Effect of Genistein on Modulating Lipid Peroxidation and Membrane-bound Enzymes in N-Nitrosodiethylamine-induced and Phenobarbital-promoted Rat Liver Carcinogenesis

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Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors worldwide and a leading cause of cancer-related death in the world. The importance of diet in the control of several major cancers is now widely recognized, and it is generally agreed that a plant-based diet may afford a significant degree of protection. The current study was designed to evaluate the effect of genistein on N-nitrosodiethylamine (DEN)-induced (200 mg/kg body weight; by single i.p injection) and Phenobarbital-promoted (0.05% through drinking water for 14 successive weeks) liver cancer in Wistar albino rats. Decreases (p < 0.001) in the activities of Na+K+ATPase and Mg2+ATPase and increases (p < 0.001) in Ca2+ATPase activity were observed in erythrocytes membrane and tissue ATPase of liver cancer-bearing animals when compared with control groups. The change in the activities of these enzymes in membrane and tissue were indicative of the persistent deteriorating effect of DEN in cancer-bearing animals. These enzyme activities were reversed to near normal value in animals treated with genistein. From our results we conclude that genistein may play an important role in preserving membrane asymmetry by suppressing free radicals implying a role for genistein against tumorigenesis.

Key words —— genistein, lipid peroxidation, membrane ATPase enzymes, N-nitrosodiethylamine Rats

INTRODUCTION

The importance of diet in the control of several major cancers is now widely recognized, and it is generally agreed that a plant-based diet may afford a significant degree of protection.1) A number of studies have reported that consumption of soy and soy products is associated with some degree of protection against either induced or spontaneous cancers in animals as well as reduced cancer risks in several human epidemiological studies. Isoflavones are among the potential chemopreventive agents that have been identified in soybeans and the principal isoflavone found in soybeans is genistein. Genistein has received much attention as a potential anticancer agent due to its wide-ranging effects on a number of cellular processes. It has also shown to be an anti-oxidant, to possess weak oestrogenic and anti-oestrogenic properties and to inhibit the activity of ribosomal S6 kinase, tyrosine kinase and topoisomerase as well as angiogenesis in vitro.2)

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors worldwide and a leading cause of cancer-related death in the world killing million people < 1.25 annually.3) N-nitrosodiethylamine (DEN) is the most important environmental carcinogen among the nitrosamines and primarily induces tumors of the liver.4) Administration of DEN to rats results in lipid peroxidation (LPO) and enhanced chemiluminescence in liver preneoplastic nodules, indicating formation of activated oxygen species.5) DEN also produces 8-hydroxyguanine (8-OH),6) an indicator of oxidative damage to DNA and the most abundant of > 20 types of modifications produced under conditions of oxidative stress. This premutagenic DNA damage
results in specific types of mutation and is likely involved in carcinogenesis. In the present study, we examined the effect of genistein on LPO and activities of membrane ATPases enzymes to assess the protective role of genistein on DEN-induced Phenobarbital (PB)-promoted hepatocellular carcinogenesis in Wistar albino rats.

**MATERIALS AND METHODS**

**Source of Chemicals** —— DEN and genistein were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. All other chemicals used for experiments were of analytical grade.

**Animals** —— Male albino rats of the Wistar strain, at the age of 6 weeks (procured from Tamil Nadu Veterinary College, Chennai, India) were divided into five groups of 6 rats each. All rats were housed in pure polypropylene cages and maintained at a diurnal 12-hr light/dark cycle with constant temperature and humidity. The animals were fed a commercial rat diet. Animals had ad libitum access to food and water. This research work on Wistar albino rats was sanctioned and approved by the Institutional animal ethical committee (vide letter No. PGIBMS/Dr.S/2002/3960/dated 27/12/2002/IAEC/No.02/021/02).

**Experimental Design** —— Group I served as control animals and was given vehicle alone for 15 days. Group II animals were induced with DEN by a single intraperitoneal injection (200 mg/kg body weight in saline). After 2 weeks recovery, carcinogenic effect was promoted by PB (0.05% PB). PB was administered to the animals through drinking water ≤ 14 successive weeks. Group III animals were treated with genistein (15 mg/kg body weight suspension in olive oil) subcutaneously for 15 days before they were treated with DEN (as in group II). Group IV animals were post-treated with genistein (as in Group III) for 15 days after the induction of HCC by DEN (after 16 weeks as in Group II). Group V animals were treated with genistein (as in group III) alone for 15 days to study the cytotoxicity (if any) induced by genistein.

**Biochemical Analysis** —— At the end of the experimental period, the animals were killed by cervical decapitation. Blood and liver tissues were collected, tissues were immediately excised, weighed and homogenized in Tris-HCL buffer 0.1 M (pH 7.4). Erythrocyte membranes were isolated according to the method of Dodge et al., with slight modification as proposed by Quist.

Na⁺/K⁺-ATPase was estimated by the method of Bonting. The activity of Ca²⁺-ATPase was assayed by the method of Hjerten and Pan. The activity of Mg²⁺-ATPase was assayed by the method of Ohnishi et al. Inorganic phosphorous was estimated according to the method of Fiske and Subbarow. Protein content was estimated by the method of Lowry et al. The level of lipid peroxides was assayed by the method of Ohkawa et al.

**Statistical Analysis** —— Data are presented as the mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) was used to detect significant changes between the groups. The Student least significant difference (LSD) method was used to compare the means of different groups and the significance was denoted by its ‘p’ value. Commercial software program (Sigma STAT, Version 7.5) was used for statistical analysis.

**RESULTS**

Figure 1 shows the level of LPO in liver of control and experimental animals. It was found that cancer-bearing (Group II) animals showed a significant (p < 0.001) increase in the LPO in liver when compared with control animals (Group I). Genistein-treated (Group III and Group IV) animals showed a significant decrease (p < 0.001) in the levels of LPO in liver when compared with HCC-bearing (Group II) animals. There was no significant difference in the LPO between the genistein alone-treated group (Group V) and control animals (Group I).

Table 1 presents the tumor incidence of control and experimental animals. Group III and Group IV animals treated with genistein show a significant (p < 0.001) decrease in tumor incidence when compared with cancer-bearing (Group II) animals. The genistein alone-treated Group V animals displayed values equivalent to those of the control group.

Table 2 shows the activities of ATPases in erythrocyte membrane of various experimental groups. Cancer-bearing animals (Group II) showed a significant decrease (p < 0.001) in the activities of Na⁺/K⁺-ATPase and Mg²⁺-ATPase with a significant (p < 0.001) increase in the activities of Ca²⁺-ATPase when compared with control animals (Group I). This change in the ATPases activities
was significantly \( p < 0.001 \) reverted in Group III and Group IV \( p < 0.001 \) genistein-treated animals to near normal values. However, genistein alone-treated animals (Group V) did not show any significant change when compared with control animals (Group I).

Table 3 shows the effect of genistein on the activities of ATPases in liver tissues of control and experimental animals. Decrease \( p < 0.001 \) in the activities of \( \text{Na}^+/\text{K}^+ \) ATPase and \( \text{Mg}^{2+} \) ATPase and increase \( p < 0.001 \) in \( \text{Ca}^{2+} \) ATPase activity was seen in cancer-bearing Group II animals when compared with control animals. There was a significant increase in the activities of \( \text{Na}^+/\text{K}^+ \) ATPase \( p < 0.001 \) and \( \text{Mg}^{2+} \) ATPase \( p < 0.001 \) and significant decrease in the activity of \( \text{Ca}^{2+} \) ATPase \( p < 0.001 \) in genistein-treated (Group III and Group IV) animals when compared with Group II animals. There was no significant difference in the activities of ATPases between Group V animals and control.

### Table 1. Effect of Genistein on the Number of Tumor-bearing Animals in Control and Experimental Groups

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>No. of tumor incidence</td>
<td>0</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>No. of tumors/rat</td>
<td>0</td>
<td>3.75 ± 0.41a</td>
<td>1.35 ± 0.15b</td>
<td>1.75 ± 0.19bc</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. for six rats in each group. a: as compared with Group I, b: as compared with Group II, c: as compared with Group III. Statistical significance: *\( p < 0.001 \).

### Table 2. Effect of Genistein on the Activities of Erythrocyte Membrane ATPases in Control and Experimental Animals

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Na}^+/\text{K}^+ )</td>
<td>2.72 ± 0.24</td>
<td>1.84 ± 0.20a</td>
<td>2.58 ± 0.32ab</td>
<td>2.37 ± 0.29abc</td>
<td>2.73 ± 0.27</td>
</tr>
<tr>
<td>( \text{Ca}^{2+} )</td>
<td>3.52 ± 0.32</td>
<td>5.11 ± 0.63ab</td>
<td>3.40 ± 0.42bc</td>
<td>2.85 ± 0.35bcd</td>
<td>3.56 ± 0.29</td>
</tr>
<tr>
<td>( \text{Mg}^{2+} )</td>
<td>1.63 ± 0.14</td>
<td>0.53 ± 0.05a</td>
<td>1.46 ± 0.18b</td>
<td>1.07 ± 0.13bc</td>
<td>1.57 ± 0.13</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. for six rats in each group. Units: moles of inorganic phosphorus formed per min/mg protein. a: as compared with Group I, b: as compared with Group II, c: as compared with Group III. Statistical significance: *\( p < 0.001 \).

### Table 3. Effect of Genistein on the Activities of Membrane Bound ATPases in Liver of Control and Experimental Animals

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Na}^+/\text{K}^+ )</td>
<td>1.87 ± 0.18</td>
<td>1.03 ± 0.11a</td>
<td>1.64 ± 0.20ab</td>
<td>1.39 ± 0.17abc</td>
<td>1.85 ± 0.16</td>
</tr>
<tr>
<td>( \text{Ca}^{2+} )</td>
<td>1.62 ± 0.14</td>
<td>2.27 ± 0.25ab</td>
<td>1.65 ± 0.20bc</td>
<td>1.81 ± 0.22bcd</td>
<td>1.63 ± 0.13</td>
</tr>
<tr>
<td>( \text{Mg}^{2+} )</td>
<td>2.65 ± 0.24</td>
<td>1.78 ± 0.19a</td>
<td>2.57 ± 0.32b</td>
<td>2.32 ± 0.29c</td>
<td>2.63 ± 0.21</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. for six rats in each group. Units: moles of inorganic phosphorus formed per min/mg protein. a: as compared with Group I, b: as compared with Group II, c: as compared with Group III. Statistical significance: *\( p < 0.001 \).
DISCUSSION

The search for new chemopreventive and antitumor agents that are more effective and less toxic than existing agents has kindled great interest in phytochemicals. A number of studies in animal models demonstrate that dietary soybean and genistein inhibit the growth of transplantable tumors in rats and dietary soybean isoflavones were shown to reduce the induction of tumors in rats by chemical carcinogens.

In the present study, we observed that treatment with genistein significantly reduced tumor incidence. Since genistein is believed to have a potential role in reducing tumor incidence and the size of the tumor nodules the effects we observed might possibly have been caused by this agent. Although it is evident that not all the hepatocytes nodules became cancerous during the lifespan of the animals, numerous observations support the concept that the nodules are precursors of HCC. Moreover, there is a large body of evidence to show a correlation between the number and size of hyperplastic nodules and hepatocarcinoma in both experimental and human disease. In view of this, inhibition of nodule growth and enhancement of their regression by genistein, as observed in our study, may be important for anticarcinogenic effect.

LPO is regarded as one of the basic mechanisms of cellular damage caused by free radicals. DEN is the most important environmental carcinogen among nitrosamine in interacting with membrane lipids and consequently inducing free radical formation. Free radicals react with lipids causing peroxidation, resulting in the release of products such as malondialdehyde, hydrogen peroxide, and hydroxy radicals. An increase in lipid peroxides indicates serious damage to cell membranes, inhibition of several important enzymes, reduced cellular function, and cell death. Administration of genistein significantly reduced membrane lipid peroxides in cancer-bearing animals. LPO can be suppressed by enzymatic inactivation of free radicals. It has been reported that the hydrogenation of double bond between carbon two and three (C2–C3) of the C-ring decreased the antiperoxidative effects. This represents the antioxidant potency of genistein and its ability to protect the membrane from free radical-mediated injuries, suggesting its anticarcinogenic effect.

In malignancy, the cell membrane plays a crucial role in stimulation and control of cell adhesiveness, mortality, and proliferation in a much-damaged condition. The protection of membranes is of potential importance in the treatment of disease processes. ATPases are membrane-bound enzymatic proteins that maintain ionic gradients between aqueous intra- and extracellular phases. Membrane-bound enzymes such as Na+/K+-ATPase, Mg2+-ATPase, and Ca2+-ATPase are responsible for the transport of sodium/potassium, magnesium and calcium ions across the cell membranes at the expense of ATP by hydrolysis. The activities of ATPases in erythrocyte membrane and liver tissues have been shown inhibited in cancer-bearing animals. These findings are similar to those reported in various cancers.

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invasion of highly invasive cancer cells is initiated by addition of serum, which induces an increase in intracellular pH as well as a transient elevation of Ca\(^{2+}\), and that addition of serum to poorly invasive cancer cells does not increase intracellular pH or Ca\(^{2+}\). There was also an increasing awareness that plasma membrane calcium (PMC) ATPase alterations are associated with tumorigenesis.\(^{37}\) There are four isoforms of PMC ATPase (PMCA1–4) with additional isoform diversity generated from the primary PMCA transcripts by alternative splicing.\(^{38,39}\) Indeed, relative greater expression of PMCA1 mRNA expression was observed in tumorigenic breast cancer MCF-7 and MDA-MB-231 cells compared with non-tumorigenic MCF-10A cells.\(^{40}\) Thus, perturbed PMCA regulation or function may be important in diseases such as cancer.

In the present study, a decrease in the activities of Na\(^+/\)K\(^+\) ATPase and Mg\(^{2+}\)-ATPase and an increase in the activities of Ca\(^{2+}\) ATPase were found in cancer-bearing animals. Genistein treatment significantly restored the tissue ATPase activities to near normal values. The results suggest that genistein protects ion pump ATPases, presumably by limiting the degree of oxidation and levels of oxidation by products due to an increase in the reduced glutathione (GSH) content in rats treated with genistein.

In conclusion, the results of our studies demonstrate that genistein can modulate ATPase enzymes and reduce free radical formation in DEN-induced PB-promoted liver cancer in albino rats. Thus inhibitory effect of genistein may play an important role in preserving membrane asymmetry by suppressing free radicals, implying efficacy of this agent against tumorigenesis.

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REFERENCES


