ABSTRACT

The present study was undertaken to evaluate the p53 and Caspase 3 gene expression potential of Allyl isothiocyanate on Human hepatoma cell line HepG2. The viability of HepG2 was significantly reduced by Allyl isothiocyanate. The lactate dehydrogenase (LDH) was significantly released from Allyl isothiocyanate (5µM and 10 µM) treated Hep G2 cells. Reduced Glutathione (GSH) was significantly reduced in Allyl isothiocyanate (5µM and 10 µM) treated HepG2 cells. Significant morphological changes were observed in fluorescent microscopic study. It indicates the apoptosis induction by Allyl isothiocyanate (5µM and 10 µM) in HepG2 cells. Noticeable changes in the intensity of pro-apoptotic protein bands were observed in Allyl isothiocyanate treated HepG2 cells. Expression of p53 and active caspase 3 in Allyl isothiocyanate (5µM and 10 µM) treated cells suggest that apoptosis induced by AITC. In conclusion, these results suggest that Allyl isothiocyanate (5µM and 10 µM) possessing significant apoptotic property with expression of p53 and active caspase 3.

Key words: Allyl isothiocyanate, MTT assay, Apoptosis, LDH leakage, Reduced GSH, Florescent microscopic study.

INTRODUCTION

Primary liver cancer is the fifth most common cancer worldwide and the third most common cause of cancer mortality (Farazi et al., 2006). At present HCC is a largest disease of the third world, especially Southeast Asia and Africa, where its prevalence exceeds by many fold (El-Serag, 2001). The established risk factors for hepatocellular carcinoma include Hepatitis B and C viral infection, aflatoxin, alcohol consumption and schistosomiasis. The host risk factors for liver cancer include cirrhosis, iron overload, obesity, diabetes, use of...
oral contraceptives and non-alcoholic steatohepatitis (London and McGlynn, 2006).

Primary cultures of rat hepatocytes have many advantages over the use of whole animals in mechanistic studies (Hammond and Fry, 1996; Melo et al., 2002). HepG2 cell line was established from liver tumor biopsies obtained during extended lobectomies of a 15 years old Caucasian male from Argentina. The cell line established after these biopsies had been cultured for several months and was designated as HepG2. Their morphological characteristics and epithelial cell shape are compatible with those of liver biopsies revealed well differentiated hepatocellular carcinoma (Aden et al., 1979). HepG2 have an obvious advantage of their readily availability and assurance of a certain reproducibility of experiments. Thus, human-derived liver cells HepG2 have been extensively used as an experimental system for the prediction of toxicity, carcinogenicity and cell mutagenicity in humans. Thus HepG2 cell line represents one of the most widely used experimental model for in vitro studies on hepatocellular carcinoma (Emanuele et al., 2002).

Natural dietary agents including fruits, vegetables and spices have drawn a great deal of attention from both the scientific community and the general public due to their demonstrated ability to suppress cancers. Research over the last decade has shown that several micronutrients in fruits and vegetables reduce cancer over the last decade has shown that several micronutrients in fruits and vegetables reduce cancer. Research on dietary chemicals can prevent chemical carcinogenesis in the laboratory and experimental animals. Isothiocyanates (ITCs) are a family of compounds derived exclusively from plants, although marine sponges and fungi also have been reported to produce a few ITCs (Fahey et al., 2001). Examples of popular crucifers that are particularly rich in certain ITCs include mustard and horseradish-allyl-ITC (AITC) (Uematsu et al., 2002), watercress phenethyl-ITC (PEITC) (Chung et al., 1992), broccoli and broccoli sprouts-sulforaphane (SF) (Zhang et al., 1992). Various biological effects of allyl isothiocyanate (AITC) have been reported which include anti-inflammatory, anti-bacterial and anti-fungal activities (Masuda et al., 1999; Shin et al., 2004). AITC extracted from mustard seeds contains both anti-angiogenic and pro-apoptotic activities (Akhilesh Kumar et al., 2009). Hence the present study was undertaken to evaluate the effect of Allyl isothiocyanate in human hepatoma cell line HepG2.

**MATERIALS AND METHODS**

**Cell culture**
Human hepatoma cell line (HepG2) was obtained from National Center for Cell Sciences (NCCS), Pune, India. The cell line was maintained at the department of Animal Bio-technology, Madras Veterinary College, Vepery, Chennai. Cells were routinely grown as monolayer cultures at 37°C in a humidified atmosphere of 5% CO₂ in 95% O₂ in Dulbecco Modified Eagles Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), penicillin (50 IU/ml) and streptomycin (50 µg/ml).

**Chemicals**
Allyl isothiocyanate (AITC), ethidium bromide were purchased from Sigma, DMEM, sodium pyruvate were purchased from Biochrom, Germany. Penicillin-streptomycin and FBS were purchased from Gibco, Germany. Trypsin-EDTA was obtained from Hi-media, India. Culture plates were purchased from TPP, Switzerland. Primary antibodies (Ab’s) such as caspase 3, P53 were purchased from Abcam, USA. Rabbit anti-mouse IgG was purchased from Bangalore Genei. PVDF membrane was purchased from Millipore, USA.

**Drug preparation**
AITC was dissolved in ethanol (final concentration of the ethanol did not affect the cell survival) prepared in DMEM and filtered by 0.045 mm syringe filter and stored at 4°C for the cell culture protocols.

**MTT assay**
Cells were seeded in 96 well microplate (1x10⁴ cells/well in 180 µl medium) and routinely cultured in a humidified incubator (37°C in 5% CO₂) for 24 hours. AITC (1µM, 2µM, 3µM, 5µM and 10µM) were added in a serial concentration and re-incubated for 24 h.

MTT (3-(4,5 dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed as described by (Cardile et al., 2004). The viability of the cells was assessed by MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. The Amount of formazan was determined by measuring the absorbance at 570 nm using an ELISA reader.

The percentage of cell survival was calculated by using the formula:

\[
\text{Mean absorbance from untreated cells} \times 100
\]

\[
\% \text{ of Cell survival} = \frac{\text{Mean absorbance from treated cells}}{\text{Mean absorbance from untreated cells}} \times 100
\]

Based on the above studies, AITC at the concentration of 5µM and 10µM were selected for further experiments.

**Morphological assessment of apoptosis**

**Fluorescent microscopic studies**
Apoptotic morphology was studied by staining the cells with a combination of the fluorescent DNA-binding dye. After treatment with AITC (5µM and 10µM), the cells were collected, washed and suspended in PBS, fixed with 70 % ethanol for 1 hour and stained with propidium iodide (30 µg/ml in PBS) containing DNAase free RNAs (1 mg/ml) for 30 min at 37°C The nuclear morphology of cells was visualized by fluorescence microscope. Fluorescent nuclei were screened for normal morphology and condensed chromatin (Belloc et al., 1994).

**Lactate dehydrogenase (LDH) leakage assay**
Lactate dehydrogenase leakage assay was performed by the method of Grivell and Berry (1994).
Estimation of glutathione (GSH)
Total reduced glutathione was determined by the method of Moron et al., (1979).

Expression of p53 and caspase -3 proteins by Western blotting

HepG2 cells (1x10^6/ml) were treated with the AITC at the concentration of 5 and 10 UM for 48 h at 37 C. Cells were lysed with 10µl of lysis buffer. Cell proteins were separated in a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). 4% stacking gel and 10% resolving gel were used to separate the proteins. After electrophoresis, gel was placed over a nitrocellulose membrane, separate blotting was done for each protein. The gel and the PVDF membrane were packed by three cut-pieces of whatmann filter paper (No.3). This set up was covered on both sides with absorbers (provided with the Medox blotting system) and clamped. The whole set up was immersed in a tank containing blotting buffer. A current of 25 mA was passed through overnight.

Then, the membrane was removed from the system and immersed in methanol for a minute. The membranes were blocked by treating with the blocking buffer for 1 h at 37 C. After washing, the membranes were incubated with anti-mouse p53 (1:100) and anti-mouse caspase-3 (1:1000) for 6h at 37 C. After three washes in PBS / 0.1% Tween 20, the membrane was incubated with Horse Raddish Peroxidase (HRP) conjugated anti-mouse IgG antibody for 1 h at 37 C. The bands developed were visualized and photographed. The band intensity for P53, caspase 3 was normalized with that of the internal control β actin.

RESULTS

CYTOTOXICITY

Recently the cytotoxic effects of various chemicals and natural substances on malignant tumor cells in culture have been extensively studied as a primary screening for anti tumor activities (Morita et al., 2003). Table 1 shows the cell viability of control, ethanol and Allyl isothiocyanate (AITC) treated (1µM, 2µM, 3µM, 5µM and 10 µM) HepG2 cells. In the present study, the AITC conspicuously inhibited the HepG2 cells at the concentration of 5µM and 10 µM after 24 h of treatment. The results showed that treatment with AITC markedly reduced the viability of HepG2 cells in a dose dependent manner.

LDH LEAKAGE

The levels of lactate dehydrogenase (LDH) released into the medium of control and AITC treated (10µM, 25µM, 50µM, 75µM, 100 µM) HepG2 cells were presented in Fig 1. From this result it was observed that LDH activities found to be significantly elevated after 48 h of exposure in the medium containing AITC when compared to the control.

GSH CONTENT

It was well known that the toxicity of anti-tumor drugs may largely depend on the intracellular level or reduced glutathione (GSH) (Troyano et al., 2001). Glutathione plays an important role in protecting cells and cellular components against oxidative stress and in detoxification. It was often found that GSH levels were increased in the drug resistant cancer cells when compared to the drug sensitive cells. Inhibition of glutathione synthesis or modulation of glutathione storages in tumors to reduce anticancer drugs resistance may contain a novel anticancer strategy (Rudin et al., 2003). The levels of GSH content in control and AITC treated HepG2 Cells were presented in figure 2. The significant depletion of GSH was observed in treated HepG2 cells at the concentration of 5 and 10 µM/ml when compared to the control cells.

APOPTOSIS

Figure 3 represents the fluorescent microscopic picture of control and AITC treated HepG2 cells at the concentration of 5 µM and 10 µM after 48 h of exposure. In the present study, normal live cells were appeared bright red in color, whereas drug treated groups (5 and 10 µM) apoptotic nuclei of dead cells appeared bright red in color with nuclear condensation, giant cell formation and fragmentation. In addition to this, 10 µM treated cells showed apoptotic nuclei with highly condensed chromatin in HepG2 cells.

P53 AND CASPASE -3 PROTEINS EXPRESSION

A lot of oncogenes and tumor suppressor genes were involved in mediating apoptosis. The p53 gene which was strongly implicated in animal and human carcinogenesis and it was a significant regulator of the process of apoptosis (Storey et al., 1997). After DNA damage in cell types, p53 can trigger the genetically altered cells to be eliminated by inducing apoptosis (Lowe et al., 1993). In addition to its DNA damage response, p53 was also involved in the response by abnormal or stress conditions such as hypoxia, Oxidative stress, the presence of genotoxic chemicals (Sun, 1990) and depletion of ribonucleotides, (Linke et al., 1996). Therefore normal p53 expression and function were crucial to prevent the propagation of genetically damaged cells and to prevent proliferation of cells under stress conditions. It was well known that after initiation of the apoptotic program, the release of cytochrome -C from mitochondria triggers the activation of caspase-3 and the consequent rapid cleavage of Poly (ADP-ribose) polymerase (PARP), a substrate of caspase 3 (Emanuele et al., 2002).

The expression of p53 and caspase-3 protein in control and AITC treated (5µM and 10 µM) HepG2 cells by western blotting was presented in Figure 4. Administration of AITC increase the band intensity of 53 kDa protein compared to the control. The accumulation of p53 protein indicates the expression of tumor suppressor protein induced apoptosis in AITC (5µM and 10 µM).
treated HepG2 cells. From this, it was observed that treatment of HepG2 cells with AITC increased intensity of 32 kDa band and appearance of low molecular weight protein below the 32 kDa in 5 and 10 µM treated HepG2 cells. This result strongly suggests that drug treatment stimulated the proteolytic cleavage of caspase-3 protein and initiate the apoptosis.

Table: 1 Effect of AITC by cell viability MTT assay on HepG2 cells

<table>
<thead>
<tr>
<th>S. No</th>
<th>CONCENTRATION</th>
<th>% OF VIABLE CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>98.9±1*</td>
</tr>
<tr>
<td>2.</td>
<td>Ethanol (solvent)</td>
<td>98 ±1*</td>
</tr>
<tr>
<td>3.</td>
<td>AITC (1µm)</td>
<td>89.4±2*</td>
</tr>
<tr>
<td>4.</td>
<td>AITC (2µm)</td>
<td>75.7±8*</td>
</tr>
<tr>
<td>5.</td>
<td>AITC (3µm)</td>
<td>65.6±7*</td>
</tr>
<tr>
<td>6.</td>
<td>AITC (5µm)</td>
<td>55.3±2*</td>
</tr>
<tr>
<td>7.</td>
<td>AITC (10µm)</td>
<td>50.1±4*</td>
</tr>
</tbody>
</table>

*Based on six observations

Figure 1. Level of LDH in control and AITC treated HepG2 cells

Figure 2. Level of GSH in control and AITC treated Hep G2 cells

Figure 3. Fluorescence microscopic photograph of control and Allyl isothiocynate (AITC) treated HepG2 Cell line

Figure 4. Western blot analysis of Active caspase3 and p53 protein expressions in control and AITC treated HepG2 cells
DISCUSSION

Assessment of the in-vitro cytotoxicity has recently become a primary screening method for evaluating the anticancer properties of various chemicals and natural substances. The cell viability (MTT assay), LDH, GSH activities, light and fluorescent microscopic examinations, expression of p53 and caspase 3 proteins were investigated in Allyl isothiocyanate (AITC) treated Hep G2 cell lines. AITC reduced the cell viability in a concentration dependent manner. The suppression of cell proliferation induced AITC may be due to the induction of cell death. Thus, the inhibitory activity of AITC provides evidence for the in vitro cytotoxicity.

Recent studies suggest that LDH is a more reliable and accurate marker of cytotoxicity, because damaged cells are fragmented completely during the course of prolonged incubation with substances (Grivell et al., 1994). In the present study, the LDH leakage increased significantly in AITC (5 µM and 10 µM) treated HepG2 cells when compared to the untreated HepG2 cells (Control). Extensive reports have documented on dietary agents and their active principles induced cytotoxicity to cancer cells. Thus, the LDH leakage in HepG2 cells may be due to the cytotoxic nature of Allyl isothiocyanate (AITC) and confirms its anti-tumor activity. Many studies have also revealed that ITCs appear to be much more toxic to transformed or malignant cells than normal cells (Musk et al., 1995; Gamet-Payrastre et al., 1998; Gamet-Payrastre et al., 2000; Xiao et al., 2003).

Apoptosis is a form of physiological cell death essential to normal tissue development and homeostasis (Lumachi and Basso, 2002). After receiving an apoptotic death stimulus, cells first enters a signaling phase followed by the first degradation phase, in which apoptosis is identifiable by chromatin condensation, cell shrinkage, caspase activation and cell fragmentation, through the formation of apoptotic bodies (Searle et al., 1974; Hsu et al., 1999).

The Fluorescence microscopic study showed apoptosis in AITC treated HepG2 cells. Morphological analysis with Propidium iodide staining of HepG2 cells showed cell clumping and formation of apoptotic bodies which are characteristic of apoptosis (Zimmerman et al., 2001). The highly condensed and fragmented nuclei that are the index of apoptosis were observed at 5 µM and 10 µM concentration of Allyl isothiocyanate (AITC). This may be due to loss of cell membrane integrity and apoptotic nature of the AITC.

It is well known that p53 acts as a guardian of the genome and is one of the major factors controlling cell proliferation, growth and transformation. The p53 tumor suppressor gene is mutated in over 50% of human cancers and the oncogenic activity of p53 dependent apoptosis by a dominant negative mechanism (Sigal and Rotter, 2000). Tumor suppressor gene p53 is one of the critical genes regulating the onset of DNA replication around G1/S boundary. Also, p53 mediated tumor suppression appears to be critical for therapeutic potential in treatment of tumors (Ryan et al., 2001). P53 contributes to apoptosis induced by a variety of cellular stresses, including DNA damage, oxidative stress and chemotherapeutic drugs (Steele et al., 1998).

Activation of p53 is often detected with natural chemotherapeutic agents and p53 negative tumors are generally less sensitive or even insensitive to these agents. In the present study, Allyl isothiocyanate (AITC) treated HepG2 cells showed upstream regulation of p53 protein expression after exposed to the concentrations of 5 µM and 10µM for 48 h. Thus it is inferred that, AITC may possibly enhance the susceptibility of HepG2 cells to apoptosis by attenuating the tumor suppressor protein.

In cells undergoing apoptosis, there is an activation of proteases known as caspases, which have an obligatory cysteine residue within the active site and cleave peptides adjacent to an aspartic acid residue (Thornberry, 1997). Caspases cascade has been demonstrated to be involved in apoptotic signal transduction and execution (Salvesen and Dixit, 1997). Human caspases 1-10 have been described and activation of the caspase cascade is involved in chemical-induced apoptosis, including degradation of DNA repair enzyme poly ADP ribosepolymerase (Lazebnik et al., 1994) and DNA-dependent protein kinase. The cleavage of chromatin at inter-nucleosomal sites mediated by caspase-activated DNase (Sakahira et al., 1998). Generally, caspases are present as inactive proenzymes, most of which are activated by proteolytic cleavage. Caspases 8, 9 and 3 are situated at pivotal junctions in apoptotic pathways (Shan and Li, 2002). Caspase-3 may then cleave vital cellular proteins or activate additional caspases by proteolytic cleavage. In the present investigation, AITC treated HepG2 cells showed a low intensity of 32-kD protein band and 17-kD protein band. This is in accordance with the Budihardjo et al., (1999) and they suggested that caspase-3 usually exits as an inactive pro-caspase 3 that becomes proteolytically activated by multiple cleavages of its 32-kD precursor to generate the 20/11 or 17/11-kD active forms in cells undergoing apoptosis. Thus, the appearance of 17-kD protein may be one of the active forms of caspase-3 protein. It may be due to proteolytic cleavage of inactive caspase-3 induced by Allyl isothiocyanate (AITC). This occurs before the further activation of caspase-3-mediated apoptosis. Recent studies have also suggested that the proteolytic degradation of specific substrates is responsible for many of the morphological features of apoptosis (Cardone et al., 1998; Ceccon, 1999).

Caspases involved in multiple apoptotic pathways. Allyl isothiocyanate (AITC) treated cells may lead to the activation of caspase. For example, in human leukemia HL60 cells, AITC at the concentration of 10µM activated caspase-9 (the mitochondria pathway), caspase-8 (the death receptor pathway), and caspase-12 (the
endoplasmic reticulum pathway), in conjunction with subsequent activation of caspase-3 (Zhang et al., 2003). Therefore, the molecular mechanisms involved in the induction of apoptosis and caspase activation by ITCs are likely complex and are only partly understood (Hu and Kong, 2004).

It is well known that the intracellular oxidative metabolites play a significant role in the regulation of apoptosis. The reduced tripeptide GSH is a hydroxyl radical and a singlet oxygen scavenger, participating in a wide range of cellular functions such as protein and DNA synthesis, intermediary metabolism and transport (Deneke and Fanburg, 1989). The maintenance of GSH levels and the reducing cells are crucial (Storey, 1996). Depletion of GSH leads to increased accumulation of lipid peroxides and loss of cell viability. It is known that the toxicity of antitumor drugs may largely depend on the intracellular level of reduced GSH. Because GSH is the main antioxidant system in cells, a possible explanation is that GSH depletion facilitates reactive oxygen species (ROS) accumulation in cells treated with antitumor drugs, which in turn increases their lethality (Miyajima et al., 1997). In the present study, the level of GSH significantly decreased in AITC treated HepG2 cells at the concentration of 5 µM and 10µM. The decrease in GSH levels may be due to redox imbalance in HepG2 cells and subsequently induces apoptosis. Depletion of GSH has been described for several agents such as oxidative and alkalyting agents in various cell types (Slater et al., 1995). Liu et al., (2000) also found that *Salvia milirohiza* inhibits human hepatoma HepG2 cell growth and induces apoptosis involving intracellular GSH depletion .In this context, Hall (1999) has demonstrated that onset of apoptosis is associated with a fall of intracellular GSH in different cellular systems. Loss of intracellular GSH is tightly coupled with a number of downstream events in apoptosis (Van den Dobbelsteen et al., 1996).

Studies in a variety of cell types suggest that cancer chemotherapeutic drugs induce tumor cell apoptosis in part by increasing the formation of ROS. In this connection, Simizu et al., (1998) have reported that some anticancer agents, including vinblastin and camptotheclin, induce cell apoptosis with the generation of ROS. Therefore, ROS may not necessarily be the direct factor to cause apoptosis induced by the drug, but intracellular ROS may modulate the genes involved in apoptosis, which may regulate apoptosis. Thus, AITC induced oxidative stress is upstream of signaling events that might alter the pro and antiapoptotic balance in HepG2 cells. Thus, it is possible that treatment of HepG2 cells with AITC can deplete the GSH levels and promote oxidation induction, which switch the mode of death via apoptosis. Therefore, the cytotoxic action of this drug may be attributed to its pro-oxidant action on the cells. This may be able to account for the discrepancy between in vitro cytotoxicity and in vivo antitumor activities of AITC.

In addition to this, induction of cell cycle arrest is another frequently observed change in ITC-treated cells. This activity was first reported by Hasegawa et al., (1993) in which a significant accumulation of cells at G2/M phase was observed at 16 h after treatment with 2.5–10µM AITC. Allyl isothiocyanate also inhibits LPS-induced Nitric Oxide (NO) production in J774.1 macrophages (IC50, 1.6 and 2.6µM, respectively), apparently by inhibiting the induction of Nitric Oxide (NO) synthase and tumor necrosis factor alpha (TNF α) (Ippoushi et al., 2002).

From the present in-vitro study, it is concluded that, AITC has profound effects on human hepatoma cell line HepG2 and exhibits its cytotoxicity of these cells and the cell death is mediated by apoptosis. The mechanism of apoptosis may be accumulation of p53 protein and proteolytic cleavage of caspase-3 protein. In addition to this, GSH depletion may also play a role in apoptosis through redox imbalance in HepG2 cells.

**CONCLUSION**

The viability of HepG2 was significantly reduced by Allyl isothiocyanate in dose and time dependent manner. The lactate dehydrogenase (LDH) was significantly released from Allyl isothiocyanate treated HepG2 cells shows the cytotoxic nature. Reduced Glutathione (GSH) was significantly reduced in Allyl isothiocyanate treated HepG2 cells shows the antioxidant nature. Significant morphological changes were observed in fluorescent microscopic study and it indicates the apoptosis induction by Allyl isothiocyanate in HepG2 cells. Thus, the expression of p53 and active caspase 3 in Allyl isothiocyanate treated cells suggest that apoptosis induced by AITC.

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