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Lycopene binds PDGF-BB and inhibits PDGF-BB-induced intracellular signaling transduction pathway in rat smooth muscle cells

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ABSTRACT

Cardiovascular diseases (CVDs) result from the sub-endothelial accumulation of inflammatory cells and smooth muscle cells (SMCs). Lycopene, a natural compound from tomato, has been suggested to play a role in CVD prevention. However, its action mechanism is still largely unknown. In this study, we examined the effect of lycopene on SMCs. We found that preincubation of PDGF-BB with lycopene resulted in a marked inhibition on PDGF-BB-induced PDGF receptor-β (PDGFR-β), PLCγ, and ERK1/2 phosphorylation in rat A10 SMCs and primary cultured aortic SMCs. In striking contrast, lycopene did not influence EGF-induced ERK1/2 phosphorylation. Surprisingly, further analysis indicates that lycopene could directly bind PDGF-BB and inhibit PDGF-BB-SMC interaction, as determined by dot binding assay and Western blotting. In functional studies, lycopene inhibited PDGF-BB-induced SMC proliferation and migration toward gelatin and collagen at concentrations ranging from 2 to 10 μM. On the contrary, lycopene did not inhibit bFGF- and VEGF-induced endothelial cell migration. Gelatin zymography demonstrated that lycopene's effect on SMC migration was not due to the inhibition of matrix metalloproteinases (MMPs). Taken together, our results provide the first evidence showing that lycopene inhibits PDGF-BB-induced signaling, proliferation and migration in rat A10 and aortic SMCs. One of the action mechanisms is that lycopene is capable of binding PDGF-BB and inhibiting its interaction with SMC, which is quite different from those previously developed PDGFR-β antagonists. The results presented here may help us to better understand the beneficial effects of lycopene in CVD prevention.

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Abbreviations: Ab, antibody; EGCG, (−)-epigallocatechin-3-gallate; mAb, monoclonal antibody; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; EGF, epidermal growth factor; ERK, extracellular matrix-regulated kinase; PDGF, platelet-derived growth factor; PDGFR-β, PDGF receptor β; SMC(s), smooth muscle cell(s)

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1. Introduction

Abnormal vascular smooth muscle cell (SMC) proliferation and growth factors such as the platelet-derived growth factor (PDGF) play an important role in the development and progression of proliferative cardiovascular diseases (CVDs), including hypertension, restenosis, and atherosclerosis [1–3].

Lycopene, responsible for the characteristic deep-red color, represents more than 80% of total tomato carotenoids [4]. Dietary intakes of tomatoes and tomato products containing lycopene and other carotenoids have been shown to be associated with decreased risk of chronic diseases such as cardiovascular and cancer diseases in numerous studies [5]. For example, several epidemiological studies have shown that low plasma lycopene concentration is associated with increased intima-media thickness (IMT) of the carotid artery wall in middle-aged men living in eastern Finland [6,7]. The Los Angeles Atherosclerosis Study also suggests that lycopene is protective against IMT progression among smokers [8].

Recently, some experimental studies using in vitro systems, animals, and human intervention trials were carried out to investigate the role of lycopene in the prevention of CVDs. For example, it has been shown that serum lycopene concentration was inversely related to soluble intercellular adhesion molecule-1, a marker of endothelial function and inflammation [9]. Lycopene inhibits IL-1β-induced expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1), reducing U937 monocytes adhesion to human endothelial cells [10]. Moreover, lycopene with α-tocopherol enhances platelet-activating factor biosynthesis in endothelial cells [11].

PDGF plays an important role in stimulating SMC proliferation and migration during atherosclerosis [3]. PDGF is increased in macrophages and SMCs [12] and its receptor in SMCs is also increased in eccentric lesions of atherosclerosis, which promotes SMC migration and proliferation and extracellular matrix (ECM) production [13]. Carpenter and co-workers [14] have shown that lycopene and other carotenoids can inhibit serum-induced human SMC proliferation, however it is still largely unknown how lycopene affects SMC's functions.

In the present study, we investigated lycopene's effects on PDGF-BB-induced signaling transduction pathway in rat A10 SMC and primary cultured aortic SMCs. Our results indicate that lycopene specifically inhibits PDGF-BB-induced signaling pathway through a novel action mechanism, i.e. binding to PDGF-BB and inhibiting its interaction with SMC. In functional studies, we also found that that lycopene inhibits PDGF-BB-induced SMC migration and proliferation.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), bovine type I collagen, gelatin, (−)-epigallocatechin-3-gallate (EGCG), protease inhibitors for Western blotting, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies raised against phospho-ERK1/2 (E-4) and PDGFR-β were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies raised against phospho-PDGFR-β at Tyr^{716} and phosphotyrosine (4G10) were from Upstate Biotech Inc. (Lake Placid, NY, USA). Ab directed against phospho-PLCγ (Tyr^{783}) was from New England Biolabs, Inc. (Beverly, MA, USA). Recombinant rat PDGF-BB and antibodies raised against native PDGF-BB, EGF and total ERK1/2 were from R&D systems, Inc. (MN, USA). Recombinant EGF was from Invitrogen Life Technologies (Carlsbad, CA, USA). α-Tubulin was purchased from Calbiochem EMD Bioscience Inc. (San Diego, CA, USA). Lycopene was purchased from Extrasynthese (Genay Cedex, France) and β-carotene was from Nacalai Tesque, Inc. (Kyoto, Japan). Lycopene used in this study contains about 93% of trans-isomer and 7% of cis-isomers by HPLC analysis using the previously described protocol [15,16]. Tetrahydrofuran (THF), containing 0.025% butylated hydroxytoluene as an antioxidant, was purchased from Sigma Chemical Co. and used as a vehicle to dissolve lycopene and β-carotene. Lycopene and β-carotene were prepared fresh prior to use.

2.2. Cell culture

Rat aortic SMCs (RASMCs) were isolated from Sprague-Dawley rat. Aortic strips were cut into small pieces and placed in six-well culture plates. These explants were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (250 ng/ml) (Invitrogen Life Technologies). RASMCs were identified by their characteristic ''hill and valley'' growth pattern and the presence of SMC-specific α-actin. Four to six passage cells were used in this study. Rat thoracic aorta smooth muscle cells (A10) and human umbilical endothelial cells (HUVECs) were purchased from Food Industry Research and Development Institute (Hsinchu, Taiwan). A10 SMCs were cultured in DMEM containing 10% FBS, antibiotics and fungizone and HUVECs were cultured in medium 199 with 25 U/ml heparin, 30 μg/ml endothelial cell growth supplement (ECGS; Upstate Biotech Inc.) containing 10% FBS, antibiotics and fungizone. Otherwise where indicated, cells reaching 80–90% of confluency were starved and synchronized in DMEM containing 0.5% FBS at 37 °C for 24 h and then subjected to further analysis.

2.3. Cell proliferation assays

Cell proliferation was determined by MTT assay, cell counting assay and luminescence assay of cellular ATP. For MTT assay, cells reaching 80% of confluency were starved and then incubated with or without lycopene in the absence or presence of PDGF-BB. After 24 h, MTT assay was performed as previously described [17]. Briefly, cells were incubated with 0.5 mg/ml MTT for 2 h at 37 °C. Formazan crystals resulting from MTT reduction were dissolved by adding 200 μl DMSO and gently agitated for 20 min. The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 550 nm. For cell counting, cells were trypsinized, collected and stained by 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 proliferation according to the manufacturer's protocol.

2.4. Cell lysate preparation and Western blot analysis

For cell lysate preparation, SMCs were washed with prechilled PBS and lysed in radioimmunoprecipitation assay buffer (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA. 1 mM sodium fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 μg/ml aprotinin and leupeptin (freshly prepared)). After sonication, the lysate was centrifuged (14,000 × *g* for 10 min at 4 °C), and supernatant was transferred to a tube. The protein content was quantified by Pierce protein assay kit (Pierce, Rockford, IL). Total protein was separated by electrophoresis on SDS-polyacrylamide gels and the proteins were electroblotted onto PVDF membranes and then probed using primary anti-phospho-ERK (0.2 μg/ml), anti-phospho-PLCγ (1 μg/ml), anti-phosphotyrosine (1 μg/ml) or anti-phospho-PDGFR-β (1 μg/ml) mAbs. Immunoblots were detected by enhanced chemiluminescence (ECL, Chemiluminescence Reagent Plus from NEN, Boston, MA). For some experiments, membranes were stripped with a striping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS and 100 mM β-mercaptoethanol), washed, and reprobed with anti-PDGFR-β (0.5 μg/ml), anti-α-tubulin (0.5 μg/ml), or anti-ERK1/2 (0.5 μg/ml) antibodies and developed as described above.

2.5. Dot binding assay

Nitrocellulose (NC) membrane (Bio-Rad Laboratories, Hercules, CA, USA) was soaked in buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 30 s. Recombinant PDGF-BB and BSA (both 2 μg/ml, 50 μl) were applied to the membrane on Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Hercules) by suction. Vehicle (THF), lycopene (2.5 μl), β-carotene (2.5 μl) and (−)-epigallocatechin-3-gallate (EGCG, in PBS) were directly spotted on the same membrane due to little volume of samples. The membrane was then blocked with BSA (5% in PBS) for 0.5 h. After washing with PBS, the membrane was incubated with PDGF-BB (0.5 μg/ml) in PBS or human plasma for 1 h at RT. A brief wash was followed and the membrane was then incubated with anti-PDGF-BB Ab (2 μg/ml in 1% BSA-containing PBS) for 1 h at RT. After a brief wash, the membrane was incubated with horse-raddish peroxidase-conjugated Ab and then developed by ECL. For some experiments, the membrane was incubated with EGF, followed by anti-EGF Ab (0.5 μg/ml) in PBS and developed as described above.

2.6. Cell migration assay

Migration assay with SMCs and HUVECs was performed using a modified Boyden chamber model (Transwell apparatus, 8.0-μm pore size, Costar) [18]. Briefly, the lower face of polycarbonate filters (Transwell insert) were coated with type

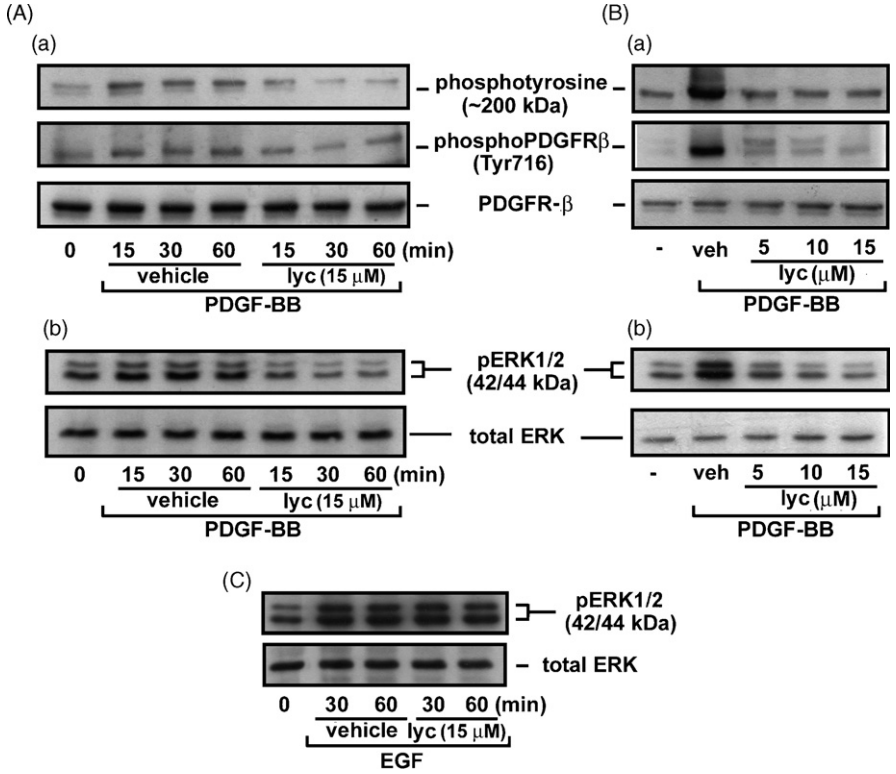

Fig. 1 – Effect of lycopene on PDGF-BB-induced signaling in A10 SMCs. Medium containing PDGF-BB (10 ng/ml) was preincubated with (A) vehicle or lycopene for the indicated times or with (B) vehicle or varying concentrations of lycopene for 30 min. After incubation, vehicle- or lycopene-treated PDGF-BB were added to rat A10 SMCs and incubated for 8 min. PDGFR-β and ERK1/2 phosphorylation were determined by Western blotting. (C) EGF (50 ng/ml) was preincubated with lycopene for the indicated times and was added to A10 SMCs for 8 min. Cell lysates were analyzed by Western blotting. Each blot was representative of three independent experiments.

I collagen (10 μg/ml) or gelatin (20 μg/ml) for 30 min in the laminar flow hood. The lower chamber was filled with serum-free or growth factor-containing medium preincubated with vehicle (THF) or lycopene for 30 min. Rat SMCs or HUVECs (2.5×10^5 cells/ml) were plated to the upper chamber in the presence of vehicle or lycopene. After 3 h of incubation, all nonmigrant cells were removed from the upper face of the Transwell membrane with a cotton swab and migrant 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) under a phase-contrast microscope (Leica DMIL®) and photographed.

2.7. Gelatin zymography

SDS-substrate zymography electrophoresis was performed using a previously described method [18]. Briefly, SMC-conditioned media were centrifuged and equal volume of each sample was separated on an 8.5% polyacrylamide gel containing 0.1% gelatin. Substrate digestion was performed by incubating the gel in 50 mM Tris–HCl, pH 7.6, containing 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 1% Triton X-100 and 0.02% NaN_3 at 37 °C for 24 h. The gel was stained with 0.1% Coomassie Brilliant Blue R250 (Sigma Co., USA), and the location of gelatinolytic activity was detected as clear bands.

2.8. Statistical analysis

Data were expressed as mean ± standard error mean (S.E.M.). Comparison of means of two groups of data was made by using the unpaired, two-tailed Student's *t* test. In some experiments, one-way ANOVA was applied and followed by the Student's *t* test.

3. Results

3.1. Lycopene inhibits PDGF-BB-induced PDGFR-β, PLCγ, and ERK phosphorylation

It has been reported that PDGF-BB binding to PDGFR (PDGF receptor) is associated with dimerization, autophosphorylation, clustering and activation of PDGFR-tyrosine kinase activity [19], subsequently causing SMC proliferation through activation of ERK1/2 and other signaling enzymes [20]. To examine lycopene's effect on PDGF-BB-induced signaling transduction pathway, the activation of PDGFR-β and its downstream kinases in A10 SMCs was determined. Preliminary results indicate that PDGF-BB-induced PDGFR-β and ERK1/2 phosphorylation was inhibited by lycopene in rat A10 SMCs. However, PDGF-BB-induced signaling was not affected if the cells were pretreated with lycopene and followed by removing of extracellular lycopene (data not shown). This suggests that lycopene possibly affects PDGF-BB-SMC interaction.

To test this hypothesis, an indirect assay for the determination of lycopene-PDGF-BB interaction was performed. Medium containing PDGF-BB was preincubated with vehicle or lycopene for a certain time and was added to SMCs. Surprisingly, the preincubation resulted in a marked inhibition on PDGF-BB-induced PDGFR-β and ERK1/2 phosphorylation in A10 SMCs. The inhibition was time- and concentration-dependent (Fig. 1A and B). In contrast, EGF-induced ERK1/2 phosphorylation was not inhibited even a longer preincubation with lycopene (Fig. 1C). We then tested whether lycopene has a similar inhibitory effect on primary cultured rat aortic SMCs (RAMSCs) isolated from Sprague-Dawley rat aorta. It was found that PDGF-BB differentially induced PDGFR-β, PLCγ, and ERK1/2 phosphorylation in RASMCs in a time-dependent manner. At the indicated time points, PDGFR-β was fully activated at 3 min after PDGF-BB stimulation, whereas PLCγ and ERK1/2 were fully activated at 10 min (Fig. 2A). As observed in A10 SMCs, medial PDGF-BB preincubated with lycopene for 30 min almost completely inhibited PDGF-BB-

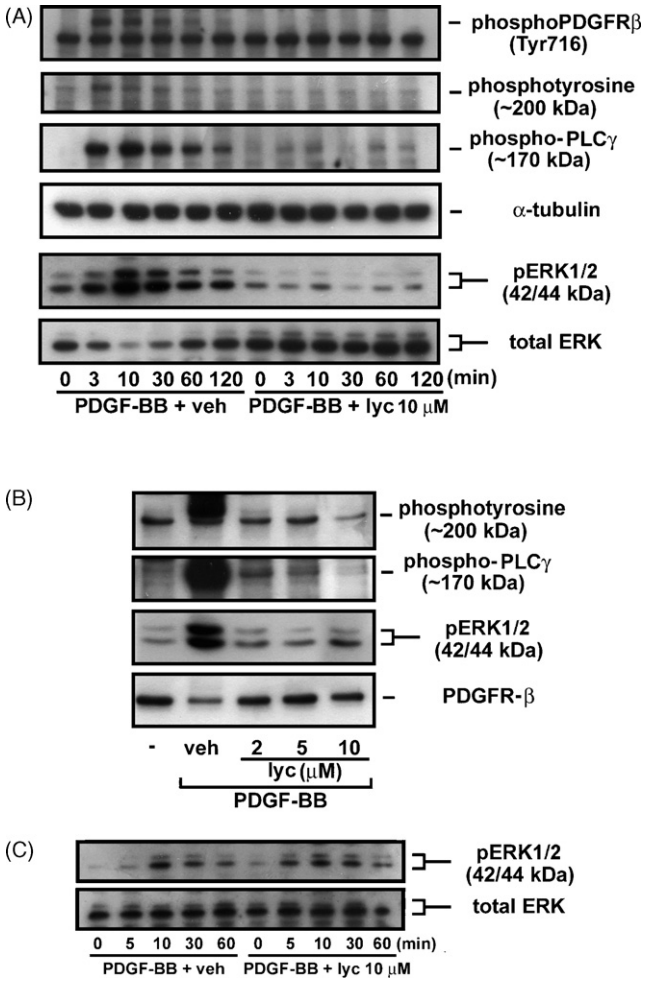

Fig. 2 – Effect of lycopene on PDGF-BB-induced signaling in RASMCs. Medium containing (A and B) PDGF-BB (10 ng/ml) or (C) EGF (50 ng/ml) was preincubated with vehicle or lycopene for 30 min. After incubation, vehicle- or lycopene-treated PDGF-BB or EGF were added to RASMCs and incubated for (A and C) the indicated times and (B) 10 min. At the end of incubation, cells were collected and PDGFR-β, PLCγ, ERK1/2 phosphorylation, α-tubulin and total ERK1/2 were determined by Western blotting. Each blot was representative of two to three independent experiments.

induced PDGFR-β, PLCγ, and ERK1/2 phosphorylation in RASMCs at the indicated time points, while expression of α-tubulin and total ERK1/2 was not affected (Fig. 2A). In parallel, lycopene pretreatment also dose-dependently inhibited PDGFR-β, PLCγ, and ERK1/2 phosphorylation (Fig. 2B). On the contrary, although EGF also induced ERK1/2 phosphorylation in a time-dependent manner in RASMCs, lycopene pretreatment did not inhibit ERK1/2 phosphorylation (Fig. 2C). Taken together, the results indirectly demonstrate that lycopene may interact with PDGF-BB but not with EGF. The interaction may result in blocking or inactivating of PDGF-BB and then inhibiting PDGF-BB-induced signaling in A10 and aortic SMCs.

3.2. *Lycopene binds PDGF-BB and inhibits PDGF-BB interacting with its receptor*

To further demonstrate that lycopene can directly interact with PDGF-BB, we performed a dot binding assay. Recombinant rat PDGF-BB, lycopene and EGF were immobilized on the membrane. After incubation with or without PDGF-BB, the membrane was incubated with Ab directed against PDGF-BB and then developed. It was shown that immobilized PDGF-BB but not EGF can be recognized by the anti-PDGF-BB Ab (Fig. 3A), suggesting Ab's specificity. A positive binding signal was only detected on the lycopene spot incubated with PDGF-BB, ruling out a nonspecific binding between Ab and lycopene spot and indicating that immobilized lycopene interacts with PDGF-BB. To determine lycopene's specificity, the same experiment was done to examine whether lycopene reacts with EGF. As shown in Fig. 3B, no binding signal was detected on lycopene spot, while immobilized EGF showed a strong binding signal (as a positive control), indicating that lycopene does not interact with EGF. To further examine lycopene's and other compounds' binding ability, the indicated doses of lycopene, β-carotene and EGCG were spotted on the membrane. As shown in Fig. 3C, β-carotene and higher doses of lycopene spots on the membrane were reddish color (upper panel). Beta-carotene is a carotenoid with a similar structure to lycopene [21] and EGCG is a tea polyphenolic compound which is known to affect PDGF-induced signaling [22]. It was shown that albumin and vehicle did not interact with PDGF-BB, whereas lycopene bound to PDGF-BB in a dose-dependent manner. Quantitative analysis showed that lycopene at 0.054 μg is sufficient to bind PDGF-BB. In contrast, immobilized β-carotene and EGCG (6.25 μg) showed a very weak binding to PDGF-BB at a higher dose (Fig. 3C, middle and lower panels).

To determine if lycopene's binding to PDGF-BB is relevant under physiological conditions, we examined whether lycopene interacts with PDGF-BB in plasma. As shown in Fig. 4A, lycopene dose-dependently bound to PDGF-BB with a similar affinity in PBS and plasma, suggesting that lycopene may interact with PDGF-BB in in vivo conditions. We next examined if the interaction affects PDGF-BB-induced signaling in SMCs, immobilized lycopene on the membrane was first incubated with medium containing PDGF-BB and followed by the removing of the membrane to avoid the presence of lycopene during coincubation with SMC. Under this condition, dot binding assay indicated that lycopene on the membrane bound PDGF-BB (Fig. 4B, upper panel), suggesting that immobilized lycopene was still on the membrane and interacted with medial PDGF-BB during coincubation. The preincubation subsequently compromised PDGF-BB-induced

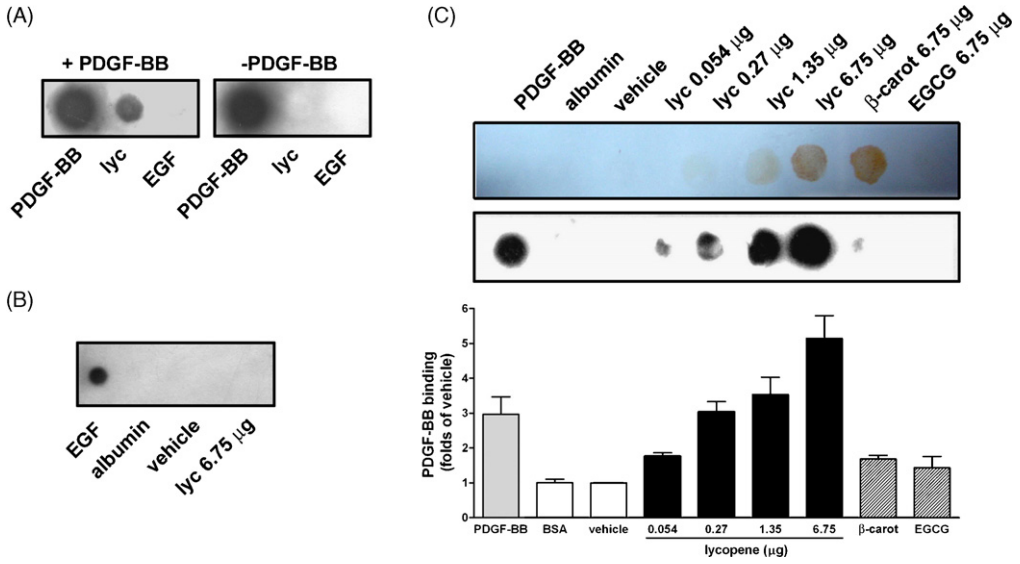

Fig. 3 – Lycopene directly interacts with PDGF-BB. (A) Rat PDGF-BB (100 ng), lycopene (lyc, 6.75 μg), and EGF (100 ng) were applied onto NC membrane. The membrane was incubated with (+) or without (−) PDGF-BB and followed by the incubation of anti-PDGF-BB Ab and developed. (B) EGF, albumin (both 100 ng), vehicle, lycopene (lyc) were applied onto NC membrane. The membrane was incubated with EGF, followed by anti-EGF Ab, and then developed. (C) Rat PDGF-BB, albumin (both 100 ng), vehicle, lycopene (lyc), β-carotene (β-carot), and EGCG were applied onto the NC membrane (upper panel). The membrane was incubated with PDGF-BB and developed (middle panel). Data were quantified by densitometry, normalized to the density of vehicle, and expressed as mean ± S.E.M. (n = 3–5) (lower panel).

PDGFR-β and ERK1/2 phosphorylation in A10 SMCs (lower panel), demonstrating that lycopene can directly bind PDGF-BB and subsequently inhibits PDGF-BB-induced signaling pathway in rat SMCs.

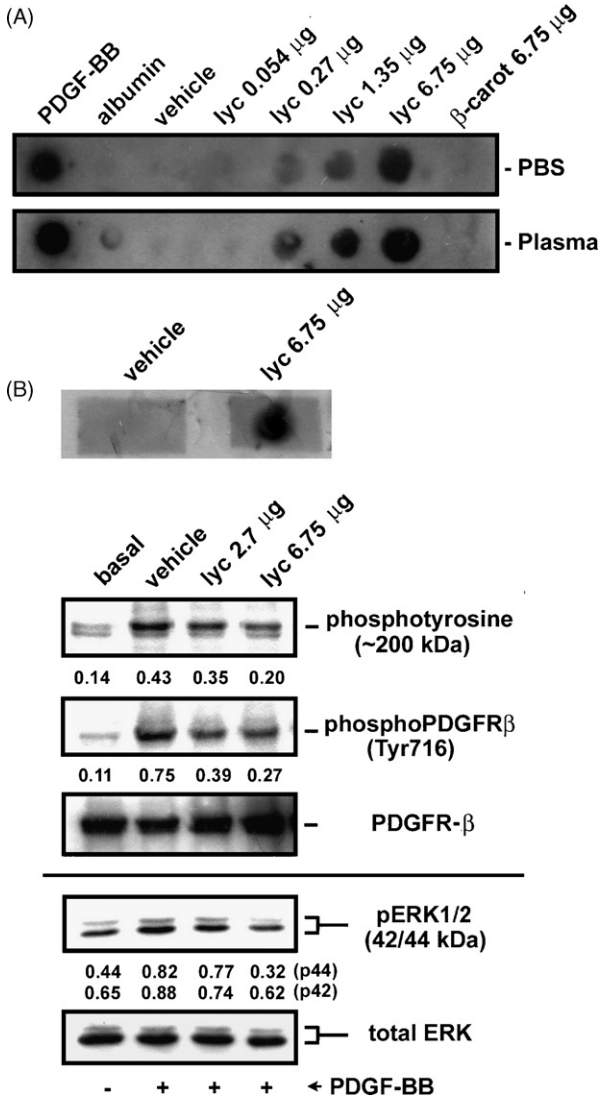

Fig. 4 – Immobilized lycopene interacts with PDGF-BB in plasma and inhibits PDGF-BB-induced signaling in SMCs. (A) Rat PDGF-BB, albumin (both 100 ng), vehicle, lycopene (lyc), and β-carotene (β-carot) were applied onto the NC membrane. The membrane was incubated with PDGF-BB in PBS or human plasma obtained by centrifugation of fresh blood. After incubated with anti-PDGF-BB Ab, the membrane was developed. (B) Vehicle or lycopene (lyc, 5 μl) were spotted on NC membrane (1.5 cm × 1 cm) and incubated with medium containing PBS or PDGF-BB (10 ng/ml). After 30-min incubation, the membrane was removed and developed (upper panel). The remaining medium was added to A10 SMC for 8 min and Western blot analysis was performed. Each number below the panel indicates the arbitrary density of pPDGFR-β and pERK1/2 ratio to total PDGFR-β and ERK1/2, respectively. Each blot is the representative of two to three independent experiments.

3.3. *Lycopene inhibits PDGF-BB-induced SMC migration*

Since lycopene elicited an inhibitory effect on PDGF-BB-induced signaling, we next examined whether lycopene functionally affects PDGF-BB-induced SMC migration. A modified Boyden chamber system was used to assay the effect of lycopene. Fig. 5A showed that gelatin alone induced little A10 SMC migration, however significant SMC migration on gelatin was observed by PDGF-BB stimulation. About 50–70 migrated A10 SMCs were found in high-powered field. Lycopene dose-dependently inhibited PDGF-BB-induced A10 SMC migration on gelatin. About 75% of SMC migration was inhibited at 2 μM of lycopene and near 100% at 10 μM of lycopene (Fig. 5A, upper panels). In parallel, significant SMC migration on collagen was observed in the absence of PDGF-BB, about 30 migrated cells were found in HPF, suggesting that collagen is also a ''chemoattractant'' for SMC. Again, PDGF-BB stimulated A10 SMC migration on collagen about four folds than collagen did alone. Lycopene did not inhibit collagen-induced SMC migration, but it inhibited PDGF-BB-induced A10 SMC migration in a concentration-dependent manner (Fig. 5A, lower panels). We then examined if lycopene affects RASMC migration. It was shown that gelatin, collagen, and PDGF-BB-induced more migration in RASMCs than in A10 SMCs, however a similar inhibitory effect elicited by lycopene was observed (Fig. 5B). In contrast, lycopene at 10 μM did not elicit an inhibitory effect on bFGF- and VEGF-induced endothelial cell migration on collagen (Fig. 5C). The results further demonstrate that lycopene has a relative binding specificity for PDGF-BB and can functionally inhibit PDGF-BB-induced SMC migration.

It has been reported that matrix metalloproteinases (MMPs) secreted by SMCs are involved in the degradation and remodeling of the connective tissue and are responsible for SMC migration in response to growth factor [23,24]. To determine if lycopene affects MMP activity, we performed gelatin zymography. As shown in Fig. 6A, zymographic analysis of SMC-conditioned media showed that control SMCs secreted several MMPs into culture media, which migrated at the molecular masses of 60, 72 and 80 kDa as clear bands under nonreduced conditions (arrows). PDGF-BB did not induce a significant increase of MMPs activity. It was found that lycopene at 10 and 25 μM did not affect activities of 60- and 80-kDa MMPs (Fig. 6B), as determined by densitometry.

3.4. *Effect of lycopene on SMC proliferation*

We next determined if lycopene affects SMC proliferation. MTT assay, measuring formazan crystals in living cells, was performed. As shown in Fig. 7, lycopene at a higher concentration (25 μM) did not affect the basal growth level of A10 SMCs. PDGF-BB-induced an increase of absorbance at 550 nm, i.e. an increase of living cells. The increase of absorbance was not affected by vehicle but was reduced by lycopene (Fig. 7A, panel a). We confirmed this result by direct hemacytometer-counting of cells, i.e. by trypan blue exclusion assay. It was shown that PDGF-BB-induced A10 SMC number increase and the increase was inhibited by lycopene (panel b). In RASMCs, we found that PDGF-AA was a weak mitogen, whereas PDGF-BB and serum could induce marked prolifera-

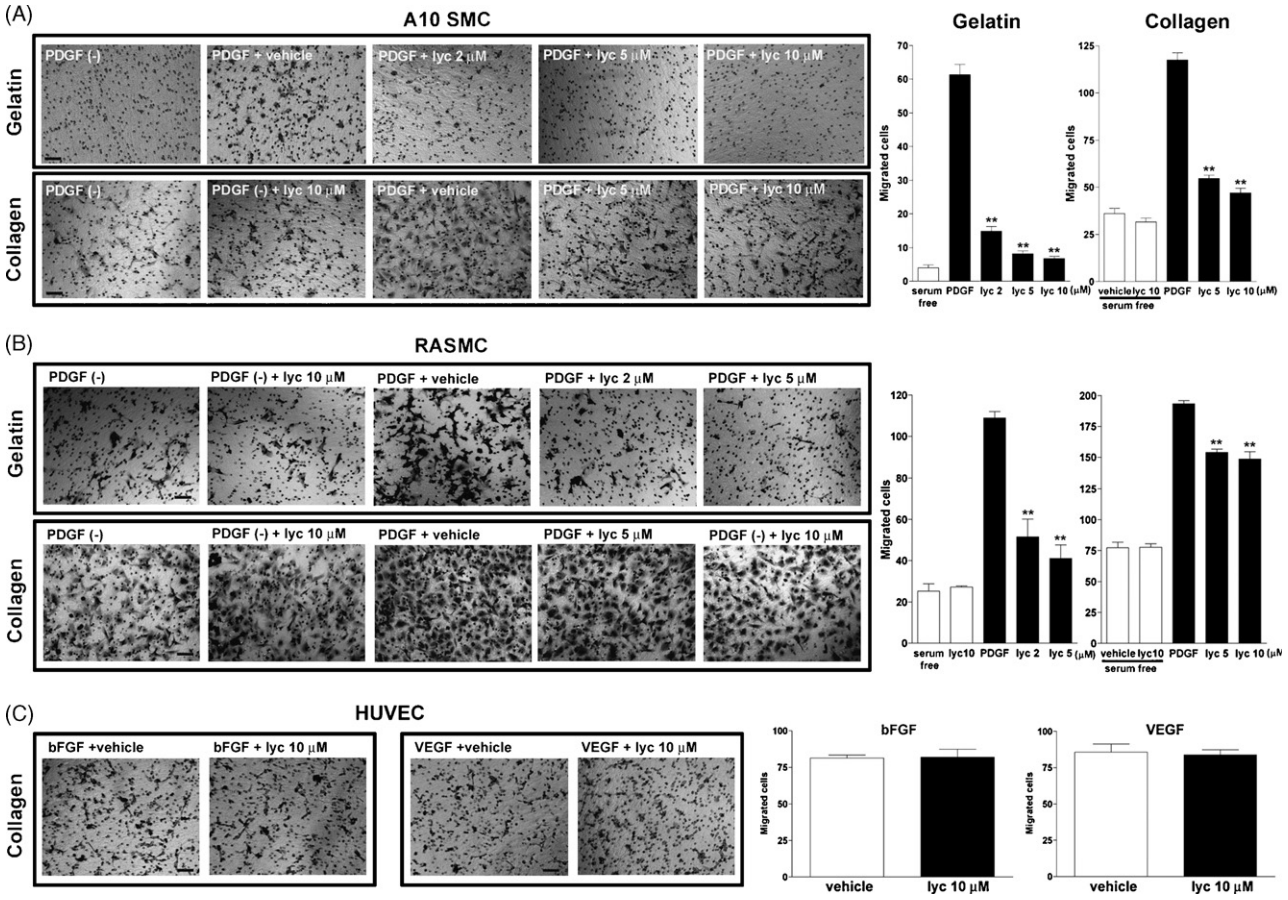

Fig. 5 – Effect of lycopene on SMC and HUVEC migration. Transwell inserts were coated with gelatin or collagen. SMCs or HUVECs were seeded in the upper chamber in the presence of vehicle or lycopene (lyc). The inserts were assembled with the lower chamber, which was filled with serum-free (PDGF (−)), PDGF-BB- (PDGF), bFGF-, or VEGF-containing medium preincubated with vehicle or lycopene for 30 min. After incubation for 3 h at 37 °C, fixation was performed and nonmigrated cells were removed. (A) A10 SMCs, (B) RASMCs (C) HUVECs that migrated to the underside of filter membrane were photographed and counted in high-power field (magnification, 100×) under a phase-contrast light microscope. Bar: 100 μm. Right panels: quantitative analysis of migrated cells. All experiments were conducted in duplicate and similar results were repeated at least three to four times. Data were presented as migrated cells/HPF and were mean ± S.E.M. (n = 5–10). Statistical comparison of data was determined using one-way ANOVA followed by the Student's t test. $^{**}P < 0.01$ vs. PBS control.

tion. Lycopene inhibited both PDGF-BB- and serum-induced RASMC proliferation (Fig. 7B, panel a), as determined by MTT assay. The inhibition of lycopene on PDGF-BB-induced RASMC proliferation was confirmed by luminescence assay of cellular ATP and cell counting assay (Fig. 7B, panels b and c).

4. Discussion

PDGF has been shown to play a prominent role in the migration of SMC into the neointima following acute injury and in atherosclerosis [19,20]. Therefore, in the past decade several selective inhibitors of PDGFR have been developed, i.e. tyrphostin AG 1296. This compound has been proposed to inhibit PDGFR via conformational changes at the ATP-binding site [25]. In addition to this compound, EGCG, a natural substance from green tea, has been shown to bind PDGFR-β and inhibit its phosphorylation and SMC proliferation [22,26,27].

In this study, we presented findings showing that lycopene, a natural compound from tomato, also inhibits PDGF-BB-induced signaling pathway, migration, and proliferation in rat A10 and primary cultured aortic SMCs. However, in contrast to the compounds described above, lycopene may influence PDGF-BB-induced signaling via a novel action mechanism by direct binding to PDGF-BB. Several lines of evidence support this hypothesis. Firstly, preincubation of medial PDGF-BB with lycopene resulted in a marked inhibition on PDGF-BB-induced PDGFR-β, PLCγ, and ERK1/2 phosphorylation in A10 and RASMCs (Figs. 1, 2 and 4). Secondly, dot binding assay demonstrated that lycopene directly interacted with PDGF-BB in a dose-dependent manner (Figs. 3 and 4). Thirdly, PDGFR-β expression and PDGF-BB-induced signaling were not affected by lycopene in A10 SMCs and in A10 SMCs pretreated

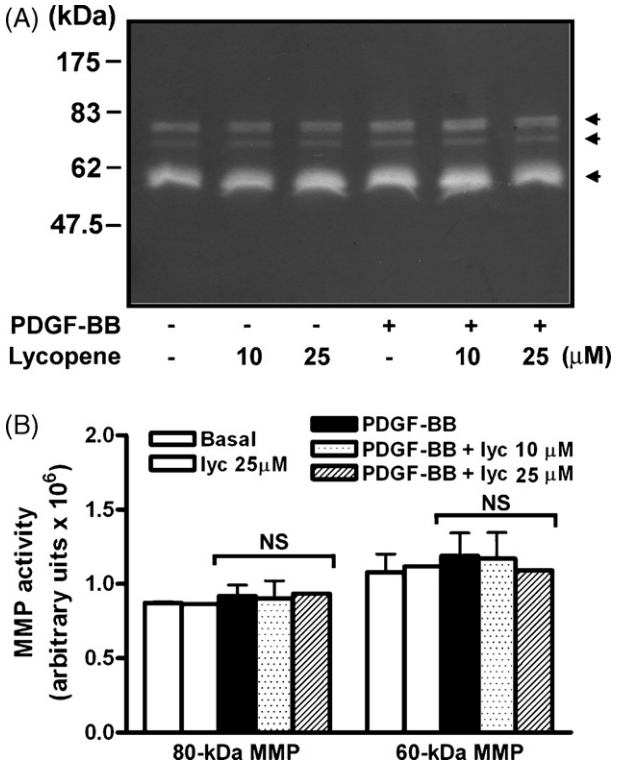

Fig. 6 – Gelatin zymography of SMC-conditioned media. A10 SMCs were treated with vehicle or lycopene in the presence or absence of PDGF-BB (10 ng/ml) at 37 °C for 24 h. The media were removed, centrifuged, and then analyzed by gelatin zymography. (A) A representative zymograph indicates that MMPs activity in SMC-conditioned media. Arrows indicate that three major MMPs migrated at 60, 72 and 80 kDa. (B) Quantitative analysis of the activities of 60- and 80-kDa MMPs ($n = 3$). NS, not significant.

with lycopene and followed by removing of extracellular lycopene, respectively (data not shown).

There are few studies that have shown that lycopene can directly interact with protein. It has been reported that incubation of LDL with carotenoids causes loss of carotenoids, especially lycopene, indicating that carotenoids may interact with free radicals in LDL [28]. Britton [29] has suggested that carotenoids may associate with hydrophobic areas in the protein or, especially, with the lipid components of lipoproteins. To date, there are few appropriate methods developed for the detection of lycopene–protein interaction. Here our study clearly demonstrates that soluble and immobilized lycopene is able to specifically bind PDGF-BB, using indirect and direct methods such as Western blotting and dot binding assay (Figs. 1–4). The binding affinity of lycopene is significant even in a small amount (0.054 μg) and at a micromolar concentration (2 μM). In contrast, EGCG and β-carotene at a higher dose showed only little binding to PDGF-BB. Lycopene may affect PDGF-BB-induced signaling through two different action mechanisms, one is to bind receptor binding site of PDGF-BB and result in blocking PDGF-BB's interaction with its cognate receptors. And the other is to inactivate PDGF-BB after binding. It has been reported that the residues in PDGF-BB responsible for receptor binding reside in loop 2, in addition to R27 and I30 [30]. The exact lycopene's binding site(s) within PDGF-BB is (are) now being examined in our laboratory.

Regarding lycopene's specificity, we observed that lycopene bound rat PDGF-BB and inhibited PDGFR-β, PLCγ, and ERK phosphorylation in rat A10 SMCs and RASMCs (Figs. 1–4). In accordance with these observations, we also found that lycopene did not inhibit SMC migration on type I collagen in the absence of PDGF-BB, but did inhibit SMC migration on collagen and gelatin upon PDGF-BB stimulation (Fig. 5). Interestingly, our lab also found that lycopene can bind human PDGF-BB and inhibit human PDGF-BB-induced signaling in human fibroblasts (Ref. [31], manuscript in preparation). Sequence alignment has shown that mature rat and human PDGF-B chain share 93% similarities in their primary sequence, suggesting that lycopene may interact with their homologous region(s). It has been reported that PDGF-A and -B are approximately 50% identical in their growth factor domain [32] and the crystal structure of VEGF is similar to that of PDGF-BB [33]. Therefore, we also tested if lycopene interacts with other growth factors. Among the tested growth factors, we found that lycopene did not inhibit EGF-induced signaling in A10 SMCs and RASMCs (Figs. 1 and 2) and bFGF- and VEGF-induced endothelial cell migration (Fig. 5C), suggesting lycopene has a relative binding specificity for PDGF-BB. However, whether lycopene interacts with other growth factors and PDGF family member needs to be determined.

It has been documented that the predominant carotenoids found in human plasma are lycopene, β-carotene, and lutein, and their concentrations vary from 0 to 8 μM depending upon dietary intake [34]. Our study showed that 2 μM of lycopene is sufficient to elicit the inhibitory effect on PDGF-BB-induced signaling and SMC migration in in vitro conditions. Most importantly, lycopene exerts the inhibitory effect on SMC migration toward gelatin and collagen by PDGF-BB stimulation, in which collagen is known to be highly expressed in developing lesions of atherosclerosis [35]. However, this inhibitory effect of lycopene is unlikely acting through MMPs because MMPs activity in SMC-conditioned media was not affected by lycopene (Fig. 6).

Numerous large cohort studies have been published on American men and women that have examined dietary intake or blood levels of total or individual carotenoids with the risk of various cardiovascular endpoints. However, results from trial of β-carotene supplementation were disappointing. Therefore, research began to transit to other specific major carotenoids [36]. In this study we provide the first evidence showing that lycopene can inhibit PDGF-BB-induced signaling, migration, and proliferation at a micromolar concentration. One of its action mechanism is through direct binding PDGF-BB. As PDGF-induced SMC migration and proliferation play an important role in angiogenesis and is relevant to disease-related vascular remodeling in conditions such as atherosclerosis and restenosis after angioplasty, the results presented here may help us to better understand the beneficial effects of lycopene in preventing CVDs.

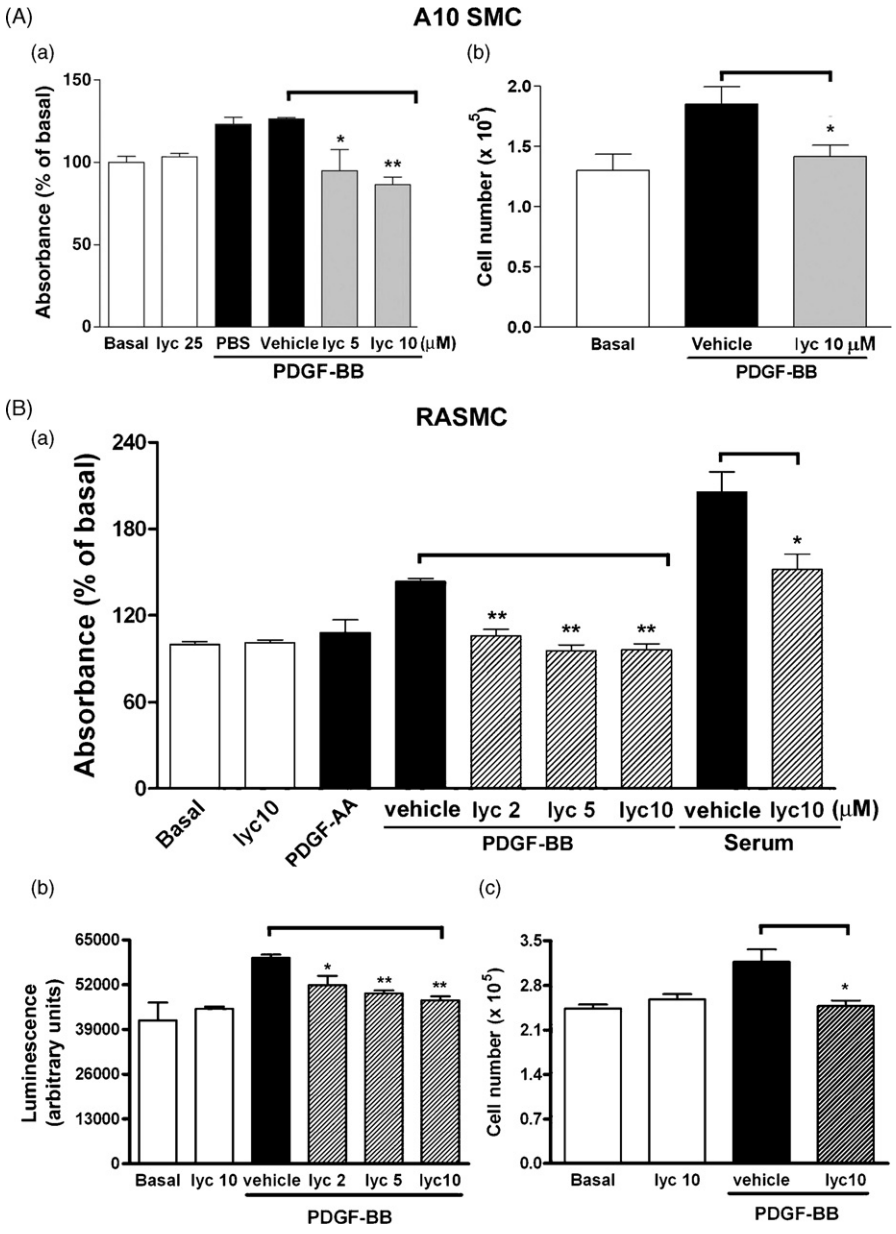

Fig. 7 – Effect of lycopene on SMC proliferation. (A) A10 SMCs were simultaneously treated with PBS, vehicle (THF), or the indicated concentrations of lycopene (lyc) in serum-free medium containing PBS or PDGF-BB (10 ng/ml). After incubation for 24 h, (a) MTT assay and (b) cell counting assay were performed to determine SMC proliferation. (B) RASMCs were simultaneously treated with vehicle (THF) or lycopene (lyc) in medium containing PBS, PDGF-AA (10 ng/ml), PDGF-BB (10 ng/ml) or 10% serum. Cell proliferation was measured by (a) MTT assay (b) luminescence assay of cellular ATP and (c) cell counting assay. Each data was expressed as mean ± S.E.M. (n = 3–5). Statistical comparison of data was determined using one-way ANOVA followed by the Student t test. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. PBS control.

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