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Report

Cycloalliin, an Organosulfur Compound in Garlic, Inhibits EMT and Invasion of the A549 Non-Small Cell Lung Cancer Cell Line

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Non-small cell lung cancer (NSCLC) accounts for 80%–90% of all lung cancers. The metastasis of NSCLC has a considerable impact on prognosis and systemic status. Cycloalliin, an organosulfur compound found in garlic, is known for its health benefits on cardiovascular disease and obesity. However, the role of cycloalliin in cancer cell invasion and metastasis remains unknown. In this study, we investigated the effect of cycloalliin on the epithelial-to-mesenchymal transition (EMT) and invasiveness of the NSCLC cell line A549. Cycloalliin inhibited transforming growth factor (TGF)-β-induced EMT and invasive potential of A549 cells. Furthermore, we found that cycloalliin suppressed Smad3 phosphorylation and expression of Snail, a transcription factor that promotes EMT, during the early stages of TGF-β-mediated EMT. This study provides valuable insights into the inhibitory potential of cycloalliin on EMT, suggesting that this compound may have a therapeutic role against EMT in NSCLC cells.

Key words cycloalliin, epithelial-to-mesenchymal transition, non-small cell lung cancer, invasion, TGF-β

INTRODUCTION

Non-small cell lung cancer (NSCLC) accounts for 80–90% of all lung cancers worldwide and is classified into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.[1,2] The frequency of distant metastases, such as those to the brain and bone, is high in advanced NSCLC, and controlling these metastases considerably affects prognosis and systemic status.[3] Therefore, new drugs are needed to inhibit the invasion and metastasis of NSCLC to the other organs.

The epithelial-to-mesenchymal transition (EMT) plays an important role in the invasion and metastasis of cancer cells. Cancer cells whose epithelial morphology changes to a mesenchymal morphology via EMT-inducing factors, such as transforming growth factor (TGF)-β, have enhanced invasive and metastatic potential.[4,5] Thus, suppressing EMT may inhibit cancer cell invasion; however, the development of drugs that effectively inhibit EMT has not yet been achieved.

Garlic (*Allium sativum*) has long been used as a spice and tonic food. In addition to its fatigue-relieving effects, garlic has been reported to prevent cardiovascular diseases by lowering blood lipid levels, preventing thrombosis, reducing blood pressure, and exerting antibacterial effects.[6] Moreover, substantial evidence suggests its preventive effects against cancer.[7,8] The organosulfur compounds in garlic are responsible for these properties, prompting research into their potential use in disease prevention and treatment. In particular, S-allyl-L-cysteine (SAC), which increases as fresh garlic matures and ferments, and diallyl sulfides, such as diallyl disulfide (DADS) and diallyl trisulfide (DATS), responsible for garlic's distinct odor, are being intensively studied. For example, SAC has been reported to inhibit cancer cell growth via apoptosis and cell cycle regulation in various cancer cells, including prostate and breast cancer.[9] Furthermore, SAC has demonstrated the ability to suppress invasion and metastasis in breast, oral, and liver cancer.[9,10] Several experimental studies have shown that DADS and DATS exhibit antitumor activity against many tumor cell types.[11,12] DADS and DATS have been reported to have anticancer effects including induction of apoptosis and regulation of cell cycle arrest, as well as inhibitory effects on carcinogenesis such as inhibition of reactive oxygen species production and suppression of DNA adduct formation.[12,13] Additionally, the effects of these compounds on NSCLC are being studied. Orozco-Morales *et al.* reported that SAC induc-

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es cytotoxic effects in NSCLC cell lines by causing oxidative damage and apoptosis.[14] Tang *et al.* found that SAC consumption inhibited NSCLC growth in a mouse xenograft model.[15] Moreover, DADS has been reported to reverse fibronectin-induced EMT in A549 cells by suppressing Wnt signaling.[16]

Cycloalliin [(1S,3R,5S)-5-methyl-3-thiomorpholinecarboxylic acid 1-oxide] is an organosulfur compound found in garlic and is associated with many garlic health benefits. Previous studies in rats have demonstrated that cycloalliin plays an important role in reducing serum triglycerides and suppressing obesity.[17,18] Additionally, cycloalliin has been reported to increase the fibrinolytic activity of human blood and inhibit platelet aggregation.[19,20] Recently, Nakayama *et al.* demonstrated that cycloalliin promotes the production of steroid hormones via activation of the PKA signaling pathway in testis-derived I-10 tumor cells.[21] These reports indicate that cycloalliin has many physiological functions; however, its role in cancer cell invasion and metastasis remains unknown. In this study, we investigate the effect of cycloalliin on EMT and the invasive potential of the NSCLC cell line A549.

MATERIALS AND METHODS

Chemicals Cycloalliin hydrochloride monohydrate was purchased from Fujifilm Wako Chemicals and dissolved in water. Recombinant human TGF-β1 was obtained from R&D systems and dissolved in 4 mM HCl containing 0.1% bovine serum albumin according to the manufacturer's instructions.

Cell Culture The human NSCLC cell line A549 was obtained from RIKEN Cell Bank and grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were treated with 0.5 ng/mL of recombinant human TGF-β1.

Cell Viability Assay A549 cells were seeded in a 96-well plate at 5 x10^3 cells/well and cultured for 24 h. The cells were then treated with cycloalliin at each concentration. After 24 h, the cell counting kit-8 (CCK-8) solution (Dojindo) was added according to the manufacturer's instructions and allowed to react for 2 h. The absorbance was measured at 450 nm using a spectrophotometer.

Western Blot Analysis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate equal amounts of total protein, which were then transferred to polyvinylidene difluoride membranes. The membranes were blocked in tris-buffered saline with tween 20 (TTBS) containing 3% nonfat powdered milk (Cell Signaling Technology) and immunoblotted with primary antibodies specific for E-cadherin, N-cadherin, Snail, phospho-Smad3, total-Smad3, and β-actin (Cell Signaling Technology). After washing with TTBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). ECL western blot substrate (Cytiva) was used to detect specific proteins, and NIH-ImageJ software was used to quantify band intensities.

Invasion Assay An invasion assay was conducted using a Transwell chamber precoated with Matrigel (Corning), as previously described.[22] In brief, A549 cells were seeded in a 12-well plate at 4 x10^4 cells/well. A549 cells treated with or without cycloalliin and TGF-β were resuspended in serum-free medium and seeded in the upper chamber (2 x 10^4 cells/well). The lower chamber was filled with high-glucose DMEM containing 10% FBS. After 24 h of incubation, noninvasive cells were removed from the upper surface of the membrane using a cotton swab. The chambers were then fixed in 2% paraformaldehyde for 10 min and stained with crystal violet. Cells that penetrated the filter were observed using a microscope and counted.

Statistical Analysis Statistical analyses were performed using R software (http://cran.r-project.org/). Statistical significance for multigroup analysis was determined using one-way analysis of variance with the Tukey–Kramer post hoc testing. *P* values less than 0.05 were considered statistically significant.

RESULTS

We first examined whether cycloalliin was cytotoxic to A549 cells. As shown in Fig. 1, cycloalliin was not cytotoxic up to a concentration of at least 200 μM.

Next, we examined the effect of cycloalliin on the TGF-β-induced EMT of A549 cells. Activation of intracellular signaling, including phosphorylation of Smad3, by TGF-β is known to be extremely rapid. Furthermore, it is unclear at which stage of TGF-β-induced EMT cycloalliin act. Therefore, in the present study, cycloalliin was treated 2 h prior to TGF-β stimulation. To investigate the effect on EMT, the expression of EMT-related genes was examined 24 h after TGF-β stimulation. The expression of the epithelial marker E-cadherin decreased with the addition of TGF-β. While the addition of 25 μM cycloalliin did not inhibit the decrease in E-cadherin expression, 100 μM cycloalliin considerably inhibited the TGF-β-induced decrease in E-cadherin expression (Fig. 2A). Conversely, the increased

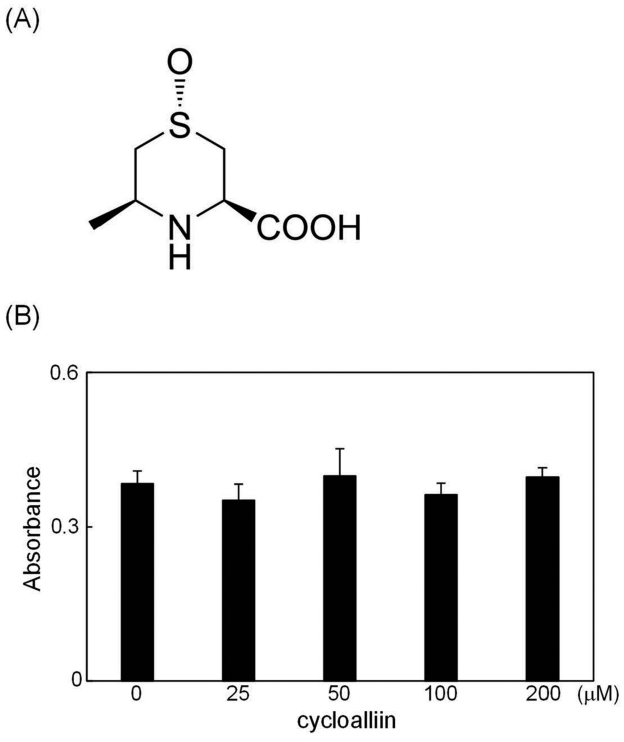

Fig. 1. Effect of Cycloalliin on the Viability of A549 Cells

(A) Chemical structure of cycloalliin. (B) A549 cells were treated with the indicated concentrations of cycloalliin for 24 h, followed by the evaluation of cell viability using a cell counting kit (CCK-8) assay. Each column shows the mean ± standard deviation (SD) (n = 3).

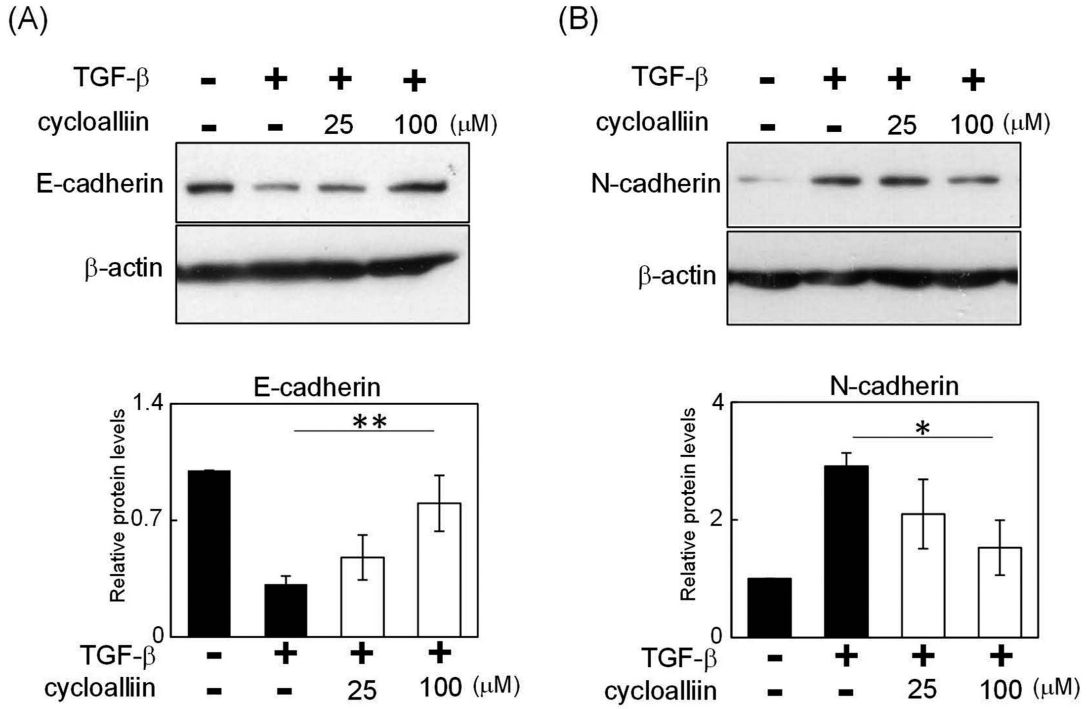

Fig. 2. Effect of Cycloalliin on the Expression Levels of EMT-Related Genes in A549 Cells

A549 cells were seeded in a 12-well plate at 4 x10^4 cells/well. The cells were pretreated with or without cycloalliin for 2 h and then treated with TGF-β with or without cycloalliin for 24 h. The cell lysates were analyzed by western blot and probed with antibodies against E-cadherin (A) and N-cadherin (B). β-Actin was used as the loading control. The protein signal intensities were quantified using NIH-ImageJ software. Each column shows the mean ± standard deviation (SD) (n = 3). Significant differences are denoted as $^{**}p < 0.01$ and $^{*}p < 0.05$.

expression of the mesenchymal marker N-cadherin induced by TGF-β was considerably suppressed by the addition of 100 μM cycloalliin (Fig. 2B). These results suggest that cycloalliin inhibits TGF-β-induced EMT in A549 cells.

The development of EMT increases the invasive potential of cancer cells. We next examined whether cycloalliin could suppress the invasive potential of A549 cells undergoing TGF-β-mediated EMT. We found that A549 cells treated with TGF-β exhibited a marked increase in invasive capacity compared with cells without TGF-β treatment. By contrast, cells co-cultured with TGF-β and cycloalliin demonstrated considerably reduced invasive potential (Fig. 3). These results indicate that cycloalliin suppresses invasion following the EMT development.

During the early stage of EMT, TGF-β binds to the receptor, enhancing the phosphorylation of Smad3 and increasing the expression of Snail, a downstream transcription factor that plays an important role in the progression of EMT and induces EMT[4]. To clarify the role of cycloalliin in the early EMT process, we next evaluated the effects of cycloalliin on the phosphorylation of Smad3 (1 h) and the expression of Snail (4 h) at the early stage of TGF-β-induced EMT. Assessment of Smad3 phosphorylation levels 1 h after TGF-β stimulation revealed that cycloalliin markedly suppressed TGF-β-induced Smad3 phosphorylation (Fig. 4A). Cycloalliin also suppressed the expression of Snail 4 h after TGF-β stimulation (Fig. 4B). These results strongly suggest that cycloalliin inhibits EMT by suppressing TGF-β/Smad signaling during the early stage of EMT.

DISCUSSION

TGF-β is an important cytokine that maintains homeostasis within the body, and its abnormal expression is an important factor involved in the development of various diseases.[23] TGF-β inhibits cancer cell growth in the early stages of cancer development, whereas it strongly enhances metastatic and invasive potential in progressive cancers.[4] Abnormalities in TGF-β receptors and signaling molecules have been implicated in diseases such as fibrosis and cancer; thus, inhibitors of TGF-β signaling are considered potential treatments for these conditions.[24] Several approaches have been attempted to inhibit TGF-β signaling, including antibodies that block TGF-β activation and compounds that inhibit phosphorylation of the TGF-β receptor. Many TGF-β inhibitors are currently being tested in clinical trials or are in preclinical development.[25] Herein, we demonstrate that cycloalliin inhibits Smad3 phosphorylation and Snail expression during the early stages of EMT in A549 cells, making it a potential new drug candidate to effectively inhibit TGF-β signaling in NSCLC.

In this study, we could not determine the precise mechanism by which cycloalliin inhibits TGF-β/Smad3 signaling. Although multiple physiological functions of cycloalliin have been reported, such as reducing obesity, lowering blood triacylglycerol levels, and promoting steroid hormones production, its molecular mechanism remains unclear, except for its activation of the PKA/cAMP response element binding protein (CREB) signaling pathway in the testis.[21] The investigation of whether cycloalliin affects intracellular signaling mechanisms other than TGF-β/Smad3, including PKA/CREB signaling, in the EMT of NSCLC is necessary. A comprehensive analysis

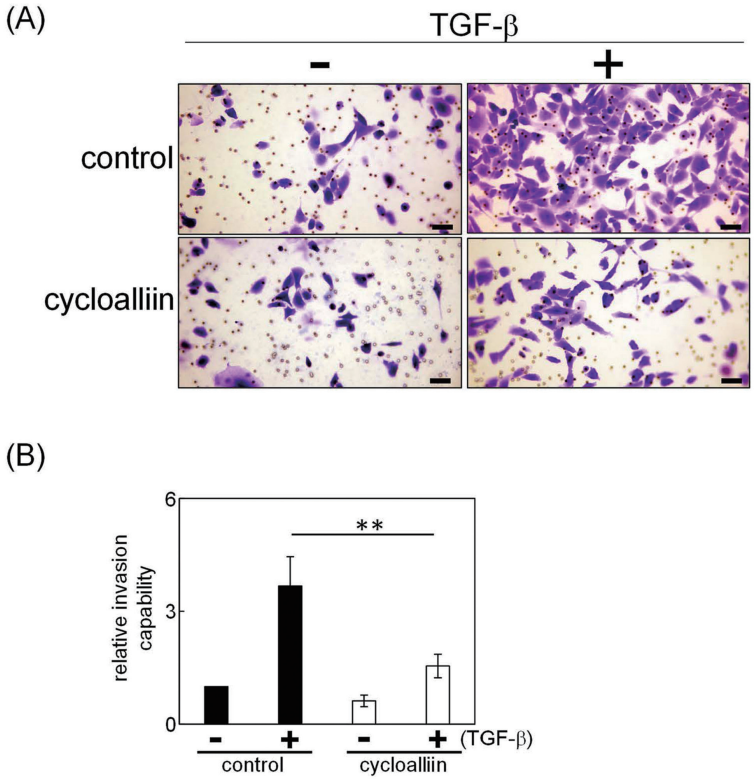

Fig. 3. Effect of Cycloalliin on the Invasion Capacity of A549 Cells Undergoing TGF-β-Induced EMT

The cells that invaded the underside of the Transwell insert were stained and counted. (A) Representative images of the invading cells. Scale bar = 100 μm. (B) The mean number of invaded cells per field was calculated. Each column shows the mean ± standard deviation (SD) (n = 3). Significant differences are denoted as **p < 0.01.

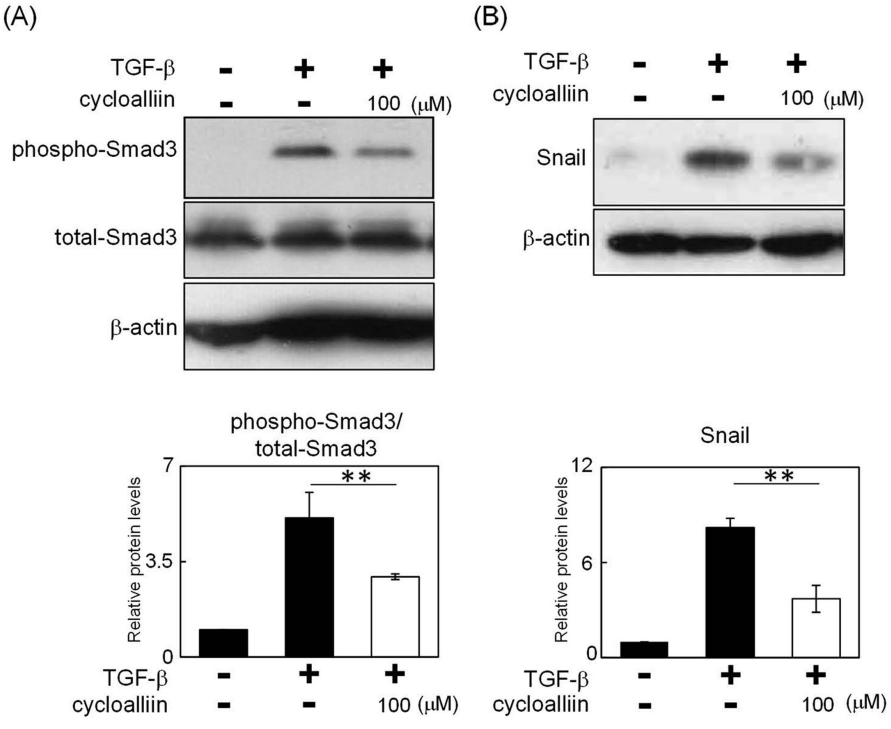

Fig. 4. Effect of Cycloalliin on the Phosphorylation of Smad3 and the Expression of Snail During TGF-β-Mediated EMT in A549 Cells

A549 cells were seeded in a 12-well plate at 4 x10^4 cells/well. The cells were pretreated with or without cycloalliin for 2 h and treated with TGF-β with or without cycloalliin for 1 h (phospho-Smad3) or 4 h (Snail). The cell lysates were analyzed by western blot probed with antibodies against phospho-Smad3 and total-Smad3 (A) and Snail (B). β-Actin was used as the loading control. The protein signal intensities were quantified using NIH-ImageJ software. Each column shows the mean ± standard deviation (SD) (n = 3). Significant differences are denoted as **p < 0.01.

of genes whose expression is altered by cycloalliin, along with the identification of proteins to which cycloalliin binds, will enhance our understanding of its cellular functions.

In conclusion, we found for the first time that cycloalliin, a sulfur compound in garlic, inhibits TGF-β-induced EMT and the subsequent invasive potential of the NSCLC cell line A549. Furthermore, we discovered that cycloalliin suppresses the progression of EMT by inhibiting the phosphorylation of Smad3 and the expression of Snail. This study provides valuable insights into the inhibitory effects of cycloalliin on EMT and suggests that this compound has therapeutic potential against EMT in NSCLC cells.

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Conflict of interest The authors declare no conflict of interest.

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