GROWTH INHIBITION BY A GREEN TEA STANDARDIZED EXTRACT (POLYPHENON E) IN PROSTATE CANCER CELLS

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Abstract
Objective: Green tea consumption has been shown to exhibit cancer-preventive activities in preclinical studies. Polyphenon E (Poly E) is a green tea standardized extract. This study was undertaken to examine the antiproliferative effect and pro-oxidant activity of Poly E on PC3 prostate cancer cells. **Experimental Design and results**: - PC3 prostate cancer cells were used as model system. Treatment of PC3 cells with 30 and 100 μg/ml Poly E significantly decreased cell viability and proliferation. At all tested concentrations, Poly E elicited pro-oxidant effect at 30 and 100 μg/ml. This effect of Poly E is consistent with the observed cytotoxicity, thus establishing a correlation between pro-oxidant activity and the antiproliferative effect of Poly E in PC3 cells. **Conclusion:** Our data showed the antiproliferative effect of Poly E and suggest Poly E-induced pro-oxidant effect involved in this activity.
Key words: *Polyphenon E, pro-oxidant effect, prostate cancer cells.*

1. INTRODUCTION

Prostate cancer (PCa) is one of the most frequently diagnosed male cancer in the Western countries and continues to represent a major cause of cancer-related mortality, despite medical advances. Asian-Americans seem to be at the lowest risk for PCa [9]. About less than 10% of PCa has been shown to be inherited suggesting that a variety of genetic and environmental factors may be important contributions to PCa development [17]. The Asians appear to have the lowest risk of developing PCa which may be due to consuming specific dietary constituents daily over many years. Over the last two decades many epidemiological studies, both cohort and case-control studies, have suggested that green tea consumption correlates with a lower risk of certain cancers such as breast, colon, and prostate [7].

Green tea contains many polyphenols, which include flavanols, flavandiols, flavonoids and phenolic acids. Most of the green tea polyphenols are flavanols, commonly known as catechins [1]. EGCG is the major catechin in green tea, which possesses antioxidant, anti-mutagenic, anti-proteolytic and anti-proliferative activity [14]. While many studies have focused on the effects and mechanism of EGCG on various cell types, the effects of Polyphenon E (Poly E) on tumor cells, as well as its mechanism of action, have to be elucidated yet. Polyphenon E is a well-defined pharmaceutical-grade mixture of polyphenols that contain about 50% EGCG and 30% other catechins [2]. Since the formulation is highly reproducible and easily prepared, Poly E is an attractive derivative of green tea for clinical chemoprevention trials [16].

In the present study, we show that Poly E, a green tea standardized extract can inhibit PC3 cell growth. We also demonstrate that Poly E can induce pro-oxidant effect, suggesting a correlation between pro-oxidant activity and the antiproliferative effect of Poly E in PC3 cells.

2. MATERIALS AND METHODS

Reagents

Polyphenon E (Poly E), a green tea standardized extract was manufactured by Mitsui Norin Co. Ltd. (Shizuoka, Japan). Poly E was dissolved in PBS plus or cell medium with 2.5% FBS.

2.1 Cell culture and treatments

PC3 human prostate cancer cells from ATCC (Rockville, MD) were cultured in Fk_{12} nutrient mixture 1X (Invitrogen, Carlsbad, CA) respectively, supplemented with 7% fetal bovine serum (FBS) and penicillin G (100 U/ml), streptomycin (100 μg/ml) and 0,25 μg/ml amphotericin B. Cells were maintained at 37°C and 5% CO_2 in a humid environment.

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The PC3 (70-80% confluent) were treated with Poly E (10, 30, and 100 µg/ml) with different time points depending on experiments. Cells used as controls were incubated with the vehicle only.

2.2. Cell viability assay (ATP assay)

Cell viability assay was assessed by using the CellTiter-Glo®Luminescent Cell Viability Assay. This is a homogeneous method to determine the number of viable cells in culture based on quantitation of the produced ATP, which signals the presence of metabolically active cells. Briefly, after treatment for 24 h, equal volume of CellTiter-Glo reagent was added directly to the wells. These contents were mixed for 2 minutes to induce cell lysis. Plates were incubated at room temperature for 10 minutes to stabilize luminescent signal. The luminescence was measured on microplate reader (Tecan). The results are expressed as percent of control.

2.3. Cell metabolic assay (MTT assay)

Cell metabolic activity was assessed in 96-well plates (BD Falcon) by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega, Madison, WI). After treatments for 24 h, cells were added with 20 µl MTT solution (5 mg/ml) in medium M199 and incubated at 37°C in a cell incubator for 60 min. At the end of the incubation period, the medium was removed and cell monolayer was washed twice with HBSS. The converted dye was solubilized with acidic isopropanol and plates were analyzed at 570 nm with background subtraction at 650 nm. Results were expressed as a percent of control.

2.4. Cell proliferation assay (BrdU incorporation Assay)

Cell proliferation was assessed by using chemiluminescent immunoassay, which based on the measurement of BrdU incorporation during DNA synthesis. Confluent cells was treated and cell proliferation was evaluated at 24 h. BrdU is added to cells cultured in microplates, followed by incubation for 10 hours. After the culture supernatant is removed, cells are fixed by Fix-Denat solution for 30 min. Fix-Denat was discarded and cells was incubated with an anti-BrdU antibody (anti-BrdU-POD) for 90 min. After rinsing three times with washing buffer, substrate solution was added and allowed to react for 6 min at room temperature. Light emission was read by using a microplate reader. Results were expressed as means ± SD.

2.5. Measurements of intracellular ROS

Intracellular ROS levels were determined by using the ROS molecular probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) [12]. After treatments, cells were incubated for 30 min with PBS plus containing 1µM H_2DCF-DA, then washed twice with PBS and fluorescence was measured with a plate reader (Tecan). Results were corrected for background fluorescence and protein concentration and expressed as a percent of untreated cells.

2.6. Statistic

Data are expressed as mean±SD of four different experiments. One-way analysis of variance followed by a post hoc Newman-Keuls multiple comparison test was used to detect differences of means among treatments with significance defined as $P< 0.05$ (GraphPad Prism version 5.00).

3. RESULTS

3.1. Dose-dependent effect of Poly E on cell viability and metabolic activity of PC3

Cell viability was evaluated by using the ATP assay. Cells were stimulated with increasing concentrations (10, 30, 100 µg/ml) of Poly E for 24 h, while untreated cells were used as control (CTRL). Although a reduction of cell viability was observed at a concentration of 10 µg/ml Poly E, it is not significant compared to the control. In contrast, the treatment of cells with the higher concentrations (30, 100 µg/ml) of Poly E, significantly lowered the viability of cells in compa rison to the untreated ones (Fig.1).

Similar to the observed cell death, a significant decrease in cell metabolic activity was induced by both 30 and 100 µg/ml of Poly E, as depicted by the data reported in Fig.1 obtained with the MTT assay. Moreover, a correlation was evident between MTT and ATP data. Results are expressed as percent of untreated controls.

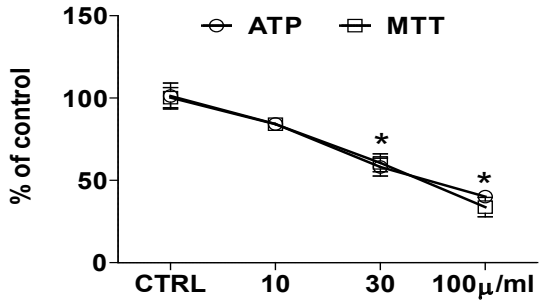

Fig. 1. Confluent PC3 cells were stimulated by Poly E for 24 h with various concentrations. Cell viability and cell metabolic activity were assessed after treatments by ATP assay and MTT assay. The results are expressed as percent of control. *Significantly different from the control ($p< 0.05$).

3.2. Dose-dependent effect of Poly E on PC3 cell proliferation

Further investigation on the cytotoxicity of Poly-E on PC3 was conducted by using the BrdU assay. Cells were treated with different concentrations of Poly E and cell proliferation was assessed after 24 h. As reported in Fig. 2, 24 h treatment of Poly E induced a dose-dependent decrease in the DNA synthesis of PC3. Consistent with data of previous experiments, this result demonstrated that Poly E is inhibiting the proliferation and inducing cell death in PC3 at both concentrations 30 and 100 µg/ml. Results are expressed as means ± SD.

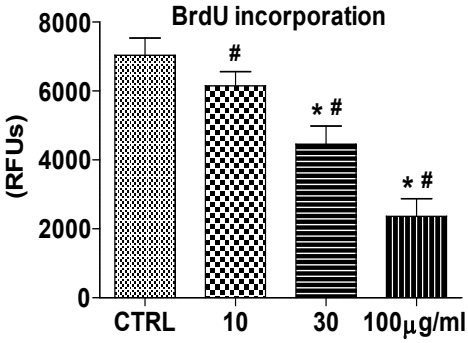

Fig. 2. Confluents PC3 were treated with different concentrations of Poly E for 24 h. Cell proliferation was evaluated by using BrdU assay. Quantification of cell proliferation in cultured PC3 in the absence (CTRL) or presence of the indicated treatments. Poly E caused dose-dependent inhibition of proliferation of PC3. *Significantly different from the control, #significantly different from each other ($p< 0.05$).

3.3. Dose and time-dependent effects of Poly E on PC3 ROS levels

To gain further mechanistic insight on the effect Poly E upon PC3 cell we assessed the potential variation of intracellular ROS levels. Intracellular ROS generation was examined in PC3 in response to Poly-E using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). This probe enters the cells and is oxidized in the presence of ROS, generating the fluorescent compound, DCF. To determine the effects of Poly E on PC3, cells were treated with the previously indicated concentrations and intracellular ROS levels were assessed after 2 h of stimulation, by a fluorescence detector. Fig. 3A shown that ROS levels were significantly increased by the Poly E treatment at dose of 30 and 100µg/ml. The observed pro-oxidant effect is consistent with the previously reported cytotoxicity results, suggesting the Poly E-induced pro-oxidant effect as responsible for the reported PC3 death.

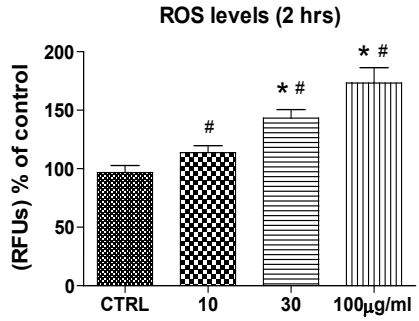

Fig. 3A. Confluent PC3 cells were stimulated with various concentrations of Poly E. ROS levels were assessed 2 h after treatment. Quantification of ROS levels in cultured PC3 in the absence (CTRL) or presence of the indicated treatments. The results are expressed as percent of control. *Significantly different from the control, #significantly different from each other ($p< 0.05$).

With dose of 30 µg/ml, we next assess ROS generation with the intent to investigate a potential time-dependent effect of Poly E at 2 h, 6 h, 12 h. Fig. 3B shown that ROS were increasingly generated after 12 h of Poly E treatment in PC3.

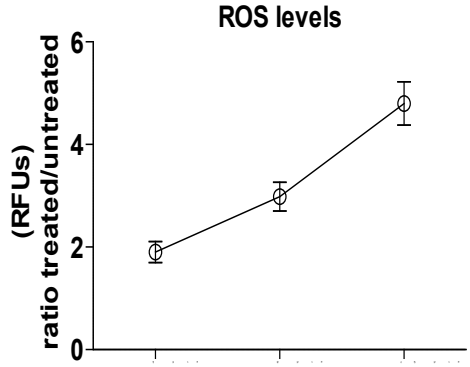

Fig. 3B. ROS levels were assessed after 2 h, 6 h and 12 h of treatment with 30 µg/ml of Poly E. The results are expressed as ratio treated and control.

4. DISCUSSION

Tea, next to water, is the most widely consumed beverage in the world. The tea plant Camellia sinensis has been cultivated in Asia for thousands of years, and green tea has been used for centuries in China, Japan, and Thailand as a traditional medicine with a variety of applications. Green tea possesses anti-carcinogenic effects, such as inhibition of growth proliferation, induction of apoptosis, induction of phase II detoxifying enzymes, and reduction of oxidative damage to DNA [11]. Several studies have more specifically shown

that consumption of green tea polyphenols is associated with decreased risk and/or slower progression of prostate cancer [7, 11]

EGCG, the most abundant catechin in green tea, has been shown to be the main effector of the anti-carcinogenic properties. For this reason, EGCG is the most commonly studied green tea catechins (GTCS). However, whole mixtures of GTCs may more accurately reflect the human consumption of green tea, possibly due to the fact that tea constituents other than catechins may also have anti-carcinogenic activity [3]. Poly E, a decaffeinated pharmaceutical preparation of tea catechins that contains approximately 50% EGCG and 30% other catechins may be preferable to EGCG. The catechins in this mixture may exert synergistic effects [15]. In addition, the effects of Poly E on tumor cells and its mechanisms of action are poorly known [16]. In the present study, we examine the effects of Poly E on PC3 prostate cancer cells. Cell viability, cell metabolic activity and cell proliferation were evaluated by ATP, MTT and BrdU assay, respectively. The results demonstrate that Poly E is able to induce loss of both viability and inhibition of PC3 DNA synthesis in dose-dependent manner (Fig.1-2). At concentration of 30 and 100µg/ml, a cytotoxic effect is shown as judged by the low viability. Similar effects of this compound were seen in studying the colon cancer, where Poly E preferentially inhibited growth of the Caco2, HCT116, HT29, SW480, and SW837 colon cancer cells compared to the FHC normal human fetal colon cell line [15]. It has been also demonstrated that Poly E inhibited proliferation of immortalized Barrett's cells as well as various adenocarcinoma cells by suppressing cyclin D1 expression through both transcriptional and post-translational mechanisms [16].

While tea and other plant polyphenols are generally considered as antioxidants [10], it is known that tea polyphenols also have pro-oxidant properties [5]. The pro-oxidant effect of green tea polyphenols has been described in vitro [6]. It has also been reported that EGCG may induce the production of H_2O_2 in the culture media [21]. Inhibition of cancer cell viability and induction of apoptosis by green tea polyphenols in vitro appear to be, in part, due to the production of ROS. Treatment of HL-60 cells with 50 µM EGCG caused the generation of ROS and a concomitant increase in apoptosis [4].

In our experiments, the pro-oxidant effect of Poly E was assessed by ROS generation. The results show that Poly E causes a rapid and significant ROS generation in PC3 cells at 2 h (Fig. 3A) and ROS reached a maximum level at 12 h (Fig. 3B). Interestingly, at cytotoxicity-inducing concentrations, Poly E causes ROS formation. ROS includes free radicals such as superoxide, hydroxyl radical, and non radical derivatives of oxygen such as hydrogen peroxide [8]. ROS are essential for normal cell function where they play key roles in regulating signal transduction events, enzyme activity, and cytokine production [20]. Indeed, ROS have been shown to be involved in regulation of both cell death and survival [18]. Cancer cells become more dependent on increased ROS levels and a highly functional antioxidant system than healthy cells, and as a consequence, they are more sensitive to agents that deteriorate antioxidant capacity or induce further oxidative stress levels [13]. When ROS reach a toxic threshold, they can trigger cancer cell death [19]. In our study, ROS level are increasingly generated and this is the evidence that Poly E elicited potential pro-oxidant effect at concentrations 30 and 100 µg/ml on PC3 cells. This activity is consistent with observed cytotoxicity, suggesting the Poly-E-induced pro-oxidant effect as responsible for the reported PC3 death.

5.CONCLUSION

Collectively, our data show the antiproliferative effect of Poly E in prostate cancer cells and suggest the pro-oxidant effect of Poly E involved in this activity. However, further investigation will be necessary to better elucidate the molecular mechanisms at the basis of the anticancer effect of Poly E.

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