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Inhibitory effect of caffeic acid phenethyl ester on mice bearing tumor involving angiostatic and apoptotic activities

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ABSTRACT

This study aims at investigating the anti-tumor effect of caffeic acid phenethyl ester (CAPE) against animal carcinogenesis. In order to substantiate this fact implanted tumor Ehrlich carcinoma cells were assessed *in vivo* to Swiss mice strain. We found that administrating of CAPE (15 mg/kg S.C.) showed that the tumor volume decreased significantly by 51%. As a result, it improved animal chances of survival and they became healthier. An anti-angiogenic effect of CAPE *in vivo* was observed, as determined by a significant serum matrix metalloproteinase (MMP-9) reduction (142.1 ng/ml), activation of endostatin serum level (1.9 ng/ml), as well as DNA fragmentation in tumor treated mice when compared with untreated ones. *Conclusion*: CAPE has a significant inhibitory effect on tumor *in vivo*. This inhibition may be related to its angiostatic and apoptotic effects. It also reduced angiogenic factors which may shift the equilibrium to the angiostatic effect of CAPE. These findings provide the possibility for the future use of CAPE as tumor therapy in human clinical trials.

1. Introduction

Cancer is considered one of the major causes of mortality in the world. Despite the recent advances in science, cancer has not been cured yet. It is estimated that by 2020 there will be 16 million new cancer cases every year [1]. It is, therefore, essential that new therapeutic options are needed for cancer therapy with attention to toxicity and side effects, besides the major treatment modalities including surgery, immunotherapy and radiotherapy [2–4].

Cancer chemoprevention is a rapidly growing area of oncology which can make a significant progress in the prevention and treatment of carcinogenesis by administration of various drugs with chemical or natural entities depending on their anti-mutagenic properties [5]. Some significant progress has been made in the understanding of pharmacological and chemical properties in this approach to provide more details which may improve and establish proper strategy for the prevention of cancer.

Caffeic acid phenethyl ester (CAPE), a phenolic antioxidant, is an active anti-inflammatory natural resinous product of honey bees—propolis (bee glue) [6–8]. It has been demonstrated that CAPE possesses cancer chemopreventive effects *in vitro* and *in vivo* [9–11]. Besides being capable of decreasing the frequency of cancer development, it also reduces the morbidity rate and supporting cancer cell survival inhibition which may be the evidence of its protective rate [12].

Several reports have demonstrated and contributed to the anti-inflammatory, cancer prevention and anti-tumor effects of CAPE which shed light on its effect on cancer immunomodulatory features, cell cycle progression, cell proliferation, tumor growth, induction of cell cycle arrest and apoptosis [13–15]. CAPE was also found to be a specific inhibitor of the transcription nuclear factor-$_k$B (NF-$_k$B), which may account for its anti-inflammatory action [16,17].

We know that carcinogenesis is closely related to angiogenesis [18,19]. The angiogenic switch is dependent on the dynamic balance between proangiogenic factors, matrix metalloproteinase (MMP-9), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α) and angiogenic inhibitors in the immediate environment of endothelial cells [20]. The prevalence of anti-angiogenic factors shifts the equilibrium to vessel quiescence or vessel regression [21]. In addition to the angiogenic factors, endogenous inhibitors of angiogenic phenotype have been identified. Endostatin is one of the better characterized endogenously produced angiogenic inhibitors [22]. It has been proved to be highly effective in inhibiting angiogenesis and tumor growth [23,24]. It may mediate these biological effects by inhibiting the proliferation and inducing apoptosis in tumor cells [25,26].

The present study is aimed at investigating the effects of the administration the CAPE compound on mice bearing tumor and the angiogenic parameters such as matrix metalloproteinase-9 as

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well as, the anti-angiogenic factor-endostatin-which is known to be associated with suppression of tumor growth. At the same time, correlate their levels with tumor regression outcome. More details and analysis on the molecular level of DNA integrity are necessary to figure out CAPE's mode of action.

2. Materials and methods

2.1. Animals

Swiss female albino strain mice which were 8 weeks old and weighing 20 ± 2 g were purchased from Cairo University. The mice were kept in the Animal Center, Faculty of Pharmacy, October University, Egypt, under constant conditions (12 h light, dark regimen, oriental chow pellet food and water *ad libitum*).

2.2. Caffeic acid phenethyl ester (CAPE)

Caffeic acid phenethyl ester (CAPE) was obtained from Sigma Chemical Company. The compound was dissolved in DMSO at 100 mM concentration, stock solution and stored at −20 °C. Serial concentrations in PBS solution were performed on the compound on the basis of its use during mice treatment.

2.3. Lethality study in Swiss albino mice

The female Swiss albino mice were brought and randomly distributed into several groups of 10 mice each. They were treated with different doses (50–300 mg/kg) of caffeic acid phenethyl ester. The number of surviving mice was recorded daily. This process was continued for 30 days. The mice were subjected to experiments in accordance with ethical standards [27].

2.4. Anti-tumor study

Ehrlich ascites carcinoma (EAC) cells were implanted subcutaneously (S.C.) by inoculation of (2×10^5 tumor cells/mice) into the left hind legs at the volume of 0.2 ml of physiological solution (for solid form). The next day the mice were randomly divided according to their weight into four groups: a control group and three experimental groups. The control group comprised mice treated with 0.9 ml sodium chloride only throughout the experiment. On the other hand, the experimental groups consisted of mice that were injected with tumor cells and treated with different doses of CAPE (5, 10 and 15 mg/kg) at the site of cells implantation for a total of 10 doses. Separate normal group of 10 mice were kept as negative control throughout the experiments.

The mice were observed carefully everyday. The tumor growth was estimated by two-dimensional measurements using a vernier calipers. The measurements were taken on the sixth day after tumor implantation became palpable. Likewise, the measurements were taken twice weekly for all groups for 50 days.

The anti-tumor activity of CAPE against Ehrlich tumor was calculated using the formula $V = 0.4 \times a(b)^2$, where (a) and (b) are the longer and shorter diameters of the tumor respectively for experimental and control groups [28].

The blood was taken from the heart punctured under anaesthetic conditions. Then, it was centrifuged at 2500 r.p.m. in order to detect matrix metalloproteinase (MMP-9) and endostatin levels using ELISA assay. The tumors were removed from the animal body and reserved for further parameters, including DNA histogram [29].

2.5. Determination of serum levels of matrix metalloproteinase (MMP-9) and endostatin

For the assessment of serum levels of MMP-9 and endostatin, blood samples were collected from each mice by puncturing the heart. They were delivered into plastic tubes which did not contain any anticoagulant. These samples were left to clot. Later, they were centrifuged to obtain serum which was stored at −70 °C. For the quantitative determination of MMP-9 and endostatin, we used competitive enzyme-linked immunosorbent assay (ELISA) which measures the natural and recombinant forms of the cytokine (Cytoimmune Science Inc., MD). For each, 100 μl of serum sample was added to their designated wells. This assay employs the quantitative sandwich enzyme immunosorbent assay technique. A monoclonal antibody specific for cytokine was pre-coated onto a microplate. Standards and samples were pipetted into the wells and cytokine bound by the immobilized antibody. After washing away the unbound substances, an enzyme-linked polyclonal antibody specific for cytokine was added to the wells. Following a wash to remove any unbound antibody, an enzyme reagent and a substrate solution were added to the wells and color developed in proportion to the amount of total cytokine (pro and/or active) bound in the initial step. The color development was stopped and the intensity of the color was measured [30].

2.6. Determination of DNA fragmentation by flow cytometry

The tumor samples were thawed and minced with a scalpel in a cold PBS solution. The samples were filtered through a 70-μm nylon mesh. After washing in the PBS solution and centrifugation, the cells chosen for analysis were collected and incubated with a solution containing propidium iodide (PI) (10 μg/ml, Sigma) and RNA-ase (1 mg/ml, Sigma). The tubes were placed at 4 °C in the dark for at least 30 min before analyzing by flow cytometry. The PI fluorescence of individual nuclei was measured using Coulter Epics XL. At least 5×10^3 cells of each sample were measured. Apoptotic cells were represented by a subdiploid peak of cells that can be easily discriminated from the peak of cells with the diploid DNA content in the red fluorescence channel. The percentage of apoptosis was indicated by the percentage of cells with subdiploid DNA content [29].

2.7. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Comparisons between two different groups were performed by Mann–Whitney's U test, and between more than two groups by Kruskal–Wallis one-way analysis of variance ANOVA followed by the Tukey–Kramer test. The Graphpad Software Instat (version 9) was used to carry out the statistical analysis.

3. Results

3.1. Toxicity of CAPE

The mice treated with CAPE (10 mg/kg S.C. or 15 mg/kg S.C.) have not shown any sign of toxicity on their body weight, general appearance and organ pathology (data not shown). The LD50 dose of CAPE was 150 mg/kg S.C. The animals cured after treatment has been surviving for almost 3 months without any symptoms of adverse effects.

3.2. Survival time

The effects of CAPE on the survival of mice bearing tumor are shown in Fig. 1. The median survival time for the untreated group