RESEARCH COMMUNICATION

Tea-induced Apoptosis in Human Leukemia K562 Cells as Assessed by Comet Formation

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Abstract

Programmed cell death or apoptosis is a physiological process by which genetically damaged cells or undesired cells can be eliminated. Various morphological and molecular changes undergoing during the process of apoptosis are the formation of apoptotic blebs of the cell membrane, cell shrinkage, condensation of chromatin and the disruption of deoxyribonucleic acid (DNA) into typical fragments of multiples of 180 base pairs. These changes can be detected in a number of ways. DNA ladder formation, which is observed following gel electrophoresis technique although is widely accepted but does not reflect the DNA breakdown in individual cell and also may miss contributions from small sub-populations in a heterogeneous cell population. Alkaline comet assay as measured by single cell gel electrophoresis, on the other hand, accurately measures DNA fragmentation on a single cell level and allows analysis of subpopulation of cells. The assay was originally developed for measuring DNA damage of cells exposed to any genotoxic agent. However, the comet image generated by an apoptotic cell is different from that obtained with a cell treated for a short time with a genotoxic agent. Correlation of comet formation with various other established parameters of apoptosis is very important. The present study aims to correlate different features of apoptosis with the formation of comet tail in human leukemia K-562 cells using tea extracts. Apoptosis as measured by formation of apoptotic bodies, flow cytometric analysis, activation of caspase 3 and 8, and expressions of apoptosis related genes such as bcl-2 and bax showed high degree of correlation with comet tail moment. This indicates that comet assay can accurately reflect measure of DNA fragmentation and hence can be used to detect a cell undergoing apoptosis.

Key Words: Human leukemia cell K-562 - comet formation - tea extracts - caspase -bax bcl-2 ratio

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Introduction

Apoptosis or programmed cell death is a highly organized, genetically controlled physiological process of cell elimination. As an antagonist of cell proliferation, apoptosis contributes to keeping the cell number in tissues and organs constant, and helps to remove superfluous and damaged cells (Roser et al., 2001). This is a crucial process for eliminating cancer cells (Guchelaar et al., 1997). Thus, induction of apoptosis may be a useful approach in the management and prevention of cancer. On a morphological level, apoptosis is distinguished by unique features including cell shrinkage, membrane blebbing, formation of pyknotic nuclei and the disruption of deoxyribonucleic acid (DNA) into fragments of multiples of 180 base pairs (Wada et al., 2003; Page et al., 2000). These apoptotic changes can be detected by a number of conventional apoptosis assays. Double stranded DNA fragmentation giving the characteristic "ladder" pattern on agarose gel electrophoresis is one of the most widely accepted criteria used to characterize apoptotic cell death because it is discernible from the random single stranded DNA breaks produced by necrosis (Yaoita et al., 2000). However, this technique is difficult to quantify and not particularly sensitive as an extensive number of cells (greater than 10^6 cells/sample) is required in synchronous fragmentation to produce oligonucleosomal ladders (Page et al., 2000). At present, enzymatic techniques are used for detecting DNA fragmentation at the individual cell level, the most common being the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay (Kockx et al., 1998; Tateyama et al., 1998). But this assay also suffers from lack of specificity and can not distinguish between apoptotic and non apoptotic cell, as it labels DNA strand breaks from any insult (Singh, 2000).

The comet assay, also called the single cell gel electrophoresis (SCGE), is a technique for detecting fragmented DNA at the level of individual cell and allows

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analysis of sub-population of cells (Bacso et al., 2000). Although it was originally developed as a qualitative and quantitative genotoxicity test to detect alkali labile sites, single strand breaks and double strand breaks induced by genotoxic agents (Henderson et al, 1998), it has been possible to show that this method could be used for detecting DNA fragmentation in a cell undergoing apoptosis (Wada et al., 2003). In this case, the comet image observed is different from that seen with genotoxic agents (Olive et al., 1998). This assay thus may be used to detect a cell undergoing apoptosis and also for the quantitative estimation of apoptosis. It is important in this context to correlate other features of apoptosis with the formation of comet tail.

There has been an increasing interest in the role of apoptosis in tumor cell turn over and response to therapy. The possibility of modifying processes involved in apoptosis could represent new avenues for cancer therapy (Olive et al., 1993). Identification of effective chemo-preventive diets or dietary supplements for human use is of much interest in this regard. Evidence indicates that intake of diet containing polyphenolic compounds reduces risk of various types of cancer (Huang and Ferraro, 1992; Ferguson., 1994; Starvic, 1994; Surh, 1998). Tea is the most ancient and popular beverage in the world, next only to water and because of its rich polyphenolic contents it has become a subject of much attention in cancer control (Roy et al., 2001).It is evident from both epidemiological and experimental studies that green tea extract, which is rich in simple catechins like (-)-epigallocatechingallate (EGCG), (-)-epicatechingallate (ECG), (-)-epigallocatechin (EGC), and (-)-epicatechin (EC), possesses significant cancer chemo-preventive properties (Roy et al., 2001; Roy et al., 2002). In black tea, the most popular form of the beverage, the simple catechins are fermented, oxidized and polymerized to form thearubigins and theaflavins. Although most of the studies on inhibition of carcinogenesis and cancer cell growth have been conducted with green tea, there are also limited studies indicating black tea as well as theaflavins having significant cancer chemo-preventive properties (Hecht et al., 1989; Hibasami et al., 1998a; Kundu et al., 2005; Yang et al., 2000).

In the present study our attempt has been to study qualitatively and quantitatively the development of comet images during apoptosis induced by tea extracts and to establish a correlation between induction of apoptosis and comet formation. Apoptosis was measured by several assays such as formation of apoptotic bodies, flow cytometric analysis, activation of caspase 3 and 8, and expressions of apoptosis related genes such as bcl-2 and bax. In this study apoptosis was induced by green tea extract and extracts of two popular varieties of black tea, i.e., Darjeeling tea and Assam tea in human leukemia cell line K-562.

Materials and Methods

Chemicals

RPMI 1640, fetal bovine serum (FBS), gentamycin, penicillin, streptomycin, normal melting point agarose, low melting point agarose, acrylamide, bis-acrylamide and Tris were purchased from GIBCO-BRL, India. RNase A , propidium iodide (PI) , ethidium bromide , Triton X-100 , sodium dodecylsulfate (SDS) , CHAPS and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich, USA. Caspase 3 and caspase 8 kits were purchased from Santacruz Biotech, USA. Antibodies (mouse monoclonal) for Bcl-2 (clone 8C8) and Bax (clone 2D2) were procured from Neomarkers, USA. Proteinase K, goat anti-mouse IgG-alkaline phosphatase conjugate and BCIP-NBT were purchased from Bangalore Genei, India. Other reagents were of analytical grade and procured locally.

Cell culture

Human leukemia cell line K562 routinely maintained in our laboratory was used in these studies. Chronic myelogenic leukemia (K-562) cells was maintained in RPMI 1640 supplemented with 15% heat inactivated FBS and gentamycin (40 μg/ml), penicillin (100 units/ml) and streptomycin (10 μg/ml). Cells were grown at 37°C in a humidified atmosphere of 5% CO2/95% air.

Preparation of tea extracts

Tea extracts were prepared by soaking tea leaves in boiling water (2.5 g per 100 ml water) for 5 minutes and then lyophilized in a rotary evaporator. Green tea (GT) and two popular brands of black tea, i.e., Darjeeling tea (DT) and Assam tea (AT) were used and for the entire study same brands were used.

Treatment of cells

For each experiment in this study 10^6 cells were seeded in 55 mm tissue culture petri dish. Exponentially growing cells were treated with various concentrations of tea extracts for 24 h. after which cells were harvested, counted, washed in sterile PBS and experiments were performed.

Comet assay

Fragmented DNA was measured using the alkaline single cell gel electrophoresis assay. The method was essentially that of Singh et al (1990). Briefly, cells (1×10^3) were suspended in 0.6 % (w/v) low melting point agarose and layered over a fully frosted microscopic slide coated with a layer of 0.75 % normal melting point agarose. The slides were then immersed in a lysing solution of pH 10 at 40˚C and left overnight. The slides were presoaked in alkaline electrophoresis buffer (pH > 13) for 20 minutes. Electrophoresis was carried out for 20 min (300 mA, 25 V). The slides were washed thrice with a neutralizing solution (pH 7.4), stained with 50 mg/ml ethidium bromide and examined under a Nikon fluorescent microscope. For each slide, 50 randomly selected cells were analyzed both visually and with the image analysis system, comet assay software program (CASP). For quantitative evaluation, the 'tail moment' (defined as the product of tail length and the corresponding DNA content as measured by the intensity) was recorded.

Microscopic Examination

Treated cells were harvested, washed with PBS and to the cell pellet PI (50 µg/ml) was added and incubated in the dark at room temperature for 10 min. Cells were spread on a slide and nuclear morphology was examined under the fluorescence microscope.

Caspase activity

Aspartic acid specific cysteine proteases caspase 3 and caspase 8 were assayed by fluorimetric technique using fluorescent tagged peptides. Cleavage of synthetic substrates DEVD-AFC and IETD-AFC by caspase 3 and caspase 8 respectively releases AFC, and free AFC emits a yellow fluorescence at 480-520 nm (peak at 505 nm) upon excitation at 400 nm. Cell lysate was used to measure the activity following the protocol submitted by the supplier.

Flow cytometric analysis

Cell cycle of the treated cells was analyzed using a flow cytometer (Lee et al., 1999). After treatment, cells were harvested by centrifugation, washed with PBS and fixed in ice cold 80% ethanol for 1 h. The cells were centrifuged, the pellets suspended in PBS and treated with RNase A (final concentration 200 µg/ml) at room temperature for 30 min. PI (50 µg/ml) was added to the sample and kept in the dark for 30 min to stain DNA. The cell cycle was analyzed by a flow cytometer (Becton Dickinson FACS calibur) using a nitrogen argon laser operating at 480/500 nm excitation / emission and the fluorescence being captured on FL2H channel with logarithmic amplification. For each data point 10,000 cells were counted.

Western blot analysis

Cells after treatment were harvested in a lysis buffer (Tris-HCl 1 mM, pH 7.5; $MgCl_2$ 600 mM; EGTA 100 mM; b-mercaptoethanol 0.065%; CHAPS 10 %; glycerol 10 %). The protein concentration of the cell lysate was determined according to Lowry's method (1951). The supernatant containing 30 µg of protein was analyzed on a 12.5 % SDS-PAGE under reducing conditions. The gel was transferred on to a nitrocellulose membrane and the membrane was probed with primary antibodies (Bcl-2 and Bax) at 1: 2000 dilutions. The affinity purified antimouse IgG conjugated to alkaline phosphatase (1:2000 dilutions) was used as secondary antibody. NBT/BCIP was used as a substrate for alkaline phosphatase to visualize the protein bands.

Results

When cultured leukemia cells (K-562) treated with tea extracts were stained with PI and examined under a fluorescence microscope, characteristic apoptotic features such as chromatin condensation and nuclear fragmentation were observed. One hundred cells were scored at random and classified into apoptotic and non-apoptotic cells based on the above criteria. Their ratio designated as apoptotic index (AI) was found to be increased with increasing concentrations of each of the tea extract. Both the green and black tea extracts were found to be equally potent in the induction of apoptosis (Fig 1a) when K-562 cells were treated with these extracts for 24h. In parallel with this finding a dose dependent increase in comet tail moment was also observed after identical treatment of cells (Fig 1b). When a time kinetic study was performed, no appreciable tail moment was observed following treatment of cells up to 20h (Table 1). The comet pattern induced by tea extracts was different from the image that is usually seen in case of genotoxic manifestation indicating that tea extracts did not induce any genotoxicity in this leukemia cell line. Treatment

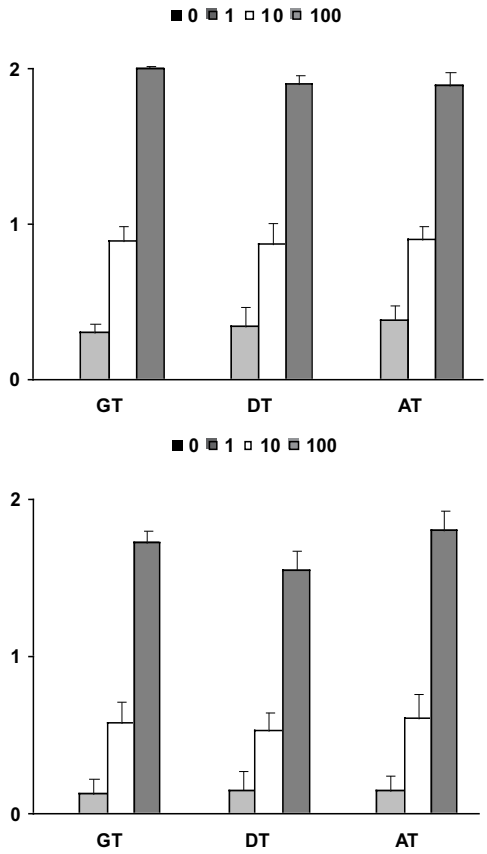

Figure 1. Response of Human Leukemic K-562 Cells to Increasing Concentrations of Tea Extract.. a) Apoptotic index; b) Comet tail formation. Data points are the means of three independent experiments.

Table 1. Comet Tail Moment in Leukemia cells by Tea Extracts at Different Time Points

Time (h)	Comet tail moment (mm) by		
	GT	DT	AT
4	0.14	0.10	0.13
8	0.12	0.12	0.15
12	0.18	0.15	0.14
20	0.54	0.49	0.55
24	2.00	1.90	1.89

Concentration of each tea extract: 100 mg/ml

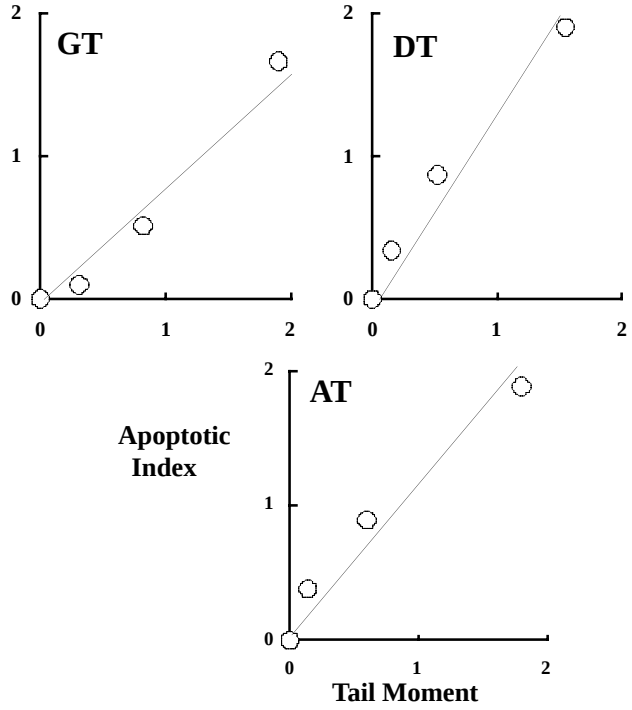

Figure 2. Linear Correlation Between Comet Tail Moment and Apoptotic Indices with Green and Black Tea Extracts in K-562 Cells

of K562 cells with tea extracts for 24 h showed the "apoptotic comets" with little DNA in the head, usually as a ring with less DNA in the center of the head (the "pin" head) followed by a large cloud (the "puffy" tail). This is characteristic of apoptotic DNA fragmentation. Necrotic DNA damage, on the other hand, shows a comet with considerable DNA both in the head and in the tail, which was not observed in our study, further confirming that the comet formation in K-562 cells by tea extracts was solely due to induction of apoptosis. Fig 2 shows a linear correlation between comet formation and apoptotic index, the correlation coefficient values (r) appear to be very near to unity. It is quite clear from the results that as the frequency of occurrence of apoptosis increases, the tail moment, the parameter correlated to the degree of DNA fragmentation measured by the comet assay, increased.

Table 2. FACS Analysis of Leukemia Cells after Treatment with Tea Polyphenols

Polyphenol	Concentration (mg/ml)	DNA(%) in sub-G1 peak
None (control)		7.59
GT	1	18.10
	10	61.12
	100	82.56
DT	1	16.20
	10	58.54
	100	83.12
AT	1	14.86
	10	60.35
	100	81.55

10,000 cells were counted per point.

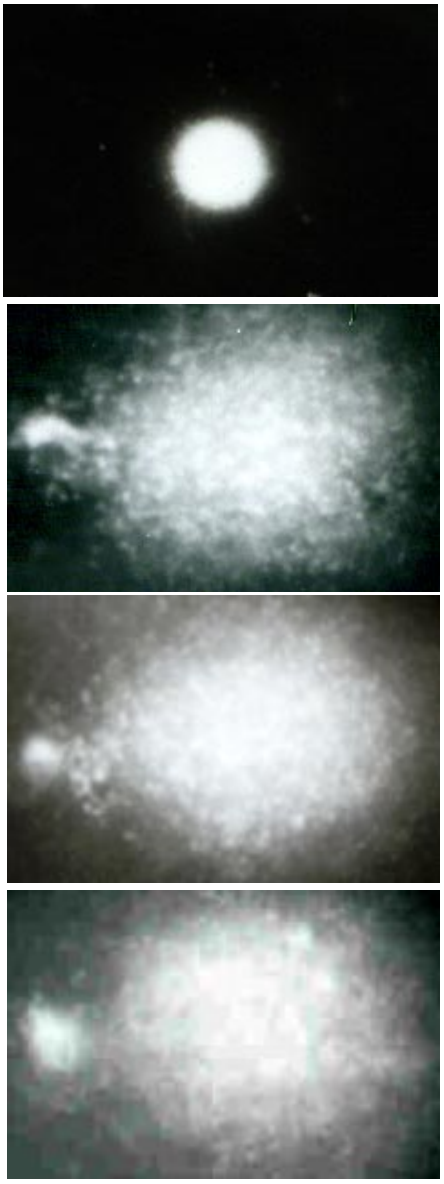

Figure 3. Apoptotic Comet Formation by Green Tea and Black Tea Extracts in K-562 cells. The photographs depict clear appearance of a fan like comet formation, which is a typical characteristic of apoptotic phenomenon.

Cell cycle distribution after treatment of K-562 cells with a range of concentrations of the three varieties of tea extracts was examined after 24 h by FACS analysis. All the tea extracts used in our study were found to induce a dose dependent increase in sub G1 peak, indicating apoptotic phenomenon, and a progressive loss of DNA content of G1 phase (Table 2). Loss of DNA is a typical feature of apoptotic cells that occurs as a result of diffusion of degraded DNA out of the cells after endonuclease cleavage and after staining with PI, cells that have lost DNA would take up less stain and would appear left to the G0/G1 peak. A good correlation (correlation coefficient, r~1) has been obtained between the DNA content in sub G1 peak and apoptotic index.

Several genes are implicated in apoptotic process. Of these the expression of pro-apoptotic gene bax and the anti-

Table 3. Apoptosis Related Gene Expression as Related to Comet Formation by Tea Extracts

Tea	Concentration (mg/ml)	bax:bcl-2	Comet tail moment (mm)
GT	0	0.21	0
	1	0.99	0.31
	10	2.63	0.82
	100	11.1	1.9
DT	0	0.24	0
	1	0.77	0.33
	10	0.97	0.86
	100	7.14	1.6
AT	0	0.26	0
	1	0.49	0.32
	10	2.17	0.85
	100	5.0	1.66

bax and bcl-2 ratio has been calculated from the band intensities as obtained by densitometric analysis.

Table 4. Comet Formation as an Indicator of Apoptosis as Measured by Different Apoptotic Parameters

Tea	Link between		Correlation coefficient (r)
GT	TM	AI	0.992
	TM	FACS	0.952
	TM	Caspase 3	0.960
	TM	Caspase 8	0.967
	CF	AI	0.979
	TM	bax:bcl-2	0.976
DT	TM	AI	0.991
	TM	FACS	0.965
	TM	Caspase 3	0.911
	TM	Caspase 8	0.964
	CF	AI	0.981
	TM	bax:bcl-2	0.902
AT	TM	AI	0.986
	TM	FACS	0.958
	TM	Caspase 3	0.904
	TM	Caspase 8	0.958
	CF	AI	0.981
	TM	bax:bcl-2	0.987

The degree of correlation (r) between two sets of data was computed statistically using appropriate software.

apoptotic gene bcl-2 were studied. The ratio bcl-2/bax ultimately decides the fate of the cell – whether it will undergo programmed cell death or continues proliferation. Using appropriate antibodies Western blot analysis was performed and we have found that the tea extracts used in the present study down-regulate bcl-2 and up-regulate the bax gene expression (data not shown). Densitometric analysis was performed with these bands and the ratio bax/bcl-2 was calculated. These parameters compared very well with the apoptotic comet tail moment (Table 3). At the highest dose of each tea extracts, the comet tail of treated cells showing the extent of DNA migration out of the nucleus due to DNA breakage and loss of structure is depicted in Fig 3.

Caspases, the cytoplasmic aspartate-specific cysteine proteases, play a very crucial role in apoptosis (Thornberry 1997). Activation of caspases appears to be responsible for several characteristic structural and molecular changes during apoptosis. Fluorimetric assay of caspase 3 and caspase 8 in cells treated with tea extracts revealed that the activity of both the proteases was elevated in proportion to the concentration. Activation of caspase 3 and 8 also showed a good correlation with comet tail formation.

Table 4 lists the correlation co-efficient values between comet formation and various parameters of apoptosis. The degree of correlation between two sets of data was computed statistically using software. In all cases the high degree of correlation further confirms our view that the comet formation can accurately reflect apoptotic cell death over the dose range studied.

Discussion

Apoptosis is a form of programmed cell death whereby a cell undergoes a controlled form of cellular suicide (Kerr et al., 1972). This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death occurs leading to inflammatory response due to leakage of the cell contents including proteases and lysozymes (Wyllie et al., 1980). On the other hand, apoptosis is a proteolytic process, whereby all apoptotic events occur before there is a loss in plasma membrane integrity (Page et al., 2000). Apoptosis leads to cytoskeletal disruption, cell shrinkage, membrane blebbing and the disruption of DNA into fragments, but does not lead to an inflammatory response in vivo (Fairbairn et al., 1996). One of the most interesting biochemical phenomena of apoptosis is the fragmentation of DNA into multiples of 180 base pairs by endonuclease activity in the linker region between nucleosomes (Olive et al., 1995). Appearance of DNA ladder has been shown to be a biochemical signature of apoptotic cell death. Because DNA fragmentation produces a characteristic ladder pattern in agarose gel electrophoresis, this technique is used as an important standard indication of apoptosis. But with heterogeneous cell population, this technique will miss contributions from small sub populations and results can not be quantified (Bacso et al., 2000; Singh, 2000). The detection of apoptosis at the single cell level requires an assay that allows analyses of individual cells for the biological changes induced by apoptosis. Comet assay or SCGE is one such technique that can detect the fragmented DNA on a cell-by-cell basis, thereby readily allowing the determination of apoptotic cells.

The current study is aimed at an attempt to find out whether comet assay can be used to detect apoptosis. In recent days various parameters of apoptosis are being compared with the comet formation and good correlations have been found between the concentrations of apoptotic cells determined by the comet assay and other methods used to measure apoptosis. Similar correlation has been observed between comet formation and TUNEL procedure. Another study finds that combination between SCGE and annexin binding offers a good quantitative method for monitoring

phosphatidyl serine exposure during programmed cell death in plants (Ning et al., 2002). Comet assay also has been demonstrated to have broad applicability for aquatic animals in terms of the organism, tissue and cell types (Lee and Steinert 2003). However, in practice, an image of a different type from the normal comet image is observed in apoptosis. As the apoptosis results in the extensive formation of double stranded breaks, apoptotic cells show massive fragmentation of cellular DNA and after the comet assay procedure these cells have almost all the DNA outside of the comet head forming large fan like tail. Since the comet assay is able to detect as few as 100 DNA strand breaks per cell, it is not surprising that this method should prove useful in detecting individual apoptotic cells which in theory could contain up o 10^7 DNA strand breaks (assuming $3x10^9$ base pairs/cell and a break between every nucleosome). The comet method is the only one currently available with sufficient sensitivity to detect, in individual cells, initial DNA damage, repair of that damage and subsequent appearance of apoptotic cells (Olive et al., 1993). For this reason, it is predicted that many DNA fragments in an apoptotic cell would facilitate detection by the comet assay.

Tea extracts and tea polyphenols have shown growth inhibitory activities in both cell lines and animal models (Ahmad et al., 1997; Dong et al., 1997; Hibasami et al., 1998b). Tea polyphenol-induced cell cycle arrest and cell apoptosis are the proposed mechanisms for inhibition of cancer cell growth (Ahmad et al., 1997; Hibasami et al., 1998b; Yang et al., 1998; Yang et al, 2000). Black tea polyphenols, green tea extract and EGCG have been shown to inhibit the growth of rat hepatoma, mouse erythroleukemia and the growth of several human cancer cell lines, such as MCF-7 breast carcinoma, HT-29 colon carcinoma, A-427 lung carcinoma and UACC-375 melanoma (Lea et al., 1993; Valcic et al., 1996). The induction of apoptosis by catechins has also been demonstrated in human epidermoid carcinoma cells A-431, human carcinoma keratinocytes HaCat and human prostrate carcinoma cells DU-145, but not in normal human epidermal keratinocytes (Ahmad et al., 1997). In another study, the growth inhibitory activity of EGCG has been demonstrated in SV-40 virally transformed human fibroblast cells W138 through apoptosis but not in normal W138 cells (Chen et al., 1998). In the same study, a differential growth inhibition was observed between a human colorectal cancer cell line Caco-2, a breast cancer cell line Hs-578T, and their respective normal counterparts. Green tea polyphenols such as EGCG and ECG also inhibit growth of human lung cancer cell line PC-9 (Okabe et al., 1997).

The study further demonstrated that growth inhibition was accompanied by G2/M phase arrest of the cell cycle (Fujiki et al., 1998). Another study presented substantial evidence that green tea extract is a more effective mixture of polyphenols than EGCG alone and that apoptosis being mediated better through enhanced incorporation of tea polyphenols into the cells (Suganuma et al., 1999). Although black tea is the most common type of tea consumed all over the world, less studies have investigated the effects of black tea components on cancer formation and growth, compared to green tea and green tea polyphenols. The inhibitory activity against tumorigenesis of black tea is comparable with that of green tea in some animal models (Yang et al., 1998 a). EGCG and theaflavins have been shown to inhibit 12-O-tetradecanoyl phorbol 13-acetate (TPA) and epidermal growth factor-induced mouse JB6 cell transformation; such effects are associated with inhibition of AP-1 activity (Dong et al., 1997). Further reports are also available on the inhibitory effect of theaflavin on NNK-induced lung tumorigenesis (Hecht et al., 1989) and on the growth of human lung cancer cell lines H441, H661 and H1299 (Yang et al., 1998 b). Results from our laboratory suggest that both black tea extract and the black tea polyphenol theaflavin induce apoptosis in human leukemic cell line HL-60 by a p53 independent pathway (Kundu et al., 2005).

The results presented in this communication unequivocally demonstrate the ability of the comet assay, a sensitive method of DNA strand breaks in individual cells, to detect apoptotic cells. Thus, the comet assay compares well with other parameters of apoptosis, suggesting a strong positive correlation between apoptosis and comet formation. Both green and black tea extracts have been shown as effective inducers of apoptosis in K-562 cells, but similar result can be expected also by other apoptotic inducers and in other cell lines.

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