Regulation of C6 glioma cell migration by thymol

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Abstract. Tumor cell motility exhibits a crucial role in tumor development. Therefore, the present study aimed to investigate whether thymol could reduce C6 glioma cell migration. Cell viability was determined using the EZ-Cytox Cell Viability kit. The scratch wound healing and Boyden chamber assays were performed to test C6 glioma cell migration in the presence of fetal bovine serum (FBS). Additionally, the study investigated whether signaling proteins relevant to C6 glioma cell migration, i.e., extracellular signal-regulated kinases (ERK)1/2, protein kinase Cα (PKCα), matrix metallopeptidase (MMP)9 and MMP2, were affected by thymol treatment. Up to 30 μM, thymol did not alter cell viability, whereas 100 μM thymol induced the death of ~20% of the cells. Furthermore, thymol (30 μM) significantly reduced FBS-induced migration. In the FBS-stimulated C6 glioma cells, thymol (30 μM) suppressed PKCα and ERK1/2 phosphorylation. MMP9 and MMP2 production was also significantly reduced by treatment with 30 μM thymol in the C6 glioma cells. Taken together, these results indicate that thymol attenuates C6 glioma cell migration. Additionally, the study suggests that the effect of thymol on the FBS-induced migration of C6 glioma cells affects PKCα and ERK1/2 signaling, and suppresses MMP9 and MMP2 production.

Introduction

Brain tumors are characterized by the presence of malignant tissues within the skull and central spinal canal. Between 2008 and 2012 in the USA, the annual age-adjusted average incidence rate for all primary brain and central nervous system tumors was 21.98 per 100,000 individuals (7.23 malignant; 14.75 benign), and 356,858 brain tumors (117,023 malignant; 239,835 benign) were reported during this time (1). Gliomas are the most common primary brain tumor, accounting for ~81% of malignant brain tumors (2). Between 2008 and 2012, the annual age-adjusted average incidence rate of this type of brain tumor was 5.83 per 100,000 individuals (1). A characteristic of glioma is that it spreads rapidly to normal brain areas so that the boundary between normal tissue and the tumor becomes indistinct (3). In particular, it has the highest mortality rate due to its specialized feature of rapid cell migration or invasion that cannot be controlled by either surgery or irradiation (4,5). Thus, patients with this brain disease have an average survival rate of 1 year from the time of tumor development.

Moreover, the diagnosis and treatment of glioma are difficult, its pathology and pattern of invasion and migration are poorly understood (6,7). Several studies have reported that the pathological response of glioma, which is the malignant process of infiltration into the extensive normal tissue, is due to the activation of mitogen-activated protein kinase (MAPK), protein kinase Cα (PKCα) and matrix metalloproteinases (MMPs) (8-11). The MAPK family consists of three types of kinases, p38 MAPK, Jun N-terminal kinase and extracellular signal-regulated kinase 1/2 (ERK1/2), which are involved in cell migration and the growth of the majority of cancer cell types (12). Notably, ERK1/2 phosphorylation is involved in the cell invasion, migration and motility coupled with the progression of brain cancer (13,14). Activation of PKCα is also implicated in the migration of glioma cells (15). MMPs are extracellular endopeptidases involved in motility and invasion (16,17).

Currently, numerous researchers are seeking novel antioxidant and anticancer agents derived from plants. Thymol is a component of a number of essential oils and is known for its anti-inflammatory, anticancer and anti-bacterial effects (18). Studies on essential oils extracted from a variety of plants have shown that they possess great nutritional value, and significant biochemical and physiological activities (18). These beneficial properties have been put to use in the development of functional and medicinal foods. The present study was performed to investigate the effect of thymol on glioma cell migration to determine whether it may have potential in glioma prevention and treatment.

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Materials and methods

Reagents. Thymol, cell culture materials and the EZ-Cytox Cell Viability Assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA), Thermo Fisher Scientific (Gaithersburg, MD, USA) and Daeil Lab Services Co., Ltd. (Seoul, Korea), respectively. Rabbit polyclonal anti-rat anti-PKCα (catalog no., #2056), rabbit polyclonal anti-rat anti-phosphorylated (P)-PKCα (catalog no., #9375) and rabbit polyclonal anti-rat anti-glyceraldehyde 3-phosphate dehydrogenase (catalog no., #2118) antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Other antibodies, such as rabbit polyclonal anti-rat anti-ERK1/2 (catalog no., sc-94), mouse monoclonal anti-rat anti-P-ERK1/2 (catalog no., sc-7383), rabbit polyclonal anti-rat anti-MMP2 (catalog no., sc-10736) and goat polyclonal anti-rat anti-MMP9 (catalog no., sc-6840), were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). All other chemicals were purchased from Sigma-Aldrich.

Cell culture and viability assay. Rat C6 glioma cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37˚C in a 5% CO_2 atmosphere. The C6 glioma cells were seeded at $5x10^4$ cells/well in a 96-well microplate containing DMEM and incubated for 24 h. The cells were then incubated with different concentrations (0, 0.1, 0.3, 1, 3, 10, 30, 100 and 200 μM) of thymol in FBS-free medium for 24 h. Cell viability was then determined using an EZ-Cytox Cell Viability Assay kit according to the manufacturer's protocol. The cell viability of thymol-treated cells was determined relative to that of control cells by measuring the absorbance at 450 nm.

Scratch wound healing assay. The C6 glioma cells were seeded at a density of $1x10^5$ cells/ml in a six-well plate and incubated in 10% FBS-containing medium for 24 h. These cells were then placed in serum-free medium for 24 h. The scratch wound was made by scratching the center of each well with a 200-μl sterile pipette tip to form a cross. This was followed by incubation with or without thymol (0, 3, 10 and 30 μm) in serum-containing medium for an additional 24 h. Images of the cells that migrated into the cell-free scratch wound area were acquired using an inverted microscope (IX71; Olympus Corp., Tokyo, Japan) and analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Boyden chamber assay. To determine the effect of thymol on the migration of the C6 glioma cells, a Boyden chamber assay was performed in a 48-well chemotaxis chamber (Neuro-Probe, Gaithersburg, MD, USA), as previously described (19). Briefly, an absence or presence of thymol (3-30 μM) in DMEM containing 10% FBS were loaded into the lower chamber. The lower chamber was covered by a polycarbonate filter membrane (pore size, 8 μm) that was coated with 0.1% collagen type-I (BD Biosciences, Franklin Lakes, NJ, USA). C6 glioma cells ($1x10^6$ cells/ml) were loaded into the upper chamber. Following incubation at 37˚C in 5% CO_2 for 90 min, the cells on the lower surface of the membrane were fixed and stained using Diff-Quick (Baxter Healthcare, Deerfield, IL, USA). The cells that had migrated through the membrane were imaged and counted under an inverted microscope (IX71; Olympus Corporation, Tokyo, Japan).

Gelatin zymography. To determine the activity of gelatinases, such as MMP2 and MMP9, in the thymol-treated C6 glioma cells, a gelatin zymography assay was performed, as previously described (8). Briefly, to test the gelatin zymography, samples of cultured media supernatant from C6 glioma cells were collected. Samples (30 μl) were loaded onto a 8% sodium dodecyl sulfate-polyacrylamide electrophoresis gel containing 0.2% gelatin (WELGENE, Daegu, South Korea). Following electrophoresis, the gel was incubated with 2.5% Triton X-100 (Sigma-Aldrich) and agitated. Following incubation at 37˚C for 24 h, the gel was stained using Coomassie Brilliant Blue R 250 (Sigma-Aldrich). Stained bands were visualized and quantified using Image J software.

Western blot analysis. To determine the expression of proteins associated with the migration of C6 glioma cells, western blotting was performed with specific antibodies. Briefly, 20 μg of protein was prepared from each treatment group. Once the proteins had been boiled at 100˚C for 10 min, they were separated by electrophoresis on 12% acrylamide gels and then transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in transfer buffer at 4˚C for 2 h. The membranes were blocked in 5% bovine serum albumin in Tris-buffered saline (TBS) at room temperature for 1 h and then washed in TBS with 0.1% Tween 20 (TBS/T). Subsequently, the membranes were incubated overnight at 4˚C with antibodies against P-ERK1/2 and P-PKCα, total ERK1/2 (T-ERK1/2) and PKC (T-PKC), MMP9 and MMP2 (all 1:1,000 dilution). The membranes were washed with TBS/T, followed by incubation with donkey anti-goat immunoglobulin (Ig) G (catalog no., sc-2033; Santa Cruz Biotechnology, Inc.), horse anti-mouse IgG (catalog no., 7076; Cell Signaling Technology, Inc.) or goat anti-rabbit IgG (catalog no., 7074; Cell Signaling Technology, Inc.) horseradish peroxidase-conjugated secondary antibodies (all 1:1,000 dilution). The protein expression levels were analyzed via electrochemiluminescence (ECL plus kit; Amersham Pharmacia Biotech). The protein bands were visualized and quantified using Image J Software.

Statistical analysis. The results are expressed as the mean ± standard error of at least three independent experiments. The differences between the test groups were examined using a one-way analysis of variance followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed with GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Effect of thymol on C6 glioma cell viability. The chemical structure of thymol is presented in Fig. 1A. Thymol cytotoxicity was tested by treating C6 glioma cells with different

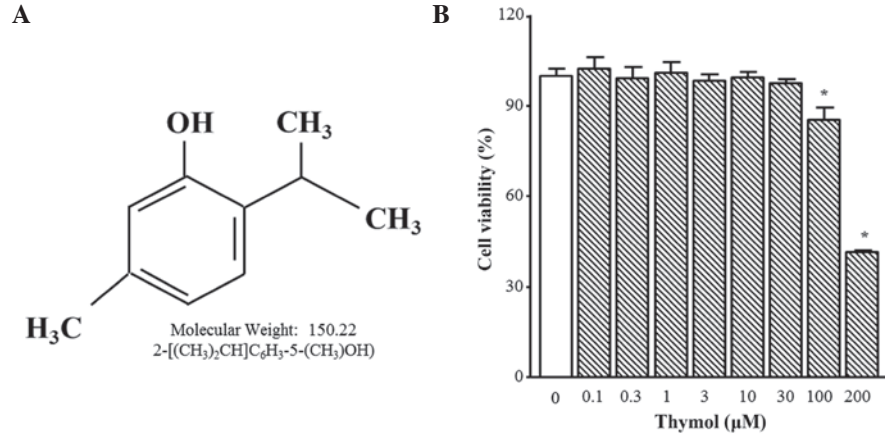

Figure 1. Effect of thymol on C6 glioma cell viability. (A) Chemical structure of thymol. (B) Effect of thymol on C6 glioma cell viability. The C6 cells were cultured in serum-free medium in the presence of thymol (0.1-200 μM) for 24 h. Post-treatment cell viability was determined using a 2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide assay. Data are expressed as the mean ± standard error (n=3). Cell viability in the absence of thymol is expressed as 100%. *P<0.05 vs. control (no thymol).

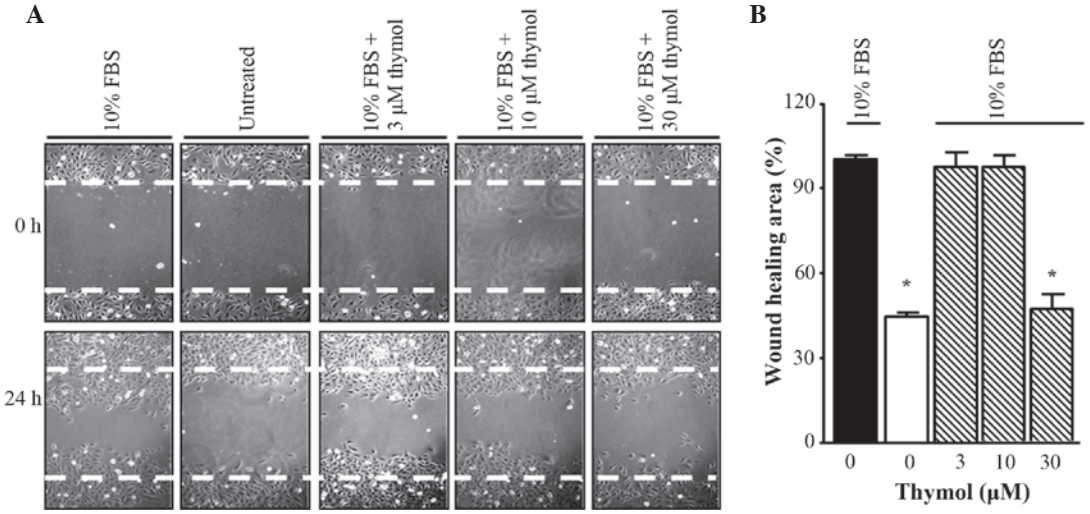

Figure 2. Effect of thymol treatment on the scratch wound healing migration of C6 glioma cells. C6 glioma cells were cultured in serum-free medium for 24 h and then scratched using a sterilized 200-μl pipette tip. The cells were cultured in the absence or presence of thymol (3-30 μM) for 1 h and then exposed to 10% FBS for 24 h. (A) The scratch distance at time 0 (the white dotted line in the upper panel) was used for normalization and the closed distance at 24 h (lower panel) was used for wound healing assay. (B) The vertical axis represents the relative healing (%) of the wounded region. Data are expressed as the mean ± standard error (n=3). Wound healing in presence of treatment (FBS alone) is expressed as 100%. *P<0.05 vs. FBS stimulation without thymol. FBS, fetal bovine serum.

concentrations (0.1-200 μM) of thymol for 24 h. Cell viability was not altered by thymol up to 30 μM, but 100 μM and 200 μM of thymol induced significant decreases in cell viability of 24.0±6.5 and 54.2±3.5%, respectively (Fig. 1B; P=0.0361 and P<0.0001, respectively). Therefore, all of the following experiments were performed using 30 μM thymol or less.

Thymol suppresses C6 glioma cell migration. To determine whether thymol could inhibit the migration of FBS-stimulated glioma cells, a scratch wound healing assay was first performed. The cells were incubated in FBS-free medium for 24 h and then stimulated with 10% FBS in the presence of different concentrations of thymol (3-30 μM) for 24 h. As shown in Fig. 2, 30 μM thymol treatment caused a decrease in scratch wound healing compared with FBS treatment alone, whereas for other concentrations of thymol (3-10 μM), the inhibition ratios indicated similar results to the untreated group. Next, in order to confirm the inhibitory effect of thymol on C6 glioma cell migration, a Boyden chamber assay was performed. Thymol (30 μM) suppressed the FBS-stimulated migration of glioma cells over 90 min. As shown in Fig. 3, FBS-stimulated C6 glioma cell migration was significantly inhibited by 30 μM thymol (P=0.0001).

Effect of thymol on the phosphorylation of PKCα and ERK1/2. To investigate the expression of protein markers for C6 glioma cell migration, the phosphorylation levels of PKCα and ERK1/2 were measured. As shown in Fig. 4, FBS-induced PKCα phosphorylation was not affected by 3 and 10 μM thymol compared with the control. However, it decreased to 26.5±1.2%, when the cells were treated with 30 μM thymol (Fig. 4B; P<0.0001). FBS induced-phosphorylation of ERK1/2 also showed a similar pattern, with no

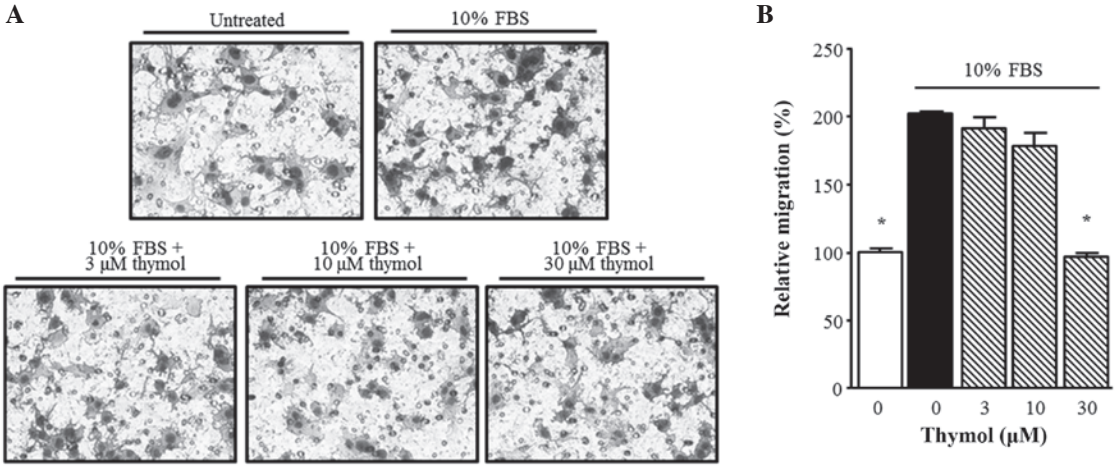

Figure 3. Effect of thymol treatment on FBS-stimulated C6 glioma cell migration. (A) C6 glioma cells were cultured in serum-free medium for 24 h. Cell migration was then tested in a Boyden chamber. The cells were incubated in the presence of thymol (3-30 μM) with 10% FBS for 90 min. (B) The data represent the relative migration compared to the untreated control cells. Data are expressed as the mean ± standad error (n=4). Untreated cell migration is expressed as 100%. *P<0.05 vs. FBS-stimulated cells (black bar). FBS, fetal bovine serum.

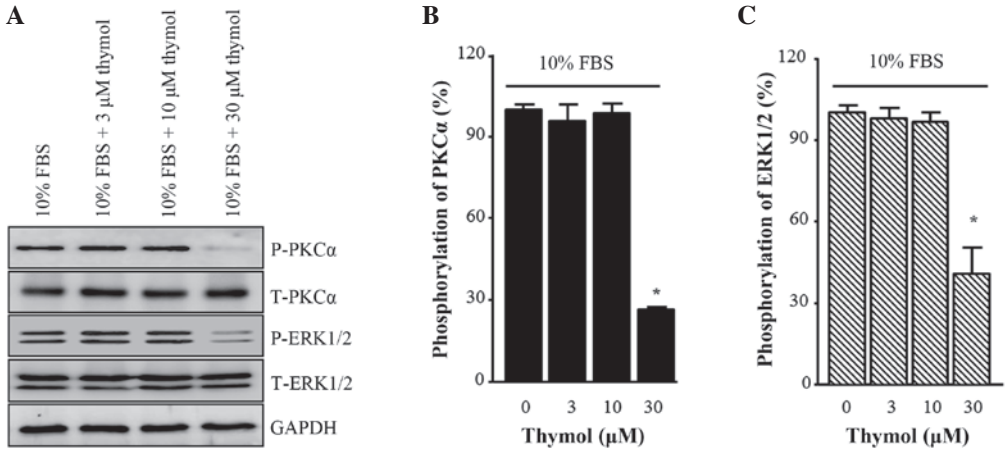

Figure 4. Western blot analysis of PKCα and ERK1/2 phosphorylation. (A) C6 glioma cells were cultured in serum-free medium for 24 h and then in the presence of thymol (3-30 μM) for 1 h, followed by exposure to 10% FBS for 24 h. Cell lysates were separated by 12% polyacrylamide gel electrophoresis and the specific protein bands were labeled using antibodies. The band intensities were measured and reflect the effect of thymol on the phosphorylation of (B) PKCα and (C) ERK1/2. These values were normalized against total PKCα (T-PKCα), total ERK1/2 (T-ERK1/2) and GAPDH. Data are expressed as the mean ± standard error (n=3). Cell response to FBS is expressed as 100%. *P<0.05 vs. FBS-stimulated cells in the absence of thymol. PKCα, protein kinase Cα; ERK1/2, extracellular signal-regulated kinases 1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FBS, fetal bovine serum.

change observed in response to 3 and 10 μM thymol and a significant reduction observed in response to 30 μM thymol (Fig. 4C; P=0.0044).

Effect of thymol on MMP9 and MMP2. To elucidate the inhibitory effect of thymol on the production of gelatinases in FBS-stimulated C6 glioma cells, the MMP9 and MMP2 levels were measured by western blotting (Fig. 5A). Thymol (30 μM) decreased the expression of MMP9 and MMP2, whereas there was no change relative to the control when 3 and 10 μM thymol was used (Fig. 5B and C; P=0.0012 and P=0.0001, respectively). Next, MMP activation was confirmed by gelatin zymography (Fig. 5D). The pattern of MMP secretion in the zymography assay was similar to that of the control group in response to 3 and 10 μM thymol, but a significant reduction was observed in response to 30 μM thymol (Fig. 5E and F; P=0.0001).

Discussion

The present study demonstrated that a natural monoterpenic phenol, thymol, inhibited the phosphorylation of ERK1/2 and PKCα, reduced the expression of MMP2 and MMP9, and decreased the migration of the C6 glioma cells. The anticancer effect of thymol on various cancers has been reported (20-24). However, the effect of thymol on the motility of glioma cells has not yet been established. Malignant glioma has been reported as an incurable tumor and represents ~50% of all brain tumors. Glioma is a highly invasive and lethal form of brain cancer, which is extremely difficult to treat via surgery or combined radiotherapy and chemotherapy (3). Consequently, the median patient survival time is limited to 12 months according to World Health Organization research (8). The relapse rate is extremely high and may lead to mortality, even with aggressive therapy. Although the strategy used in the

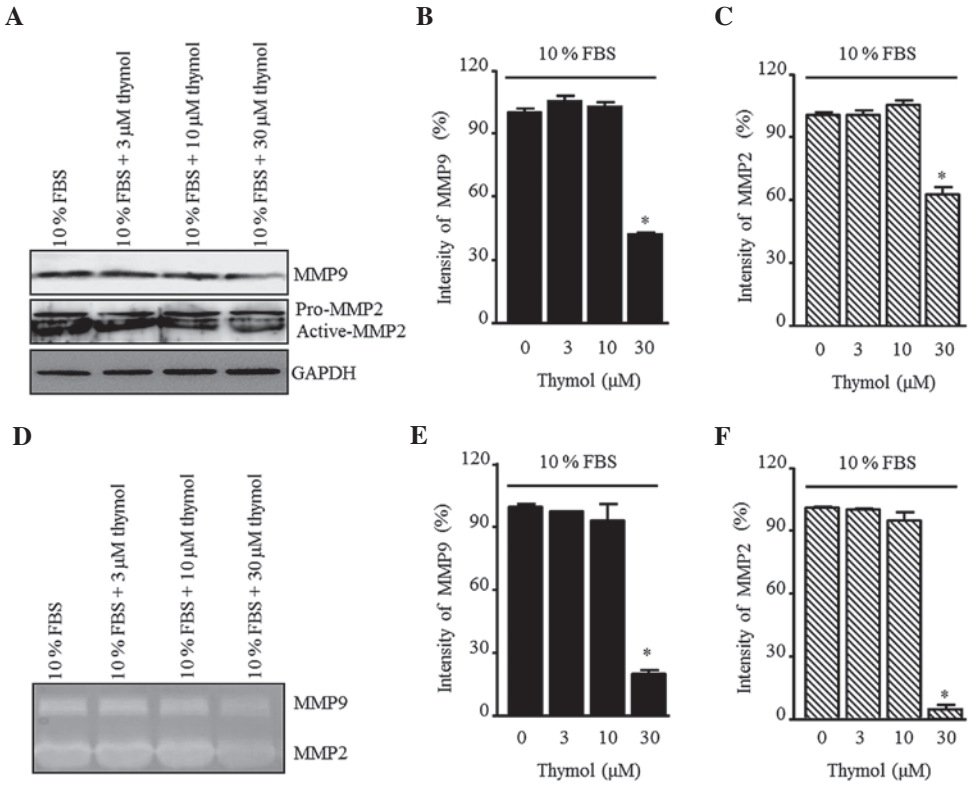

Figure 5. Effect of thymol on MMP2 and MMP9 activity in C6 glioma cells. (A) The C6 glioma cells were cultured in serum-free medium for 24 h and then in the presence of thymol (3-30 μM) for 1 h, followed by exposure to 10% FBS for 24 h. Cell lysates were separated by 12% polyacrylamide gel electrophoresis and the specific protein bands were labeled using antibodies. The band intensities were measured and reflect the effect of thymol on (B) MMP9 and (C) activated MMP2. These values were normalized to GAPDH. (D) The harvested supernatants were also used for gelatin zymography assay. The band intensities were measured and reflect the effect of thymol on (E) MMP9 and (F) activated MMP2. Data are expressed as the mean ± standard error (n=3). Cell response to FBS is expressed as 100%. *P<0.05 vs. FBS-stimulated cells in the absence of thymol. MMP, matrix metalloproteinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FBS, fetal bovine serum.

present study is not a direct clinical method, this approach, in addition to those focusing on apoptosis or cell death, may prove useful for improving patient survival and slowing down cancer progression. Hence, we speculated that the inhibition of glioma cell migration could be a therapeutic strategy. Thus, a component from plant essential oils that could be effective for the treatment and, more importantly, the prevention of glioma was searched for. As a result, the present study was focused on thymol, which has been widely reported as a component of plant essential oils (18).

In the present study, the scratch wound healing and Boyden chamber assays were used to assess glioma migration. The results indicated that thymol inhibits FBS-induced C6 glioma cell migration. Furthermore, thymol significantly inhibited the phosphorylation of PKCα and ERK1/2, as well as the activation and expression of MMP9 and MMP2. PKC is an intracellular signaling protein of the protein kinase family that regulates cell survival, proliferation and differentiation, and the cell cycle (25-27). PKC phosphorylation is involved in malignant transformation and tumor promotion, as well as in invasion and metastasis. The high motility of glioma cells is key to the inability to manage the disease, preventing complete tumor removal and leading to therapeutic failure and recurrence (15). Moreover, elevated MMP2, which is regulated by PKC, is tightly involved in glioma invasion (9). Notably, Tam *et al* studied the role of PKCα in cancer treatment and reported that it has been a major focus for various types of cancer and even cancer stem cells (28). In addition, Hu *et al* reported that activation of PKCα is also implicated in glioma cell migration (15). ERK1/2 also contributes to cell migration and proliferation in cancer cells (29,30). Hence, the expression and phosphorylation of ERK1/2 was measured in the C6 glioma cells of the present study. The results showed that 30 μM thymol attenuated ERK1/2 phosphorylation, but did not alter T-ERK1/2 expression. Recently, the role of ERK1/2 in the expression of the MMP family (MMP2 and MMP9) has been reported. MMP2 and MMP9 are also involved in tumor invasiveness and migration (10).

Taken together, the present results suggest that thymol inhibits glioma cell migration through PKCα and ERK1/2 phosphorylation, which consequently results in a decrease in MMP9 and MMP2 expression. The study indicates that thymol is a potential candidate for the treatment of malignant gliomas, as it reduces the FBS-induced motility of C6 glioma cells. The results of the present study shed light on the mechanism underlying the inhibitory effects of thymol on C6 glioma cell migration. Further studies are warranted to address whether the inhibitory effect of thymol on PKCα and ERK1/2 phosphorylation is associated with neuroprotective effects in normal cells.

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