5-Caffeoylquinic acid inhibits invasion of non-small cell lung cancer cells through the inactivation of p70S6K and Akt activity: Involvement of p53 in differential regulation of signaling pathways

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Abstract. In the present study, we investigated the effects and molecular mechanism of 5-caffeoylquinic acid (5-CQA), a natural phenolic compound isolated from Ligularia fischeri, on cell invasion, proliferation and adhesion in p53 wild-type A549 and p53-deficient H1299 non-small cell lung cancer (NSCLC) cells. 5-CQA abrogated mitogen-stimulated invasion, but not proliferation, in both A549 and H1299 cells. In addition, 5-CQA inhibited mitogen-stimulated adhesion in A549 cells only. Anti-invasive activity of 5-CQA in A549 cells was mediated by the inactivation of p70S6K-dependent signaling pathway. In contrast, in H1299 cells the inactivation of Akt was found to be involved in 5-CQA-mediated inhibition of cell invasion. Collectively, these findings demonstrate the pharmacological roles and molecular targets of 5-CQA in regulating NSCLC cell fate, and suggest further evaluation and development of 5-CQA as a potential therapeutic agent for the treatment and prevention of lung cancer.

Introduction

Lung cancer is one of the most aggressive types of cancer, and the leading cause of cancer-related mortality. Approximately 85% of all diagnosed cases of lung cancer are non-small cell lung cancer (NSCLC). Extremely low survival rates in patients with lung cancer are attributable to lack of potent therapeutic targets and drugs against recurrent and metastatic phenotypes (1,2). Matrix metalloproteinases (MMPs) have been known to control cancer metastasis-related processes including cell detachment, invasion, proliferation and angiogenesis by degrading all components of extracellular matrix (ECM) and cell surface molecules. High expression and activity of MMPs are well correlated with aggressive phenotypes and poor survival of a variety of cancers (3-6). However, strategy to regulate MMP activity for the treatment of cancer has been disappointing in clinical trials (7,8). Therefore, further understanding molecular mechanisms of lung cancer growth and progression is required for the identification of therapeutic targets and development of potent anticancer agents.

Ligularia fischeri (L. fischeri) (Ledeb.) Turcz. (Compositae) has been used as a traditional medicine for the treatment of rheumatoid arthritis, scarlet fever and jaundice in eastern Asia including Korea, China and Japan. Previous investigations demonstrate that L. fischeri extract and its bioactive components such as caffeic acid and chlorogenic acid isomers possess anti-oxidant, anti-inflammatory, anti-angiogenic and anticancer properties (9-11). Chlorogenic acid isomers including 5-caffeoylquinic acid (5-CQA), which have been identified in a variety of plants including green coffee beans, walnut (Juglans regia) leaves, yerba-mate (Ilex paraguariensis) extract, Petasites japonicus extract and Marrubium vulgare extract as well as L. fischeri extract, have been known to exert a variety of biological functions such as anti-oxidant, anti-microbial, anti-diabetic, anti-inflammatory and anticancer activities (12-19). However, no detailed mechanisms of 5-CQA responsible for regulation of NSCLC cell fate has been clearly elucidated to date. In the present study, the regulatory effects and action mechanisms of 5-CQA on cell proliferation and differentiation were investigated in p53 wild-type A549 and p53-deficient H1299 NSCLC cells.

Materials and methods

Cell culture conditions. Human NSCLC cell lines (A549 and H1299) from the American Type Culture Collection (ATCC; Manassas, VA, USA) were grown in 10% fetal bovine serum-Dulbecco's modified Eagle's medium (FBS-DMEM; Hyclone Laboratories, Logan, UT, USA).

Reagents. 5-Caffeoylquinic acid (5-CQA) was isolated from the ethanolic extract of L. fischeri. The structure of 5-CQA is presented in Fig. 1A. The following pharmacological agents and antibodies were purchased from commercial sources: mTOR/p70S6K inhibitor, rapamycin (Sigma-Aldrich, St. Louis,

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MO, USA); PI3K/Akt inhibitor, LY294002 (Merck Millipore, Billerica, MA, USA); anti-phospho-ERK (T202/Y204), anti-phospho-Akt (S473), anti-phospho-p70S6k (T421/S424), anti-phospho-p38MAPK (T180/Y182) and anti-p38MAPK (Cell Signaling Technology, Beverly, MA, USA); anti-integrin β1 (BD Biosciences, Bedford, MA, USA); anti-ERK, anti-Akt, anti-p70S6k, anti-EGFR, anti-integrin α3, anti-ILK, anti-actin antibodies, and mouse and rabbit IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell cycle analysis. The upper side of the Transwell insert (Costar, 6.5 mm diameter insert, 8 µm pore size; Corning Inc., Corning, NY, USA) was coated with 50 µl of 1 mg/ml Matrigel® (BD Biosciences) diluted in serum-free DMEM. Aliquots (100 µl) of cells (5x10⁴ cells/ml) resuspended in serum-free DMEM were added to the upper compartment of the Matrigel-coated Transwell and 600 µl of serum-free DMEM were added to the lower compartment. After serum starvation for 2 h, cells were pretreated with 5-CQA (1-50 µM) for 30 min in the presence or absence of rapamycin (50 nM) or LY294002 (10 µM), followed by 10% FBS stimulation for 16 h. The inserts were fixed with methanol and using a cotton-tipped swab the non-invasive cells were removed from the top of the membrane. After staining with 0.04% Giemsa staining solution (Sigma-Aldrich), the numbers of invasive cells (mean ± standard deviation) were determined from six different fields using x200 objective magnification (20).

Zymogram analysis. Activities of MMPs were measured by zymography (21). Aliquots of conditioned medium were diluted in sample buffer, and applied to 10% polyacrylamide gels containing 1 mg/ml gelatin (Sigma-Aldrich) as a substrate. After electrophoresis, the gels were incubated in 2.5% Triton X-100 for 1 h to remove SDS and allow re-naturalization of MMPs, and further incubated in developing buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM CaCl₂, and 150 mM NaCl for 16 h at 37°C. The gels were stained with 0.5% Coomassie brilliant blue R-250 in 30% methanol-10% acetic acid for 2 h, and followed by destaining with 30% methanol-10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of the Coomassie blue-stained gelatin.

Cell viability and proliferation assay. Subconfluent A549 and H1299 cells, plated on 6-well plates (5x10⁴ cells/well; SPL Life Sciences Co., Ltd., Gyeonggi-do, Korea), were serum-starved for 24 h in basal DMEM to synchronize cells in the G₀/G₁ phase of the cell cycle, and treated with 5-CQA (1-50 µM) for 30 min prior to 10% FBS stimulation for 24 h. Following culture for 24 h, cell viability was determined by a Muse™ cell analyzer using cell count and viability assay kit (Merck Millipore), and the cell proliferation was quantified as previously described (22). The results from triplicate determinations (mean ± standard deviation) are presented as the number of adherent cells (25,26). Statistical analysis was performed using the Student's t-test, and was based on at least three different experiments. The results were considered to be statistically significant at P<0.05.

Results

5-CQA inhibits NSCLC cell invasion. We first analyzed the effect of 5-CQA on cell invasion which plays pivotal roles in cancer progression. 5-CQA treatment dose-dependently blocked mitogen-stimulated cell invasion in p53 wild-type A549 and p53-deficient H1299 NSCLC cells (Fig. 1B and C). H1299 cells appeared to be more responsive to 5-CQA-mediated inhibition of cell invasion, as compared with A549 cells, indicating that anti-invasive activity of 5-CQA might be dependent on p53 expression status. We next examined the activity of matrix metalloproteinases (MMPs) in 5-CQA-treated NSCLC cells. As shown in Fig. 2, the conditioned media from cell cultures had high levels of MMP-2 activity relative to those of MMP-9. 5-CQA treatment did not alter activity of MMP-2 and MMP-9 in response to mitogenic stimulation. In addition, the levels of MMP-2, MMP-9 or tissue inhibitor of metalloproteinase-2, an endogenous inhibitor of MMP, were not changed in 5-CQA-treated cells (data not shown), suggesting the inhibitory effect of 5-CQA on cell invasion might be medi-
ated through an MMP-independent mechanism. However, the possibility that 5-CQA may regulate the expression and activity of other MMPs and their endogenous inhibitors cannot be excluded (3,27-29).

5-CQA does not alter viability and proliferation in NSCLC cells. Based on 5-CQA-mediated inhibition of cell invasion, we investigated the possibility that cytotoxicity or anti-proliferative effect of 5-CQA might mediate anti-invasive activity. 5-CQA treatment did not significantly alter cell viability and proliferation in A549 or H1299 cells (Fig. 3A and B). To ascertain that 5-CQA had little or no effect on cell proliferation, we next examined the ability of 5-CQA to regulate cell cycle progression (Fig. 3C). Mitogenic stimulation for 24 h increased the percentage of cells in S and G2/M phases, and simultaneously decreased the percentage of cells in G1 phase.
compared with untreated controls. 5-CQA treatment did not alter the percentage of G1, S and G2/M phases of the cell cycle associated with mitogenic stimulation. Moreover, our initial experiments indicate that 5-CQA treatment did not change the percentage of live, apoptotic or dead cells in either cell line at the highest concentration used in this study (data not shown).

Collectively, these findings demonstrate that 5-CQA directly regulates cell invasion without any effect on cell cycle progression, cell proliferation or cell viability in NSCLC cells.

5-CQA inhibits adhesion in A549 cells. Cell adhesion and migration associated with cancer cell growth and progression are controlled by interactions with ECM molecules and cellular components (4). We next investigated the ability of 5-CQA to regulate cell adhesion. As shown in Fig. 4, 5-CQA treatment dose-dependently suppressed mitogen-stimulated cell adhesion in A549 cells, but not in H1299 cells. Although

5-CQA inhibits adhesion in A549 cells. Cell adhesion and migration associated with cancer cell growth and progression are controlled by interactions with ECM molecules and cellular components (4). We next investigated the ability of 5-CQA to regulate cell adhesion. As shown in Fig. 4, 5-CQA treatment dose-dependently suppressed mitogen-stimulated cell adhesion in A549 cells, but not in H1299 cells. Although
the inhibitory effect and functional consequences of 5-CQA on cell adhesion in A549 cells remain to be addressed, 5-CQA-mediated inhibition of cell adhesion did not appear to affect the cell viability and proliferation (Fig. 3A and B), and may contribute to the modulation of cell invasion (Fig. 1B and C).

**Discussion**

Differential regulation of 5-CQA in mitogen-stimulated signaling pathways in NSCLC cells. To investigate the molecular mechanisms and therapeutic targets of 5-CQA in regulating cell invasion, we first examined the changes in the expression of cell surface signaling-related molecules such as epidermal growth factor receptor (EGFR), integrin α3β1 and integrin-linked kinase (ILK) in 5-CQA-treated NSCLC cells (30,31). 5-CQA treatment did not significantly change the expression of EGFR, integrin α3β1 and ILK in either cell line (Fig. 5), raising the possibility that 5-CQA-mediated inhibition of cell invasion might be mediated through the regulation of downstream signaling pathways of receptor tyrosine kinases (RTKs) and integrins. Therefore, we next analyzed the changes in activation of mitogen-stimulated signaling pathways including phosphatidylinositol 3-kinase (PI3K)/Akt, mammalian target of rapamycin (mTOR)/p70S6K, extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38MAPK) in 5-CQA-treated cells (10,20,32). As shown in Fig. 6A, mitogenic stimulation increased the phosphorylation/activation of Akt, p70S6K, ERK and p38MAPK, as compared with unstimulated controls. 5-CQA treatment markedly inhibited mitogen-stimulated activation/phosphorylation of p70S6K, but not Akt, ERK and p38MAPK, in A549 cells. Pretreatment of A549 cells with rapamycin, an inhibitor of mTOR/p70S6K pathway, mimicked the suppressive effect of 5-CQA on cell invasion (Fig. 6B). In contrast, 5-CQA treatment inhibited mitogen-stimulated phosphorylation of Akt in H1299 cells (Fig. 6A). Inhibition of PI3K/Akt signaling pathway by LY294002 suppressed cell invasion similarly in 5-CQA-treated H1299 cells (Fig. 6C). Co-treatment with 5-CQA did not enhance the inhibitory effect of these chemical inhibitors on cell invasion, suggesting that 5-CQA and these inhibitors may share similar roles and mechanisms of action in regulating NSCLC cell invasion.

**References**


