0.1%, which did not affect the assays as indicated by comparison with the control medium. In the ECIS and Transwell migration assays as well as the Western blot analysis, the serum-free cell culture medium with various concentrations of lycopene were all pre-incubated with or without PDGF-BB (20 ng/ml) at 37 °C for 30 min.

ECIS migration assay. We used the electric cell–substrate impedance sensing (ECIS) system (Applied Biophysics, Inc., Troy, NY) to conduct this migration assay. ARPE19 cells were cultured in 8W1E ECIS arrays (Applied Biophysics), in which each well for cell culture contains a small gold film circular electrode (5×10^{-4} cm^2) and a larger (0.15 cm^2) counter electrode. We seeded ARPE19 cells at a density of 70,000 cells/well in the arrays which were incubated for 24 h. After applying an elevated voltage pulse with a frequency of 40 kHz, amplitude of 4 V for 10 s, dead and detached cells were present on the small active electrode. The medium was then changed to serum-free cell culture medium with various concentrations of lycopene pre-incubated with or without PDGF-BB (20 ng/ml) at 37 °C for 30 min. Cells surrounding the small active electrode that had not been submitted to the elevated voltage pulse then migrated inward to replace the killed cells. Cell migration was assessed by continuous resistance measurements for 30 h.

Transwell migration assay. Transwell migration assay with ARPE19 cells were performed by using a modified Boyden chamber model (Transwell apparatus, 8.0 mm pore size, Costar). For detection of RPE cell migration in the Transwell, the lower face of each polycarbonate filter (Transwell insert) was coated with fibronectin (0.3 mg) for 30 min in the laminar flow hood. The lower chambers filled with 0.6 ml of serum-free medium or PDGF-BB (20 ng/ml)-containing medium were pre-incubated with various concentrations of lycopene. RPE cells (5×10^4 cells, 200 μl) were plated to the upper chamber. After 5 h of incubation, all non-migrant cells were removed from the upper faces of the Transwell membranes with a cotton swab and migrated cells were fixed and stained with 0.5% toluidene blue in 4% PAF. Migration was quantified by counting the number of stained cells per 100× field (high power field, HPF) in images taken with a phase-contrast microscope (Leica DMIL1).

Cell viability assays. Cell viability of lycopene was studied by a trypan blue exclusion assay (cell counting assay) and a luminescence assay of cellular ATP. For the trypan blue exclusion assay, cells were trypsinized, collected and stained with trypan blue. Hemacytometer-counting viable cells were determined under the phase-contrast microscope. The luminescence assay of cellular ATP was performed by using ATPlite 1step kit (Perkin–Elmer, MA, USA) for the quantitative evaluation of cell viability according to the manufacturer's protocol. Briefly described, ARPE19 cells were cultured on 96-well plates (Costar, Cambridge, MA) at a concentration of 10,000 cells/well for 24 h and then starved for another 24 h. After being washed with serum-free cell culture medium, the cells were treated with 0.1% THF or different concentrations of lycopene and incubated at 37 °C for 24 h. The cells were then mixed with 100 μl ATPlite per well and shaken at 700 rpm for 2 min. Afterwards, the 96-well plates were subjected to measurements with a Wallac Victor 3 1420 multilabel counter (Perkin–Elmer, Turku, Finland) using excitation and emission wavelengths at 485 and 535 nm, respectively.

Adhesion assays. 96-well plates were coated with 50 μl fibronectin (15 μg/ml in PBS, pH 7.4) per well and incubated at 37 °C for 24 h. After being washed with PBS three times, unspecified binding was blocked by 100 mg/ml bovine serum albumin (Sigma–Aldrich) in PBS at room temperature for 1 h. ARPE19 cells were trypsinized and resuspended in serum-free cell culture medium and labeled with BCECF/AM (10 mg/ml) for 30 min at 37 °C. After being washed with serum-free cell culture medium, the labeled cells were resuspended in serum-free cell culture medium with different concentration of lycopene to a density of 1.0×10^5 cells/ml and incubated for another 30 min at 37 °C. The suspended cells were then applied onto 96-well plates containing 100 μl serum-free cell culture medium with different concentrations of lycopene per well and incubated at 37 °C for 1 h. After gentle washes with PBS three times, the non-adherent cells were aspirated and the 96-well plates were subjected to measurements by Wallac Victor 3 1420 multilabel counter (Perkin–Elmer, Turku, Finland) using excitation and emission wavelengths at 485 and 535 nm, respectively. Afterwards, the cells were photographed to record their morphological change through a phase-contrast microscope (Olympus IX71).

Cell lysate preparation and Western blot analysis of JNK, ERK, p38, PI3K, Akt and anti-phosphotyrosine antibodies (4G10). ARPE19 cells cultured on 6 cm dishes were starved for 24 h and then treated with various concentrations of lycopene which were pre-incubated with or without PDGF-BB (20 ng/ml) at 37 °C for 30 min. 4G10, PI3K, Akt, ERK, JNK and p38, phosphorylations were analyzed. They were then lysed in a radioimmunoprecipitation assay buffer. After sonication, the lysate was centrifuged (14,000*g* for 10 min at 4 °C), and the supernatant was removed. The protein content was quantified by a Pierce protein assay kit (Pierce, Rockford, IL). Total protein was separated by electrophoresis on 8% SDS–polyacrylamide gels. The proteins were then electroblotted onto PVDF membranes and probed using the specific antibodies mentioned. Immunoblots were detected by enhanced chemiluminescence (Chemiluminescence Reagent Plus from NEN, Boston, MA).

Statistical analysis. All data are analyzed with SigmaPlot 2002 for Windows (Version 8.00). Data are expressed as mean ± standard error (SE). Comparison of the means of two groups of data is made by using the unpaired, two-tailed Student *t* test.

Results

Lycopene inhibits PDGF-BB-induced ARPE19 cell migration

In the ECIS migration assays, we observed that ARPE19 cell migration was enhanced by PDGF-BB but the enhancing effect was significantly suppressed by lycopene. However, lycopene did not inhibit ARPE19 cell migration when PDGF-BB was not present (Fig. 1A). In the Transwell migration assays, a significant number of ARPE19 cell migrations (about 20 migrated cells in HPF) on fibronectin was observed in the absence of PDGF-BB (Fig. 1B, upper panel). This suggests that fibronectin is a "chemoattractant" for ARPE19 cells. PDGF-BB stimulated ARPE19 cell migration was about three to four times more frequent than that elicited by fibronectin alone. However, we observed that lycopene suppressed PDGF-BB-induced ARPE19 cell migration on fibronectin (Fig. 1B, middle panel). Quantitative analysis indicates that nearly 100% of migration was inhibited with 10 μM of lycopene (Fig. 1B, lower panel). These observations indicate that lycopene is effective in the prevention of PDGF-BB-induced ARPE19 cell migration.

Lycopene shows no cytotoxicity on ARPE19 cells

Fig. 2A shows that lycopene (1–15 μM) treatment did not significantly affect cell viability in the trypan blue exclusion (cell counting) assays. In the luminescence assay of cellular ATP, no effect on cellular ATP metabolism in ARPE19 was observed during the 24 h of incubation with lycopene (Fig. 2B). The results indicate that the effects of lycopene on ARPE19 cell migration do not decrease cell viability.

Lycopene shows no effect on ARPE19 cell adhesion

As shown in Fig. 3C, the fluorescence value was increased about 2.5-fold in the wells coated with fibronectin. The photograph shown in Fig. 3B also reveals obvious morphological change in these wells. These observations show that the presence of fibronectin induces ARPE19 cell adhesion to the plate. However, lycopene showed no influence on both the morphological change and the amount of cell adhesion (Fig. 3B). The results indicate that the inhibitory effect of lycopene on ARPE19 cell migration is not by interfering with the attachment of the cells to fibronectin.

Lycopene inhibits PDGF-BB-induced PDGFR phosphorylation and downstream PI3K/Akt and MAPK pathway activation

To determine whether PDGF-BB-induced signaling pathway is affected by lycopene, the extent of phosphorylation of PDGFR and its downstream components in ARPE19 cells were investigated. Fig. 4A shows that stimulation of ARPE19 cells with PDGF-BB resulted in PDGFR phosphorylation, as determined by Western blotting with antibody directed against phosphotyrosine. PI3K and Akt phosphorylation were also increased by PDGF-BB stimulation. Pre-incubation of PDGF-BB with lycopene resulted in a marked inhibitory effect on PDGFR, PI3K and Akt phosphorylation in a concentration-dependent manner (Fig. 4A). Among MAPKs, ERK and p38 phosphorylation were also increased after the cells were stimulated with PDGF-BB and the increases were suppressed by pre-incubation with lycopene in a concentration-dependent manner (Fig. 4B). By

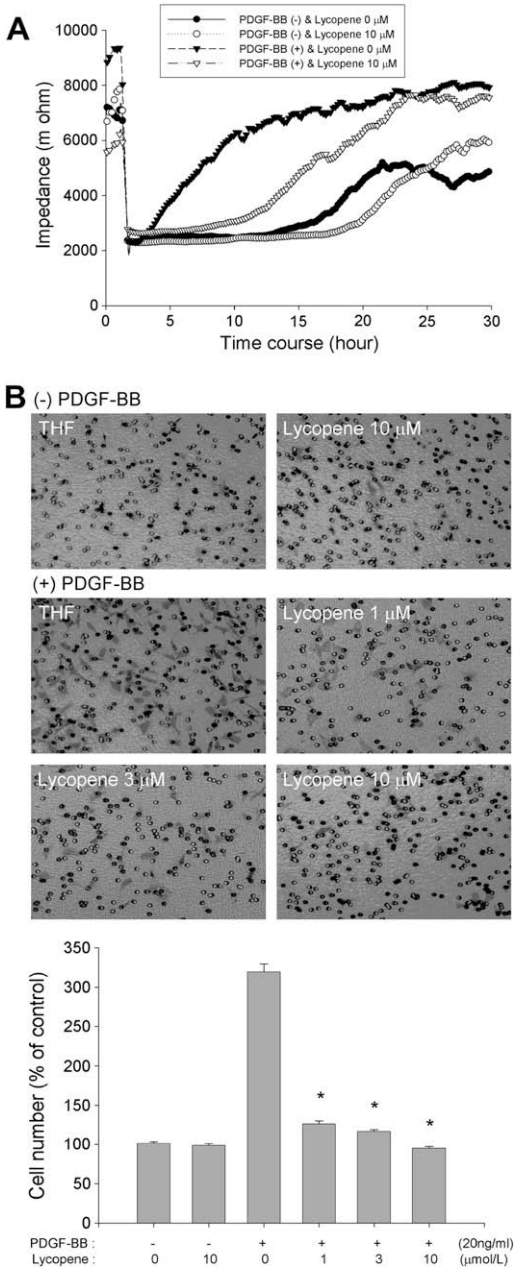

Fig. 1. PDGF-BB-induced ARPE19 cell migration was inhibited by lycopene in ECIS and Transwell migration assays. (A) In ECIS migration assay, the ARPE19 cells were treated with different combinations of PDGF-BB (20 ng/ml) and lycopene (0 μM indicates that it contained only THF) before being pre-incubated together at 37 °C for 30 min. Cell migration was then assessed by continuous resistance measurements for 30 h. In the well containing PDGF-BB but not lycopene, the impedance, which represents the number of migrated cells, increased sharply during the first 10 h. By contrast, the impedance in the well containing both PDGF-BB and lycopene increased slowly during the same time. Comparing to these two wells, the impedance in the well containing no PDGF-BB increased mildly and gradually regardless of whether it contained lycopene. (B) In the Transwell migration assay, ARPE19 cells (5×10^4 in 200 μl) were seeded in the upper chambers in the absence or presence of lycopene. The Transwell inserts coated with 15 μg/ml fibronectin were assembled in the lower chambers, which were filled with 600 μl serum-free ((−)PDGF-BB) (upper panel) or 20 ng/ml PDGF-BB-containing medium ((+)PDGF-BB) (middle panel) and pre-incubated with various concentration of lycopene for 30 min at 37 °C. After incubating for 5 h at 37 °C, ARPE19 cells that had migrated to the underside of the filter membranes were photographed (upper and middle panels) and counted with a phase-contrast light microscope under high power field (magnification, 100×) (lower panel). All experiments were conducted in duplicates and similar results were obtained at least two to three times. The results are expressed as percentage of control and shown as mean ± standard errors (SE). *Significantly different from cells stimulated with PDGF-BB alone ($P < 0.05$).

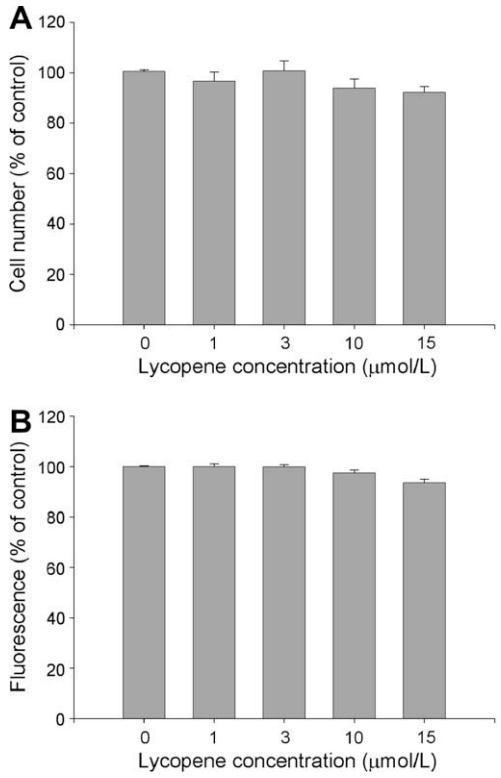

Fig. 2. Viability of ARPE19 cells was not markedly influenced by lycopene. The cells were treated with different concentration of lycopene for 24 h after being starved for 24 h. Cell viability was determined by (A) trypan blue exclusion assay and (B) luminescence assay of cellular ATP. The results are expressed as percentage of control and shown as mean ± standard errors (SE) determined from three independent experiments.

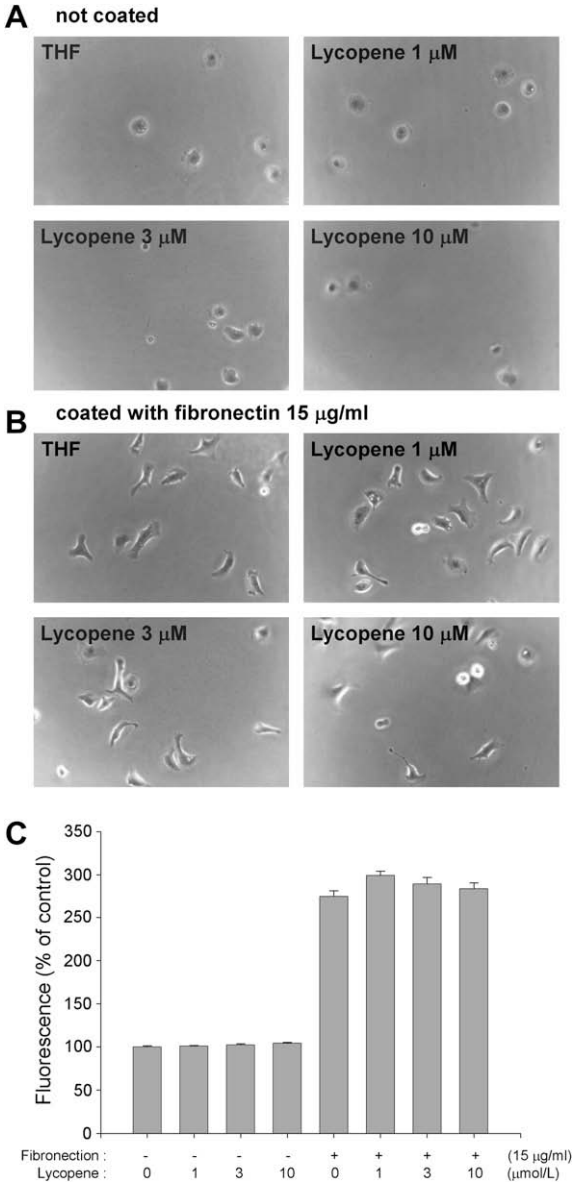

Fig. 3. ARPE19 cell adhesion was not affected following lycopene treatment. BCECF labeled cells were treated with THF or lycopene for 30 min and then seeded and allowed to undergo adhesion on plates (A) with or (B) without fibronectin (15 μg/ml) precoated at 37 °C for 1 h. Cells photographed under a phase-contrast microscope were used for morphological analysis. (C) Fluorescence was measured using excitation and emission wavelength at 485 and 535 nm, respectively. The results are expressed as percentage of control and shown as mean ± standard errors (SE) determined from three independent experiments.

contrast, we found that JNK phosphorylation was not influenced by PDGF-BB stimulation.

Discussion

Lycopene, responsible for the characteristic deep-red color of tomatoes, represents more than 80% of total tomato carotenoids [19]. A focus of current carotenoid researches is on the possible role of this group of compounds in the protection against age-related macular degeneration (AMD) [20]. The antioxidants including lycopene, zeaxanthin and lutein significantly reduce lipofuscin formation and the development of age-related macular degeneration [21]. The two most well-known hypotheses for the protective role are based on the ability of these carotenoids to filter out phototoxic short-wavelength visible light and their efficient capacities to quench light-induced free radicals such as singlet oxygen [22].

In PVR, RPE cells are stimulated by chemotactic factors, such as PDGF, to migrate away from monolayer into a provisional extracellular matrix (ECM) where they participate in epiretinal membrane formation [1,23,24]. In the early stage of PVR, provisional ECM components, including fibronectin, are synthesized and deposited on the retinal surfaces [25]. Studies have shown that PDGF is a strong chemotactic factor for RPE cells in the presence of fibronectin [26,27]. The current study provides evidence that lycopene results in a significant inhibition of PDGF-BB-induced RPE cell migration without cytotoxicity. Chemotactic migration is a complex phenomenon involving adhesion to ECM, cell motility, and the effects of chemotactic factors [28–30]. Our results indicate that RPE cell adhesion with morphological change was significantly increased on fibronectin coated plates compared with non-coated ones. However, we found that lycopene did not affect the RPE cell adhesion and morphological change on fibronectin. The inhibition in PDGF-BB-induced cell migration coincided with reduced activation of PDGF-BB-induced Akt, ERK and p38, suggesting that lycopene inhibits cell migration via inhibition of the PI3K and MAPKs cascade.

In our system, we found that pre-incubation of PDGF-BB with lycopene results in a marked inhibitory effect on its signaling in RPE cells, including phosphorylation of PDGFR-β. Recently we have found that lycopene can directly bind to rat PDGF-BB and inhibit PDGF-BB-induced signaling in aortic smooth muscle cells and skin fibroblast [17,31]. Therefore, lycopene may also influence the function of PDGF-BB through similar mechanisms in human RPE cells. It binds to PDGF-BB, subsequently blocks the interaction of PDGF-BB with its receptors and inactivates PDGF-BB functions.

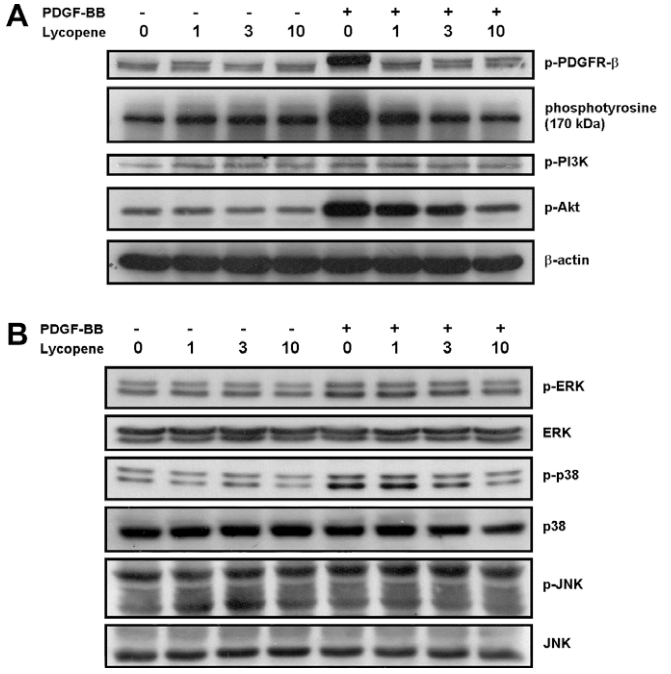

Fig. 4. Effects of lycopene on PDGF-BB-induced PDGFR, PI3K/Akt and MAPKs phosphorylation. (A) Lycopene inhibited PDGF-BB-induced PDGFR and PI3K/Akt activations in a concentration-dependent manner. (B) Lycopene inhibited PDGF-BB-induced ERK and p38 activation in a concentration-dependent manner, but PDGF-BB did not induce JNK activation. ARPE19 cells were treated with various concentrations of lycopene and pre-incubated with or without PDGF-BB (20 ng/ml) at 37 °C for 30 min. After further incubation, the cells were collected and lysates were analyzed by Western blot analysis. The result is representative of three to four experiments.

This study provides the first evidence that lycopene inhibits PDGF-BB-induced ARPE19 cell migration, and phosphorylation of PDGF receptor, PI3K/Akt, ERK1/2 and p38 signaling. The findings presented here provide an important basis for further explorations toward understanding the mechanisms by which lycopene affects RPE cells and possibly its beneficial effect in the preventions of AMD and PVR.

Acknowledgments

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