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Curcumin induces apoptosis and inhibits growth of orthotopic human non-small cell lung cancer xenografts☆,☆☆

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Abstract

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality. Curcumin is involved in various biological pathways leading to inhibition of NSCLC growth. The purpose of this study was to evaluate the effect of curcumin on expression of nuclear factor κB-related proteins *in vitro* and *in vivo* and on growth and metastasis in an intralung tumor mouse model.

H1975 NSCLC cells were treated with curcumin (0–50 μM) alone, or combined with gemcitabine or cisplatin. The effects of curcumin were evaluated in cell cultures and *in vivo*, using ectopic and orthotopic lung tumor mouse models. Twenty mice were randomly selected into two equal groups, one that received AIN-076 control diet and one that received the same food but with the addition of 0.6% curcumin 14 days prior to cell implantation and until the end of the experiment. To generate orthotopic tumor, lung cancer cells in Matrigel were injected percutaneously into the left lung of CD-1 nude mice. Western blot analysis showed that the expressions of IkB, nuclear p65, cyclooxygenase 2 (COX-2) and p-ERK1/2 were down-regulated by curcumin *in vitro*. Curcumin potentiated the gemcitabine- or cisplatin-mediated antitumor effects. Curcumin reduced COX-2 expression in subcutaneous tumors *in vivo* and caused a 36% decrease in weight of intralung tumors (P=.048) accompanied by a significant survival rate increase (hazard ratio=2.728, P=.036). Curcumin inhibition of COX-2, p65 expression and ERK1/2 activity in NSCLC cells was associated with decreased survival and increased induction of apoptosis. Curcumin significantly reduced tumor growth of orthotopic human NSCLC xenografts and increased survival of treated athymic mice. To evaluate the role of curcumin in chemoprevention and treatment of NSCLC, further clinical trials are required.

Keywords: Curcumin; NSCLC; Cisplatin; Apoptosis; Synergistic effect

1. Introduction

Lung cancer is the second most common cancer in both genders and the leading cause of cancer death, accounting for 28% of all cancer deaths expected to occur in 2013 [1]. About 85% to 90% of lung cancers are non-small cell lung cancer (NSCLC), with a 5-year survival rate of only 16% [1]. Standard treatment regimens for NSCLC depend on the stage of the disease. Early-stage tumors are treated primarily with surgery or radiotherapy [2]. Radiation may also be used postoperatively when surgical margins are close or positive. More advanced cancers often require multimodality therapy consisting of surgery, radiation and chemotherapy. Chemotherapy for NSCLC uses a combination of two drugs, most frequently combination of carboplatin–taxol or gemcitabine–cisplatin [2]. Both antitumor agents gemcitabine and cisplatin interfere with DNA replication and repair and induce cell apoptosis [3,4]. Cisplatin induces its cytotoxic properties through binding to nuclear DNA and subsequent interference transcription and/or DNA replication mechanisms [4]. Kim et al. [5] found that activation of NF-κB is required for cisplatin-induced apoptosis in HNSCC cell lines. Cisplatin was shown to induced IkBα degradation and NF-κB-dependent transcriptional activation prior to cell death [5]. The transcription factor nuclear factor κB (NF-κB) is a crucial regulator in oncogenesis. It promotes proliferation, inhibits apoptosis and, by doing so, maintains the balance between normal cell division and cell death. NF-κB, a multifunctional transcription factor, is activated by numerous extracellular stimuli, including cytokines, growth factors, carcinogens and tumor promoters that lead to the expression of defined target genes for diverse biological functions [6]. NF-κB is a heterodimeric protein composed of five subunits [RelA (p65), RelB, c-Rel, NF-κB1 (p50 and p105) and NF-κB2 (p52) proteins], and it is retained in the cytoplasm by the inhibitory subunit, IkBa [6]. The extracellular stimulus that initiates activation of NF-κB is dependent on the degradation of IkBa proteins, which is

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mediated through the activation of the IkBa kinase (IKK) complex that subsequently releases the active NF-κB to translocate into the nucleus where it regulates numerous gene expressions, including cyclooxygenase 2 (COX-2) [7]. Since NF-κB is involved in cancer development, modulating NF-κB activation pathways has important implications in cancer prevention and therapy. NF-κB, which may play an important role in COX-2 induction, is mediated through activation of the canonical NF-κB pathway [8]. Charalambous et al. [9] have shown that up-regulation of COX-2 was accompanied by increased expression of NF-κB-p65 and IκB-kinase-alpha (IKKα) in human colorectal cancer epithelial cells. Those authors suggested that these were early postinitiation events involved in tumor progression [8]. COX-2 is induced by proinflammatory or mitogenic stimuli and is overexpressed in a variety of human cancers, including NSCLC [10–12]. Elevated tumor COX-2 expression is associated with increased angiogenesis, tumor invasion and promotion of tumor cell resistance to apoptosis [11,12]. COX-2 was found to be up-regulated at most stages of tumor progression in lung cancer [13]. Takahashi et al. [14] demonstrated an association of up-regulation of COX-2 with tumor invasion and metastasis in human lung tumors. Moreover, the proportion of adenocarcinoma cells with marked COX-2 expression is reportedly much greater in lymph node metastases than in the corresponding NSCLC primary tumors [10]. Other studies have shown that elevated expression of COX-2 is associated with poor prognosis and a worse overall survival rate in NSCLC [11]. Consistent with these findings, several epidemiologic studies have shown a positive association between consumption of nonsteroidal anti-inflammatory drugs and the incidence of lung cancer [11,15].

Selective COX-2 blocking (COXIB) agents and celecoxib in particular have a strong potential for the chemoprevention of human lung cancer [16]. Long-term use of COXIBs has, however, been associated with increased risk of serious cardiovascular events [17], and clinical trials have shown that the addition of celecoxib to chemotherapy failed to benefit the survival of NSCLC patients [13]. Taken together, these findings establish the need to find a better therapeutic strategy for NSCLC.

Curcumin (diferuloyl methane) is a natural yellow-pigmented polyphenol component of the spice turmeric, derived from the roots of the *Curcuma longa* plant indigenous to Southeast Asia. Curcumin has been used as an anti-inflammatory agent in traditional Indian Ayurvedic medicine for centuries [18]. Numerous *in vitro* and *in vivo* studies have shown that curcumin possesses anticancer activities via its effect on a variety of biological pathways. Additionally, curcumin affects growth factor receptors and cell adhesion molecules involved in tumor growth, angiogenesis and metastasis, all of which are relevant to cancer. Curcumin also has antioxidant and anti-inflammatory properties and is able to modulate various signaling mechanisms among which is the ability to inhibit COX-2 at the transcriptional level [19], leading to the suppression of prostaglandin synthesis [20–23]. Moreover, curcumin is an inhibitor of the transcription factor, NF-κB, and downstream gene products, such as COX-2. Curcumin blocks the IκK-mediated phosphorylation and degradation of IκBα, thus NF-κB remains bound to IκBα in the cytoplasm and is not able to enter the nucleus to activate transcription, thereby constraining the expression of the COX2 gene [24]. Curcumin can potentiate the antitumor effects of gemcitabine on human cancer cells [25–27]. It has been shown to potentiate the antitumor effects of gemcitabine in an orthotopic model of human bladder cancer through suppression of proliferative and angiogenic biomarkers [26]. It enhances the effect of cisplatin via inhibition of IKKβ protein of the NF-κB pathway [28]. Combined therapy of curcumin and cisplatin by means of differing mechanisms exhibited a synergistic effect leading to cell death and growth suppressive effect [29]. While curcumin exerted its effect through the inhibition of cytoplasmic and nuclear IKK, leading to inhibition of NF-κB activity, cisplatin mediated its effect via increased expression of p16 and p53, leading to cellular senescence [29]. Curcumin's inhibitory effect on carcinogenesis has been demonstrated in several animal models of various tumor types [18,25,30–32]. *In vitro* studies of NSCLC have shown that curcumin activated cell cycle arrest and apoptosis through different pathways [18].

In the present study, we investigated the effects of curcumin on survival and apoptosis of human NSCLC carcinoma cell lines and sought to determine whether this effect is associated with down-regulation of COX-2, p-ERK1/2 and EGFR expression. We further investigated the pattern of growth and metastatic processed and the response to curcumin of subcutaneous and orthotopic NSCLC tumors. To the best of our knowledge, this is the first study to assess the effect of curcumin in an orthotopic intralung NSCLC model. This study may pave the way to the implementation of curcumin treatment in combination with chemotherapy for patients with NSCLC.

2. Materials and methods

2.1. Cell culture and reagents

The human NSCLC cell lines H1975, H358 and H1299 were obtained from the American Type Culture Collection (ATCC). The human lung carcinoma PC-14 cell line was kindly provided by Prof. I. Fidler (M. D. Anderson Cancer Center, Houston, TX, USA). All cell lines were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Beit HaEmek, Israel) supplemented with 10% fetal calf serum, L-glutamine, sodium pyruvate, 1% penicillin and 1% streptomycin (full medium) at 37 °C, in an atmosphere of 95% oxygen and 5% CO_2. Curcumin (97% purity) was purchased from Merck (Whitehouse Station, NJ, USA) and gemcitabine from Eli Lilly (Indianapolis, IN, USA). Specific antibodies against p65, IκBα and COX-2, EGFR and p-EGFR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), against phospho-IκBα from Cell Signaling Technology (USA) and against actin from MP Biomedicals (USA).

2.2. Cell viability assay

Cell viability was evaluated by XTT assay as described previously [31]. The cells ($1–2\times10^3$ cell/well) were seeded in 96-well microwell plates, incubated at 37 °C for 24 h and then treated with the tested drugs. After 72 h, cell viability was assessed by the ability of metabolically active cells to reduce the tetrazolium salt to colored formazan compounds. The optical density was read at 450 nm. Each variant of the experiment was performed in triplicates. The data were expressed as the mean values of at least three different experiments. Cell survival following treatment was expressed as a percentage of viable cells relative to control value.

2.3. Synergism between curcumin and chemotherapy

To determine whether the addition of curcumin to gemcitabine or cisplatin is synergistic, additive, or antagonistic, cytotoxicity data were analyzed using the combination index (CI) values calculated by the CalcuSyn software (Biosoft, Ferguson, MO, USA), which is based on multiple-drug effect equations [33].

2.4. Flow cytometry analysis

Cell cycle and apoptosis were assessed by flow cytometry. The cells were plated at a density of 0.5×10^6 per 10-cm dish. The tested drugs were added 24 h later, at selected concentrations. Cells ($1–2\times10^6$) were washed in phosphate-buffered saline (PBS), and the pellet was fixed in 3 ml ethanol for 1 h at 4 °C. The cells were pelleted, resuspended in 1 ml PBS and incubated for 30 min with 0.15 mg/ml RNAse at 37 °C, then stained with 5 μg/ml propidium iodide for 1 h before flow cytometry analysis. Data acquisition was performed on a FACScan and analyzed by CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Data for at least 10,000 cells were collected for each data file. Necrotic cells that had been detected by counting cells following staining with trypan blue before fixation were excluded from the calculation of apoptotic cells.

2.5. Protein extraction and Western blot

COX-2, p-ERK1/2, EGFR and p-EGFR expression was evaluated by Western blot analysis. Exponentially growing cells were collected, washed three times in ice-cold PBS and resuspended in lysis buffer [20 mM Tris-HCl pH 7.4, 2 mM EDTA, 6 mM β-mercaptoethanol, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS) and 10 mMNaF, plus the protease inhibitors, leupeptin 10 μg/ml, aprotinin 10 μg/ml and 0.1 mM phenylmethylsulfonyl fluoride]. The nuclear extracts of H1975 cells that had been cultured for 72 h in curcumin-supplemented media were prepared according to the manufacturer's protocol with NucBuster Protein Extraction Kit (Novagen, EMD Biosciences). The protein concentration of each sample was estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Actin expression was used to verify that equal amounts