Induction of apoptosis in human lung cancer cells by curcumin

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Abstract

Curcumin, a phenolic compound from the rhizome of the plant Curcuma longa has anti-inflammatory, antioxidant and anti-cancer activities. Although the precise mode of action of this compound is not yet elucidated, studies have shown that chemopreventive action of curcumin might be due to its ability to induce apoptosis and to arrest cell cycle. This study investigated the cellular and molecular changes induced by curcumin leading to the induction of apoptosis in human lung cancer cell lines—A549 and H1299. A549 is p53 proficient and H1299 is p53 null mutant. The lung cancer cells were treated with curcumin (0–160 μM) for 12–72 h. Curcumin inhibited the growth of both the cell lines in a concentration dependent manner. Growth inhibition of H1299 cell lines was both time and concentration dependent. Curcumin induced apoptosis in both the lung cancer cell lines. A decrease in expression of p53, bcl-2, and bcl-XL was observed after 12 h exposure of 40 μM curcumin. Bak and Caspase genes remained unchanged up to 60 μM curcumin but showed decrease in expression levels at 80–160 μM. The data also suggest a p53 independent induction of apoptosis in lung cancer cells.

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1. Introduction

Cancer causes significant morbidity and mortality and is a major public health problem worldwide. An effective cancer prevention program, diet, and exercise may decrease the incidence of cancer. Plant-derived compounds are known to have curative potential. Curcumin (diferuloylmethane) is a phenolic compound from the plant Curcuma longa (Linn). It is widely used as a coloring and flavoring agent in food [1]. Its anti-inflammatory activity is well documented. Curcumin is not toxic to mammals at very high doses (5–10% by weight of diet) [2].

Curcumin is found to have inhibitory function towards a broad range of tumors such as mammary adenocarcinoma, fore stomach, duodenal and colon cancer as well as 12-O-tetradecanoyl-13-phorbol ester (TPA) induced skin tumors in mice [2,3]. Curcumin is a potent anti-cancer agent and affects cells in a cell type dependent manner. Its ability to induce apoptosis in different cancer cells indicates the possibility of developing curcumin as a universal cancer prophylactic agent.
The protein p53 has an important role in pathogenesis of neoplasia [4]. The mechanism involved entails a rapid increase in p53 protein levels and the mediation of several cellular responses including G1 arrest, DNA damage repair and induction of apoptosis [5]. One of the major signaling pathways involved in apoptotic cell death includes the intracellular caspases, a family of structurally related cysteine proteases [6]. Caspase activity is responsible, either directly or indirectly, for the cleavage of cellular proteins, which are characteristically proteolyzed during apoptosis. For example, caspases -2, -3, -6, -7 and -9 can cleave poly (ADP ribose) polymerase (PARP) [7]. Bcl-2 family proteins are one of the already identified regulators of apoptosis. Bcl-2 family of homologous proteins represents a critical checkpoint within most apoptotic pathways, acting upstream of such irreversible damage to cellular constituents [8]. At least 15 bcl-2 family members have been identified so far in mammalian cells. They function either as proapoptotic (Bax, Bak, Bad) or anti apoptotic (Bcl-2, Bcl-XL) regulators. These proteins form heterodimers of anti and pro-apoptotic members, thereby titrating one another’s function [8]. The ratio of anti-apoptotic and proapoptotic proteins determines in part how cells respond to apoptotic or survival signals [9]. Despite extensive analysis of anti-tumor activities of curcumin, its ability to modulate lung cancer growth has not yet been well characterized. We used lung cancer cell lines to study the effect of different concentrations of curcumin on cell viability and genes related to apoptosis. Our results demonstrated that curcumin causes growth arrest and apoptosis in lung cancer cell lines and the growth inhibitory effects of curcumin appeared to be mediated by the regulation of bcl-2, bcl-xL, bax, caspase-1, caspase-3, c-myc and p53 genes. Curcumin caused induction of apoptosis and the induction was independent of p53 status of the cell lines.

2. Materials and methods

2.1. Cell lines

The human lung adenocarcinoma cell line A549 (ATCC, Manassas, VA, USA) were used for this study. Cell line A549 is p53 wild type and H1299 is devoid of endogenous p53. The cells were grown in RPMI1640 culture medium supplemented with 2 mM l-glutamine and 10% FCS, penicillin (50 IU/ml) and streptomycin (50 (g/ml) at a temperature of 37 °C in a humidified incubator with a 5% CO2 atmosphere.

2.2. Curcumin treatment of cells

Curcumin (Sigma Chemical Co, St Louis MO, USA) was dissolved in DMSO at a concentration of 10 mM and was stored in a dark-colored bottle at 4 °C as a stock solution. The stock was diluted to the required concentration immediately before use with growth media. The cells were exposed to curcumin at different concentration (0–160 (M) and for different time durations (6–72 h). Cells grown in media containing equivalent amount of DMSO without curcumin served as control.

2.3. Cell viability assay

The viability of the cells was assessed by MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product [9,10]. Cells (1 × 105) were plated in a 96-well plate (Costar from Corning, NY). After 24 h they were treated with different concentrations (0–160 (M) of curcumin for different time intervals (12–72 h). After the treatment, media containing curcumin were carefully removed by aspiration. Hundred microlitre of 0.5 mg/ml MTT in cell culture medium was added to each well and incubated for 4 h as described in Ref. [10]. Hundred microlitre of 1% SDS was added to each well after 4 h. The plates were covered with aluminum foil and kept in an incubator for 12 h for dissolution of the formed formazan crystals. Amount of formazan was determined measuring the absorbance at 540 nm using an ELISA plate reader.

2.4. PARP cleavage

The apoptosis was determined by measuring the cleavage of poly (ADP) ribose polymerase (PARP).
PARP is a highly conserved nuclear enzyme present in higher eukaryotes. The enzyme is a DNA binding protein that recognizes DNA strand breaks and is implicated in DNA repair and in the apoptotic response of cells [11]. PARP functions by adding poly (ADP-ribose), in an ATP and NAD+ dependent manner, to various proteins in response to DNA damage and has been shown to directly interact with DNA polymerase. As a marker for apoptosis, PARP cleavage has been shown to occur early in the apoptotic response as a result of caspase-3 activity [12]. PARP cleavage correlates well with chromatin condensation and has been shown to be associated with the condensed chromatin in apoptotic cells, as a measure of apoptosis appearing as early as 3 h post apoptosis inducing event, and precedes the ability to detect actual DNA fragmentation. PARP cleavage was tested by using an anti PARP (rabbit polyclonal) cleavage site (214/215) specific antibody-FITC conjugate (Biosource, Camarillo, CA, USA). The protocol supplied by the manufacturer was strictly followed. After antibody labeling, the cells were analyzed by flow cytometry (Coulter-Epics XL, Coulter, Krefeld, Germany).

2.5. Gene expression studies

Expression of apoptosis related genes, bcl2, bcl-xL, bax, bak, caspase-3, p53 and c-myc was studied using reverse transcriptase-PCR (RT-PCR). The housekeeping genes β-actin/GAPDH were used as control. Total RNA was isolated using trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was generated by reverse transcription of 3 μg of total denatured RNA. Reverse Transcription was performed at 37 °C for 3 min followed by a PCR cycle of denaturation at 95 °C for 1 min, annealing and extension step at 58 °C for 3 min. The final step included incubation at 70 °C for 10 min followed by a soaking at 25 °C. Statistical analyses were carried out using ANOVA and student’s ‘t’ test.

3. Results

3.1. Effect of curcumin on the growth of A549 and H1299 cells

A549 and H1299 lung cancer cell lines were used as a model system to examine the effect of curcumin on their growth. The growth inhibitory effect of curcumin was concentration dependent in both the cell lines (Figs. 1 and 2). H1299 cells also showed time dependent effect of curcumin and were more sensitive to curcumin treatment than A549 cells. The IC50 s at 24 exposure of curcumin were 50 and 40 μM for A549 and H1299 cells, respectively.

3.2. Induction of apoptosis

We analyzed PARP cleavage in H1299 cells by flow cytometry. PARP cleavage site-specific MoAb (rabbit polyclonal anti PARP cleavage site (214/215) specific antibody-FITC conjugate (Biosource International, USA)) was used. The data (Fig. 3) correlated with the results of cell viability assays (Figs. 1 and 2). PARP cleavage started at a lower concentration (10 μM) and increased with the increasing concentration of curcumin. At 24 h, a concentration of 100 μM of curcumin induced PARP cleavage in ~90% of treated cells (Fig. 3).

3.3. Expression of apoptotic genes

Effect of curcumin on the mRNA expression of selected apoptosis related genes was analyzed by
Table 1
PCR primers used in the gene expression studies

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward (nt)</th>
<th>Reverse (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>X00351</td>
<td>5'-CTGTCATGGCGCACCCAT-3'</td>
<td>5'-GCACATGCTGATCCGC-3'</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>M13994</td>
<td>5'-AAGCTGAGCGAGTTCAGCTGAC-3'</td>
<td>5'-TGACGCAAAAAGATGTGCACG-3'</td>
</tr>
<tr>
<td>Bak</td>
<td>X84213</td>
<td>5'-AAGCTAGCGGAGAATGCACTGAC-3'</td>
<td>5'-TTGCCGCAAGATGTGCACG-3'</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>Z23115</td>
<td>5'-ATGGCAGCAGTAAAGCAAGCG-3'</td>
<td>5'-TTCTGTGTTGGCGCAATGCGG-3'</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>U26943</td>
<td>5'-TTGTGTTGTGCTTGGAGCC-3'</td>
<td>5'-ATTCTGTGACCCATTTGGC-3'</td>
</tr>
<tr>
<td>C-myc</td>
<td>V00568</td>
<td>5'-CCAGGACTGTATGTGGAGCG-3'</td>
<td>5'-CTTGAGACCCAGTGGGCTGT-3'</td>
</tr>
</tbody>
</table>

RT-PCR. A decrease in expression of p53, bcl-2, and bcl-XL was observed. A 12 h exposure to 40 μM of curcumin decreased the expression of Bcl-XL by 40%, Bcl-2 by 35% and p53 by 25% of normal levels (Fig. 4). Exposure to 160 μM of curcumin for 12 h decreased expression of these genes to below detectable levels. Bak, and Caspase genes remained unchanged up to 60 μM curcumin but showed decrease in expression levels at 80–160 μM.

4. Discussion

Our results, for the first time, demonstrate that curcumin induces apoptosis in human lung cancer cells. Curcumin is a hydrophobic molecule and passes easily through the plasma membrane into the cytosol [14]. The results of this study demonstrate that this phenolic substance has the ability to reduce the viability of lung cancer cells through induction of apoptosis. Forty to fifty micromoles of curcumin decreased the cell viability to 50%, whereas 160 μM Curcumin reduced the viability by 95%. It is known that curcumin is poorly absorbed and it will not be possible to achieve these high levels of curcumin in blood [15]. Therefore, the studies should also be concentrated on increasing the bioavailability of curcumin [15]. This induction of apoptosis occurred within several hours; consistent with the view that curcumin induces apoptosis by activating the pre-existing apoptosis machinery. The cell line, H1299, is p53 deficient and A 549 is p53 proficient. Inhibition of growth of both cell lines (p53 deficient and proficient) suggest that growth inhibitory effect of curcumin is independent of p53 expression. Induction of apoptosis in both the cell lines also suggest that a p53 independent pathway is operative in this system.
It has been reported that when p53 gene is mutated in A549 cells, it has lost the pro-apoptotic function [16]. Strasser et al. reported the existence of a p53 independent pathway in leukemic and lymphatic cell lines [17]. In a previous report a growth arrest and apoptosis of B cell lymphoma by down regulation of c-myc, bcl-XL and p53 on exposure to curcumin was documented [18]. Hipp and Baver [19] reported that p53 is not necessary for intercellular induction of apoptosis, as p53 negative transformed cells are sensitive to intercellular induction of apoptosis and p53 negative non-transformed cells are able to induce apoptosis. It was also reported in human breast cancer cell line that CD437 induces G0-G1 arrest and apoptosis via regulation of p21\(^{WAF/CIP1}\), Bcl-2 and Bax in a p53 independent manner [20]. Adachi et al. and Miyashita et al. reported that CD437-induced apoptosis in some lung cancer cells did not necessarily require p53 [20–22]. It therefore appears that in certain cells apoptosis can be induced in a p53 independent pathway either by inducing downstream genes of p53 (e.g. p21, Bax etc.) or by an as yet unknown mechanism. It was also reported that mutant p53 has a dominant negative effect on a wild type p53 and a presence of mutant p53 renders various cell lines resistant to the chemotherapeutic agents or ionizing radiation [23].

In our experiments, curcumin showed increased expression of c-myc, another immediate early gene, intimately involved in the control of cell growth and apoptosis [24]. The proto-oncogene c-myc is known to regulate neoplastic development and apoptotic cell death, and a relationship between p53 abnormalities and c-myc activation has been suggested previously [25]. There is a controversy regarding the relationship between p53 and c-myc in the process of apoptosis. For example, several investigators have reported that c-myc-mediated apoptosis requires wild-type p53 [26]. In contrast, other investigators reported that c-myc-mediated apoptosis does not require wild type p53 [27]. The exact mechanism of c-myc-mediated apoptosis via the p53 independent pathway is unknown.

In order to clarify whether cell death following curcumin treatment is by necrosis or apoptosis, we studied the PARP cleavage. Caspases are activated during apoptosis and that, in-turn cleave PARP. PARP cleavage is known to be a good index for apoptosis [12]. The stability of p53 is regulated by several factors. The interaction of p53 with PARP represents one of several alternative pathways regulating...
the stability of p53 protein. PARP forms tight complexes with p53 and colocalizes with wild-type as well as with mutant p53 protein [28,29]. The result of this study confirmed curcumin-induced apoptosis in lung cancer cell lines.

To obtain further insight into the mechanism of curcumin action, we studied the expression of caspase-3, a major player in apoptotic cell death. Results of this experiment suggest the possibility that the mechanism of curcumin-induced apoptosis in cancer cells involve caspase-3 activation and the resultant cascade of reactions. Caspases have been shown to be activated during apoptosis in many cell systems and play critical roles in both the initiation and the execution of apoptosis [30]. Recently it was reported that caspase-3 is essential for DNA fragmentation and the morphological changes associated with apoptosis [31]. Caspase-3 activation and site-specific proteolysis of PARP were evident in cells treated with curcumin. It has been postulated that activated caspases-3 cleaves the inhibitor of caspase-activated DNase (ICAD/DEF-45), releasing from the complex the caspases activated DNase (CAD/CPAN). Once the ICAD is cleaved, CAD enters the nucleus and degrades chromatin into oligonucleosomal fragments [32]. This, in turn leads to apoptosis.

We observed a down-regulation of bclXL gene. It is reported that bclXL is down-regulated by c-myc protein [33,34]. Since we observed an increase in c-myc expression, the role of c-myc in the down regulation of bcl XL can be postulated. Bcl XL is an anti-apoptotic gene and hence its down-regulation is associated with apoptosis induced by curcumin [35,36]. The effect of curcumin on the bcl-2 gene appears to be less pronounced. Proteins of the bcl-2 family turned out to be important regulators of apoptosis [37]. This model demonstrated that homo-dimers of death agonists induced apoptosis, while hetero-dimers or homo-dimers of death antagonists prevented apoptosis [38]. Several studies indicated that these pro- and anti-apoptotic proteins (Bcl-2, bcl XL and bax) might function independently and without formation of hetero-dimers [39]. The high basal level of bcl-2 expression is consistent with p53 inactivation because p53 inhibits bcl-2 gene expression [22]. A high level of apoptosis, even in the presence of higher basal level of bcl-2 may suggest the possibility of a treatment-associated phosphorylation of that protein. Treatment associated bcl-2 phosphorylation, was reported to cause a loss of its anti-apoptotic function [40].

In summary, we have demonstrated that the cancer chemopreventive agent curcumin induces apoptosis in lung cancer cell lines. Induction of apoptosis by curcumin involves multiple pathways. Curcumin up-regulates different pro-apoptotic genes and at the same time down-regulate some of the anti-apoptotic genes. Homo-dimerisation or hetero-dimerisation of this pro- and anti-apoptotic genes in favor of apoptosis is one of the suggested mechanisms of curcumin-induced apoptosis.

References


