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Indomethacin Induces Apoptosis in NCI-H1299 Human Lung Carcinoma Cells

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Recently, nonsteroidal anti-inflammatory drugs (NSAIDs) have been found to be useful in the chemoprevention of colon cancer. To investigate whether indomethacin, an NSAIDs, induces apoptosis and thus assess the possibility of its application in the chemoprevention of human lung cancer, we have performed MTT assay, TUNEL assay, DAPI staining, and flow cytometric analysis using human lung carcinoma cell line NCI-H1299. Through morphological and biochemical analyses, it was demonstrated that NCI-H1299 cells treated with indomethacin (0.5 mM) exhibit classical apoptotic features. These results suggest that indomethacin induces apoptosis in NCI-H1299 cells and that NSAIDs, including indomethacin, may be a useful tool for the chemoprevention of human lung cancer.

Key Words: Chemoprevention, Indomethacin, Lung neoplasms, Apoptosis

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed and have antipyretic, analgesic and anti-inflammatory activities (Katzung & Furst, 1998). Epidemiological studies have showed the beneficial effects of NSAIDs in the prevention of cancers of the colon (Thun et al, 1991), breast (Harris et al, 1996), and lung (Schreinemachers & Everson, 1994). Experimental studies have also shown that NSAIDs have chemopreventive activity against human colon cancer cell lines (Hanif et al, 1996), human breast carcinoma cell lines (Han et al, 1998), and mouse lung adenomas (Castonguay et al, 1998). The chemopreventive activity of NSAIDs has been ascribed to their inhibition of cyclooxygenase (COX) (Smalley & DuBois, 1997; Subbaramaiah et al, 1997; Dannenberg & Zakim, 1999), to their growth-inhibitory actions (Shiff et al, 1995), and their ability to induce apoptosis (Chan et al, 1998; Klampfer et al, 1999).

Lung cancer is one of the most malignant and has a high incidence rate worldwide. However, relatively few studies have been reported on the chemopreventive effects of NSAIDs on lung cancer. In this paper, we have investigated whether indomethacin induces apoptosis in human lung carcinoma cells.

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METHODS

Cell culture

Human lung carcinoma cells (NCI-H1299) were purchased from American Type Culture Collection (ATCC, Rockville, MI). Cells were cultured in RPMI 1640 medium (GibcoBRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (GibcoBRL, Grand Island, NY) at 37°C in 5% CO_2, 95% air in a humidified cell incubator.

Chemicals and reagents

Indomethacin, diaminobenzidine (DAB), 4,6-diami-

dino-2-phenylindole (DAPI) and propidium iodide (PI) were obtained from Sigma (St. Louis, MO). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was purchased from Boehringer Mannheim (Mannheim, Germany). The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay kit was obtained from a commercial supplier (Intergen, Purchase, NY).

MTT assay

MTT assay was performed according to the manufacturer's instructions. In brief, NCI-H1299 cells were cultured in a 96-well plate (Corning Incorporated, Corning, NY) at a density of 5×10^3 cells per well. Cells were treated with varying concentrations of indomethacin. After 24 h, the cells were incubated with the MTT labeling reagent for 4 h. After the cells were incubated with the solubilization solution for 24 h, absorbance at 595 nm was measured using a microtiter plate reader (Bio-Tek, Winooski, VT). Percent viability was calculated as (absorbance of drug-treated sample/control absorbance) $\times$ 100. For morphological analysis, following a 24-h exposure to indomethacin (0.5 mM), NCI-H1299 cells were observed by phase-contrast microscopy (Olympus, Japan).

TUNEL assay

For *in situ* detection of apoptotic cells, TUNEL assay was performed using ApoTag® peroxidase *in situ* apoptosis detection kit (Intergen, Purchase, NY). In brief, NCI-H1299 cells were cultured in 4-chamber slides (Nalge Nunc International, Naperville, IL) at a density of 2×10^4 cells/chamber. After a 24-h exposure to indomethacin (0.5 mM), cells were fixed by incubating in 1% paraformaldehyde for 10 min. The fixed cells were incubated with digoxigenin-conjugated dUTP in a TdT-catalyzed reaction for 60 min at 37°C and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min. The DNA fragments were stained using DAB as a substrate for the peroxidase.

DAPI staining

DAPI staining was performed according to previous protocols (Luo et al, 1998). Cells were cultured in 4-chamber slides. After exposure to indomethacin (0.5 mM) for 24 h, the cells were washed twice with phosphate-buffered saline (PBS) and fixed by incubation in 4% paraformaldehyde for 30 min. The fixed cells were again rinsed twice with PBS and were incubated in 1 μg/ml DAPI solution for 30 min. The apoptotic cells were observed with a fluorescence microscope (Zeiss, Oberkochen, Germany).

Flow cytometric analysis

Flow cytometric analysis was performed as previously described (Ormerod, 1998). Cells were collected by centrifugation and were washed in ice-cold PBS. Approximately 10^6 cells were resuspended in 200 μl of PBS, and 2 ml of ice-cold 75% ethanol solution was added. The cells were recentrifuged and resuspended in 1 ml of PBS containing 20 μg of PI and 100 μg of RNase. The stained cells were incubated for 30 min at 37°C and were analyzed using FACScan (Becton Dickinson, San Jose, CA).

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Science software SAS (version 6.1.2). Data were analyzed by Student's t test. Differences were considered significant if $P < 0.05$.

RESULTS

A dose-related pattern was seen in the viability of NCI-H1299 cells when treated with various concentrations of indomethacin. Viabilities of cells treated with indomethacin at concentrations of 0.1 mM, 0.3 mM, 0.5 mM, and 1 mM were about 99%, 78%, 59%, and 17% of that of the untreated control (100%), respectively (Fig. 1). These results indicate that indomethacin induced cell death in NCI-H1299 cells.

For the analysis of morphological changes, cells were examined by phase-contrast microscopy. Indomethacin-treated cells (0.5 mM) showed cell shrinkage, cytoplasmic condensation, and irregularity in shape (Fig. 2; Top). These morphological characteristics suggest that indomethacin induces apoptotic cell death in NCI-H1299 cells. Next, to examine the biochemical findings of apoptosis in NCI-H1299 cells induced by indomethacin (0.5 mM), TUNEL assay, DAPI staining, and flow cytometric analysis were performed. As shown in Fig. 2, some nuclei of the

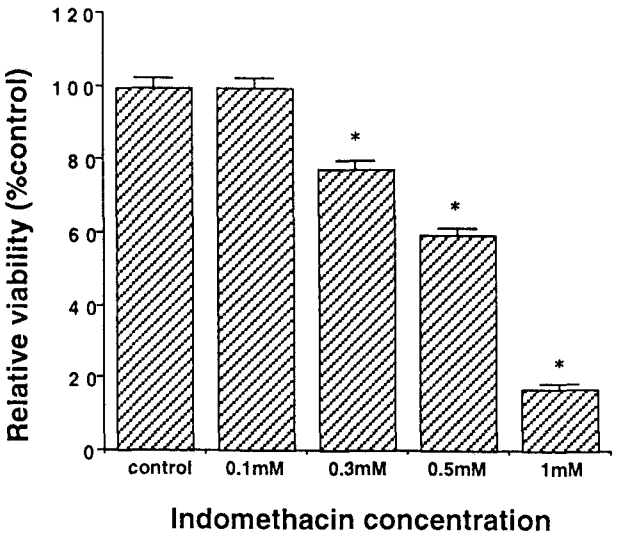

Fig. 1. Dose-dependent effect of indomethacin on cell viability. NCI-H1299 cells were incubated with indomethacin at the indicated concentrations for 24 h. Cellular viability was determined via MTT assay. Relative viability (% control) is shown as the percentage absorbance of the sample with respect to that of the control (without indomethacin). Results are represented as mean ± standard error (bars) for two independent experiments, each with a minimum of three cultures. *Statistically significant decrease ($P < 0.01$) compared to the control.

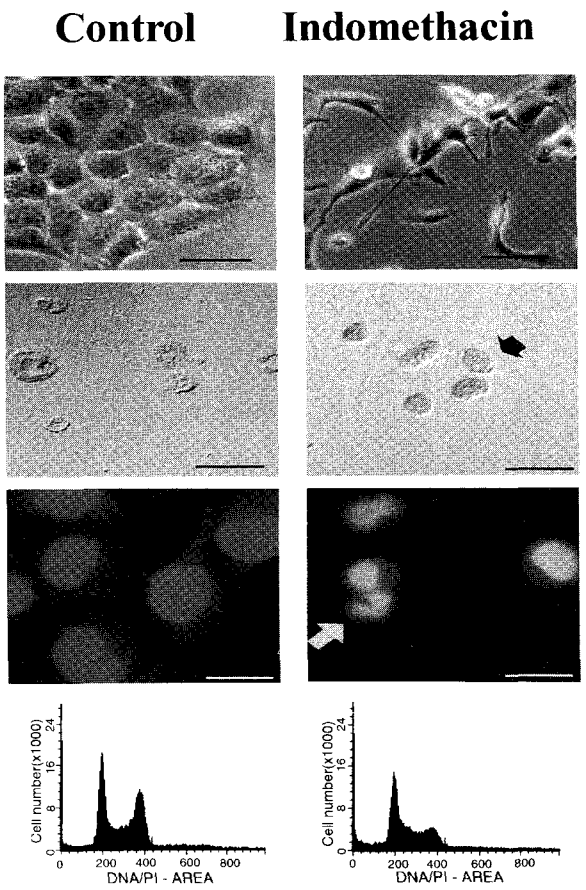

Fig. 2. Characterization of indomethacin-induced cell death in NCI-H1299 cells. Cells were cultured without indomethacin (Control) or with 0.5 mM indomethacin for 24 h (Indomethacin). Top) Morphology. Phase-contrast microscopy revealed cell shrinkage, irregularity in shape, and detachment of some cells from the culture dish in indomethacin-treated culture, but not in the control. Scale bars represent 100 μm. Upper-middle) TUNEL assay. The black arrow indicates labeling of condensed and marginated chromatin. Scale bars represent 40 μm. Lower-middle) DAPI staining. The white arrow indicates condensed nuclei. Scale bars represent 25 μm. Bottom) Flow cytometric data. The number of cells in the sub-G_1 phase was increased after treatment with 0.5 mM indomethacin. All experiments were triplicated independently.

indomethacin-treated cells exhibited typical features of apoptosis, such as nuclear condensation and fragmentation. Through flow cytometric analysis, it was shown that there were an increased proportion of cells in the sub-G_1 phase among the indomethacin-treated cells (27.3%) compared to that of the control (8.6% of total cells).

DISCUSSION

In this study, we reported that human lung carcinoma cells (NCI-H1299) undergo apoptosis upon treatment with pharmacological concentrations of indomethacin.

Chemoprevention is especially important in cancer patients because some cancers have resistance to most therapeutic methods. Although studies on chemoprevention hold interest in this respect, there are few studies to date, and only a few agents are known to be useful for chemoprevention in cancer patients. Through epidemiological studies, NSAIDs have been shown to possess the chemopreventive activity against cancers of the colon, lung, and breast (Thun et al, 1991; Schreinemachers & Everson, 1994; Harris et al, 1996). To date the chemopreventive effects of NSAIDs have been studied mainly in the context of colon cancer (Rao et al, 1995; Hanif et al, 1996; Smalley & DuBois, 1997). Lately, more attention has been given to such effects on leukemia (Bellosillo et al, 1998; Klampfer et al, 1999), breast cancer (Han et al,

1998), abdominal neoplasm (Picariello et al, 1998), and pancreatic cancer (McDade et al, 1999). However, relatively few studies have focused on the chemopreventive effects of NSAIDs on lung cancer. Castonguay et al (1998) reported that NSAIDs inhibit lung tumorigenesis induced by the tobacco-derived carcinogen NNK in A/J mice. They proposed the hypothesis that NSAIDs induce apoptosis by inhibiting both the activation of NF-B and the enzymatic activity of COX-2. Their report, however, included no data regarding NSAIDs-induced apoptosis. Hereby, we investigated whether indomethacin induces apoptosis in human lung cancer cells.

From MTT assay, the cytotoxicity of indomethacin was apparent at a concentration of 0.5 mM. Indomethacin-induced apoptosis of NCI-H1299 cells was determined according to the morphological and biochemical criteria of apoptosis. Through morphological analyses, it was shown that cells undergoing indomethacin-induced apoptosis exhibit classical visual markers of apoptosis, such as cell shrinkage, cytoplasm condensation, and irregularity in shape. Biochemical analyses revealed the occurrence of DNA fragmentations and accumulation of cells in the sub-G_1 phase upon treatment with indomethacin.

The definitive molecular mechanisms of chemopreventive action of NSAIDs are still unknown. However, it is well known that NSAIDs are potent inhibitors of both NF-B activation (Kopp & Ghosh, 1994) and the enzymatic activity of COX (Katzung & Furst, 1998). Previous studies have reported that NSAIDs activate caspases (Bellosillo et al, 1998; Klampfer et al, 1999) and interfere with mitogen-activated protein kinase (MAPK) signal pathways (Schwenger et al, 1997). Klampfer et al (1999) also reported that sodium salicylate inhibits the expression of MCL-1 but does not alter the expression of BCL-2, BAX and BCL-X_L in human myeloid leukemia cells. RT-PCR analysis of apoptosis-related genes was performed in this study, and it was shown that the expression of *bcl-2* in NCI-H1299 cells treated with either indomethacin (0.5 mM) or sodium salicylate (5 mM) was decreased, compared to those of the untreated control (data not shown). Interestingly, although indomethacin is a more potent inhibitor of COX-1 and sodium salicylate is a poor inhibitor of COX-1 and COX-2, the expression of *cox-2* in NCI-H1299 cells treated with either indomethacin or sodium salicylate was decreased compared to that of the control (data not shown). For the precise molecular mechanism of the chemopreventive effects of NSAIDs on human lung cancer, further studies such as caspase assay, and studies regarding MAPK signal pathways and apoptosis-related genes are called for.

In the present study, we reported that indomethacin induces apoptosis in NCI-H1299 human lung carcinoma cells, and we propose that NSAIDs, including indomethacin, may be useful for the chemoprevention of human lung cancer.

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