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Theaflavins induce G2/M arrest by modulating expression of p21$^{waf1/cip1}$, cdc25C and cyclin B in human prostate carcinoma PC-3 cells

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Abstract

Cancer of the prostate gland (PCA) is the most common invasive malignancy and is the second leading cause of cancer-related death in males. The polyphenolic constituents of black tea have gained considerable attention as chemopreventive agents. Many studies have shown that black tea reduces the risk of several cancer types. In the present study, we studied the effect of a black tea polyphenol, theaflavin (TF), on cellular proliferation and cell death in the human prostate cancer cell line, PC-3. We showed that TF inhibits cell proliferation in a dose- and time-dependent manner. Studies on cell cycle progression have shown that the anti-proliferative effect of TF is associated with an increase in the G2/M phase of PC-3 cells. Western blot results showed that TF-induced G2/M phase arrest was mediated through the inhibition of cyclin-regulated signaling pathways. TF induces cyclin kinase inhibitor p21$^{waf1/cip1}$ expression and inhibits cdc25C and cyclin B expression. Increased exposure time to TF caused apoptosis of PC-3 cells, which was associated with up-regulation of the pro-apoptotic proteins Bax, caspase-3 and caspase-9 and down-regulation of anti-apoptotic protein Bcl-2. The role of caspase-induced apoptosis was further confirmed by a reduction in mitochondria membrane potential and the appearance of a DNA laddering pattern. Thus, it can be concluded that TF acts as an effective anti-proliferative agent by modulating cell growth regulators in prostate cancer cells.

Keywords: Prostate cancer; TF; PC-3; Cell cycle; Apoptosis

Introduction

Prostate cancer (PCA) is a major public health concern and a leading cause of cancer-related deaths among males in the United States (Adhami et al., 2003). It has been estimated that 218,890 new cases of PCA will be diagnosed in the USA alone in 2007, and 27,050 men will die from this disease in 2007 (Jemal et al., 2007). Although Asians have the lowest incidence and mortality rates of prostate cancer in the world, the rates have risen rapidly in the past two decades in many Asian countries (Pu et al., 2004). In India, the incidence of PCA ranges from 5.0–9.1 per 100,000 (Hebert et al., 2006). Despite the substantial morbidity and mortality associated with the disease, advances in radical prostactomy and external radiotherapy techniques have not been fully effective in the treatment of the disease.

These reports have led to the hypothesis that aspects of life style, such as dietary habits, are capable of altering the progression of PCA. Understanding the mechanisms involved in the progression of PCA from a latent to a clinically relevant form and discovering compounds that affect these pathways will be helpful in the management of PCA (Klein and Fischer, 2002). In recent years, many dietary agents have been described that show a wide range of anti-cancer effects on in vitro and in vivo prostate carcinogenesis systems. One such agent is tea (*Camellia sinensis*, a member of the botanical family theaceae), which is the most popularly consumed beverage in the world next to water (Saleem et al., 2003).

Tea polyphenols comprise about one-third of the weight of the dried leaf, and they exhibit biochemical and pharmacological properties that include antioxidant activities, inhibition of cell proliferation, induction of apoptosis, cell cycle arrest and modulation of carcinogen metabolism (Kalra et al., 2005;

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Ahmad et al., 1997). Studies conducted on cell culture systems and animal models reported that black tea polyphenols provide protection against a variety of cancer types (Siddiqui et al., 2006; Banerjee et al., 2005; Chandra Mohan et al., 2005; Larsson and Wolk, 2005; Wu et al., 2003; Zhou et al., 2003; Mukhtar and Ahmad, 2000) Treatment of human leukemic cell lines, HL-60 and K-562, with tea polyphenols resulted in an induction of apoptosis through activation of caspases-3 and -8, down-regulation of Bcl-2 and up-regulation of Bax proteins (Kundu et al., 2005). The tea polyphenols have been shown to be involved in cell signaling transduction events associated with the MAP kinase, AP-1, and NF-kappa B pathways. Recently, we showed that TF can induce apoptosis of androgen-sensitive LNCaP cells through induction of p53 and down-regulation of NF-kappa B and MAP kinases (Kalra et al., 2007).

Several epigenetic alterations that lead to constitutively active mitogenic and cell survival signaling pathways, as well as loss of the apoptotic response, are involved in uncontrolled growth of PCA, leading to androgen-independent growth, apoptosis resistance and increased expression and secretion of angiogenic factors (Kish et al., 2001). Therefore, one targeted approach for PCA prevention, growth control and/or treatment could be the inhibition of molecular events involved in PCA growth, progression and apoptosis resistance. In light of the above, we examined the anti-proliferative apoptotic effects of TF on androgen-independent PC-3 cells. Next, we employed Western blots to gain further insight into protein expression patterns that might play a role in the regulation of these cellular events.

Materials and methods

Cell cultures and chemicals

Human prostate cancer cells PC-3 were obtained from National Centre for Cell Science (Pune, India) and cultured in Dulbecco's Modified Eagle Media (DMEM) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Rhodamine 123, propidium iodide (PI), purified TF (>98% pure) and β-actin (clone AC-74) were purchased from Sigma (St Louis, USA). The cdc2, cdc25C, $p21^{waf1/cip1}$, cyclin B, caspase-3, caspase-9, Bcl-2 and Bax antibodies were procured from Cell Signaling Technology, Inc (Danvers, MA, USA). The anti-rabbit horseradish-peroxidase conjugated secondary antibodies were obtained from Bangalore Genei (Bangalore, India). The polyvinylidene fluoride (PVDF) membrane was obtained from Millipore (Bedford, Massachusetts, USA). The rest of the chemicals were of analytical grade and were procured locally.

Treatment of cells

TF dissolved in DMEM was employed for the treatment of cells. The stock solution (1 mg/ml) was filtered under sterile conditions. Working solutions were prepared by diluting the stock solution in DMEM to get the desired concentrations. For dose-dependent studies, the cells (50% confluent) were treated with different concentrations of TF in complete cell medium. Cells with no TF treatment served as a control.

Cell viability assay

The effect of TF on the viability of PC-3 cells was determined with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay (Mosmann, 1983). The cells were plated at 1×10^4 cells per well in 200 μl of complete culture medium and treated with various concentrations of TF in 96-well microtiter plates. Each concentration of TF (10–100 μg/ml) was repeated in ten wells. After incubation for 24, 48 and 72 h at 37 °C in a humidified incubator, cell viability was determined. MTT (5 mg/ml in phosphate buffered saline) was added to each well and incubated for 5 h; after this, the plate was centrifuged at 1800 rpm for 5 min at 4 °C. The supernatant was removed from the wells by aspiration. After careful removal of the medium, 0.1 ml buffered DMSO was added to each well, and the plates were shaken. Absorbance was recorded on a microplate reader (Biotek Instruments Inc. Winooski, VT) at a wavelength of 530 nm. The effect of TF on growth inhibition was assessed as percent cell viability, where cells with no treatment were considered 100% viable.

Cell cycle analysis

The cells were grown at a density of 1×10^6 cells in 75 cm sq culture dishes and then treated with different concentrations of TF (40–80 μg/ml) for 24 and 48 h. The cells were trypsinized, washed twice with cold PBS and centrifuged. The cell pellet was re-suspended in 50 μl cold PBS and fixed in 2 ml 70% ice cold ethanol. Cells were centrifuged from the fixative and treated with 0.1% Triton X-100 for 5 min. After incubation, cells were again centrifuged and re-suspended in 1 ml PBS; ribonuclease (100 μg/ml) was added, and the cells were incubated at 37 °C for 30 min. After further centrifugation, cells were re-suspended in 1 ml PBS and 50 μg/ml PI and incubated for 30 min at 4 °C. The data were acquired and analyzed on flow cell cytometer (BD-LSR II) using Cell Quest software (Nicoletti et al., 1991).

Mitochondrial membrane potential determination (Δψ)

The untreated and treated PC-3 cells were incubated with rhodamine 123 (5 μg/ml) for 60 min in the dark at 37 °C, harvested and suspended in PBS. The mitochondrial membrane potential was measured using flow cytometry by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells (Bai et al., 1999).

Cell lysate preparation

After treatment with or without TF, $3–5 \times 10^6$ cells were washed with 0.5 ml PBS and suspended in cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, pH 7.4) with freshly added protease inhibitor cocktail (Protease inhibitor Cocktail Set III; Calbiochem, La Jolla, CA, USA) on ice for 30 min for total cell lysate preparation. After incubation, cells were centrifuged at 12,000×g for 10 min at 4 °C. The