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Indole-3-carbinol induces apoptosis through p53 and activation of caspase-8 pathway in lung cancer A549 cells

Hee-Sook Choi [a,1], Min-Chul Cho [a,1], Hee Gu Lee [b,*], Do-Young Yoon [a,*]

[a] Department of Bioscience and Biotechnology, BMIC, Konkuk University, Seoul 143-701, Republic of Korea
[b] Medical Genomics Cell Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Republic of Korea

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ABSTRACT

Indole-3-carbinol (I3C) has anti-tumor effects in various cancer cell lines. However, the anti-tumor effect of I3C on human lung cancers has been rarely reported. We investigated the anti-tumor effects and its mechanism of I3C on human lung carcinoma A549 cell line. Treatment of the A549 cells with I3C significantly reduced cell proliferation, increased formations of fragmented DNA and apoptotic body, and induced cell cycle arrest at G0/G1 phase. I3C increased not only the protein levels of cyclin D1, phosphorylated p53, and p21 but also the expression of Fas mRNA. Cleavage of caspase-9, -8, -3 and PARP also was increased by I3C. Treatment with wortmannin significantly suppressed both I3C-induced Ser15 phosphorylation and accumulation of p53 protein. The inhibition of caspase-8 by z-IETD-FMK significantly decreased cleavage of procaspase-8,-3 and PARP in I3C-treated A549 cells. Taken together, these results demonstrate that I3C induces cell cycle arrest at G0/G1 through the activation of p-p53 at Ser 15 and induces caspase-8 mediated apoptosis via the Fas death receptor. This molecular mechanism for apoptotic effect of I3C on A549 lung carcinoma cells may be a first report and suggest that I3C may be a preventive and therapeutic agent against lung cancer.

1. Introduction

Lung cancer is one of the most common causes of cancer-related deaths in the United States (Jemal et al., 2008). Lung cancer is divided into small and non-small cell lung cancer (NSCLC), and 80% of all lung cancer patients had NSCLC (Chen et al., 2008; Kuang and Chen, 2004). Lung cancer mortality has steadily increased because it is highly metastatic and because of the side effects which are frequently accompanied by surgery and radiotherapy. Thus, an understanding of the molecular mechanisms of more effective and less harmful therapies are needed to reduce lung cancer mortality. Cancer cells escape apoptosis by a number of mechanisms, among which overexpression of anti-apoptotic genes, including members of the Bcl-2 family, heat shock protein (HSP) gene family and the inhibitor of apoptosis protein (IAP) family, has been shown to play a critical role (Beere and Green, 2001; Deveraux and Reed, 1999; Xanthoudakis and Nicholson, 2000). HSP is highly expressed in lung tumor (Malusecka et al., 2001) and this overexpression allows cells to survive in otherwise lethal conditions, suggesting that cellular transformation can modulate the basal level of these stress proteins and participate in conferring chemoresistance (Jaattela, 1999). Resistance can be overcome by the use of sensitizing agents that modify the deregulated apoptosis signaling pathways in cancer cells (Zhuang et al., 2009).

Various natural products including flavonoid derivatives, indole-3-carbinol (I3C), 3,3′-diindolylmethane (DIM), curcumin, (-)-epiglallocatechin-3-gallate, etc, have reported as natural agents to inhibit cancer development through perturbing multiple cellular signaling (Sarkar et al., 2009). I3C, a compound from vegetables, such as cabbage, brussels sprouts and broccoli, is a well-known anti-cancer agent for prostate, breast and colon cancer cells (Chinni et al., 2001; Garcia et al., 2005; Howells et al., 2008; Sarkar and Li, 2004). I3C is biologically active, and it is converted into the 3,3′-diindoylmethane (DIM) under pH 5–7, which is also biologically active (Safe et al., 2008). In various cancer cells, I3C has exhibited anti-cancer effects, including cell cycle inhibition, apoptosis and decreasing tumor invasion (Brew et al., 2006; Chung et al., 1993; Kuang and Chen, 2004; Souli et al., 2008). I3C regulates cellular signals, including the Akt, NF-κB, JNK and MEK pathways, in various cell lines (Safe et al., 2008; Sarkar and Li, 2004; Sarkar et al., 2009; Weng et al., 2008).

The p53 tumor suppressor gene prevents tumorigenesis in response to physiological and environmental stress and plays a role in cell cycle progression, apoptosis and repair of DNA damage. p53

Abbreviations: CDKI, cyclin-dependent kinase inhibitor; I3C, indole-3-carbinol; PARP, poly (ADP-ribose) polymerase; PI3K, phosphatidylinositol 3-kinase; z-IETD-FMK, benzyloxycarbonyl-Ile-Glu (OMe)-Thr-Asp (OMe)-fluoromethyl ketone.

* Corresponding authors. Tel.: +82 42 860 4182; fax: +82 42 860 4593 (H.G. Lee), tel.: +82 2 450 4119; fax: +82 2 444 4218 (D.Y. Yoon).
E-mail addresses: hglee@kribb.re.kr (H.G. Lee), ydy4218@konkuk.ac.kr (D.-Y. Yoon).

[1] These authors share equal authorship.

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has may be involved in transcriptional regulation of pro-apoptotic genes associated with intrinsic and extrinsic pathways (el-Deiry, 1998). The activation of the p53 pathway leads either to cell cycle arrest or to apoptosis. Activated p53 increases the expression of p21 in DNA damaged cells and affects expression of p27 (Roy et al., 2007; Safe et al., 2008; Weng et al., 2008). The up-regulation of cyclin-dependent kinase inhibitors (CDKI), such as p21 and p27, is related to cell cycle arrest and contributes to down-regulation of cyclin D1, cyclin E, cyclin-dependent kinases (cdks) 2, 4 and 6 (Safe et al., 2008; Weng et al., 2008). I3C-mediated G1 arrest is mainly associated with an up-regulation of p21 and p27 levels in prostate cancer and HaCaT cells (Safe et al., 2008). It also induces cell cycle arrest via activation of p53 phosphorylated serine 15, 20, 33, 37 and 46. In particular, the phosphorylation of serine 15 affects DNA damage and growth inhibition functions of p53 (Agarwal et al., 1998; Brew et al., 2006; Chen et al., 2008; el-Deiry, 1998; Suzuki et al., 2007). The activation of p53 induces up-regulation of pro-apoptotic Bax but not anti-apoptotic Bcl-2. Also, p53-mediated apoptosis involves the activation of Fas and the intrinsic mitochondrial pathway, which results in activation of caspase-8 and -9 (Agarwal et al., 1998; Liebermann et al., 2007).

Anti-tumor effects of I3C have been reported in various cancer cell lines. However, the anti-tumor effect of I3C on human lung cancer has not yet well described. In addition, it has been reported that anti-proliferative effect of I3C in A549 was independent of induction of apoptosis (Kuang and Chen, 2004). In this study, we investigated the effects of I3C and the molecular mechanism of I3C on growth and apoptosis in human lung epithelial carcinoma cell line A549. Our results suggest that I3C-induced apoptosis through phosphorylation of p53 at serine 15 and activated the caspase-8 dependent apoptosis pathway in human lung epithelial carcinoma A549 cells line.

2. Materials and methods

2.1. Reagents

Indole-3-carbinol (I3C) and PI3-kinase inhibitor wortmannin were obtained from Sigma Chemical Co. (St. Louis, MO), and caspase-8 inhibitor (z-IETD-FMK) was from R&D System, Inc. (Minneapolis, MN). The respective antibodies against phospho-p53 (Ser 15), caspase-3, -8, -9, Bax, Bcl-2, Bcl-$_X$L, cytochrome C and PARP were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The antibodies specific to p21, p27 and β-tubulin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and anti-cyclin D1 and anti-cyclin E antibodies were from BD Biosciences Pharmingen, Inc. (San Jose, CA). p53 (Ab-6) antibody was obtained from Calbiochem (La Jolla, CA). The anti-cIAP-1 and anti-cIAP-2 antibodies were purchased from Abcam (Cambridge, MA).

2.2. Cell proliferation assay

The human A549 lung cancer cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The cells were maintained in RPMI 1640 medium (Hyclone, UT) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), and were cultured in a humidified CO_2 incubator at 37 °C. In order to examine the effects of I3C in A549 cells, A549 cells (0.5×10^4) were seeded in 96-well microplate in 200 μl of complete medium with 10% heat-inactivated FBS and the cells were incubated without or with I3C in 96-well plate for 24 and 48 h. The cell viability was measured using an MTS assay (Celltiter 96 Aqueous one solution cell proliferation assay, Promega, Madison, WI) according to the manufacturer's instruction. Briefly, MTS solution was added to each well and the cells were incubated for a final 1 h. The absorbance at 492 nm was measured using a microplate reader (Apollo LB 9110, Berthold Technologies GmbH, Germany).

2.3. Detection of apoptosis

For DNA laddering assay, A549 cells (1×10^5) were treated with I3C (100, 200, 300, 400 and 500 μM) for 48 h and were washed with ice-cold phosphate-buffered saline (PBS) and then lysed by a lysis buffer containing 20 mM Tris–HCl (pH 8.0), 20 mM EDTA, 300 mM NaCl, and 2% SDS. The cell lysates were extracted with an equal volume of phenol, followed by an equal volume of a mixture of chloroform/phenol/isoamylalcohol, precipitated with an equal volume of isopropanol, and then washed twice with 70% ethanol. DNA electrophoresis was carried out in 1.6% agarose gel and DNA was stained with ethidium bromide.

For Hoechst 333258 staining, after being treated with I3C, the A549 cells were washed with PBS and fixed in 4% paraformaldehyde for 1 h. The cells were stained with Hoechst 333258 (10 μg/ml) for 20 min at 37 °C and the cells were photographed under a fluorescent microscope.

For TUNEL assay, A549 cells on the coverslips were fixed in 4% paraformaldehyde for 1 h and washed with PBS. Cell membrane was permeabilized by exposing to 0.1% Triton X-100 in PBS for 30 min. Apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assays using the *In Situ* Cell Death Detection Kit (Roche-Diagnostics, Indianapolis, IN) as described by the manufacturer.

2.4. Flow cytometric analysis

Cells were collected and washed twice with PBS. The cells were resuspended in PBS and then fixed using cold ethanol on ice for at least 1 h. The cells were pelleted by centrifugation and incubated with 1 ml of DNA staining solution (200 μg of propidium iodide (PI) in 10 ml of PBS containing 2 mg of DNase free RNase) for at least 30 min. Acquisition and analysis were performed by FACScan using Cell Quest Pro software (Becton Dickinson Labware, Franklin Lakes, NJ) with excitation at 488 nm.

For the measurements of Annexin-V-PI binding, the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) was used according to the manufacturer's instructions. In brief, the cell pellets were suspended in 100 μl of Annexin binding buffer, followed by incubation for 15 min in the dark, and then analyzed by the flow cytometry (Becton Dickinson Labware, Franklin Lakes, NJ).

2.5. Immunoblot analysis

Cytosolic and total cell lysate extraction were carried out as previously described (Cho et al., 2008). Briefly, cells were lysed in cytosolic buffer (10 mM HEPES-KOH pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.2 mM NaF, 0.1 mM EDTA, 0.1 mM Na_3VO_4, and 1 mM DTT) containing protease inhibitors. After addition of NP-40 to a final concentration of 0.15%, the lysate was vigorously mixed for 15 s and then centrifuged at 12,000 rpm for 1 min at 4 °C. The resulting supernatant was store at −80 °C as the cytoplasmic extract. Total lysates were prepared by RIPA buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% SDC, 1 mM EDTA, 1 mM EGTA, 1 mM orthovanadate, aprotinin (10 μg/ml) and 0.4 mM phenylmethylsufonyl fluoride (PMSF). Collected cells were maintained for 1 h in RIPA buffer and after centrifugation at 12,000 rpm for 30 min at 4 °C, the supernatant was collected and preserved at −80 °C until use. Equal amounts (60 μg) of total cell lysate was subsequently applied to SDS–PAGE and transferred into poly-vinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was blocked with 5% skimmed milk for overnight and then incubated with specific antibodies. After washing with PBS containing 0.05% Tween-20 (PBST), the membrane was reacted with horseradish peroxidase (HRP) conjugated secondary antibody. The immune complex in PVDF membrane was detected with enhanced chemiluminescence (ECL) solution (Amersham Bioscience, Buckinghamshire, UK) and X-ray film.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR

In order to conduct RT-PCR, total RNA was isolated using easy-BLUE™ total RNA extraction kit (iNtRon Biotechnology, Korea) as described by the manufacturer's protocol. Reverse transcription was performed using the ProSTAR™ (Stratagene, LaJolla, CA). After cDNA was synthesized by using Oligo (dT), the cDNA was amplified by PCR. GAPDH primer has been previously described (Cho et al., 2008) and primers specific for Fas are synthesized according to the sequences (Sawanobori et al., 2003). The specific primers to perform SYBR green-based real-time quantitative PCR were designed as follow: 5′-TGA AGG ACA TGG CTT AGA AGT G-3′/5′-GGT GCA AGG GTC ACA GTG TT-3′ for human Fas. Real-time PCR was performed as previously described (Lee et al., 2009). The resulting gene mRNA data were normalized against the reference gene (GAPDH) mRNA and compared to normal cells of the appropriate strain for relative expression values.

2.7. Statistical analysis

Statistical analysis was done using the student's *t*-test, with the following significance levels: $^*P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Effects of inodole-3-carbinol (I3C) on A549 lung carcinoma cells

To examine the effect of I3C on the growth of A549 cells, a cell proliferation assay using MTS assay was performed with various