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# Anti-angiogenic effects of lycopene through immunomodualtion of cytokine secretion in human peripheral blood mononuclear cells☆,☆☆,★

Chin-Shiu Huang[a,*], Cheng-Hung Chuang[b], Tsue-Feng Lo[a], Miao-Lin Hu[c,*]

[a]Department of Health and Nutrition Biotechnology, Asia University, Taichung 41354, Taiwan ROC
[b]Institute of Biomedical Nutrition, Hungkuang University, Taichung, 43302, Taiwan ROC
[c]Department of Food Science and Biotechnology, National Chung-Hsing University, Taichung 40227, Taiwan ROC

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**Abstract**

The carotenoid lycopene has been reported to possess anti-metastatic activity which may be associated with immunomodulation. However, the anti-angiogenic effects and mechanisms of action of lycopene have not been reported. In this study, we investigated the immunomodulatory effect on in vitro and ex vivo angiogenesis of lycopene. We found that the proliferation, migration and the matrigel tube formation of human umbilical vein endothelial cells (HUVECs) was remarkably inhibited by conditioned medium (CM) of human peripheral blood mononuclear cells (MNC-CM) stimulated with various dose (1-10 μmol/L) of lycopene (LP-MNC-CM). LP-MNC-CM treatment inhibited ex vivo angiogenesis, as revealed by chicken egg chorioallantoic membrane assay. We further examined the effects of lycopene stimulation on cytokine levels in MNC and showed that, as compared to the control, lycopene (10 μmol/L) significantly ($P<.001$) up-regulated interleukin (IL)-12 by 163% and interferon (IFN)-γ by 531%. Furthermore, pre-treatment of HUVECs with dexamethasone, an IL-12 inhibitor, blocked the anti-angiogenic effects of LP-MNC-CM in parallel with inhibition of IL-12 and IFN-γ induction in MNC. These results demonstrate that lycopene has a potent anti-angiogenic effect and that these effect may be associated with its up-regulation of IL-12 and IFN-γ.
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*Keywords:* Lycopene; Angiogenesis; Interleukin-12; Immunomodulation; Mononuclear cells

## 1. Introduction

Angiogenesis, the generation of new capillaries from preexisting vessels, is one of the most important events in tumor growth, progression and metastatic dissemination [1,2]. As considerable experimental evidence suggests that tumor growth is dependent on angiogenesis [3], theoretically, obliterating the vessels feeding a tumor should cause its shrinkage or death [4]. Therefore, anti-angiogenesis remains a prime therapeutic target and anti-angiogenic therapy may be a promising cancer treatment modality [5,6].

Tumor angiogenesis consists of several steps including the secretion of angiogenic factors by tumor and host cells, activation of proteolytic enzymes, endothelial cell migration, proliferation and capillary formation [7]. Angiogenesis is tightly controlled by a wide variety of regulators which include cytokines [8-14] and matrix metalloproteinases (MMPs) [15,16] by tumors or the immune system. Interleukin (IL)-12 is a heterodimeric cytokine produced mainly by macrophages/monocytes, with pleiotropic activities. Treatment with IL-12 promotes a cytotoxic anti-tumor immune response [17] and an anti-angiogenic effect [8,10]. The anti-angiogenic actions of IL-12 are thought to be largely due to the local interferon (IFN)-γ production [8,10,18]. IL-8, a chemokine, may also exert proangiogenic actions, while the reverse is true for IL-12 [10]. It has been demonstrated that IL-8 functions as an important autocrine and a growth and angiogenic factor in regulating multiple biological activities, such as MMP-2, in endothelial cells [12,19].

Dias et al. [20] reported that IL-12 down-regulates vascular endothelial growth factor (VEGF) and MMPs in a murine breast cancer model. Inhibition of VEGF, an endothelial cell (EC)-specific mitogen and also known as a vascular permeability factor, would reduce the vessel abnormality and increase the permeability of the tumor to chemotherapies [21]. MMPs are a closely related mutigene family of zinc-dependent proteolytic enzymes. They play a role in

*Abbreviations:* APC, antigen-presenting cells; BC, β-carotene; CM, conditioned medium; DE, dexamethasone; EC, endothelial cell; ECGS, endothelial cell growth supplement; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HUVECs, human umbilical vein endothelial cells; IFN, interferon; IL, interleukin; LP, lycopene; MMPs, matrix metalloproteinases; MNC, mononuclear cells; $PGE_2$, prostaglandin $E_2$; PHA, phytochemagglutinin; THF, tetrahydrofuran; VEGF, vascular endothelial growth factor.

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* Corresponding author. Tel.: +886 4 2281 2363; fax: +886 4 2281 2363.
*E-mail addresses:* cshuang@asia.edu.tw (C.-S. Huang), chchuang@sunrise.hk.edu.tw (C.-H. Chuang), xs1213@yahoo.com.tw (T.-F. Lo), mlhuhu@dragon.nchu.edu.tw (M.-L. Hu).

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normal physiological tissue remodeling and are capable of degrading all components of the extracellular matrix. There is increasing evidence that MMPs potentiate neovascularisation of tumor tissue [22-24], with both MMP-2 and MMP-9 expression being strongly correlated with angiogenesis [15,25].

Carotenoids including β-carotene and lycopene possess several common biological functions such as photoprotection, antioxidant effects, and immunomodulatory and anticancer activity [26,27]. It has been reported that the role of carotenoids in gene regulation, apoptosis and angiogenesis is correlated with the possible mechanism by which carotenoids regulate immune function and cancer [27]. Lycopene was also shown to enhance the immune response in tumorigenesis of mice [28]. Bessler et al. [29] have recently reported that lycopene modulates the cytokine production for reducing the risk of certain diseases. In addition, lycopene was found to attenuate the impairment of lungs and by increasing the phagocytic function of pulmonary alveolar macrophages and restraining the secretion of tumor necrosis factor α (TNF-α) and IL-8 in rats with acute lung injury induced by lipopolysaccharide [30]. In rats with gastric cancer induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, lycopene was shown to increase blood IL-2, IL-4, IL-10 and TNF-α levels and reduce the IL-6 level [31]. However, contradictory results exist in human studies regarding the effect of lycopene on immune systems [32, 33]. In patients with Type 2 diabetes mellitus, lycopene supplementation (10 mg/day) was found to enhance innate immunity and attenuate T cell-dependent adaptive (pro-atherogenic) immune response. In contrast, it was shown that prolonged tomato juice consumption (containing 47.1 mg/d lycopene) increases plasma lycopene concentrations without significantly affecting cell-mediated immunity in well-nourished elderly subjects.

Lycopene has been shown to significantly decrease endothelial cell proliferation, migration and tube formation; the latter result suggests that lycopene may inhibit angiogenesis [34]. We previously showed that oral supplementation with lycopene decreases the number of lung tumors and the tumor cross-sectional area in athymic nude mice after tail vein injection with SK-Hep-1 cells, and this effect involves angiogenic factors, including up-regulation of IL-12 secretion [35]. To better understand the effect of lycopene on immune responses and the relation with its anti-angiogenic effect, the present study examined the production of cytokines in human peripheral blood mononuclear cells (MNC) stimulated with lycopene.

## 2. Material and methods

### 2.1. Chemicals

Lycopene (Wako, Japan) was delivered to the cell using tetrahydrofuran (THF, Merck) solvent, containing 0.025% butylated hydroxytoluene (BHT) to avoid formation of peroxide. Ficoll-Hypaque solution (1.077 g/ml) was the product of Pharmacia Fine Chemicals (Sweden). Phytochemagglutinin (PHA) and dexamethasone (DE) were from Sigma (USA). Commercial kits for IL-12, IFN-γ, IL-8, prostaglandin $E_2$ ($PGE_2$) and VEGF were purchased from R&D (USA). In vitro angiogenesis assay kit (ECMatrix) was from Chemicon (USA). All chemicals used are of reagent or higher grade.

### 2.2. Preparation of conditioned media (CM)

Human peripheral blood was obtained from four normal adult volunteers (aged between 24 and 28) with informed consent, and mononuclear cells (MNCs) of each person were separated by density centrifugation (400×*g*, 30 min) in a Ficoll-Hypaque solution (1.077 g/ml) [36]. MNCs recovered at the interface were resuspended in RPMI 1640 medium containing 10% FBS. To investigate the effect of lycopene on stimulating the immune cells, total MNCs ($1\times10^6$ cells/ml) were incubated in RPMI 1640 medium containing 10% FBS with or without various concentrations of lycopene at 37°C for 1 d. After that, all CM were collected, filtered, and stored at −70°C until use. They were named LP-stimulated-MNC-CM (LP-MNC-CM) in the presence of lycopene or non-stimulated-MNC-CM in absence of lycopene, respectively. PHA (5 μg/ml) and THF (0.1%, v/v) were also used to prepare MNC-CM of PHA (PHA-MNC-CM) (positive group) and of THF (THF-MNC-CM) (solvent control), respectively. Some cultures were pre-treated with DE (50 nmol/L), an IL-12 inhibitor [37], prior to lycopene treatment.

### 2.3. Treatment of human umbilical vein endothelial cells and cell proliferation assay

Human umbilical vein endothelial cells (HUVECs) were purchased from the Cell culture Center of the Food Industry Research and Development Institute (Hsinchu, Taiwan), and were cultured in M199 (Gibco, USA) supplementation with 10% fetal bovine serum (FBS), 30 μg/ml EC growth supplement, 25 U/ml heparin, 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate and 1% antibiotic-antimycotic (Gibco, USA). Cells were seeded onto 10-cm dish or 96-well plate coating 1% gelatin and incubated at 37°C in a humid atmosphere containing 5% $CO_2$. The cells were cultured at an initial concentration of $1\times10^5$ cells/ml in the presence of 30% (v/v) LP-MNC-CM or non-stimulated-MNC-CM. After pre-incubation with LP-MNC-CM for 24 h, cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol terazolium bromide colorimetric assay.

### 2.4. Cytokine assay

IL-12, IFN-γ, IL-8 and $PGE_2$ in the various preparations of MNC-CM were quantified separately with the commercial kit by a solid-phase ELISA (R&D Systems) at a wavelength of 450 nm. MNC-CM was diluted 1–5-fold with commercial dilute-solution just before assay. Three separate experiments were each tested in duplicate.

### 2.5. Tube formation assay

The effect of lycopene on in vitro angiogenesis was estimated by the tube formation assay, as described previously [8]. Briefly, HUVECs ($1\times10^5$ cells/ml) were cultured into 24-well culture plates, which were precoated with the 50 μl/well ECMatrix (Chemicon, USA). Then, each well was added a medium containing VEGF (50 ng/ml) and various concentrations of LP-MNC-CM or DE-treated LP-MNC-CM and incubated for 12 h, after which the tube formation was observed by microscopy. For each replicate, the cells in 10 randomly selected fields were determined.

### 2.6. Cell migration assay

Cell migration was assayed in transwell chambers (Costar) according in the methods reported by Repesh [38] with some modifications. Briefly, transwell chambers (Costar) with 6.5 mm polycarbonate filters of 8 μm pore size. After pre-incubation with LP-MNC-CM for 24 h, HUVECs ($5\times10^5$ cells/ml) were finally suspended in M199 (100 μl, serum free, 5 ng/ml VEGF) and placed in the upper transwell chamber, and then incubated for 12 h at 37°C. The medium in the lower transwell chamber contained 50 ng/ml VEGF and 10% FBS. After incubation for 12 h at 37°C, the cells on the upper surface of the filter were completely wiped away with a cotton swab. The cells on the lower surface of the filter were fixed in methanol, stained with Giemsa and then counted under a microscope. For each replicate, the tumor cells in 10 randomly selected fields were determined, and the counts were averaged. For each replicate, the tumor cells in 10 randomly selected fields were determined, and the counts were averaged. The percentage inhibition of invasion was calculated by the formula: [1−(treatment/control)]×100%.

### 2.7. Gelatin zymography

MMP-2 activity was assayed using gelatin zymography according to the methods as described previously [39]. HUVECs ($5\times10^4$ cells/ml) were treated with LP-MNC-CM for 24 h in M199 medium containing 10% (v/v) FBS and incubated for 24 h at 37°C in serum-free M199, then the culture medium was harvested and stored at −20°C until use. For the assay of gelatin zymography, the culture medium of an appropriate volume (adjusted by vital cell number) was electrophoresed in a 10% sodium dodecyl sulfate-PAGE gel containing 0.1% (w/v) gelatin. The gel (MMP-gel) was washed for 30 min at room temperature in a solution containing 2.5% (v/v) Triton X-100 with two changes and subsequently transferred to a reaction buffer for enzymatic reaction containing 1% $NaN_3$, 10 mmol/L $CaCl_2$, and 40 mmol/L Tris-HCl pH 8.0 at 37°C with shaking overnight (for 15 h). Finally, the MMP-gel was stained for 30 min with 0.25% (w/v) Coomassie blue in 10% acetic acid (v/v) and 50% methanol (v/v) and de-stained in 10% acetic acid (v/v) and 50% methanol (v/v). The relative MMP-2 activity was quantitated by Matrox Inspector 2.1 software.

### 2.8. Chorioallantoic membrane (CAM) assay

Ex vivo anti-angiogenic activity of LP-MNC-CM was measured by CAM assay as described elsewhere [40] with minor modification. A group of ten 7-day-old fertilized eggs was incubated at 37.5°C with 55% relatively humidity. On day 8, a 1-$cm^2$ window was carefully created on the broad side of the egg, which can candle the egg to assure existence of embryonic blood vessels. A volume of LP-MNC-CM (20 μl containing 1,5 or 10 μmol/L per egg) or MNC-CM and THF-MNC-CM was applied on a filter paper disk and then placed in CAM, after which a permeable sticky tape was immediately appended to the windows. After incubation for 3 days (until Day 11), the egg shell was pushed aside around the window, and the blood vessels were photographed. Anti-angiogenic effect of LP-MNC-CM on CAMs was quantified by counting the number of blood vessel branch points which were marked using artistic software on the photos.

Table 1
Effects of LP or in combination with DE on cytokine levels in mononuclear cells isolated from human peripheral blood[a,b]

| Treatment pg/ml | IL-12 | IFN-γ | IL-8 | $PGE_2$ |
|---|---|---|---|---|
| Control | 559±49[c] | 29±17[d] | 349±72[a] | 71±3[a,b] |
| THF[3] | 669±91[b,c] | 45±30[d] | 329±30[a] | 79±1[a] |
| LP1 | 717±103[b,c] | 86±34[c] | 316±39[a] | 65±0[b,c] |
| LP2.5 | 797±16[a,b] | 104±4[b,c] | 313±2[a] | 71±8[a,b] |
| LP5 | 840±138[a,b] | 138±14[a,b] | 311±48[a] | 69±6[a,b,c] |
| LP10 | 912±134[a] | 154±23[a] | 316±46[a] | 59±13[c] |
| PHA[4] | 931±123[a] | 156±14[a] | 284±115[a] | 76±0[a,b] |
| + DE[5] | | | | |
| Control | 474±44[b] | 3±4[e] | 384±38[a] | 8±4[b] |
| THF | 476±36[b] | 28±3[e] | 378±17[a] | 75±10[a] |
| LP1 | 511±32[a,b] | 33±3[d,e] | 374±10[a] | 72±11[a] |
| LP2.5 | 518±20[a,b] | 43±8[c,d] | 375±11[a] | 70±7[a] |
| LP5 | 525±30[a,b] | 49±6[b,c] | 372±16[a] | 68±4[a] |
| LP10 | 531±24[a,b] | 59±9[a,b] | 357±22[a,b] | 69±5[a] |
| PHA | 562±37[a] | 68±12[a] | 329±26[b] | 74±12[a] |

[a] Values are means±S.D., $n>3$; means in a column without a common letter differ, $P<.05$.
[b] Mononuclear cells were incubated with LP (1, 2.5, 5 and 10 μmol/L), β-carotene (10 μmol/L), or PHA (5 μg/ml) at 37°C for 1 day.
[c] THF is the solvent for lycopene.
[d] PHA is the positive control.
[e] DE is the IL-12 inhibitor.

*2.9. Statistical analysis*

Values are expressed as means±S.D. and analyzed using one-way analysis of variance followed by Duncan's Multiple Range Test for comparisons of group means. The statistical analysis was performed using SPSS for Windows, version 10 (SPSS); a $P<.05$ was considered statistically significant.

## 3. Results

### *3.1. Effects of lycopene on the levels of cytokines in MNC-CM*

MNC were incubated with lycopene (1–10 μmol/L) for 24 h, and the levels of IL-12, IFN-γ, IL-8 and $PGE_2$ in the CM were determined. As shown in Table 1, lycopene treatment significantly and dose-dependently increased IL-12 levels in MNC-CM, , and the highest level of IL-12 was achieved at 10 μmol/L (163%, $P<.001$), as compared to control. Lycopene also significantly and dose-dependently increased the level of IFN-γ in LP-MNC-CM (with a 4.3-fold increase at 10 μmol/L lycopene). Lycopene slightly, but not significantly, decreased the level of IL-8 in MNC-CM. PHA (5 μg/ml), added as a positive control, significantly increased the levels of IL-12 and IFN-γ. Although PHA decreased IL-8 production by ca. 20%, the extent of reduction was not statistically significantly ($P>.05$), as compared to the control (without DE) level.

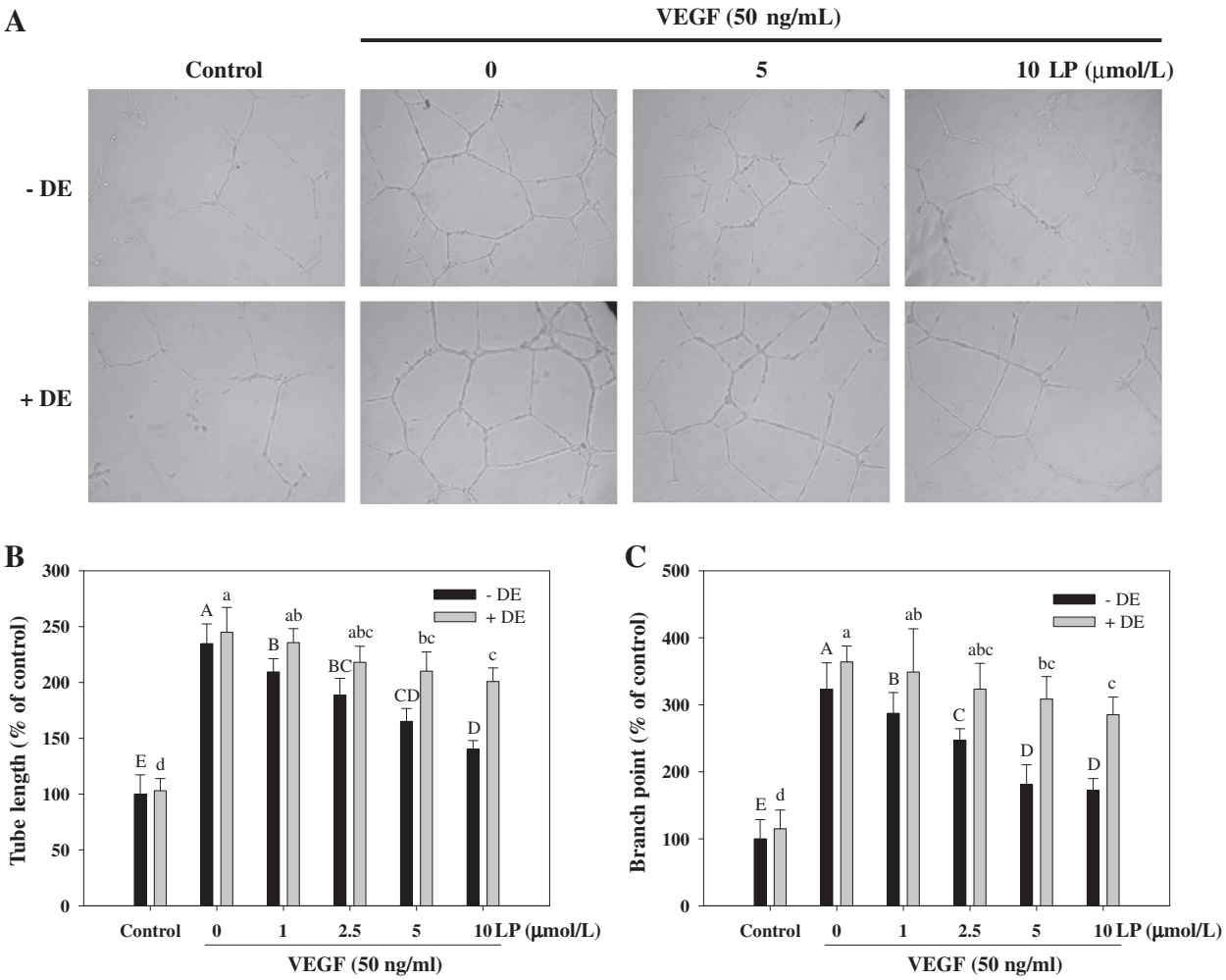


Fig. 1. Effects of LP alone or in combination with DE on tube formation of HUVECs. HUVECs ($1\times10^4$ cells/well) in a medium containing 10% serum were seeded into matrigel pre-coated 24-well plate and treated with 30% (v/v) LP-MNC-CM and stimulation with VEGF (50 ng/ml). Representative phase contrast photomicrographs (100× magnification) taken at 12 h after seeding (A) and evaluated the length of complete tubes connecting points (B) and the number of branch point (C) of individual polygons of the capillary network by AlphaEaseFC. Values are mean±S.D., $n>3$; means not sharing a common letter (upper case for –DE and lower case for +DE) differ significantly ($P<.05$).

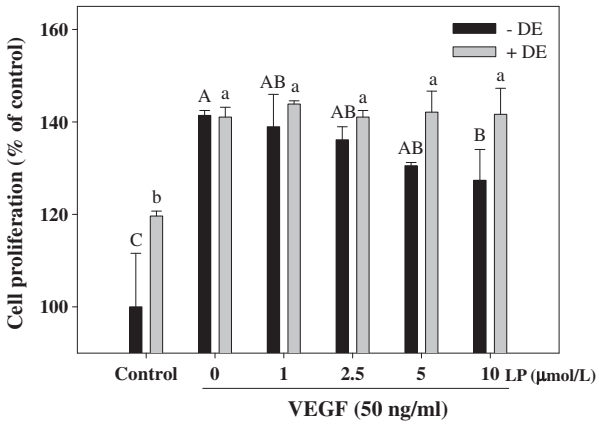


Fig. 2. Effects of LP alone or in combination with DE on cell proliferation of HUVECs. HUVECs were treated with 30% (v/v) LP-MNC-CM and stimulated with VEGF (50 ng/ml) for 24 h. Values are mean±S.D., $n$>3; means not sharing a common letter (upper case for −DE and lower case for +DE) differ significantly ($P$<.05).

We also examined the inflammatory effect of lycopene on the level of $PGE_2$, a biomarker of inflammation [41], in MNC-CM. We found that lycopene tended to exert a concentration-dependent decrease in the $PGE_2$ level, although the $PGE_2$ level was only significantly decreased at 10 μmol/L lycopene (17%, $P$<.05). Neither THF nor PHA significantly affected the level of $PGE_2$ in MNC-CM.

### 3.2. Effects of LP-MNC-CM on VEGF-induced HUVECs tube formation

The three-dimensional (3D) tube formation assay of HUVECs that mimics the angiogenic process was used to demonstrate the anti-angiogenic activities of lycopene. The cells quickly attached to the matrix and morphologically differentiated into a capillary-like network in about 12 h. HUVECs incubated with 50 ng VEGF/ml for 12 h showed a marked increase in the endothelial tubes, as compared with control (without DE) (Fig. 1). When compared with the non-stimulated-MNC-CM control, LP-MNC-CM-treated HUVECs formed relatively incomplete and narrow VEGF-induced tube-like structures at 12 h (Fig. 1A). The LP-MNC-CM obtained from incubation with 10 μmol/L lycopene significantly decreased the number of branch point and tube length by 55% and 40%, respectively (Fig. 1B, C). PHA-MNC-CM exhibited strongly inhibitory effect on tube formation.

To explore whether IL-12 and IFN-γ are responsible for inhibition of tube formation by LP-MNC-CM, we pre-treated MNC with DE (50 nmol/L), an IL-12 inhibitor. Data reveal that DE inhibited the production of IL-12 and IFN-γ induced by lycopene in MNC (Table 1). Notably, DE antagonized the ability of lycopene by blocking tube formation of HUVECs, whereas DE alone did not show any significant

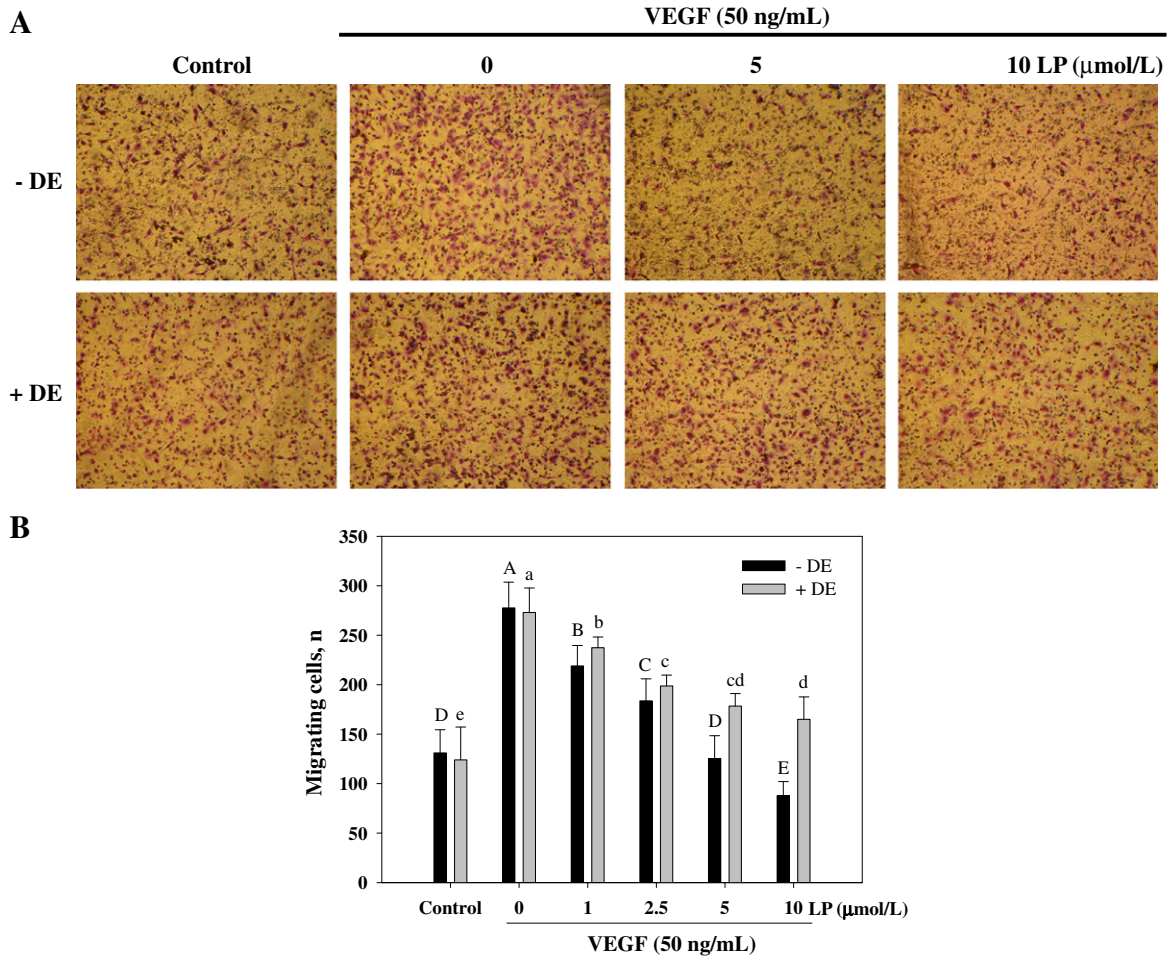


Fig. 3. Effects of LP alone or in combination with DE on VEGF-induced cell migration of HUVECs. HUVECs were treated with 30% (v/v) LP-MNC-CM and stimulated with VEGF (50 ng/ml) for 24 h. Cells migration was assayed using transwell chamber (Millipore). Representative phase contrast photomicrographs (100× magnification) taken at 12 h (A) and migration ability of HUVECs was quantified by counting the number of cells that invaded to the underside of the membrane under microscopy (B). Values are mean±S.D., $n$>3; means not sharing a common letter (upper case for −DE and lower case for +DE) differ significantly ($P$<.05).

effect (Fig. 1). The results suggest that IL-12 and IFN-γ in the LP-MNC-CM play an important role in the anti-angiogenic effect of lycopene.

### 3.3. Effects of LP-MNC-CM on VEGF-induced proliferation of HUVECs

We then examined the effect of LP-MNC-CM on VEGF-induced HUVECs proliferation. HUVECs were treated for 24 h with LP-MNC-CM obtained from incubation of MNC with 1–10 μmol/L LP. We found that lower concentrations of LP (1–5 μmol/L) had no effect on VEGF-induced HUVECs proliferation (Fig. 2). LP-MNC-CM (10 μmol/L) significantly inhibited VEGF-induced proliferation of HUVECs (ca. 10%, $P<.05$), as compared with non-stimulated-MNC-CM control. However, DE antagonized the ability of lycopene to block anti-angiogenic effect of proliferation in HUVECs.

### 3.4. Effects of LP-MNC-CM on VEGF-induced migration of HUVECs

The migration of HUVECs through basement membranes, which is a crucial step in the development of new blood vessels [36], was examined by using Transwell chamber. The results showed that LP-MNC-CM significantly inhibited VEGF-induced migration in a dose dependent manner, with 68% inhibition for LP-MNC-CM obtained from incubation of MNC with 10 μmol/L LP (Fig. 3). However, DE antagonized the anti-angiogenic effect of LP-MNC-CM on migration of HUVECs, whereas DE alone did not show any significant effect.

### 3.5. Effects of LP-MNC-CM on VEGF-induced MMP-2 activity in HUVECs

The activity of MMPs, which is linked to tumor metastasis for various cancers, was measured using gelatin zymography assay. We found that the activity of MMP-2 was significantly induced by VEGF (ca. 27% of control without DE), whereas LP-MNC-CM obtained from 10 μmol/L lycopene significantly ($P<.05$) inhibited VEGF-induced MMP-2 activity by ca. 13% and 22% at 5 and 10 μmol/L of LP, respectively (Fig. 4). However, DE antagonized the anti-angiogenic effect of LP-MNC-CM on MMP-2 activity of HUVECs, whereas DE alone did not show any significant effect.

### 3.6. Effect of LP-MNC-CM on ex vivo anti-angiogenesis in CAM assay

We used an ex vivo CAM assay to further examine the anti-angiogenic effect of LP-MNC-CM. The concentrations of LP in MNC-CM for CAM assay were selected from the LP-MNC-CM that were effective against tube formation of HUVECs in vitro. The control CAMs showed well-developed neovascularization, whereas LP added at 5 and 10 μmol/L for 72 h significantly inhibited the neovascularization (Fig. 5A). LP-MNC-CM obtained from 10 μmol/L lycopene significantly inhibited the number of blood vessels (by 43%, $P<.01$), as compared to solvent control (THF-MNC-CM, Fig. 5B).

## 4. Discussion

Lycopene has been shown to enhance the immune response in tumorigenesis of mice [28]. In addition, oral supplementation with lycopene was found to decrease the number of lung tumors and the tumor cross-sectional area in athymic nude mice after tail vein injection with SK-Hep-1 cells, and this effect involves angiogenic factors, including up-regulation of IL-12 secretion [35]. To better understand the effect of lycopene on immune responses and the

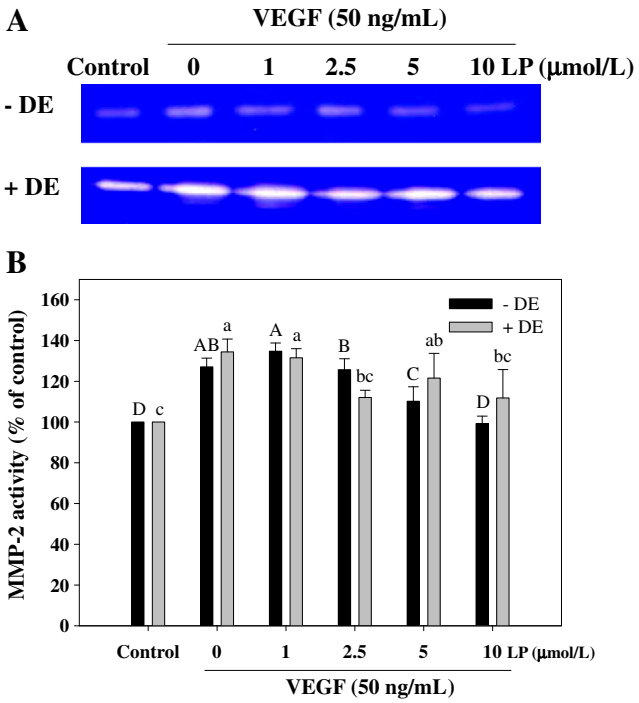


Fig. 4. Effects of LP alone or in combination with DE on the VEGF-induced cell MMP-2 activity in HUVECs. HUVECs were treated with 30% (v/v) LPLP-MNC-CM and stimulated with VEGF (50 ng/ml) for 24 h and then washed twice in phosphate-buffered saline before incubation with serum-free medium M199. (A) Gelatin zymography of MMP-2. (B) Densitometric analysis of Panel A. Values are mean±S.D., $n>3$; means not sharing a common letter (upper case for –DE and lower case for +DE) differ significantly ($P<.05$).

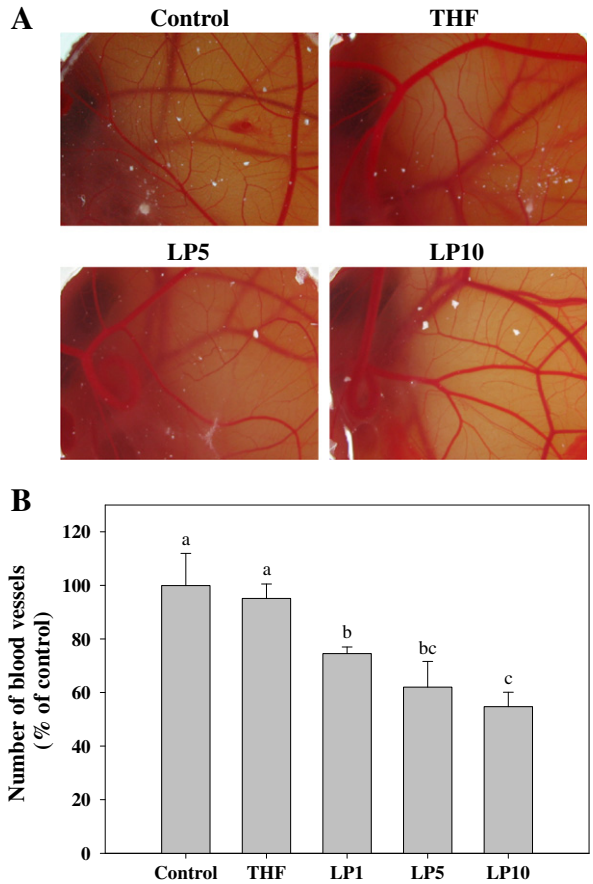


Fig. 5. Effects of LP on angiogenesis of chicken eggs by chorioallantoic membrane assay. Eight days after fertilization, LP-MNC-CM or THF-MNC-CM (solvent control) was added to a filter paper disk. After incubating the eggs for 3 days, CAMs were peeled off and photographed (A). (B) Quantitative analysis of neovascularization from the photographs. Data expressed as mean±S.D., $n>3$; means without a common letter differ significantly ($P<.05$).

relation with its anti-angiogenic effect, the present study examined the production of cytokines in human peripheral blood MNC stimulated with lycopene. Data reveal that LP-MNC-CM inhibited angiogenic features of HUVECs in vitro, as demonstrated by assays of tube formation and cell migration. In addition, LP-MNC-CM treatment significantly inhibited neovascularization ex vivo by CAM assay. To the best of our knowledge, this is the first report showing the anti-angiogenic effects of lycopene through immunomodulation both in vitro and ex vivo. Mechanistically, we presented data to show that the anti-angiogenic effect of lycopene involved regulation of cytokine secretion and reduction of MMP-2 activity in HUVECs.

Several lines of evidence support the hypothesis that lycopene may exert their anti-angiogenic activity indirectly through immunomodulation. First, we showed that LP-MNC-CM significantly increased secretion of IL-12 and IFN-γ in MNC-CM, indicating that lycopene enhances the host immune response. Augmentations of cytokine secretion are known as the important function of lycopene [29,33,42]. A human intervention study has also demonstrated that tomato juice consumption for 2 weeks significantly enhances IL-12 secretion [33]. Previously, we demonstrated that lycopene inhibits metastasis of hepatocarcinoma in nude mice and that this effect is associated with an induction of IL-12 expression by ca. 1.8–2.1-fold, supporting an immunomodulatory function of lycopene [35]. Furthermore, IL-12 has been shown to inhibit the secretion of VEGF and bFGF [43] and has been demonstrated in many studies to have potent anti-angiogenic effects and anti-tumor activities [44]. In addition, our present results show that HUVECs incubated with IL-12 (100–1500 pg/ml) for 6, 12 and 24 h developed relatively incomplete and narrow tube-like structures (Supplemental Figure 1). Indeed, it has been suggested that lycopene may exert its anti-angiogenic effects through immunomodulation [27].

A second line of evidence that lycopene may exert their anti-angiogenic activity indirectly through immunomodulation is that DE, an IL-12 inhibitor, antagonized the anti-angiogenic ability of lycopene. Furthermore, DE in combination with lycopene to MNC also reversed the immunomodulatory effects of lycopene on IFN-γ and IL-8. These results indicating that IL-12 plays a pivotal role in the anti-angiogenic effect of lycopene. The decrease in the angiogenesis-associated phenotypes, such as tube formation, cell proliferation cell migration and MMP-2 expression in HUVECs, induced by LP-MNC-CM treatment may, at least partially, be mediated by IL-12 cascade present in MNC-CM induced by lycopene, including up-regulation of IFN-γ and down-regulation of IL-8 and $PGE_2$.

Human IFN-γ has been shown to inhibit proliferation and migration of human endothelial cells and capillary tube formation in vitro and represses lymphocyte-induced tumor angiogenesis [45]. Moreover, IL-8 has been suggested to induce proliferation and migration of HUVECs, angiogenesis in human coronary atherectomy tissue [46], and tube formation of human dermal microvascular endothelial cell, whereas treatment with anti-IL-8 antibody inhibits tube formation and neovascularization in endothelial cells [19]. In this context, Wysoczynski et al. [46] have suggested that anti-IL-8 therapy could be a potential therapeutic strategy against tumor angiogenesis. Our results are in accord with those of Simone et al. [47], which show that lycopene pre-treatment results in a significant inhibition of cigarette smoke extract-induced IL-8 expression at both mRNA and protein levels in human THP-1 cells. In addition to IL-8, we showed that $PGE_2$ treatment stimulated the tube formation of HUVECs and enhanced the expression of VEGF mRNA [48]. Indeed, it has been suggested that the development of agents that lower cellular levels of $PGE_2$ or that specifically inhibit the $PGE_2$ downstream signaling pathway may be useful for cancer prevention [49].

Overall, the present study demonstrates that lycopene has significant anti-angiogenic effects both in vitro and ex vivo and that these effects are likely mediated by modulation of cytokine secretion of MNC, leading to decreased phenotype of angiogenesis and secretion of MMP-2 in HUVECs. These results provide a potentially new mechanism underlying the anti-angiogenic action of lycopene.

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