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

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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 an active research area due to its potential to reduce the incidence of cancer. 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. CAPE possesses cancer chemopreventive effects which shed light on its effect on cancer immunomodulatory 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-κB (NF-κB), 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-α (TNF-α) and angiogenic inhibitors in the immediate environment of endothelial cells [20]. The prevalence of antiangiogenic 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...
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 × 10⁶ 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 measured using the formula

\[ V = \frac{a \times b^2}{2}, \]

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 × 10⁴ 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 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...
of mice was 21 days. On the other hand, the group of mice bearing tumor and treated with 10 mg/kg S.C./each 5 days had a median survival time of 29 days. Two mice were found to be completely cured. The median survival time of the group treated with 15 mg/kg S.C./each 5 days was found to be 43 days and 3 mice were found to be completely cured. However, those mice treated with 5 mg/kg S.C./each 5 days did not show any remarkable changes in their survival percentage. It has also observed giving treatment more than once a week caused sores and increased irritability in the mice.

Table 1

<table>
<thead>
<tr>
<th>Group parameters</th>
<th>Normal group (n = 10)</th>
<th>Tumor group (n = 10)</th>
<th>Treated group (n = 10)</th>
<th>P1</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9 (ng/ml)</td>
<td>130.9</td>
<td>181.9</td>
<td>142.1</td>
<td>P&lt;0.001</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Median range</td>
<td>79.6–166.4</td>
<td>88.2–216.1</td>
<td>84.5–196.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endostatin (ng/ml)</td>
<td>2.2</td>
<td>1.4</td>
<td>1.9</td>
<td>P=0.01</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Median range</td>
<td>1.1–6.3</td>
<td>1.0–5.4</td>
<td>1.3–9.3</td>
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</tbody>
</table>

P1: tumor group vs. normal group.
P2: treated group vs. tumor group.

Table 2

<table>
<thead>
<tr>
<th>Correlation between investigated angiogenic factors and, hemoglobin (HB), white blood cells count (WBCs) and platelets count in tumor treated mice.</th>
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<tr>
<td>MMP-9</td>
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<tr>
<td>Endostatin</td>
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3.3. Solid tumor regression

To explore the effect of CAPE 15 mg/kg S.C./each 5 days, on the growth of transplanted Ehrlich carcinoma into Swiss mice, the solid tumor volume has been shown to grow more slowly in CAPE-treated mice and appreciably smaller volume (1.9 ± 0.46) mm$^3$, with respect to the untreated group (3.7 ± 0.82) mm$^3$. This value was significant (P<0.01). The difference was observed from the beginning of tumor measurement, i.e. since the 6th day after tumor implantation to the host, and was maintained until the end of observation (Fig. 2).

3.4. In vivo evaluation of serum metalloproteinase-9 and endostatin

Untreated mice bearing Ehrlich tumor elicited a highly significant increase of serum MMP-9 level (181.9 ng/ml), which was reduced (142.1 ng/ml) in mice treated with CAPE at a dose 15 mg/kg (P<0.01) close to the normal mice serum level. However, in the untreated mice bearing tumor serum endostatin (sE) was significantly lowered (1.4 ng/ml) compared with the normal mice. In CAPE-treated mice serum endostatin level was significantly higher (1.9 ng/ml) than the serum level in the untreated group (Table 1). On the other hand, there was a negative correlation between (sMMP-9 and sE) and the total white blood cells (WBCs), hemoglobin (HB) and the platelet count of mice (Table 2).
3.5. Flow cytometry for tumor cell apoptosis

The percentage of apoptotic cells with hypodiploid DNA content was determined from DNA histograms. Untreated tumor-bearing mice showed a peak pattern which represented proliferative and high metastatic tumor activity (Fig. 3A). However the mice which had their tumor treated at 15 mg/kg S.C. did not alter the relative size of the peak, but induced a significant parallel shift to less-intense fluorescence (D-area). This decrease in the intensity and shift may be termed as nuclear apoptosis and fragmentation (Fig. 3B).

4. Discussion

Propolis obtained from honeybee hives has been used since time immemorial in folk medicine as an anti-carcinogenic, anti-inflammatory, anti-bacterial and immunomodulatory agent. CAPE is an active component of propolis which attracts the attention of investigators and researchers to ascertain its potential effects for the treatment of a number of disorders and illnesses [31–33]. Studies conducted earlier have shown that CAPE inhibits the growth of C6 glioma cells transplanted in nude mice. Recent studies suggest that the CAPE combined with MG-123 had a strong anti-proliferative effect on a lymphoblastoid B-cell line via NF-Kappa B inhibitor [34].

Angiogenesis is regulated by the net balance between positive (angiogenic) and negative (angiostatic) regulators of blood vessel growth. Therefore, the improved regulation of angiogenesis is often associated with the development of angiogenesis–dependent diseases and plays a fundamental role in metastasis in the neoplastic process and solid tumor [35]. Among these factors are the so-called endogenous inhibitors of angiogenesis which maintain metastases in a non-proliferating quiescent state for a number of tumors. It has been shown that this dormant state is mediated through the inhibition of angiogenesis. This dormant state is characterized by normal proliferation, increased apoptosis and insufficient neovascularization. Removal of inhibiting anti-angiogenic factors leads to the growth of dormant metastases [36].

In this study, to gain more insights into the effects and mechanism of the action of the CAPE, we performed an in vivo study of mice bearing Ehrlich tumor. Our results showed that CAPE, associated in this work, induced reduction in the tumor growth. This reduction proved beneficial to the host in two ways: it increased the lifespan of EAC bearing animals treated and it prevented the EAC cell-growth and induced marked tumor regression in tumor treated group compared to the untreated group of mice. This finding is one of the characteristic effects of anti-tumor drugs. It also agrees with the other findings which suggest that subcutaneous administration of an aqueous crude water–soluble propolis (CWS) resulted in marked regression of transplanted tumors [37].

Moreover, we found highly significant reduction of serum MMP-9 (142.1 ng/ml, P < 0.01) in the same treated group; this plays a critical role in angiogenesis, tumor invasion and metastasis. Also the level of MMP-9 in the serum of untreated mice was significantly higher (181.9 ng/ml, P < 0.001) as compared to the normal mice serum (130.9 ng/ml). This finding seems to be in accordance with other similar findings reported by Melani et al. who revealed that suppression of MMP-9 activity breaks the vicious loop linking the tumor growth and the myeloid cell expansion [38].

Based on the findings of this study, we can conclude the following: the endogenous inhibitor of the angiogenic serum (endostatin) has been shown to be overexpressed, significantly higher in treated mice compared to untreated ones (1.9 ng/ml), and nearly to the value of normal serum mice (Table 1). These findings prove that endostatin itself may be used effectively in the therapy. It also indicates that CAPE has the potential of an anti-metastatic agent. It may mediate CAPE effects by inhibiting the cell proliferation. The findings of this study are in accordance with Schuch et al. [39] claim that endostatin microbeads significantly inhibit the growth of subcutaneous choromas SCCID mice as compared to control ones. Furthermore, our data showed fragmentation of DNA in the treated tumor-bearing mice (Fig. 3B) compared to untreated group of mice (Fig. 3A). This fact helped us confirm our results of CAPE’s apoptotic effects in vivo. On the other hand, MMP-9 and endostatin did not correlate with the white blood cells (WBCs), hemoglobin (HB) and platelet count (Table 2). It may be concluded that MMP-9 and endostatin are independent factors.

5. Conclusion

These results suggest that CAPE is a potent apoptosis-inducing agent which has anti-metastatic and anti-angiogenic properties. The experimental data support that CAPE could be potentially useful in the control of tumor growth in experimental models. Its action is accompanied by the shifting and elevating of the angiostatic and inhibiting angiogenic factors. It has been demonstrated that CAPE has many biological and pharmacological properties with predictive future applications in human clinical trials.

Conflict of interest
None.

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