

Inhibitory Effects of Lycopene on the Adhesion, Invasion, and Migration of SK-Hep1 Human Hepatoma Cells

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Lycopene, which is the predominant carotenoid in tomatoes and tomato-based foods, may protect humans against various cancers. Effects of lycopene on the adhesion, invasion, migration, and growth of the SK-Hep1 human hepatoma cell line were investigated. Lycopene inhibited cell growth in dose-dependent manners, with growth inhibition rates of 5% and 40% at 0.1 μ M and 50 μ M lycopene, respectively, after 24 hrs of incubation. Similarly, after 48 hrs of incubation, lycopene at 5 μ M and 10 μ M decreased the cell numbers by 30% and 40%, respectively. Lycopene decreased the gelatinolytic activities of both matrix metalloproteinase (MMP)-2 and MMP-9, which were secreted from the SK-Hep1 cells. Incubation of SK-Hep1 cells with 110 μ M of lycopene for 60 mins significantly inhibited cell adhesion to the Matrigel-coated substrate in a concentration-dependent manner. To study invasion, SK-Hep1 cells were grown either on Matrigel-coated Transwell membranes or in 24-well plates. The cells were treated sequentially for 24 hrs with lycopene before the start of the invasion assays. Cell growth and death were assessed under the same conditions. The invasion of SK-Hep1 cells treated with lycopene was significantly reduced to 28.3% and 61.9% of the control levels at 5 μ M and 10 μ M lycopene, respectively ($P < 0.05$). In the migration assay, lycopene-treated cells showed lower levels of migration than untreated cells. These results demonstrate the antimetastatic properties of lycopene in inhibiting the adhesion, invasion, and migration of SK-Hep1 human hepatoma cells. *Exp Biol Med* 231:322–327, 2006

Key words: lycopene; SK-Hep1 cell; liver cancer; matrix metalloproteinase; adhesion; invasion; migration; metastasis; extracellular matrix

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Introduction

Lycopene is the main carotenoid in tomatoes and tomato products and is responsible for the red coloration of tomatoes. Although lycopene lacks provitamin A activity, it has efficient singlet oxygen quenching and radical scavenging activities (1, 2). Recent animal and cohort studies have shown lower risks for liver (3) and prostate cancer (4, 5) in people with either high serum levels of lycopene or with a regular high intake of lycopene or tomatoes. Suggested inhibitory mechanisms for lycopene include enhancing gap junctional intercellular communication (6), cell cycle arrest (7), suppression of tumor cell proliferation (8), and apoptosis (7, 9).

Metastasis is one of the major causes of mortality in cancer patients and occurs as a complex multistep process that involves cancer cell adhesion, invasion, and migration (10, 11). Inhibition of the invasion and metastasis of cancer cells is of great significance in cancer treatment.

Tumor cell invasion and metastasis are fundamental and characteristic properties of carcinogenesis. In the multiple stages of these processes, the degradation of environmental barriers, such as the extracellular matrix (ECM) and basement membrane, is the initial step, and several proteolytic enzymes participate in the degradation of these barriers (12, 13). Among these enzymes, matrix metalloproteinases (MMPs) play a major role.

MMPs constitute a family of zinc-binding endopeptidases, which collectively degrade most of the components of ECM and have been implicated in cancer invasion and metastasis (11). In particular, MMP-2 (M_r 72,000 type IV collagenase, gelatinase-A) and MMP-9 (M_r 92,000 type IV collagenase, gelatinase-B) degrade components of the basement membrane and are strongly implicated in the invasion and metastasis of malignant tumors (11, 14, 15). Therefore, the inhibition of invasion mediated by MMP-2 and MMP-9 may be crucial for the inhibition of cancer metastasis. Recent clinical studies have shown that MMP activity is required for rapidly proliferating and invading tumors rather than for already established tumors (16–18). Thus, the inhibition of MMP activity is important for the

prevention of early stage carcinogenesis, particularly the tumor promotion process.

Hepatocellular carcinoma (HCC) is the most frequent malignant tumor of the liver. The human HCC cell line SK-Hep1 is invasive and expresses the gelatinase activity required for migration and invasion (15, 19). Few studies have been reported on the potential link between liver cancer and lycopene. Liver cancer is the second most common fatal cancer in Southeast Asia and Korea (20).

The aim of this study was to elucidate the potential of lycopene as an inhibitor of MMP-2 and MMP-9 activities using gelatin zymography. We also investigated the influence of lycopene on cancer cell viability, adhesion, invasion, and migration using SK-Hep1 cells.

Materials and Methods

Chemicals. Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), and RNase A were obtained from Sigma Chemical Company (St. Louis, MO). Trypsin-EDTA and penicillin/streptomycin were from Gibco Life Technologies Inc. (Paisley, UK), and fetal bovine serum (FBS) was obtained from Gemini Bio-Products (Calabasas, CA). All of the solvents were analytical high-performance liquid chromatography (HPLC)-grade from Fisher Scientific (Los Angeles, CA), and cell culture supplies were purchased from Costar (Corning, Cypress, CA). Lycopene (99%) was purchased from Sigma Chemical.

Cell Culture. SK-Hep1 human hepatocellular carcinoma cells were obtained from the Korean Cell Line Bank (KCLB; Seoul, Korea). The SK-Hep1 cells were cultured in DMEM that was supplemented with 10% FBS and penicillin/streptomycin in a 37°C humidified incubator (Forma Scientific Co., Marietta, OH) in an atmosphere of 5% CO₂ and 95% air.

Lycopene Treatment and Determination. Lycopene was dissolved in tetrahydrofuran (THF) to a final concentration of 2 mM to ensure the complete dispersion of crystals. This stock solution was prepared with a minimum of exposure to air and light and stored at -70°C. Immediately before the experiment, aliquots from the stock solution were added to the cell culture medium, and the final concentration of lycopene was determined by HPLC as described by Hwang and Bowen (21) with some modification. Reverse-phase HPLC was carried out on a JASCO HPLC system consisting of an intelligent HPLC pump PU-2086 and wavelength absorbance detector MD-2010 (JASCO, Tokyo, Japan). The final THF concentration of 0.5% did not affect cell growth compared with control media.

Cell Proliferation Assay. Cell viability was measured by the MTT assay. Cells were cultured for 24 hrs in 96-well plates at a concentration of 1000 cells/well. Each well was filled with fresh FBS-free medium that contained various amounts of sample. The cells were then incubated

for a further 48 hrs at 37°C. Each well was then incubated with MTT for 4 hrs. The liquid was removed, and dimethylsulfoxide (DMSO) was added to dissolve the solid residue. The optical density of each well at 570 nm was determined using a microplate reader (Molecular Devices, San Diego, CA).

Gelatin Zymography. Cells were grown to subconfluence, rinsed with phosphate-buffered saline (PBS), and incubated in serum-free medium for 48 hrs. The conditioned media were collected, and the cell numbers were determined. Cell number-standardized conditioned media were resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that contained 1 mg/ml gelatin. The gel was washed twice with 2.5% Triton X-100 for 30 mins to remove the SDS. The gel was subsequently incubated for 18 hrs at 37°C in a buffer that contained 50 mM Tris-HCl (pH 7.6), 200 mM NaCl, 5 mM CaCl₂. The gel was stained with 0.5% Coomassie blue for 1 hr and then destained in water that contained 10% glacial acetic acid and 30% methanol. Proteolysis was detected as a white zone under a dark field, and the intensities of the bands were measured.

Cell Adhesion Assay. The 24-well tissue culture plates were coated with 25 µg/well Matrigel and left to air-dry for 40 mins. Cells (5×10^4) suspended in DMEM that contained 0.5% bovine serum albumin (BSA) were dispensed into each well of the 24-well culture plate, incubated in 5% CO₂ at 37°C for 1 hr and gently washed three times with PBS to remove the unattached cells. The cells that remained attached to the bottom of the plate were stained with hematoxylin-eosin and counted under a microscope (Olympus IX70; Olympus, Okaya, Japan). The experiments were performed at least four times.

Cell Invasion Assay. A Transwell system that incorporated a polycarbonate filter membrane with a diameter of 6.5 mm and pore size of 8 µm (Corning Inc., Corning, NY) was used to assess the rate of cell invasion. The Matrigel (12.5 µg in 50 µl PBS) was added to the filter to form a thin gel layer, dried in a laminar hood overnight, and reconstituted in 100 µl PBS at 37°C for 2 hrs. The cells at 90% confluency were harvested using a cell-dissociation solution. The cells (1×10^5) were suspended in 100 µl of serum-free medium in the presence or absence of different concentrations of lycopene and added to the upper chamber of the Transwell insert. The lower chamber was filled with 500 µl of the same medium as in the upper chamber. After 24 hrs of incubation at 37°C, the cells on the upper surface of the filter were removed using a cotton swab. The cells that penetrated to the lower surface of the filter were stained with hematoxylin and counted under an Olympus IX70 microscope (Olympus) in 13 randomized fields at $\times 400$ magnification. The assay was performed on at least four separate occasions.

Wound Migration Assay. Cell motility was examined using the wound migration assay. Cells were cultured to 100% confluency in a six-well plate. The cells were

pretreated with mitomycin C (25 $\mu\text{g/ml}$) for 30 mins before the injury line was made by applying a plastic pipette tip to the cells across the center of the well, thereby producing a clean 1-mm-wide wound area. After rinsing with PBS, the cells were allowed to migrate in the medium. A computer-based microscopy imaging system (Olympus) was used to determine wound healing at 0 hr with a microscope at $\times 200$ magnification. At the indicated time points, migration was assessed by counting the number of cells across the wound, which was then compared with the same frame at 0 hr. Several wound areas (at least five per plate) were quantified for cell migration. The migration distances between the leading edge of the migrating cells and the edge of the wound were compared. Migration rate = (migration distances of lycopene-treated cells/migration distances of untreated cells) $\times 100\%$. The experiments were performed at least four times.

Statistical Analysis. All of the experiments were replicated three times. The mean standard deviation, mean square errors, two-factor ANOVA, correlation and interaction of main effects were calculated using the SigmaStat computer software, version 1.0 (Jandel Corp., San Rafael, CA). Appropriate comparisons were made using the Student-Newman-Keuls' method for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

The MTT assay was used to determine the effects of lycopene on SK-Hep1 human hepatoma cell growth. Figure 1 illustrates the effects of 0.1–50 μM lycopene on the growth of SK-Hep1 cells after 24 and 48 hrs. After 24 hrs of incubation, lycopene inhibited the proliferation of SK-Hep1 cells in a dose-dependent fashion ($n = 4$). At lycopene concentrations of 0.1–50 μM , the cell numbers were significantly reduced by 5%–40%. Similarly, after 48 hrs of incubation, the cell numbers were decreased by 30% with

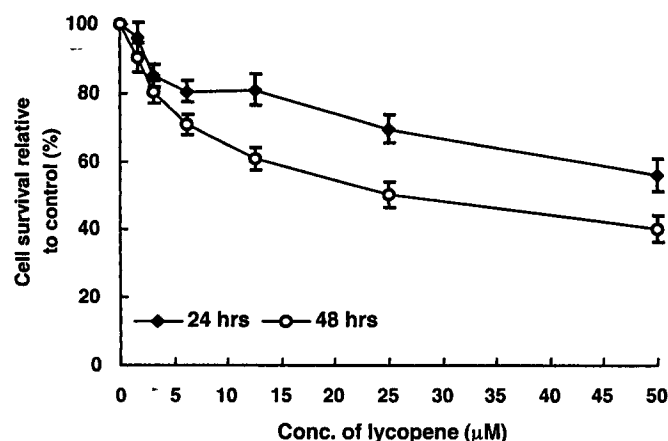


Figure 1. Effect of lycopene on SK-Hep1 cell proliferation, as measured by the MTT assay. MTT assay was determined at 570 nm and expressed as cell survival relative to control. Results are presented as mean \pm SD of four independent experiments.

5 μM lycopene and by 40% with 10 μM lycopene. This compound also dose-dependently inhibited LNCaP cell proliferation.

Gelatinases/type IV collagenase play a major role in the facilitation of cancer metastasis. Thus, gelatin zymography was used to study the effects of lycopene on MMP-2 and MMP-9 activities. The activities of MMPs are regulated at various levels, such as during secretion and the activation of pro-MMP to the enzymatically active MMP. To examine the inhibitory effects of lycopene on MMP-2 and MMP-9, conditioned media from SK-Hep1 cell cultures were subjected to gelatin zymography in the presence of various concentrations of lycopene. The SK-Hep1 cells constitutively secreted high levels of MMP-9 and low levels of MMP-2. As shown in Figure 2, the gelatin lysis activity in the zymogram was inhibited by lycopene in a dose-dependent manner. Lycopene at 1.25 μM and higher concentration clearly suppressed MMP-2 activity, and MMP-9 activity was inhibited in a dose-dependent manner by 2.5–20 μM lycopene. Thus, in the SK-Hep1 cell system, both MMP-2 and MMP-9 were downregulated by lycopene treatment.

Tumor metastasis comprises multiple steps. Therefore, tumor cells need to express a variety of properties, including altered adhesiveness, increased motility, and invasive capacity, to complete the metastatic process. Because the adhesion to and motility of tumor cells in the ECM are considered important steps in the invasive processes of metastatic tumor cells, the effects of lycopene on cell adhesion were examined. Incubation of SK-Hep1 cells with 1–10 μM lycopene for 60 mins significantly inhibited cell adhesion to the Matrigel-coated substrate in a concentration-dependent manner (Fig. 3).

We further evaluated the antimetastatic activity of lycopene using the Transwell assay. We tested the ability of SK-Hep1 cells to invade through a reconstituted basement membrane barrier (Matrigel) with or without lycopene. Similar to the migration assay, lycopene inhibited the invasion of SK-Hep1 cells in a dose-dependent manner in the range of 1–10 μM . When the SK-Hep1 cells were grown on Matrigel, a significant reduction in the number of invasive cells was seen when the cells were treated with 5–10 μM lycopene for 18 hrs, as compared with the control (fresh medium alone), with the levels of invasion being reduced to $61.9 \pm 5.09\%$ of the control levels at 10 μM lycopene (Fig. 4). No significant reduction in invasiveness

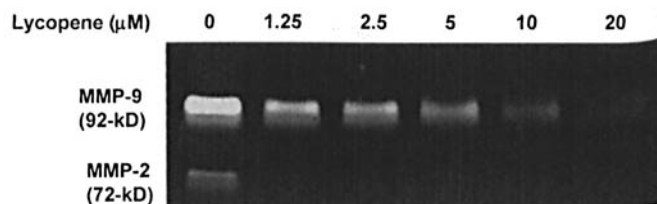


Figure 2. Effects of lycopene on the MMP activities of SK-Hep1 cells.

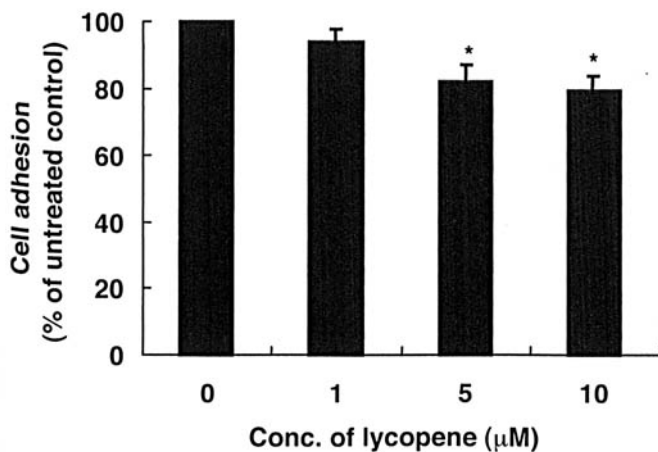


Figure 3. Effect of lycopene on cell adhesion using Matrigel. SK-Hep1 cells in wells that were precoated with Matrigel were treated with or without different concentrations of lycopene for 24 hrs. After a 60-min incubation at 37°C, the numbers of adhering cells were counted under a microscope. The data are presented as the mean \pm SD of three separate experiments. The asterisk symbol indicates that the means are significantly different from the control ($P < 0.05$).

was observed when the cells were treated with the lower dose of 1 μ M lycopene or when the cells were treated for shorter time periods (1 hr or 4 hrs).

To evaluate the antimetastatic activity of lycopene, we assessed the effect of lycopene on the migration of SK-Hep1 cells in the wound migration assay. The migration distances between the leading edge and the wound line were compared between lycopene-treated cells and untreated cells. As shown in Figure 5, cellular motility was controlled in a time-dependent manner by 10 μ M lycopene, with up to 94% and 82% motility at 24 hrs and 48 hrs incubation, respectively ($P < 0.001$).

Discussion

Chemoprevention involves the use of natural or synthetic chemicals to block, reverse, and suppress the process of carcinogenesis (22). Because many studies have demonstrated the inhibitory action of lycopene against carcinogenesis in animals and humans, lycopene is considered a useful chemopreventive agent (7, 23).

Hwang and Bowen (24) showed that lycopene and a tomato paste hexane extract inhibited LNCaP human prostate cell growth in a dose-dependent (0.1–50 μ M lycopene) manner, and that growth inhibition after 48 hrs was 55% and 35% with 1 μ M lycopene and tomato paste hexane extract, respectively.

In animal models, lycopene has shown protective effects on liver cancer, with two out of four investigations showing protection. Toledo *et al.* (3) treated rats with 70 mg/kg/body weight lycopene or corn oil by gavage on alternate days for eight consecutive weeks. The lycopene treatment resulted in increases in the respective liver carotenoid concentrations ($P < 0.05$) and reduced the incidence, total number, and multiplicity of hepatocyte nodules, in addition to reducing the numbers of hepatic

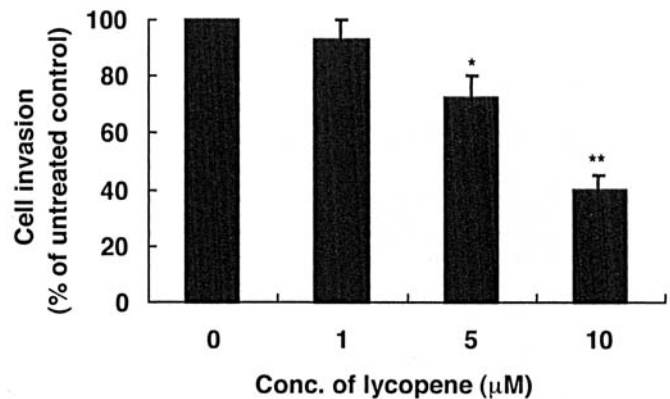


Figure 4. Effect of lycopene on the invasiveness of SK-Hep1 cells. The invasiveness of SK-Hep1 cells toward endothelial cell-conditioned medium was measured using Transwell chambers and tissue culture-treated filters with 8 μ m pores. Each bar represents the mean \pm SD of three separate experiments. The symbols * and ** indicate that the means are significantly different from those obtained for other treatments ($P < 0.05$).

placental glutathione-S-transferase (GST)-positive preneoplastic lesions and hepatic DNA strand breaks when compared with the control group. Feeding the rats lycopene (300 mg/kg diet) for a 3-week period significantly decreased the sizes of the γ -glutamyl transpeptidase- and GST-positive foci induced by diethylnitrosamine by 64% and 65%, respectively, as well as decreasing the fraction of the liver volume occupied by foci (25). However, Watanabe *et al.* (26) found that administration of 0.005% lycopene (originally the product of tomato oleoresin that contained 13% lycopene) for 70 weeks did not reduce the rate of HCC, the level of serum α -fetoprotein, or the histologic grade of HCC in rats. Although these results are apparently inconsistent, many epidemiological and animal studies have shown some bioactivity in the prevention of cancer in humans and animals.

Lycopene is very sensitive to light, oxygen, and incubation temperature and is rapidly oxidized under these conditions. Several oxidation products of lycopene have been reported (27, 28). However, little is known about their biological effects, in terms of the inhibition of cell proliferation and the enhancement of gap junctional communication. Kotake-Nara *et al.* (29) have reported that lycopene drastically increases the inhibitory effect on cell growth when it is oxidized before cell supplementation.

Kozuki *et al.* (30) examined eight types of carotenoid (lycopene, α -carotene, β -carotene, β -cryptoxanthin, zeaxanthin, lutein, canthaxanthin, and astaxanthin) to evaluate their abilities to inhibit the invasion of AH109A rat ascites hepatoma cells. All of the tested carotenoids, including lycopene, decreased AH109A invasion in a dose-dependent manner (2.5–20 μ M) at concentrations of 0–5 μ M, and inhibitory activity reached a plateau at 5 μ M or more. The conjugated double bonds and ROS-quenching properties may be important for the actions of carotenoids that inhibit the invasion processes of cancer cells. These results suggest

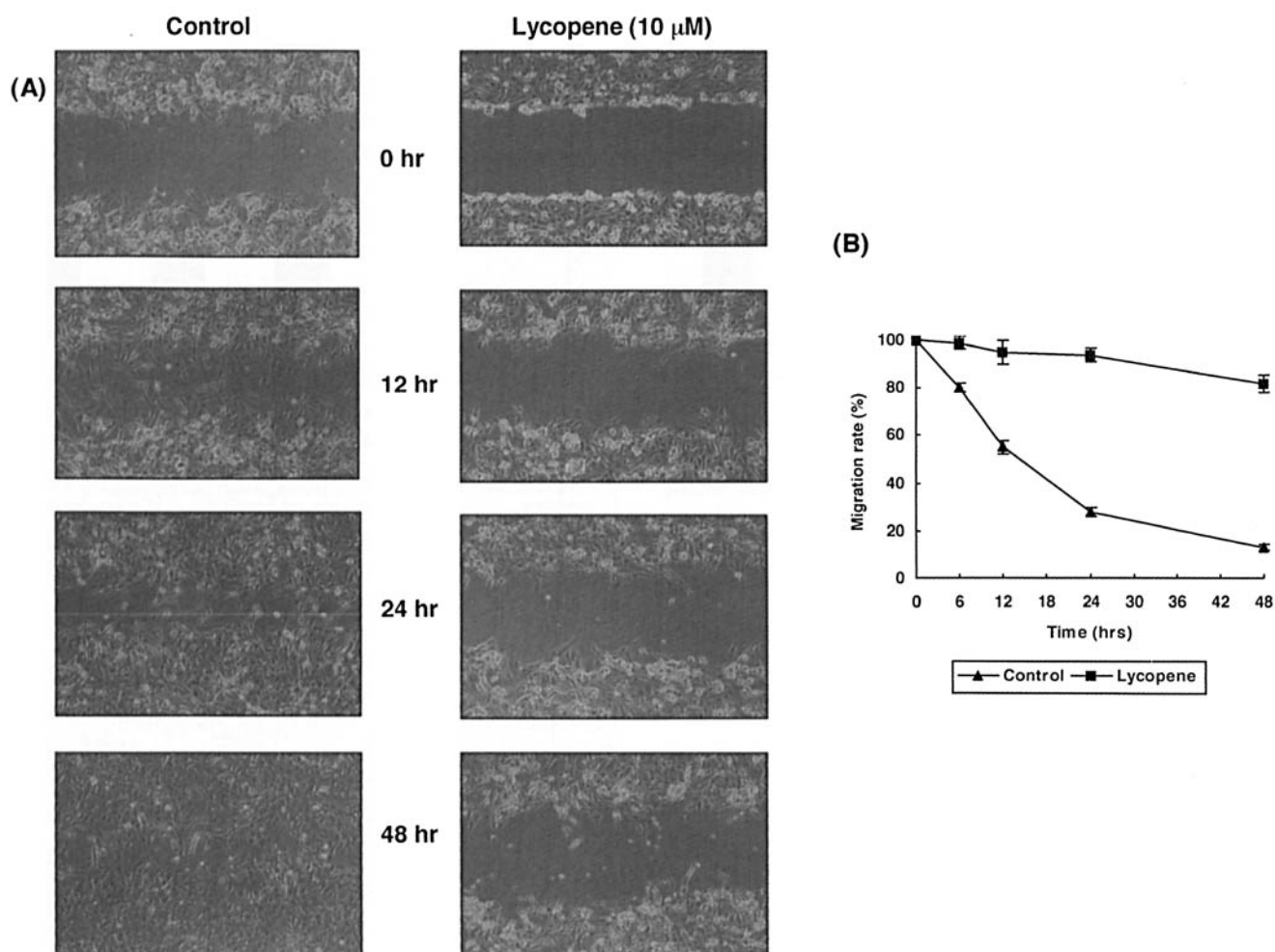


Figure 5. Effects of lycopene on wound healing migration of SK-Hep1 cells. A wound was introduced by scraping the confluent cell layers with a pipette tip. (A) Representative photographs of invading cells that received either control treatment or lycopene. (B) The migration distances were measured at the indicated time-points. Migration rate was determined as follows: migration rate = (migration distances of lycopene-treated cells/migration distances of untreated cells) \times 100%. Results are presented as mean \pm SD of four independent experiments.

that the antioxidative properties of carotenoids are linked to their anti-invasive actions.

Metastasis is one of the most important factors related to cancer therapeutic efficacy and prognostic survival (31). In cancer research, one of the most active fields is the development of novel antimetastatic drugs with low toxicity and high efficacy. In this study, using adhesion, wound healing, and invasion assays, we have shown that lycopene effectively inhibits the migration of tumor cells *in vitro*. In addition, we have demonstrated that lycopene suppresses the activities of MMP-2 and MMP-9 and eventually inhibits cancer cell metastasis. This conclusion is in agreement with those of previous studies that inhibitors of MMP-2 significantly suppress tumor metastasis in experimental animals (32). Nevertheless, the underlying molecular mechanisms of this action of lycopene remain unknown and require further research.

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