The Effects of Catechin on Superoxide Dismutase Activity and Its Gene Expression in Pheochromocytoma Cells

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Key Words
-catechin;
-manganese superoxide dismutase;
mRNA;
pheochromocytoma cells

Background. Overexpression of manganese superoxide dismutase (MnSOD) cDNA via plasmid transfection leads to growth inhibition in vitro and in vivo in various human cancers. Polyphenolic compounds such as catechin isolated from tea bush *Camellia sinensis* has been shown to have anticancer effect in vitro. This study evaluated the effect of catechin on the MnSOD activity and mRNA level of pheochromocytoma cells (PC-12).

Methods. PC-12 cells were incubated with different concentrations of catechin at short-term (2 days) and long-term (7 days) in Dulbecco-modified Eagle medium. The activity of superoxide dismutase (SOD) was measured and its mRNA was assayed by Northern blotting.

Results. After incubation for 2 days, catechin slightly but significantly increased the activity of copper/zinc superoxide dismutase (CuZnSOD). However, it did not show any significant effect at 7 days. The MnSOD activity showed significant changes in both short-term and long-term. The amount of mRNA also showed similar changes.

Conclusions. Catechin is a natural antioxidant which has been shown to have antitumor effect in basic and epidemiological studies. The present data suggest that catechin can increase MnSOD gene expression in PC-12, which might have beneficial effect in tumor prevention.

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Over the past two decades, much evidence has accumulated linking reactive oxygen species (ROS) and many types of cancer.¹ ROS are molecules that contain oxygen and have higher reactivity than ground state molecular oxygen. These species include not only the oxygen radicals (like superoxide, hydroxyl, and peroxyl radicals), but also non-radical molecules like singlet oxygen and hydroperoxide. ROS are generated during normal aerobic metabolism, and in increased levels of these species are produced during various forms of oxidative stress. ROS are known to react with various intracellular targets, including lipids, proteins, and DNA. ROS-induced damage can result in cell death, mutations, chromosomal aberrations, or carcinogenesis.¹

Cells contain a large number of antioxidants in dants to prevent or repair the damage caused by ROS. These antioxidants include several small molecular weight compounds such as vitamins E, C, and A, as well as the larger molecular weight antioxidant enzymes.²
There are four types of primary intracellular antioxidant enzymes in mammalian cells: copper- and zinc-containing superoxide dismutase (CuZnSOD), manganese-containing superoxide dismutase (MnSOD), catalase (CAT), and peroxidase, of which glutathione peroxidase (GPX) is the most prominent. The superoxide dismutases (SOD) convert superoxide radical into hydrogen peroxide, while catalase and peroxidases convert hydrogen peroxide into water. In this way, two toxic species -- superoxide radical and hydrogen peroxide -- are converted to the harmless product water. These antioxidant enzymatic functions are thought to be necessary for life in all oxygen-metabolizing cells. SOD and CAT need no co-factors to function, but GPX requires several co-factors and secondary enzymes. Two such proteins, glutathione reductase (GR) and glucose-6-phosphate (G6PDH), are considered secondary antioxidant enzymes since they do not act directly on ROS but enable the GPX to function. The antioxidant enzyme scheme is shown in Fig. 1.

An important feature of these enzymes is that they are highly compartmentalized. In general, MnSOD is localized in the mitochondria, CuZnSOD in the cytoplasm, CAT in peroxisomes and cytoplasm, and GPX in many subcellular compartments.

Recently, several lines of evidence on the antitumor activities of tea catechins have emerged from animal models and human epidemiological studies. However, the exact mechanisms underlying these activities are still elusive and mostly speculative. Antioxidant activities and interactions with certain enzymes or proteins have been implicated in tumor biology. Our recent study has shown that the natural antioxidant vitamin E could result in down-regulation of MnSOD gene after long-term incubation with pheochromocytoma cells (PC-12). Therefore, it is possible that tea catechins may have effects on an antioxidant gene expression. This study was undertaken to evaluate whether another natural antioxidant catechin extracted from green tea has any beneficial effect on MnSOD gene expression on PC-12.

**Methods**

**Preparation of PC-12 cells**

PC-12, a transplantable rat adrenal medullary pheochromocytoma cell line was originally cloned by Greene & Tischler in 1975. This well-characterized catecholamine-secreting neoplastic cell line is responsive to nerve growth factor (NGF) and is a useful tool for research on tumor. In our study, PC-12 was provided by the laboratory of Dr. Juei-Jang Cheng of National Cheng-Kung University. These cells were incubated in Dulbecco modified Eagle medium (DMEM), which contains 10% fetal bovine serum. Cells were washed in phosphate-buffered saline (PBS) containing 0.1% EDTA and 0.5% trypsin and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and a cell suspension was obtained after adding culture medium. Cell suspension (100 µL) was mixed thoroughly with 100 µL PBS containing 0.04% trypan blue. The number of surviving cells was counted using a light microscope and Coulter counter. Surviving cells were divided into four groups: a control group incubated with 100 µL PBS (without catechin) and treatment groups (catechin) incubated...
with culture medium containing catechin at 50 µM, 100 µM, and 200 µM, respectively.

**Assay of SOD activity**

The activity of SOD was determined by a commercial assay kit (SOD test 435-7061, Wako, Tokyo, Japan). Cells from one dish were harvested and homogenized in 1 mL of 0.9% NaCl. The crude homogenate was centrifuged at 10,000g for 1 h to get the supernatant (cytosolic) and pellet (particulate). For assay of MnSOD activity in the pellet, 1 mM potassium cyanide was added to the incubation mixture to inhibit CuZnSOD activity. The activity of CuZnSOD was derived by the subtraction of MnSOD from total SOD activity in the pellet. The SOD protein amount was determined according to the method developed by Lowry in 1951. 20 µl sampling protein (SOD) was added to 1 mL reagent (Protein Assay Kit, Bio-Rad Lab., Richmond, Can ada) and the increase in absorbance at 595 nm was monitored. The units of SOD activity were derived from a standard curve constructed using purified SOD from bovine erythrocytes (S-2515; Sigma Chemical, St. Louis, MO). Results are expressed as unit/mg of protein determined.

**Northern blotting analysis**

Cells for RNA isolation were frozen in liquid nitrogen immediately after removal and then stored at -80 °C. Total RNA was isolated by a single extraction with an acid guanidinium-thiocyanate-phenol-chloroform mixture (Ultraspec™-II RNA Isolation System, Biotecx, Houston, TX) described previously. The extracted RNA was then transferred to Hybond N nylon membranes (Amersham Int. Ltd., Buckinghamshire, UK) and fixed by UV-crosslinker. The blot membrane was stained with 0.05% methylene blue. The filters were rapidly prehybridized at 65 °C in hybridization solution (Quickhyb®, Stratagene, CA). The cDNA probes were pre pared by Megaprime™ DNA labeling systems (Amersham Int. Ltd., Bruchinghamshire, UK). Plasmids containing cDNA of SOD were supplied by Dr. Y. S. Ho and plasmids containing cDNA of catalase and glutathione peroxidase (GPX) were obtained from Dr. T. S. Chiou. Transformation in Escherichia coli, plasmid preparation, and cDNA purification were performed according to standard methods. cDNA were pre pared labeled with [α-32P]-dCTP using the multiprime DNA labeling system (Amersham Int. Ltd, Bruchinghamshire, UK). The cDNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes were added directly into the prehybridization solution (Quickhyb®, Stratagene) at a radioactivity of 1 × 10⁶ cpm/(min.mL). Hybridization was performed at 68 °C for 70 min. After washing, the wet blot membrane were sealed in plastic foil and exposed to medium-sensitive medical X-ray film (Biomax MR-1, Kodak, NY) at -70 °C using in ten sifier screens. Exposure times were 2-3 days for Northern blots. Hybridization in ten sity of autoradiographic signals was measured using two-dimensional densitometry (Vilber Lourmat, Cedex 1, Marne LA Vallee, France). The obtained densiticy (optical unit) was calculated vs the value of Northern blot for GAPDH to quantify the mRNA.

**Statistics**

All values are presented as means ± SEM from each group. Statistical significance was evaluated using one-way ANOVA when multiple groups were compared; where only 2 groups were compared, Student t-test was used. A value of p < 0.05 was considered to be significant.

**Results**

**Effect of catechin on cell number of cultured PC-12 cells**

In the present study, the number of surviving cells was counted under a light microscope with a counter. The PC-12 cells were divided into 4 groups: the control group incubated with culture medium containing 0.5% EtOH (vehicle) and treatment groups (catechin) incubated with culture medium containing catechin at 3 concentrations. After 7 days of culturing, the cell number of PC-12 cells in the control group was (18.9...


± 1.6) \times 10^6 \text{ cells and 1} \, \mu M \text{ catechin with (19.2 ± 1.8) \times 10^6 \text{ cells, at 10} \, \mu M \text{ catechin with (19.6 ± 1.4) \times 10^6 \text{ cells, and at 100} \, \mu M \text{ catechin with (19.4 ± 1.2) \times 10^6 \text{ cells. There was no significant difference in cell number of PC-12 between groups in cultured with catechin and control group after 7 days culture.}

Effect of catechin on the activity of superoxide dismutase in cultured PC-12 cells

The activities of SOD in PC-12 were influenced by catechin measured from eight experiments. After two days of incubation, the SOD activity did not reveal any significant change at a concentration of 1 \, \mu M. However, at concentrations of 10 and 100 \, \mu M, catechin slightly increased the activity of SOD, including MnSOD and CuZnSOD (data not shown). After 7 days of incubation, catechin slightly increased the activity of MnSOD in PC-12 in a concentration-dependent manner, and this in increase of MnSOD activity was more significant than that of CuZnSOD (Table 1).

Effect of catechin on the mRNA of SOD in cultured PC-12 cells

Representative responses of mRNA levels in PC-12 cells to the incubation of catechin were measured from eight experiments and are shown in Fig. 2.

![Fig. 2. Northern blot of total RNA (30 \mu g/ lane) from control and catechin-incubated samples probed for copper zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), catalase (CAT) and glutathione peroxidase (GPX) to compare with the internal standard GAPDH. Lane 1 shows the control, and lanes 2-4 indicate the response to 2-day incubation with catechin at 1, 10 and 100 \mu M, respectively.](image)

Table 1. Changes of SOD activity (IU/mg protein) in PC-12 cells after 7 days of incubation with catechin

<table>
<thead>
<tr>
<th>Enzymatic activity</th>
<th>Catechin (0 \mu M)</th>
<th>Catechin (1 \mu M)</th>
<th>Catechin (10 \mu M)</th>
<th>Catechin (100 \mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuZnSOD</td>
<td>64.6 ± 4.6</td>
<td>60.8 ± 3.9</td>
<td>63.2 ± 5.3</td>
<td>59.8 ± 4.2</td>
</tr>
<tr>
<td>MnSOD</td>
<td>14.2 ± 2.2</td>
<td>18.2 ± 2.4(^a)</td>
<td>22.2 ± 3.2(^b)</td>
<td>25.6 ± 2.8(^b)</td>
</tr>
</tbody>
</table>

\(^a\) p < 0.05; \(^b\) p < 0.01 compared with 0 \mu M catechin. CuZnSOD = copper zinc superoxide dismutase; MnSOD = manganese superoxide dismutase.

Table 2. Changes of SOD mRNA of PC-12 cells after 2 days and 7 days of incubation with catechin

<table>
<thead>
<tr>
<th>SOD mRNA</th>
<th>Catechin (2 days)</th>
<th>Catechin (7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 \mu M</td>
<td>1 \mu M</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>100</td>
<td>98.7 ± 4.5</td>
</tr>
<tr>
<td>MnSOD</td>
<td>100</td>
<td>96.5 ± 6.5</td>
</tr>
<tr>
<td>CAT</td>
<td>100</td>
<td>99.1 ± 5.6</td>
</tr>
<tr>
<td>GPX</td>
<td>100</td>
<td>101.5 ± 5.4</td>
</tr>
</tbody>
</table>

\(^a\) p < 0.05; \(^b\) p < 0.01 compared with 0 \mu M catechin. CuZnSOD = copper zinc superoxide dismutase; CAT = catalase; MnSOD = manganese superoxide dismutase; GPX = glutathione peroxidase.
The responses of SOD were quantified using GAPDH as an internal standard as indicated (Table 2). Similar to the changes of activity, the mRNA levels of SOD, for both CuZnSOD and MnSOD were slightly elevated by catechin with two days in culture at concentrations 10 and 100 µM. However, after seven days in culture with 10 and 100 µM catechin, the CuZnSOD mRNA level did not show any changes, whereas the MnSOD showed further increase (Table 2).

Discussion

It has been two decades since the first report was published demonstrating that the activity of MnSOD was diminished in transformed cells when compared to an appropriate normal cell control. Since that time, numerous papers have been published showing altered levels of antioxidant enzymes in tumor cells. Tuor cells are nearly always low in MnSOD and CAT activity, and usually low in CuZnSOD activity.

Mitochondrial manganese superoxide dismutase (MnSOD) is a defensive antioxidant enzyme which protects cells towards oxy-radical species generated during oxidative metabolism and exposure to several chemical (redox-cycling drugs), physical (radiation) and biological (cytokines) agents. Usually, tumor cells, which are generally characterized by areduction in the expression of this enzyme, are more sensitive than normal cells to treatments per formed with such agents of ten used in the therapy of hu man neoplasia. Thus, a decrease in MnSOD appears to be unfa vorable, and con sequently an in increase should be benefi cial to tu or cells. In deed, several reports have been published showing that several classes of tumors, in stead of showing a reduction in the activity of MnSOD, are char ac ter ized by an over-expression of this en zyme, often in a grade-related fashion.

Green tea, oolong tea and black tea are origi nally derived from the same plant, Camellia sinensis, green tea being the non-oxidized/nonfermented product that contains several tea polyphenolic compounds such as (-)-epigallocatechingallate and catechin. An in vitro antioxidemolecular study conducted at Saitama, Japan, beginning from 1986, evaluated the effect of green tea consumption and cancer prevention. During the first 9 years, 384 of the cohort members, 220 males and 164 female, were found to have cancer. These caner patients, male and female, were divided into three groups, by the amounts of daily green tea consumption: under three cups, four to nine cups and over 10 cups per day. Data showed that the first evidence that cancer on set of the patients who had consumed over 10 cups of green tea per day was 3.0 years later among males and 8.7 years later among females than that of patients who had consumed under 3 cups per day. This sug gests that many human cancers can be pre vented, or at least the onsets delayed, by daily consumption of a beverage familiar to all of us.

Catechin is a hydrophilic naturally existing antioxidant that can penetrate human cells well into the mitochondria, so it could probably act effectively on MnSOD and potentiate its activity. We propose that this phenomenon should be beneficial to human cells, which could further explain the presently existing evidence that tea catechins might have tumor-preventive effect.

In conclusion, the present data reveal that tea catechin can slightly increase the MnSOD activity and mRNA level in PC-12 cells. This finding may shed further light on this natural antioxidant and its related tumor prevention effect.

References

6. Fujiki H, Suganuma M, Okabe S, Komori A, Sueoka E,


