Anticancer property of gallic acid in A549, a human lung adenocarcinoma cell line, and possible mechanisms

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Gallic acid is widely distributed in plants, fruits and foods with a range of biological activities. In the present study the possible mechanisms of gallic acid anticancer properties were explored in A549, a human lung adenocarcinoma cell line. Our study shows that it inhibited the A549 cell growth and decreased cell viability monitored at 24 h. It also inhibited cell proliferation in dose- and time-dependent manner as measured by 3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide assay at 24 and 48 h. Morphological examination of the cells after gallic acid treatment showed the typical feature of cell death such as cell shrinkage and rounding up of the cells. Clonogenic assay indicated that gallic acid treatments inhibited the colony formation. DNA fragmentation assay indicated the disappearance of the genomic DNA in dose-dependent manner. To find out possible mechanisms, mitochondrial potential and intracellular reactive oxygen species were measured. It was observed that gallic acid treatment decreased mitochondrial membrane potential and increased intracellular reactive oxygen species. Further caspases activity was measured and it was found that gallic acid activated the caspase-3 but not caspase-8 indicating the involvement of intrinsic pathway of cell apoptosis.

Key Words: apoptosis, adenocarcinoma, DNA damage, reactive oxygen species

Worldwide, lung cancer is the most common cancer in terms of both occurrence and mortality, with the highest rates in Europe and North America. Despite rapid advances in diagnostic and operative techniques, lung cancer remains one of the most difficult human malignancies to treat. The American Cancer Society estimates that 214,440 persons in the United States developed lung cancer in 2009, with 159,390 deaths. Lung cancer—Society estimates that 214,440 persons in the United States

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were seeded in 6-well plate and were incubated at 37°C for 12 days without any disturbances. Following incubation, the medium was removed and colonies were fixed and stained with 0.5% methylene blue (dissolved in 50% ethanol). Colony counting was performed with cellAnalyst software (AssaySoft, Inc., CA). The experiment was carried out twice in triplicates.

**DNA Fragmentation analysis.** In brief, 7.5 × 10^5 cells per well of 6-well plate were seeded and kept overnight for attachment. Next day old medium was replaced with medium containing 0, 100, 200 and 400 µM GA and incubated for 24 h. Following incubation, cells were collected and washed with 1 × PBS (phosphate buffered saline), resuspended in 100 µl lysis buffer and incubated for 1 h at 50°C. 10 µl of RNase (10 mg/ml) was added to the lystate and further incubated for 1 h at 50°C. The DNA samples were electrophoresed in a 1.8% agarose gel in TBE (Tris-borate-EDTA) buffer (pH 8.0). The DNA bands were visualized using a gel-doc system. The profile of the DNA band was analyzed using Scion Image analysis software.

**Detection of intracellular ROS levels.** The intracellular ROS (Reactive oxygen species) generation in A549 cells was investigated using the 2',7'-dichlorofluorescein-diacetate (DCFH-DA) dye. For measuring the intracellular ROS in individual cells 5 × 10^5 cells were seeded on cover-slip in 6-well plate and left for 2 h at 37°C. At the end of incubation the cover-slip was removed from the culture plate and stained with 40 µM DCFH-DA for 30 min. Excess of the dye was removed by washing with 1 × PBS. Cover-slip was mounted on glass slide and images of the cells were captured using 40× objectives under fluorescence microscope.

For measuring total ROS level in the cells, 5 × 10^5 cells were seeded in a 6-well plate and allowed to attach overnight. The next day, medium was replaced by fresh medium containing 0, 100 and 200 µM GA and incubated to allow for 1 and 2 h. Following incubations, the cells were harvested and resuspended in 10 µM DCFH-DA containing PBS were distributed in each well of 96-well fluorimetry plate and incubated at 37°C for 45 min. The plate was read using an excitation of 485 nm and emission at 520 nm and graph was plotted denoting the change in ROS level.

**Assessment of MMP.** For assessing the mitochondrial membrane potential, A549 cells (5 × 10^5/well) were seeded in 6-well plates and incubated overnight for attachment. Next day old medium was replaced with fresh medium containing 200 µM GA and incubated for 1 h. Cells were collected by trypsinization and resuspended in 1 ml of DiO6 (3,3'-dihexyloxacarbocyanine iodide) solution (40 nM DiO6 in 1 × PBS) and incubated for 30 min at room temperature. Fluorescence intensity (Ex/Em: 488/525) was measured using microtiter plate reader.

**Detection of caspase-3 levels.** In brief, 1 × 10^6 A549 cells
were seeded in a 6-well plate and allowed to attach overnight. The next day medium was replaced by fresh medium containing 0, 100 and 200 µM of GA and incubated for 1, 3 and 6 h. At the end of incubations, cells were collected by trypsinization and washed twice in PBS. Cell pellet was resuspended in 75 µl of 1 × lysis buffer and incubated on ice for 15–20 min. Cell lysate were centrifuged at 20,000 rpm for 15 min at 4°C. Pellet was discarded and supernatant was transferred to new tubes and stored at −80°C till analysis. The supernatants were used for estimation of caspase-3 as instructed by the manufacturer in 96-well microtiter plate.

**Statistical analysis.** All values are expressed as means ± SE. Statistical significance was set at *: p<0.05; **: p<0.005; ***: p<0.01.

**Results**

GA inhibits A549 cells growth and proliferation. As shown in Fig. 1 GA inhibited A549 cell proliferation in dose- and time-dependent manner. From Fig. 1 it is also clear that this cell growth inhibition was limited to 200 µM. After 200 µM there was not much cell growth inhibition. From examination of A549 cells morphology at 24 h after 100, 200 and 400 µM GA treatment it was observed that cells started shrinking showing the symptoms of the cell death. Further, total cell count and the cell viability were measured after treating cells with GA for 24 h. From Fig. 2A, it was clear that the total cell count started decreasing in the GA treated group and this effect was observed in a concentration-dependent manner. As shown in Fig. 2B, the percent viability of cells decreased with GA treatment but not in a concentration-dependent manner.

GA inhibited A549 colony growth. The anticancer property of GA was also examined by clonogenic assay. The colony is defined to consist of at least 50 cells. As shown in Fig. 3, GA significantly decreased A549 colony growth in a concentration-dependent manner.

GA induces cellular DNA fragmentation. From Fig. 4, it is clear that GA leads to a concentration dependent decrease in the total genomic DNA. However, no clear DNA ladder pattern was observed in this cell line.

GA induced intracellular ROS in A549 cells. As shown in Fig. 5A–C, GA treatment induced the ROS level in cell as measured by fluorescence microscopy study at individual cell level. From these cell images it is clear that the fluorescence intensity of individual cells increased in a concentration-dependent manner indicating ROS generation by GA. Further, total cellular ROS was measured fluorimetrically at 1 and 2 h post treatment with GA and this observation collaborated well with fluorescence microscopy results (Fig. 5D).

GA disrupts mitochondrial membrane potential in A549 cells. Since it is known that increase in ROS level disrupt the MMP to execute the apoptosis, the third experiment was carried out to find out the possible mechanisms was the measurement of MMP. As shown in Fig. 6, 1 h incubation of cells with GA marginally disrupted the MMP.

GA induces caspase-3 in A549 cells. Since disruption of MMP leads to induction of apoptosis by intrinsic pathway via caspase-3 induction, in the next experiment the level of caspase-3 was measured and it was found that treatment of A549 cells with GA induced the caspase-3 (Fig. 7) but not caspase-8 (data not shown) to execute the process of apoptosis.

**Discussion**

The relationship between diet and cancers has been implicated in several epidemiological studies. The cancer incidence is significantly lower in people whose diet consists largely of fruits and vegetables than people whose diet consists of mainly animal products.(15) The present paper focused on evaluating the effects of GA, a dietary phytochemical on A549, human lung adenocarcinoma cell line in relation to cell growth inhibition, apoptosis and their underlying mechanisms.

To determine the inhibitory concentration of GA on A549 cells, cell proliferation and cell viability was determined. It was observed that exposure to GA for 24 h, A549 cell growth was inhibited as it is clear from MTT assay and direct cell count (Figs. 1 and 2). The morphological changes in cells clearly indicate that cells undergo apoptosis at 24 h after incubation with the concentration of GA chosen based on the MTT assays (Fig. 1C). To study reproductive cell death further clonogenic assay was carried out. Our finding shows that GA dose dependently inhibited the A549 colony formation as compared to the
control cells (Fig. 3). This indicates that GA decreases the potential of individual cells to form a colony and thereby acts as an anti-cancer drug. This finding well corroborated with our cell proliferation and cell count studies. Further DNA fragmentation assay was performed for study of the apoptosis induction by GA. Here we did not find clear DNA ladder pattern in A549 cells but the concentration dependent degradation of genomic DNA was observed in cells treated with GA (Fig. 4).

To gain insight into the molecular mechanism involved in GA induced apoptosis, the level of ROS, change in MMP and the activation of caspase-3 were assessed in A549 cells. GA has been reported to have both pro-oxidant and antioxidant properties. Accumulation of ROS coupled with an increase in oxidative stress has been implicated in the pathogenesis of several disease including cancers. Increasing evidence suggests that apoptosis induced by GA is associated with oxidative stress derived from ROS and mitochondrial dysfunction. Our result indicates that the intracellular ROS levels were significantly increased in A549 cells treated with GA for 1 and 2 h (Fig. 5). Although cancer cells have an increased ROS as compared to healthy cells, the increased ROS is believed to be responsible for many novel cancer cell properties including loss of cell senescence. However, an increase of ROS beyond threshold would result in irreversible DNA damage that would translate in the activation of apoptosis pathway. GA has shown a 2 fold increase in ROS as compared to the control cells. This is in correlation with other reports that provide substantial evidence that apoptotic cells generally exhibit an increase in ROS levels. Loss in membrane potential is another major biochemical change in cells undergoing apoptosis. Our study showed that there is a 31% decrease in the MMP in control cells compared to 200 µM GA treated cells after 1 h of incubation (Fig. 6). Caspases activity was quantified by colorimetric assay. Although we estimated both caspase-3 and -8, our studies have shown a clear increase in the caspase-3 activity (Fig. 7) but not the caspase-8 (data not shown), indicating that GA induced apoptosis in A549 cells is by the activation of caspase-3 pathway (intrinsic pathway). The maximum activation of caspase-3 was seen at 1 and 3 h and showed a slight decrease compared to earlier values at 6 h.

**Fig. 4.** DNA fragmentation assay. Lane 1: control cells, Lane 2, Lane 3, Lane 4 are 100, 200 and 400 µM gallic acid treated cells respectively depicting loss in genomic DNA.
The radioprotective properties of GA have been determined by an independent study in our laboratory.\(^{(19)}\) Even administration of GA alone has been shown to have anti-tumor effect and provides evidence that GA can be used alone or as a combination therapy with radiation. Being a dietary constituent it is readily available in natural form and is not expensive in terms of purification of the compound. Since GA is found in micro molar concentrations in the human urine, it rules out any possible cytotoxicity to neighboring healthy cells. Hence GA provides with an ideal choice in the treatment of lung cancer.

In conclusion our studies prove that GA induces cell death in A549 lung carcinoma cells in a dose dependent manner and induced apoptosis by elevation ROS, disrupting MMP, and activating caspase-3.
Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| GA | 3,4,5-trihydroxyl-benzoic acid |
| MTT | 3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide |
| DMEM | Dulbecco’s modified Eagle’s medium |
| FBS | fetal bovine serum |
| PBS | phosphate buffered saline |
| TBE | Tris-borate-EDTA |
| ROS | reactive oxygen species |
| DCFH-DA | 2',7'-dichlorfluorescein-diacetate |
| Dio6 | 3,3'-dihexyloxacarboxyanine iodide |

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