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Original Article

Humic Substances Suppresses the Proliferation of TC-1 Cells, the Lung Cancer Cell

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In humic substances, fulvic acid (FA) is a subclass of diverse compounds known as humic substances, which are by-products of organic degradation from microorganisms. FA can suppress the proliferation of tumor cells. Despite numerous studies, the exact mechanism for the various effects of FA is not clearly understood. Based on results demonstrating anti-proliferation effects on human cancer, we investigated whether FA has similar effects on lung cancer in this study. Firstly, the anti-cancer effect of FA in pulmonary epithelial tumor cell lines (TC-1 cells) was examined by confirming its inhibitory effect on the cell proliferation of TC-1 cells. TC-1 cell proliferation was reduced by FA on a dose-dependent and time-dependent manner. After 24 hours of FA treatment, cell morphological changes such as cell volume decrease, non-adherence and increased number of apoptotic cells were clearly observed. In addition, FA induced a DNA ladder pattern by increased of DNA fragments in TC-1 cells. In the intracellular regulatory pathway by FA, we confirmed that FA induced the reduction of the anti-apoptotic protein, Bcl-2 protein levels. These results indicate that FA has anti-cancer effect by inducing intracellular apoptotic pathway. Further research on the mechanism of anticancer effects will be basic data for the development of potential anticancer drugs.

Key Words: Fulvic acid, TC-1 cells, Proliferation, Apoptosis, Anti-cancer effect

INTRODUCTION

Tumor cells may respond to chemo- or radiotherapy by activation of several cellular signaling cascades that influence cell survival and cell death, including activation of cell cycle arrest, senescence, or triggering of several cell death types (i.e., mitotic catastrophe, necrosis, or apoptosis) (Kristina and Rolf, 2007). However, tumor cells derived from solid tumors are often refractory to therapy or develop resistance during the treatment course. This is illustrated by non-small cell lung cancer (NSCLC), which shows a high degree of intrinsic resistance, and by small cell lung cancer (SCLC), which often develops resistance to treatment during disease (Joseph et al., 2000). Lung cancer is diagnosed in more than 25,000 people every year in the Republic of Korea alone and is the common cause of deaths associated with cancer (Table 1) (Statistical Office, 2022).

Although radiotherapy and chemotherapy employed for the treatment of lung cancer have undergone drastic improvement over the past decade, the prognosis of patients is still dismal. The of 5-year survival rate for patients with lung carcinoma is around 15% (Tengchaisri et al., 1998). Radical resection followed by administration of adjuvant

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Table 1. The incidence and mortality of lung cancer in the Republic of Korea from 2017 to 2020

Year	2017	2018	2019	2020
The incidence of lung cancer (cases)	27,464	28,891	29,960	30,612
The mortality of lung cancer (cases)	17,980	17,852	18,574	18,673

chemotherapy to patients with primary tumors has been reported to induce recurrences in most cases (Asamura et al., 2008). There is urgent need for improvement in lung carcinoma treatment through identification of molecular targets and by discovering novel anti-cancer compounds.

The most effective treatment for lung cancer requires the discovery of a substance that specifically acts only on lung cancer cells and induces apoptosis of lung cancer cells without adverse effects on surrounding tissues. Apoptosis or programmed cell death is a naturally occurring event found in healthy organisms, and it is indispensable mode of physiological cell death to gain the maintenance of proper number and types of cell (Schultz and Harrington, 2003). Apoptosis is the important biological process for physiological death of cells. It is maintained through evolution and programmed at the genetic level and required for cell recycling, normal organ homeostasis and regulation of immune system (Johnson and Sarosiek, 2023).

Cytoplasm condensation and cell shrinkage with structure integrity of mitochondria, chromatin compaction, membrane blebbing and further cell fragmentation into apoptotic bodies constitute the most accepted cell morphology of the process (Kerr et al., 1995). In addition, morphological changes in apoptosis are usually accompanied by intracellular nucleosomal DNA fragmentation (Majtnerová and Roušar, 2018), and produces ladders of DNA fragments (Lu et al., 2023). All these characteristics are effects of selective proteolysis of proteins involved in cell signaling, DNA repair, or structural maintenance of DNA integrity, carried out by caspases (a group of cystein-aspartate enzymes).

Cells have mechanisms to converse various extracellular signals into intracellular common signals during apoptosis (Bortner et al., 1995). In the extrinsic caspase activation pathway, TNF superfamily ligands bind to DRs, causing oligomerization of death receptorsa (DRs) and recruitment of adaptor proteins via a death domain. In the intrinsic pathway, apoptotic signals trigger increased mitochondrial outer membrane permeability (MOMP), followed by selective release of apoptogenic proteins from the itochondrial inter membrane space to the cytosol (e.g., cytochrome c, Smac/DIABLO, and HtrA2/omi), all which promote caspase activation. In part, MOMP is controlled by Bcl-2 family proteins, and the anti-apoptotic members Bcl-xL and Bcl-2 both inhibit MOMP. Accordingly, pro-apoptotic members such as Bak or Bax, both which are activated by some BH3-only proteins (Bid, Bim, Bad, PUMA, and NOXA), can promote MOMP (Kristina and Rolf, 2007). The Bcl-2 protects cells from apoptosis mediated by endogenic stimulation. A member of the Bcl-2 family, Bcl-xL, Bcl-w, and Bcl-2 is able to protect cells from a wide variety of apoptotic stimuli. Bax is a proapoptotic protein of Bcl-2 family that is involved in the pathogenesis of cell death (Desagher et al., 1999). Based on these findings, we can be sure that apoptotic process is a valuable mechanism for the treatment of cancer cells. Antitumor drugs also cause apoptosis in some cancer cells, indicating that apoptosis plays a very key role in cancer chemotherapy. Therefore, for cancer treatment, it is important to discover substances that do not act on normal cells but specifically act on cancer cells.

Fulvic acid (FA) is a subclass of diverse compounds known as humic substances, which are by-products of organic degradation from microorganisms. FA is primarily studied for its effects on plants and soil (Senesi and Loffredo, 1998). FA has been indirectly utilized in traditional Indian medicine ("Ayurveda") for roughly 3000 years (Wilson et al., 2011). Because FA decrease proinflammatory markers but also activate the immune system to kill bacteria. It is shown to reduce oxidative stress and even induce apoptosis in hepatic cancer lines, it is considered an antiseptic and analgesic (Meena et al., 2010; Winkler and Ghosh, 2018). Despite many studies, the exact mechanism for the various effects of FA is not clearly understood. However, it is already available as a nutraceutical to the modern public (Peña-Méndez et al., 2005). Since FA has been reported to have an anti-inflammatory effect and apoptosis-inducing effect of liver cancer cells, it is necessary to investigate various

cancer cell inhibitory effects and anti-inflammatory effects based on this.

In this study, we used a lung epithelial tumor cell line (TC-1 cells) and investigated the effect on cell survival and proliferation after treating FA. And then, we investigated the effect of FA on the regulations of DNA fragment and apoptosis-related molecules.

MATERIALS AND METHODS

Cell culture and growth condition

We purchased cancer cell lines TC-1 from American Type culture collection (ATCC, Rockville, MD, USA). Cancer cells (TC-1 cells) were cultured in RPMI 1640 (Welgene, Korea) containing 10% fetal bovine serum (Corning Cellgro, USA), Trypsin EDTA (Sigma) and 1% antibiotics (Invitrogen) and incubated at 37℃ in a humidified incubator with a 5% CO_2 atmosphere. Cells were seeded in 6-well plates and treated with GBE (0~100 μg/mL).

Trypan blue exclusion test

Cell viability was examined by the dye exclusion test. Dyes were mixed in the cell suspension and then n visually checked whether cells absorbed or excluded the dye. Viable cells have a clear cytoplasm, while dead cells have a blue cytoplasm.

Cell proliferation analysis

TC-1 cells were seeded in 96-well culture plates (a density of 3×10^3 cells/well). After 24 h, the cancer cells were treated with fulvic acid (FA) for 24 h. In each well, WST plus-8 cell proliferation assay reagent (GenDEPOT, TX, USA) was added and determined the cell proliferation ratio. Cell viability was measured using ELISA reader at 450 nm. The cell viability was observed as percentage of control, comparing with the untreated cells.

DNA fragmentation analysis

The apoptotic properties of the cells were examined by agarose gel electrophoresis of nuclear DNA, using the method of Waring (Waring, 1990). Cells in a culture dish (100 mm) were lysedin pH 8.0 buffer containing 5 mM Tris, 20 mM EDTA and 0.5% Triton X-100. After incubation for 15 min on ice, samples were centrifuged for 10 min at 10,000 rpm to separate the intact chromatic (pellet) from the fragmented DNA (supernatant). Pellets were resuspended in the buffer and supernatant fractions were analyzed for DNA content using the diphenylamine reagent containing 1.5% sulfuric acid, 1.5% diphenylamine and 0.008% acetaldehyde in glacial acetic acid. DNA fragmentation was quantified by measuring the ratio of the DNA content in supernatant fraction to the total DNA content. For visualization, the supernatant fraction containing fragmented DNA was extracted two times with phenol and once with chloroform. The extracted DNA fragments were precipitated in 67% ethanol and 0.3 M sodium acetate at -70℃ for overnight, and then resuspended in a buffer containing 10 mM Tris, 1 mM EDTA and RNase. And then, extracted DNA fragments was electrophoresis in 1.8% agarose gel (Nakvasina et al., 2023).

Western blot analysis

Cell lysate extractions were isolated with RIPA buffer. Cell lysates were incubated for 20 min on ice and centrifuged at 13,200 rpm for 25 min at 4℃. Supernatant protein concentration was analyzed by protein assay reagent (Thermo Scientific, MA, USA). Protein samples were loaded into wells of the SDS-PAGE gel and transferred to PVDF (Merck Millipore, MA, USA). The membranes were blocked with 5% skim milk for 1 h at room temperature. After blocking, membranes were incubated with primary anti-bodies overnight at 4℃. The membranes were washed 3 times for 10 min with PBST and incubated with HRP conjugated secondary antibodies (Bethyl Laboratories, TX, USA) for 1 h at room temperature. The membranes were washed 3 times for 10 min with PBST. The FUSION SOLOS (Vilber, Eberhardzell, Germany) was used for image detection according to manufacturer's instructions. Antibodies used were anti-β-actin, anti-Bcl-2. (Santa Cruz, TX, USA).

Statistical analysis

All data were expressed as mean ± SD. Data were analyzed by student's *t*-test using SPSS statistical software package (Version 10.0, Chicago, IL). A *P* values less than

Table 2. The cytotoxicity of Fulvic acid (FA) on TC-1 cells

FA (μg/mL)	0	6.25	12.5	25	50	100
Mean ± SD	98.9±0.6	81.5±0.8	74.6±0.8*	59.0±1.3**	35.3±3.0**	22.7±3.0**

Table 3. The inhibitory effect of Fulvic acid (FA) on the cell proliferation of TC-1 cells

FA (time)	0 h	6 h	12 h	24 h	48 h
Con	100.0±0.0	99.5±0.5	99.1±0.2	98.1±0.9	96.1±0.3
FA (25 μg/mL)	100.0±0.0	84.9±0.6	78.8±0.6*	58.6±1.2**	49.1±1.2**

0.05 was considered statistically significant.

RESULTS

Fulvic acid reduces cell proliferation of TC-1 cells

In the present study, we firstly examined the effect of Fulvic acid (FA) on the cell proliferation of TC-1 cells. To determine the optimal concentration of FA for cell treatment, the cytotoxicity of FA on the TC-1 cell line was investigated through Trypan blue exclusion test as explained in the Materials and Methods. FA was treated for 24 h in dose dependent manner (6.25, 12.5, 25, 50, 100 μg/mL). The results are shown in (Table 2). Data are expressed as the means ± SD in three individual experiments. *$P < 0.05$ and **$P < 0.01$ were considered a significant difference between the untreated group and FA-treated group. We found that the cell viability decreased by about 50% at a concentration of 25 μg/mL of FA. Since it is good to observe various intracellular changes in cells when the cell viability is reduced by about 50%, 25 μg/mL of FA was used in the subsequent experiment.

And then, we determined the inhibitory effect of FA on the TC-1 cell proliferation. After the cells were treated with FA (25 μg/mL) in time dependent manner, cell proliferation was analyzed by WST plus-8 cell proliferation assay as explained in the Materials and Methods. Data are expressed as the means ± SD in three individual experiments. *$P < 0.05$ and **$P < 0.01$ were considered a significant difference between the untreated group (Con) and FA-treated group (FA) at same incubation time. As shown Table 3, FA inhibits cell proliferation of TC-1 cells in a time-dependent manner. These results suggested that FA has potently inhibitory effect of TC-1 cells proliferation.

Fulvic acid affects morphological changes of TC-1 cells

When cell death is induced by cell death-induced substance, it causes a morphological change in the cell. TC-1 cells (the tumor cell line) were derived from primary lung epithelial cells of C57BL/6 and grow in the monolayer attached to the surface (adherent cell line) (Morrison et al., 2012).

In this study, mode of the TC-1 cells death induced by FA treatment was assessed by characteristic morphological criteria. After treated with 25 μg/mL FA in a time-dependent manner (6, 12, and 24 h), morphology changes of TC-1 cancer cells were observed by light microscopy. The control TC-1 cells appeared normal configuration, typical adherent and homogeneous cells (Fig. 1A). But, in the FA -treated group, the morphological change gradually increased over time (Fig. 1B, 1C and 1D). The characteristic microscopic changes were a decrease in cell volume and an increase in nonadhesion and death numbers.

Fulvic acid treatment induces apoptosis in TC-1 cells

Cleavage of chromosomal DNA into oligonucleosomal size fragments is a biochemical hallmark of apoptosis. DNA fragmentation factor (DFF) is a major apoptotic endonuclease for DNA fragmentation *in vitro* (Zhang and Xu, 2000). We examined the DNA fragmentation of FA-treated TC-1 cell by agarose gel electrophoresis of their nuclear DNA. DNA fragmentation during apoptosis in the cells treated to FA is shown in Fig. 2. After treatment with 25 μg/mL FA,

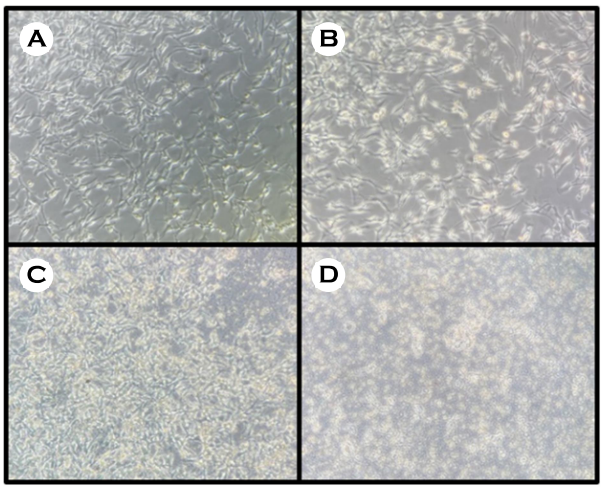

Fig. 1. Fulvic acid treatment induces apoptotic morphological changes in TC-1 cells. The morphological changes were detected by light microscopy (A: control cells). After treated to Fulvic acid (25 μg/mL) for 6 h (B), 12 h (C) and 24 h (D), the TC-1 cell showed reduced volume, non-adherence, shrinkage and increased death number of cells. Magnification 200×.

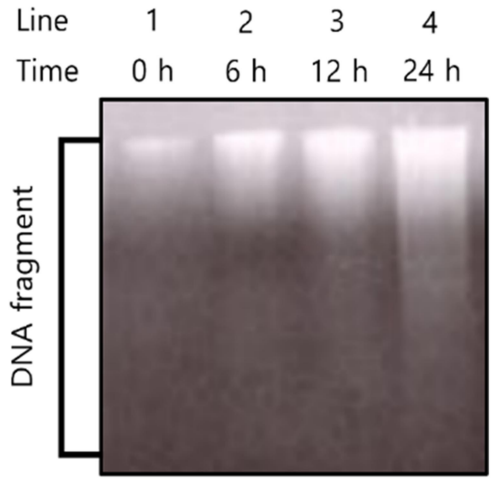

Fig. 2. DNA fragmentation patterns of TC-1 cells treated to Fulvic acid. Cells were exposed to 25 μg/mL Fulvic acid and then incubated for 6, 12, 24, and 48 h. DNA fragments extracted from the cells at different time points of post-incubation were separated in 1.8% agarose gel. Lane 1 represents the control.

cells were incubated for various times (0, 6, 12 and 24 h). DNA ladder pattern treated to FA was shown at 24 h (4 line on Fig. 2).

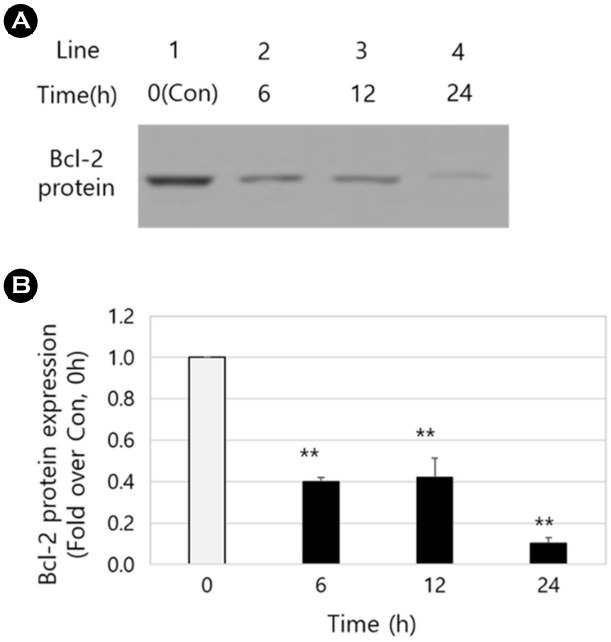

Fig. 3. Effect of Fulvic acid treatment on the expression of Bcl-2 in TC-1 cells. Cells were treated to Fulvic acid (FA) and then incubated for 0, 12 and 24 h. Harvested cells were lysed and were performed Western blotting as described in the materials and methods. The expression of Bcl-2 protein was detected with anti-Bcl-2 antibody (A). FA treatment for 0 h represents the control (Con). And then, to accurately compare the Bcl-2 expression level shown in the WB film, the density of the con (0 h) band was converted to 1, and the density of the remaining bands (6, 12 and 24 h) was calculated proportionally (B). Data are expressed as the means ± SD in three individual experiments. $^{**}P < 0.01$ were considered a significant difference of Bcl-2 protein level between the control group (0 h) and FA-treated group (6, 12 and 24 h).

The anti-proliferation effect of Fulvic acid by TC-1 cell apoptosis

To confirm the mechanism of inhibition of proliferation of TC-1 cells by FA, we investigated the changes in the expression of Bcl-2 protein (apoptosis-related protein) acting on the mitochondrial pathway. Apoptosis related protein was detected by western blot analysis. Fig. 3 represents the expression levels of Bcl-2 proteins in TC-1 cells treated with FA for 0, 6, 12 and 24 h. The expression of Bcl-2 proteins was decreased after FA treatment. The representative protein expression of anti-apoptotic protein Bcl-2 was decreased. These results suggest that FA treatment induced apoptosis of TC-1 cells, and this apoptosis is related to decrease of Bcl-2 proteins.

DISCUSSION

In this study, we investigated the effect on cell survival and proliferation after treating Fulvic acid (FA) in TC-1 cells. Cell survival was confirmed by treating FA concentration as a cell inhibitory effect, and the effect on apoptosis was to be investigated.

Various physical and chemical agents can induce cell death that provides some promising strategies for cancer treatment. One of the physical agents is FA. There are many studies focused on the targeted induction of apoptotic cell death to control the cancer cells. Our results show that FA treated TC-1 cells were decreased cell viability in a dose-dependent and time-dependent manner (Table 2 and 3). In this study, FA treatment induces a significant dose-dependent reduction in cell proliferation and an increase of apoptotic cell death. Apoptosis involves the condensation of chromatic, restructuring of the cytoplasm and blebbing of cytoplasmic membranes. And finally, apoptotic cells are induced fragmentation and the apoptotic bodies are phagocytosed by neighboring cells. These features are distinguished from the process of necrotic cell death (Lu et al., 2023). In the beginning of apoptosis, it is characterized by morphological changes in dying cells. Typical changes in apoptosis include several morphological and biochemical features (Zakeri et al., 1995). A number of structural proteins are processed on initiation of the apoptotic cascades. One of the most prominent of these proteins is actin (Mashima et al., 1997). Actin forms microfilaments and regulates the cell shape in the cortical cytoskeleton. Examples of other proteins that are cleaved by caspases during apoptosis are spectrin, fodrin, E-catenin, gelsolin, growth arrest-specific 2, and p21-activated kinase 2. These proteins are involved in the maintenance, organization, and attachment of the cytoskeleton (e.g., gelsolin, Gas2, PAK2) and play a role in cell-to-cell junctions (e.g., spectrin, fodrin, β-catenin) (Ziegler and Groscurth, 2004). As shown in Fig. 1, the control TC-1 cells appeared normal configuration, typical adherent and homogeneous cells (Fig. 1A). But, in the FA-treated group, the characteristic microscopical changes were a decrease in cell volume and an increase in nonadhesion and death numbers (Fig. 1B, 1C and 1D). Although the nucleosomal DNA ladder is not always associated with apoptosis, the morphological changes during apoptosis are associated with double-strand cleavage of nuclear DNA in the linker regions between nucleosome. Agarose gel electrophoresis displays the inter-nucleosomal DNA of apoptotic cells in a typical ladder pattern, while DNA cleavage of necrotic cells is random (Aleksandrushkina and Vanyushin, 2012). DNA ladder by various concentrations of FA treatment was observed. In this study, DNA ladder induced by treatment with FA treatment in TC-1 cells was shown at 24 h (Fig. 2).

Apoptosis can be triggered in various intracellular signaling pathways (Lowe et al., 1993). In apoptotic pathway, calcium concentration is controlled by apoptosis-associated proteins. The anti-apoptotic Bcl-2 has been reported to exert an inhibitory effect on apoptosis by blocking the release of cytochrome *c* and the loss of MMP, and this protein acted in the inhibition of apoptosis in the pathogenesis of cancer (Sovilj et al., 2023). In contrast, Bax that is a pro-apoptotic protein integrates into the outer mitochondrial membrane and causes cytochrome *c* release (King et al., 2023). In the present study, we confirmed that FA have potential pro-apoptotic effects in the TC-1 cell line through decreased expression of Bcl-2 (Fig. 3).

Taken together, our results indicate that FA-induced apoptosis in TC-1 cell is dependent on the regulation of intracellular signaling pathway. Our findings suggest that the FA has a potent pro-apoptotic activity, and it may provide a therapeutic index of lung cancer.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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