**Apoptogenic Effect of Green Tea Polyphenon-60 against Ehrlich Ascites Carcinoma Cells in Swiss Albino Mice**

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**ABSTRACT**

The mechanism of apoptogenic impact of green tea polyphenon-60 (PP-60) on Ehrlich ascites carcinoma (EAC) cells was studied. Mice were implanted with \(2 \times 10^6\) EAC cells to serve as the control group. Antitumor activity of PP-60 was evaluated 12 days after tumor implantation. Oral administration of PP-60 to EAC-bearing mice produced a significant decrease in tumor volume and increased survival time. Flowcytometric analysis showed an increase in the number of cells in the sub-G0/G1 population of the cell cycle that demonstrated apoptosis. These results were confirmed by the double labeling system Annexin V/PI, signifying tumor cell apoptosis by PP-60 and DNA fragmentation using the comet assay method. A decrease in the proliferating factor ki-67 and an increased expression of pro-apoptotic protein p53 in EAC cells was demonstrated. These results were followed by an increased expression of Bax with down-regulation of the anti-apoptotic protein Bcl-2. These effects led to the activation of initiator caspases-9 and effector caspase-3, thus, supporting the notion that PP-60 inhibited cell growth via induction of intrinsic p53-dependant apoptosis. These observations together suggest that PP-60-induced apoptotic signals lead EAC cells towards programmed cell death, and could be used in the future for the development of chemotherapeutic drugs against cancer.

**Keywords:** Apoptosis; Green tea; Polyphenols; Caspases; P53; Bcl-2; cancer

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INTRODUCTION

Complementary medicine using natural products, including green tea, has been used progressively worldwide for the improvement of chemotherapeutic/preventive efficacy and reduction of adverse effects. Epidemiological studies show a reduced risk of cancer with increased green tea intake [1]. Green tea contains a high concentration of polyphenolic catechins such as polyphenols-60 and epigallocatechingallate (EGCG), which are responsible for its pharmacological effects and its benefits with regard to cancer prevention and inhibition [2]. The green tea catechins have been reported to be effective in several cancer types [3]. An inverse association between the risk of breast cancer and the intake of green tea has also been reported in Asian Americans [4].

Polyphenon-60 (PP-60) contains 60% pure catechin extracted from green tea. It induces modification of the breast cancer miRNA expression profile, which contributes to the efficacy of green tea treatment. The resulting decrease in carcinogenesis is further supported by the altered miRNA regulation of potential oncogenes and tumor-suppressor genes [5]. Green tea catechins showed an inhibitory effect on pancreatic cancer growth by acting as an inhibitor of lactate dehydrogenase A, which is essential for cancer cell metabolism [6]. Another in vitro study revealed the role of green tea catechins in targeting cancer stem cells in both hepatoma and colon cancer cells suggesting its role in reducing the incidence of tumor recurrence [7]. Kobalka et al illustrated the synergism of green tea catechins and flavins on diminishing growth and induction of apoptosis in human prostate cancer cell line DU 145 [8].

Ehrlich ascites carcinoma (EAC) is one of the first models to study the growth and proliferation behavior of cancer cells [9]. Due to its inability to adhere to a synthetic surface, EAC cells have to be passed from mouse to mouse via i.p transplantation [10]. The intra-peritoneal proliferation of EAC cells stimulates vascular permeability of the surrounding blood vessels and plasma accumulation in the peritoneal cavity [9, 10].

Cancer cells are characterized by dysfunction of normal mechanisms of cell cycle regulation, with an over proliferation of cells and/or decreased removal of cells [11]. The inhibition of apoptosis is the primary pathway in the development and progression of cancers. In fact, there are several molecular mechanisms that tumor cells adopt to suppress apoptosis. The cellular mechanisms underlying the oncostatic action of PP-60 are not fully unknown in EAC cells. Since PP-60 has anticancer activity, we considered the possibility that PP-60 may stimulate the sensitivity of EAC cells to apoptosis. Therefore, the aim of the present work was to identify the apoptotic influence of PP-60 on intraperitoneal proliferation of Ehrlich ascites carcinoma model and its sensitization for cell apoptosis in situ.

MATERIAL AND METHODS

Cell line, Chemicals, antibodies

Ehrlich ascites Carcinoma cell line (EAC) was obtained from the National Institute of Cancer, Cairo University, Egypt. Polyphenon-60 (PP-60) from green tea was purchased from Sigma (St. Louis, MO, USA). Phosphate buffer saline (PBS) was purchased from Gibco (USA). Anti-mouse FITC conjugated primary antibodies of caspases 3, 9 and Bax, Bcl2, Ki67, P53 were from Santa Cruz, CA, USA.

Animals and experimental design

Female adult Swiss albino mice were randomly divided into three groups of 10 mice each: 1) The control tumor-bearing group, in which mice were intraperitoneally injected with 2x10⁶ exponentially grown EAC, 2) the PP-60 treated group, for which EAC-bearing mice were treated orally with 250 mg/kg of PP-60 starting from day 1 until the end of the experiment at12 days, and 3) the protected group: Mice received an oral dose of 250 mg/kg PP-60 for three weeks prior to EAC i.p implantation and the treatment continued until the end of the experimental period at day 12 post-implantation. All experimental procedures were carried out in accordance with the guide for care and use of laboratory animals published by the US National institute of Health [NIH publication, 1985].
At the end of the experimental period, the EAC cells were isolated from the peritoneal cavity of tumor-bearing mice. The peritoneal fluid containing the tumor cells was withdrawn and collected in sterile tubes. The EAC cells were washed repeatedly with PBS and processed for further experiments.

**EAC growth response:**

The antitumor effect of PP-60 was assessed by change the body weight, ascites tumor volume, mean survival time, and percentage increased life span. Mean survival time of each group was monitored by recording mortality daily for 12 days. Before harvesting the EAC cells, the peritoneum was examined for angiogenesis and photographed in both control and PP-60-treated tumor bearing mice.

**Cell cycle and apoptosis:**

For the determination of cell cycle phase distribution, EAC cells harvested from tumor-bearing mice were permeabilized and nuclear DNA was labeled with propidium iodide (PI) using Cycle Test Plus DNA reagent kit. Cell cycle phase distribution of nuclear DNA was determined on FACS, a fluorescence detector equipped with a 488 nm argon laser light source and a 623 nm band pass filter (linear scale) using Cell Quest software (Becton Dickinson). A total of 10 000 events were acquired and analysis of flow cytometric data was performed using Mod Fit software. A histogram of DNA content (x-axis, PI fluorescence) versus counts (y-axis) has been displayed.

A double labeling system was used to distinguish between apoptosis and necrosis. EAC cells from all groups of tumor-bearing mice were harvested and PI and annexin V were added directly to the medium. The mixture was incubated for 15 min at 37°C. Excess PI and annexin V were then washed off, cells were fixed and then analyzed on a flowcytometer (equipped with a 488 nm argon laser light source, 515 nm band pass filter for FITC-fluorescence, and 623 nm band pass filter for PI-fluorescence) using CellQuest software. A total of 10 000 events were acquired and the cells were properly gated for analysis.

**Assessment of proliferation markers and pro- and anti-apoptotic proteins**

For the determination of the expression of pro-apoptotic proteins p53, Bax, and caspases 9 and 3 or anti-apoptotic protein Bcl-2, EAC cells from mice were fixed and permeabilized as described earlier. Cells (1x10⁶) from each group were incubated for 30 min with anti-Bcl-2 [100/D5] antibody and anti-Bax antibody for flow cytometry analysis of Bcl-2 and Bax, respectively. A mouse anti-P53 FITC, Clone: DO-1, was used for P53 analysis by flow cytometry. For caspases-3 and -9: FITC rabbit anti-active caspase-3 (CPP32; Yama; Apopain, BD Bioscience), anti-caspase-8 (E6) antibody or rabbit monoclonal anti-caspase-9 (E23) antibody (Abcam, USA) were used respectively. Cells were washed thoroughly with PBA/BSA, centrifuged at 400xg for 5 min, re-suspended in 0.5% paraformaldehyde in PBS/BSA and analyzed on a Flow cytometer equipped with a 488 nm Argon laser light source and a 515 nm band pass filter for FITC-fluorescence. A total of 10 000 events were acquired and the cells were properly gated for analysis.

**Comet assay (Single cell gel electrophoresis):**

Evaluation of DNA damage using single-cell gel electrophoresis was performed for EAC collected from the harvested ascites of all groups as previously described [12]. One hundred cells of each group were analyzed using the Comet Assay II automatic digital analysis system. The quantification of the DNA damage in the obtained images was performed with CASP software to directly obtain the percentage of DNA in the tail, the tail length and the tail moment.

**Statistical Analysis**

Differences between mean values were assessed for statistical significance using a Student's t-test (GraphPad Prism 4.0 software, La Jolla, CA). Values of P< 0.05 were considered statistically significant.
RESULTS

The antitumor effect of PP-60 against EAC cells intraperitoneally implanted in mice was demonstrated by determining body weight (Fig. 1A), survival time (Figure 1B) and ascetic volume (Figure 1C) 12 days after EAC cell inoculation. The EAC-bearing mice demonstrated an increase in body weight due to ascites formation. The abdominal wall of these animals showed intense dilated vascularization (Figure 1D). These findings associated with increased mortality of tumor-bearing mice. Treatment with PP-60 either before or after EAC implantation showed a lower body weight and improved survival time during the experimental period compared with EAC-bearing mice. In addition, treatment with PP-60 reduced the harvested ascetic fluid by 54% and 60% in the treated and protected mice respectively, compared with the control EAC-bearing mice. Furthermore, Figure 1D demonstrates markedly less vascularization in the abdominal layer in the PP-6 treated and protected mice compared with untreated animals.

Figure 1: Effect of PP-60 on (A) body weight changes (B) percentage of survival (C) volume of ascetic fluid and (D) abdominal vascularization. EAC cells (1x10^6 cells / mouse, i.p.) were injected into Swiss albino mice; PP-60 was administered daily orally. Values are mean ± SEM of three determinations. *P < 0.05, **P < 0.01 ***P < 0.001 vs control.

Flow cytometry was used to demonstrate whether the decrease in ascetic volume was due to PP-60-induced inhibition in cell cycle progression of EAC cells (Figure 2A). Cell cycle phase distribution analysis showed a significant inhibitory effect of PP-60 on cell cycle phases in the EAC obtained from treated and protected groups compared with the untreated EAC group (Figure 2A). Both PP-60-treated and protected groups showed cell cycle arrest at G1 phase with a significant increase in the number of apoptotic cells in sub-G1 phase, compared with untreated EAC group.

An assessment of the percentage of the expression of the cell proliferation marker Ki-67 in the different EAC-bearing mice was conducted using flowcytometry approach. The resulting data revealed a highly significant PP-60 inhibition in Ki-67 expression levels in both PP-60-treated and protected groups in comparison with the control untreated EAC (Figure 2B). Furthermore, there was insignificant difference in Ki-67 expression levels between PP-60-treated and protected mice.

To verify the induction of apoptosis by PP-60, cells obtained from tumor-bearing mice were stained with annexin V/PI. The flowcytometric data revealed that compared to control untreated EAC cells (Figure 3), the percentage of apoptotic and necrotic cells was higher in both PP-60treated and protected EAC-bearing mice.
Figure 2: (A) The cell cycle distribution was determined by propidium iodide staining in EAC bearing-mice in different animal groups. The DNA histogram shows the distribution and the percentage of cells in phases of the cell cycle. Values are expressed as percentage of the cell population in the sub-G1, G0/G1, S and G2/M phases of cell cycle. The PP-60 caused cell cycle arrest at G0/G1 phase. (B) Flowcytometry detection of proliferative marker Ki-67 in EAC. PP-60 administration decreased the Ki-67 expression in EAC cells. Values are mean ± SEM of three determinations. *P < 0.05, **P < 0.01 ***P < 0.001 vs control.

Figure 3: Flow cytometer analysis of apoptosis in different animal groups. In a double label system, EACs from control, PP-60-treated and protected tumor-bearing mice were labeled with annexin V/PI and analyzed by a Flowcytometer. The events shown in the lower left quadrant represent viable. Events in the upper left quadrant are indicative of necrotic cells that have taken up the PI. The percentage of early apoptotic cells that are annexin positive only, are represented in the lower right quadrant and events depicting late apoptotic cells that are both annexin and PI positive are shown in the upper right quadrant. Values are mean ± SEM of three determinations. *P < 0.05, **P < 0.01 ***P < 0.001 vs control.
In order to understand the molecular pathways by which PP-60 causes apoptosis in EAC, expression of some regulating proteins involved in apoptosis were determined by flow cytometer. The present data demonstrated that levels of pro-apoptotic p53 (Figure 4), Bax (Figure 5A) as well as caspases-3 and 9 (Figures 6 A&B) were higher in EAC cells obtained from animals receiving PP-60 compared with cells from control EAC-bearing mice (Figure 5). Concurrently, the anti-apoptotic protein Bcl-2 significantly decreased in EAC-bearing mice injected PP-60 (Figure 5B).

Figure 4: Percentage of p53 of EAC cells from EAC-bearing animals of all groups. Flow cytometer histograms from control (A) and PP-60 treated groups (B) and PP-60 protected groups. The PP-60 treatment induced an increase in p53 expression. Values are mean ± SEM of three determinations. *P < 0.05, **P < 0.01 ***P < 0.001 vs control.

Figure 5: Effects of PP-60 administration before and after EAC implantation on the expression of Bax (A) and Bcl-2 (B). PP-60 administration enhances the expression of Bcl-2 and decreases Bax in EAC cells implanted ip in mice. Values are mean ± SEM of three determinations. *P < 0.05, **P < 0.01 ***P < 0.001 vs control.
Figure 6: Effects of PP-60 administration before and after EAC implantation on the expression of caspase 3 (A) and 9 (B). PP-60 administration enhances the activity of caspase-3 and 9. Values are mean ± SEM of three determinations. *P < 0.05, **P < 0.01 ***P < 0.001 vs control.

Figure 7: Effect of PP-60 on the percentage of DNA damage in EAC cells in different groups using the comet assay that detects DNA fragmentation. Comet parameters include Percent of tail DNA, tail length, and tail moment. The microscopic images of representative comets for the different groups are shown. (A) Untreated EAC-bearing mice, (B) PP-60 treated EAC-bearing mice and (C) PP-60-protected EAC-bearing mice. Values are mean ± SEM of three determinations. *P < 0.05, **P < 0.01 ***P < 0.001 vs control.
Figure 8: Molecular mechanism of apoptogenic action induced by PP-60 in EAC cells. PP-60 activates the proapoptotic marker p53 and Bax, down-regulates antiapoptotic marker Bcl-2, triggers initiator caspase-9 and 3 and produces G0/G1 arrest and apoptosis in EAC cells leading to tumor suppression.

The contribution of DNA damage to the anticancer activity of PP-60 is vital. To determine whether DNA damage contributes to PP-60-induced cell death observed in EAC cells, DNA damage was assayed by the comet assay in EAC cells harvested from the different animal groups. Figure 7 shows the percentage of comet parameters. PP-60 caused injuries in DNA and drastically increased comet tail lengths. Figure 7 shows that comet formation increased in EAC cells obtained from both PP-60-treated and protected EAC-bearing mice compared with comet formation seen in EAC obtained from EAC-bearing animals. In cells obtained from mice receiving PP-60, the percentage of damaged DNA, tail length and tail moment were significantly increased.
when compared with these parameters of untreated EAC cells. From these data, it appears that PP-60 causes DNA damage in EAC cells.

**DISCUSSION**

In this study, the oncostatic effect of polyphenon-60 (PP-60) on Ehrlich ascites carcinoma (EAC) was demonstrated. A rapid decrease in ascetic tumor volume with improved survival time was demonstrated when compared to EAC implanted mice and mice who received PP-60. This supports the concept that a reliable benchmark for establishing oncostatic impact of an antitumor drug is the increased survival of animals, decreased tumor volume and enhanced quality of life. The implantation of EAC cells, into the peritoneal cavity caused inflammation and enhanced vascularization, which in turn resulted in progressive edema and increased ascet fluid formation [13, 14]. Ascet fluid is the essential nutritional source for proliferation of tumor cells [15]. PP-60 decreased ascetic fluid volume, which was subsequently supported by a decrease in body weight with prolongation of survival time. Current results indicate that EAC cells respond significantly to PP-60 treatment. Not only did treatment with PP-60 reduce the tumor volume, it also increased the survival of EAC-bearing mice, confirming the inhibition of EAC cell growth and proliferation. These results suggest that treatment with PP-60 significantly decreases tumor mass.

On the basis of these experiments, a hypothesis of the anticancer action of green tea-derived PP-60 was put forward. The data revealed a significant effect of PP-60 in inducing a cell cycle arrest of EAC cells at G1 phase, with an increasing percentage of apoptotic EAC cells in PP-60-treated and protected EAC-bearing mice. The arrest of the cell cycle is complemented by a decrease in expression of Ki-67, a proliferation marker in EAC cells. The decrease in Ki-67 in EAC cells in the PP-60-treated and protected groups demonstrated statistical significance when compared to EAC cells from the EAC-bearing control group. Ki-67 is a nuclear protein associated with cellular proliferation and expressed in the growth and synthesis phases of the cell cycle but not in the G0 phase. It is also considered a prognostic parameter in breast cancer patients [16] and is increased in many other cancer types [17]. The expression of Ki-67 provides information about the proportion of active cells in the cell cycle. This finding indicates that green tea PP-60 reduced cell proliferation, confirming the antitumor effects reported using green tea catechins in human melanoma and breast cancer [4, 18].

The bioactive polyphenol in green tea can prevent tumor development not only through scavenging free radicals but also by inducing pro-oxidative effects on biomolecules including DNA in the cell [19]. These data were further investigated utilizing comet assay. The comet parameters indicated a significant effect of PP-60 on stimulation of cell death signals of EAC cells, represented via a significant increase in percentage of tail length of both PP-60 treated and protected groups. This indicates a potential cytotoxic effect of green tea PP-60 on genomic integrity of EAC cells.

Because the primary pathway followed by cancer cells is to avoid apoptosis, appropriate anticancer treatments would stimulate apoptotic mechanisms to inhibit cancer proliferation. In support of this finding, we found the apoptotic effect of PP-60 on EAC cells was statistically significant, and may play a major role in reducing tumor growth, since the number of apoptotic and necrotic cells were high in both treated and protected groups. In addition, the treatment of EAC-bearing mice with PP-60 significantly decreased the anti-apoptotic protein Bcl-2 and elevated pro-apoptotic proteiins including p53, Bax, and caspases-3 and 9. The results suggest that PP-60 activates P53, Bax, and caspases-3 and 9 thereby activating the intrinsic signaling apoptotic pathway and inhibiting cell growth and proliferation. It is well known that p53 functions to stimulate protective mechanisms against tumor growth by regulating the expression of both Bax and Bcl-2 [20, 21]. It also acts as a tumor suppressor through its capacity to induce cell cycle arrest and apoptosis in response to a variety of chemotherapeutic drugs. These results confirm other studies in which induction of apoptosis by tea polyphenols mediated in mouse skin tumors [22] and human fibrosarcoma HT-1080 cells through intrinsic cell death pathways in mouse skin tumors [22] and human fibrosarcoma HT-1080 cells [23]. Similarly, Epigallocatechin-3-gallate induces apoptosis in estrogen receptor-negative human breast carcinoma cells via modulation in protein expression of p53, Bax and caspase-3 activation [24]. It can be concluded that the expression of p53, Bcl-2, Bax and caspases-3 and 9 are main steps in the regulation of PP-60 induced apoptosis in EAC cells of treated and protected mice.

In conclusion, PP-60 from green tea had significant anticancer activity on EAC cells. Our results indicate that PP-60 suppresses EAC ascetic tumor volume and cell proliferation. Eventually, PP-60 could induce
cell cycle arrest and apoptosis via activation of p53 and various pro-apoptotic proteins with DNA fragmentation, while inhibiting Bcl-2 in the mitochondrial pathway (Figure 10). Thus, the apoptogenic activity of PP-60 could be utilized for future development of chemotherapeutic drugs against cancer.

Conflict of interest:

The authors declare that there is no conflict of interest

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