RESEARCH COMMUNICATION

Black Tea Polyphenols Restrict Benzopyrene-induced Mouse Lung Cancer Progression through Inhibition of Cox-2 and Induction of Caspase-3 Expression

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Abstract

Lung cancer is one of the leading causes of cancer related death in most developed and many developing countries of the world. Due to lack of validated screening methods and poor prognosis, treatment of lung cancer has not improved significantly over the last two decades. Therefore the risk of the disease needs to be minimized by preventive measures. One approach for lung cancer prevention envisages reversal or restriction of precancerous lesions by chemopreventive intervention. It demands a deeper understanding of the pathogenesis of the disease and identification of the ideal point of intervention. In the present investigation, tea components, epigallocatechin gallate (EGCG) and theaflavins (TF) were assessed for their chemopreventive potential when administered in the post initiation phase of lung carcinogenesis in an experimental mouse model. Histopathological changes in lungs of mice administered benzo(a)pyrene (BP) were followed serially and correlated with the expression of Cox-2, caspase-3 and caspase-7, which play key roles in histopathogenesis of neoplasia. The observations strongly indicate that both EGCG and TF can influence the expression of these genes to modulate the process of carcinogenesis, resulting in delayed onset and lowered incidence of pre-invasive lung lesions.

Key Words: Theaflavins - epigallocatechin gallate - lung carcinogenesis - chemoprevention - gene expression - Cox-2 - caspase-3 - caspase-7

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Introduction

Lung cancer is the leading cause of cancer mortality in most countries (Boring et al., 1992). It has reached alarming proportions both in developed and developing countries (Parkin et al., 2001). Reports from our institute also revealed that cancer of lung and bronchus tops the list of major male cancers of this region (Population Based Cancer Registry, 2001). Tobacco smoking is the major risk factor for lung cancer (Heeht et al., 1988) and benzo (a) pyrene (BP), one of the polycyclic aromatic hydrocarbons in tobacco smoke, is associated with human lung cancer and known to be carcinogenic in experimental systems (Zedeck MS, 1980; Hecht et al., 1988; Hecht et al., 1993; Yun et al., 1995).

Surgery and chemotherapy is the main treatment for most cancers but in case of lung cancer treatment outcome by these procedures is limited due to frequent recurrence. Therefore current approaches for control of lung cancer focus on prevention. Although tobacco cessation is important for lung cancer prevention, ex-smokers are still at risk. Management of lung cancer by chemoprevention through recommended intake of selected food items and beverages would be another practical alternative.

Tea (*Camellia sinensis*) is one of the most commonly consumed beverages worldwide. Tea polyphenols have superior antioxidant properties compared to any other antioxidant present in fruits and vegetables, known to mankind (Jhawar, 2000). Most of the green tea polyphenols are flavonols, commonly known as catechins (Ahmad and Hassan, 1999). These catechins, especially epigallocatechin gallate (EGCG), the major polyphenolic constituent of green tea, have been studied as a preventive substance for carcinogenesis (Fujiki et al., 1999). Black tea is more widely consumed than green tea and hence its role in prevention of diseases particularly cancer also needs to be investigated. Theaflavins are the characteristic polyphenolic constituents in black tea which are formed from oxidation of green tea flavanols (catechins and gallocatechins) by polyphenol oxidase during processing (Balentinc, 1992).

The present study was designed to evaluate the influence of black tea compounds EGCG and TF on the expression of proliferation associated gene Cox-2 and apoptosis associated genes caspase-3 and caspase-7 during progression of lung carcinogenesis in an experimental mouse model.

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Materials and Methods

Animals and chemicals

Newborn Strain A mice (outbred) 24-48 hours old were obtained from the animal colony of Chittaranjan National Cancer Institute (CNCI). They were maintained in plastic cages at an ambient temperature of 25±2°C, on 12 hour light / dark cycle with their mothers. After weaning, the male and female animals were identified and caged separately. All animals were maintained, handled and sacrificed following the guidelines of the animal ethical committee of the institute (Regn. No.: IAEC-1.2/SD-1/2001-2003).

Benzo (a) pyrene (BP), epigalocatechin gallate (EGCG) and theaflavins (TF) were procured from Sigma Chemical Co.; USA. Caspase-3 and Cox-2 rabbit polyclonal antibodies, caspase-7 goat polyclonal antibodies (Primary antibody), and anti-rabbit and anti goat IgG-HRP (Secondary antibody), were purchased from Santa Cruz Biotechnology, Inc. Other chemicals were purchased from Sigma Chemical Co.; USA., Merck India Ltd. and Roche Molecular Biochemicals, Manheim, Germany.

Induction of lung carcinogenesis in mice (Yun et al., 1995) and treatment with tea components

Each newborn mouse (24-48 hours) received subcutaneous injection in the sub scapular region with 0.02 ml of a suspension containing 0.2 mg of BP in 1% aqueous gelatin solution (single dose). The carcinogen was used within 1 hour after emulsification. The newborn animals were allowed to grow with their mothers, provided with water and food pellets (Lipton India Ltd.; India) *ad libitum*. After weaning (5th week) animals were divided into three groups:Group I, the carcinogen control group (BP administered) receiving distilled water (i.p) every day from fifth week of BP administration and continued up to the twenty-sixth week; Group II, the EGCG treated group (BP+EGCG) receiving (i.p) EGCG at a dose of 0.01 mg per mouse per day from the fifth week of BP administration up to the twenty-sixth week; Group III, the TF treated group (BP+TF) receiving (i.p) TF at a dose of 0.02 mg per mouse per day from fifth week of BP administration and continued upto twenty-sixth week.

Animals from each group were sacrificed in the 8th, 17th and 26th weeks for study of different parameters. The number of animals was 30 for each group (N=30; n-10 for each time point). Randomly selected six animals were used for histopathological analysis and the remaining mice were used for Western blotting analysis.

Preparation of tissue sections and histopathology

After sacrificing the mice from each group at the different time points, all five lung lobes from each mouse were collected, washed in PBS, soaked in blotting paper to remove the blood and fixed in 10% neutral buffered formalin for 24 hrs. The tissue samples were dehydrated in ascending concentrations of ethanol, cleared in xylene, and embedded in paraffin to prepare the block. All five lobes of lung were sectioned, mounted on slides, stained with hematoxylin-eosin (HE) and examined under light microscope.

Protein extraction and Western blot analysis

Protein was isolated for the analysis by Western blotting. Lungs were collected from both groups, homogenized in suspension buffer (20mM HEPES, 2mM EDTA, 2mM EGTA, 10% glycerol); PMSF (1mM); Aprotinin (1 mg/ml); Leupeptin (0.1M). Particulate fraction was prepared by centrifugation (13000 rpm for 1 hr.) and the amount of protein was quantified (Lowry et al., 1951). Polyacrylamide gels were loaded with 50μg of protein/lane and subjected to electrophoresis. The separated proteins were then transferred to Millipore Immobilion-P-membranes (PVDF), blocked with blocking buffer (100mM Tris-cl and 150 mM Nacl and 0.1% Tween 20) and incubated with a 1: 250 dilution of a specific primary antibody followed by 1:500 dilution of secondary antibody. Hybridized protein bands were then detected using luminol reagent (Santa Cruz Biotechnology, Inc) and immunoreactive proteins were quantified with a densitometer.

Statistical analysis

The data obtained from expression analysis of different proteins were tested using MS Excel and values among different groups were expressed as mean ±SD.

Results

BP induced early lung lesions in strain A mice had been studied previously in our laboratory and we had histopathologically identified hyperplasia, dysplasia and carcinoma in situ (CIS) in 8th, 17th and 26th week respectively (Banerjee and Das, 2004). In the present study we evaluated the effect of these important black tea polyphenols, EGCG and TF, on the incidence of hyperplasia, dysplasia, and CIS and the expression of proliferation associated genes Cox-2 and apoptosis inducing gene

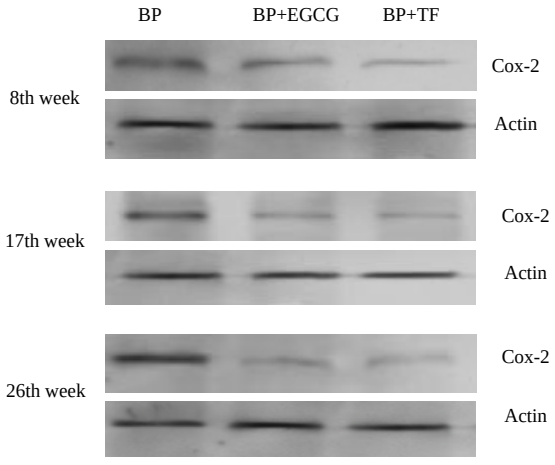

Figure 1. Inhibitory Effects of EGCG and TF on Benzo-[a]-pyrene Induced Cox-2 expression in Mouse Lung

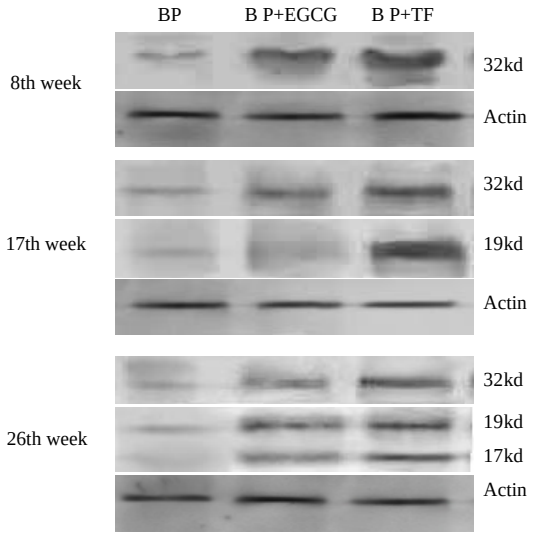

Figure 2. Effect of EGCG and TF on Caspase-3 Expression in Mouse Lung

caspase-3 and caspase-7.

While 83.3% of BP exposed mice had hyperplasia in lung tissue; this was reduced to 66.7% in the EGCG treatment group and 50% in the TF treatment group. Incidences of dysplasia noted on the seventeenth week were found to be 66.7% in BP exposed group, 50% in both EGCG and TF treatment groups indicating an inhibition by 25.0% after such treatment. Incidence of CIS in carcinogen control group was 66.7% whereas there was a reduction of incidence to 33.3% in EGCG treatment group and 16.7% in TF treatment group resulting 50% and 75% inhibition by such treatments, respectively.

The expression profiles of proliferation associated gene Cox-2 and apoptosis inducing genes caspase-3 and caspase-7 were analyzed at the same time point used for histopathological study.

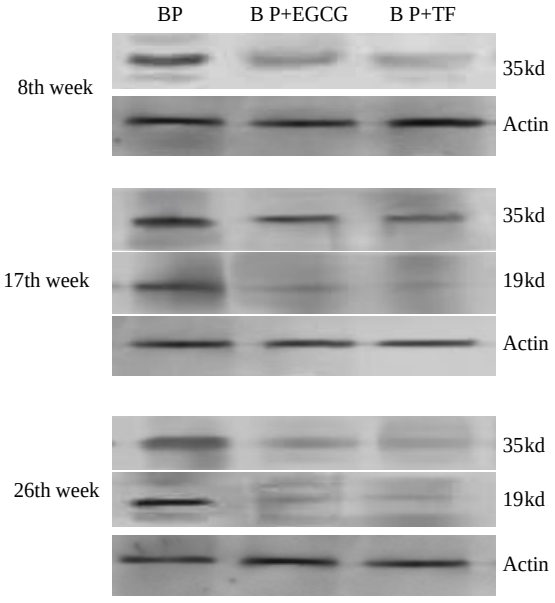

Figure 3. Effect of EGCG and TF on Procaspase-7 Expression in Mouse Lung

It may be noted from Fig1 that the Cox-2 expression in BP exposed mouse lung was reduced following treatment with EGCG and TF by 54.6% and 55.3% on 8th week; 26.9% and 65.99% on 17th week and 63.55% and 78.71% on 26th week, respectively.

Black tea components EGCG and TF not only upregulated the expression of precursor caspase-3 but also increased its activation. On the eighth week there was increase in expression of procaspase-3 by 60.5% and 72.8% respectively in EGCG and TF treated groups in respect of carcinogen control (Figure 2) but no active band was found at this time point. On seventeenth week and twenty -sixth weeks EGCG and TF not only increased the expression of procaspase-3 but its active band was also detected at both these time points. The increase in expression of procaspase-3 was by 56.3% and 61.3% on 17th week and 51.0% and 68.9% on 26th week respectively in EGCG and TF treated BP exposed mouse lung (Figure 2).

The expression of procaspase-7 was higher in BP administered group in comparison to the EGCG and TF treated groups in all respective weeks. Moreover, prominent active form of caspase-7 (19Kd) was found in BP administered group whereas very faint active band was noted in EGCG and TF treated groups on 17th week and 26th week (Figure 3).

Discussion

Experimental studies have revealed that the process of carcinogenesis can be modulated by timely intervention by administration of chemopreventive agent or by intake of protective agents through regular food and drinks. Tea is the second most popular drink in the world. Abundant experimental and limited epidemiological evidences have suggested that polyphenolic antioxidants present in green and black tea can reduce cancer risk (Katiyar and Mukhtar, 1996; Kohlmeier et al.,1997). Most of the studies showing the preventive effects of tea were however conducted with green tea; only a few studies assessed the effectiveness of black tea (Stoner and Mukhtar, 1995).

During BP induced lung carcinogenesis in mice distinct cellular and histopathological changes could be identified as hyperplasia, dysplasia, and carcinoma in situ and invasive carcinoma (Banerjee et al., 2005). Black tea compounds were found to inhibit or delay this progression. The incidence of hyperplasia was reduced after treatment with the two active components EGCG and TF. Likewise a significant inhibition was also observed on the incidence of dysplasia in the treatment groups. Impact of such restrictive effect was reflected as reduction of incidence of CIS in the treatment groups. Histopathological analyses of the different stages of lung carcinogenesis thus suggest a protective role of black tea component by restriction of the process of carcinogenesis. Significant inhibition of progression of NNK induced adenoma to adenocarcinoma was reported (Yang et al., 1997). Earlier investigation from our laboratory

demonstrated that black tea could protect from azoxymethane induced aberrant crypt formation, a precancerous condition in rat colon (Sengupta et al., 2003) and reduced the number of skin papiloma a benign precancerous growth induced by DMBA-croton oil in mouse (Saha and Das, 2002). The present investigation provides further evidences to suggest that active tea compounds viz. EGCG and TF significantly restricted progression of BP induced changes viz hyperplasia, dysplasia as well as carcinoma in situ and may prove to be useful agents for chemopreventive intervention for prevention of lung cancer.

Cyclooxygenase-2 (Cox-2) overexpression is found in a wide variety of human cancers, including lung cancer (Dannenberg and Subbaramaiah, 2003; Dubinett et al., 2003). Increased Cox-2 expression is associated with tumour invasion (Tsujii et al., 1998; Dohadwala et al., 2001; Dohadwala et al., 2002) and promotion of tumour cell resistance to apoptosis (Krysan et al., 2004). Regulation of Cox-2 over expression may be a reasonable target for chemoprevention of lung cancer. A study reported that benzo(a)pyrene can itself can up-regulate Cox-2 expression and prostaglandin E2 (PGE2) production (Kelley et al., 1997) and thereby enhance the rate of cell proliferation. Gradual elevation of Cox-2 expression during preneoplastic stages (hyperplasia, dysplasia and carcinoma in situ) of lung carcinogensis in BP exposed untreated group observed in the present investigation which is in accordance with observation made by others. There are reports to show that Ras/ ERK-MAPK, JAK-STAT, and P38 kinase signal transduction pathways are involved in the induction of gene expression of Cox-2 (Subbaramaiah et al.,1996; Yamaoka et al., 1998; McVicar et al., 1998) and that both tea polyphenols EGCG and TF inhibit all of these signal transduction pathways (Chung et al., 1998) which possibly suppress Cox-2 expression. Another investigation indicated that theaflavin 3_ monogallate supress the expression of Cox-2 (Lu et al., 2000). Inhibition of Cox-2 expression by EGCG and TF noted here is quite compatible with our observations. And the modulatory action of EGCG and TF during lung carcinogenesis is likely to be due to thus influence on Cox-2 expression.

Caspase plays an important role in cell death, is synthesized as proenzymes and must be proteolytically activated to produce the mature, catalytically active heterodimers, which specifically cleave target proteins (Barge et al., 1997). Both caspase–3 and caspase-7 is known to play a role during execution phase of apoptotic pathway as an effector caspase. (Kumar S, 1995; Patel et al., 1996; Nicholson and Thornberry,1997). Therefore upregulation of caspase-3 and -7 expressions is an important phenomenon for induction of apoptosis. Caspase-3 is generally present as an inactive p32 zymogen or procaspase-3 (Cohen GM, 1997), on induction of apoptosis it is processed to yield p19 and p17 subunit. In the present investigation expression of caspase-3 has been shown to be elevated in the treated groups on 8th week with respect to the BP administered group (untreated group). Further increase in both expression and activation of caspase-3 was also noted in EGCG and TF treatment groups indicating that the induction of apoptosis by active chemicals of black tea may be via the elevation of expression and or activation of caspase-3 protein. It is documented in literature that expression of caspase-3 is associated with apoptosis in non small cell carcinoma (Tormanen-Napankangas et al., 2001) and EGCG is capable of activating caspase-3 in human chondrosarcoma cells (Chen et al., 2000; Islam et al., 2000). Moreover EGCG can directly bind to the FAS death receptor to initiate Caspase-8 activation (Hayakawa et al., 2001) and ultimately activation of caspase-3, which leads to apoptosis. In spite of this FAS dependent pathway of apoptosis, EGCG may increase the mitochondrial membrane permeability and induce cytochrome C release, resulting in the activation of caspase-3 via Caspase-9 activation (Chen et al., 2003), which leads to apoptosis. Observations made in the present study do suggest that EGCG and TF may increase the rate of apoptosis via the elevation of caspase-3 expression and or activation. Previous investigation from our laboratory also revealed that black tea and its active compounds i,e EGCG and TF can induce apoptosis via caspase-3 activation (Das et al., 2004) during DMBA induced skin carcinogenesis. The results of the current study imply that EGCG and TF interfere in the process of programmed cell death through modulation of caspase-3 expression. But the result of caspase-7 contradicts previous / other studies where expression of caspase-7 was found to be increased as an executioner caspase to induce apoptosis (Nicholson et al., 1995; Fernandes-Alnemri et al., 1995; Thornberry et al., 1997).

Caspase-7 exists as an inactive p35 zymogen or procaspase-7 (Fernandes-Alnemri et al., 1995; Duan et al., 1996; MacFarlane et al., 1997). On induction of apoptosis it is activated to yield a large 19KD and small 10KD subunit (Fernandes-Alnemri et al., 1995; Duan et al., 1996; MacFarlane et al., 1997). Experimental evidences suggest that Fas induced apoptosis could activate the procaspase-7 (Chandler et al., 1998) and EGCG can induce the Fas mediated apoptosis which may activate or upregulate the expression of caspase-7 (Hayakawa et al., 2001). Therefore it was expected that the expression of caspase-7 would be increased in the EGCG, and TF treated groups in the present study. Interestingly the expression of procaspase-7 was found to be elevated in carcinogen control group along with their active p19 subunit but EGCG and TF treated groups showed lesser expression of caspase-7. There is no other report to draw a reference for this observation. It is likely that some other mechanism may be involved which upregulate the expression of caspase-7 in BP exposed untreated group and downregulate of its expression in treated group. Further studies are required to establish this contention.

The observations of our study strongly support the chemopreventive efficacy of black tea component; EGCG and TF along with a preliminary insight into the possible mechanisms through which it may exhibit its chemopreventive activity during post initiation stage of lung carcinogenesis in experimental mice.

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