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Plant polyphenols mobilize nuclear copper in human peripheral lymphocytes leading to oxidatively generated DNA breakage: Implications for an anticancer mechanism

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Abstract

It was earlier proposed that an important anti-cancer mechanism of plant polyphenols may involve mobilization of endogenous copper ions, possibly chromatin-bound copper and the consequent pro-oxidant action. This paper shows that plant polyphenols are able to mobilize nuclear copper in human lymphocytes, leading to degradation of cellular DNA. A cellular system of lymphocytes isolated from human peripheral blood and comet assay was used for this purpose. Incubation of lymphocytes with neocuproine (a cell membrane permeable copper chelator) inhibited DNA degradation in intact lymphocytes. Bathocuproine, which is unable to permeate through the cell membrane, did not cause such inhibition. This study has further shown that polyphenols are able to degrade DNA in cell nuclei and that such DNA degradation is inhibited by neocuproine as well as bathocuproine (both of which are able to permeate the nuclear pore complex), suggesting that nuclear copper is mobilized in this reaction. Pre-incubation of lymphocyte nuclei with polyphenols indicates that it is capable of traversing the nuclear membrane. This study has also shown that polyphenols generate oxidative stress in lymphocyte nuclei which is inhibited by scavengers of reactive oxygen species (ROS) and neocuproine. These results indicate that the generation of ROS occurs through mobilization of nuclear copper resulting in oxidatively generated DNA breakage.

Keywords: *Plant polyphenols, pro-oxidant DNA breakage, nuclear copper, Comet assay*

Introduction

Plant-derived polyphenolic compounds that include flavonoids, tannins, curcuminoids, gallocatechins such as epigallocatechin-3-gallate (EGCG) and stilbenes such as resveratrol possess a wide range of pharmacological properties, the mechanisms of which have been the subject of considerable interest. They are recognized as naturally occurring antioxidants and have been implicated as antiviral and anti-tumour compounds [1,2]. In recent years, a number of reports have appeared which have shown that gallocatechins found in green tea and which include tannic acid, gallic acid, epigallocatechin, epicatechin-3-gallate and epigallocatechin-3-gallate (EGCG) induce apoptosis in various cancer cell lines [3,4]. Similarly, curcumin [5] from the spice turmeric and resveratrol [6] which is found in red grapes and red wine have also been shown to be inducers of apoptosis in cancer cells. The consumption of green

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tea is considered to reduce the risk of various cancers such as that of bladder, prostate, oesophagus and stomach [4]. Of particular interest is the observation that EGCG was found to induce internucleosomal DNA fragmentation in cancer cell lines such as human epidermoid carcinoma cells, human carcinoma keratinocytes, human prostate carcinoma cells and mouse lymphoma cells. However, such DNA fragmentation was not observed in normal human epidermal keratinocytes [4]. Similarly, gallic acid showed cytotoxicity for a number of tumour cell lines but primary cultured rat hepatocytes and macrophages were found to be refractory to the cytotoxic effect [3]. Resveratrol was also shown to induce apoptotic cell death in HL60 human leukaemia cell lines but not in normal peripheral blood lymphocytes [6].

Studies in our laboratory have shown that flavonoids [7], tannic acid and its structural constituent gallic acid [8], curcumin [9], gallocatechins [10] and resveratrol [11] cause oxidative strand breakage in DNA either alone or in the presence of transition metal ions such as copper. Copper is an important metal ion present in chromatin and is closely associated with DNA bases, particularly guanine [12]. It is also one of the most redox active of the various metal ions present in cells. Most of the plant polyphenols possess both antioxidant as well as pro-oxidant properties [3,7] and we have earlier proposed that the pro-oxidant action of polyphenolics may be an important mechanism of their anti-cancer and apoptosis inducing properties [13]. In fact, some recent interesting studies have suggested that increasing reactive oxygen species generation over an established threshold by lowering antioxidant defences may contribute to selective killing of cancer cells [14,15]. Such a mechanism for the cytotoxic action of these compounds against cancer cells would involve mobilization of endogenous copper ions, possibly chromatin bound copper and the consequent pro-oxidant action. Using a cellular system of peripheral lymphocytes isolated from human blood and alkaline single cell gel electrophoresis (Comet assay), we have confirmed that the polyphenol resveratrol in the presence of Cu(II) is indeed capable of causing oxidative DNA degradation in cells and thus this reaction could be of biological significance [16]. Further, we have also shown that plant polyphenols alone are capable of oxidatively degrading lymphocyte DNA through mobilization of cellular copper [17].

As a further confirmation of our idea, we have used a lysed version of the comet assay to show that plant polyphenols are capable of mobilizing copper ions from lymphocyte nuclei. The use of a lysed version eliminates the cell membrane barrier and any putative interaction of polyphenols with cytoplasmic components. Thus, the polyphenols are able to directly interact with the nuclei. The results are in further support of our hypothesis that the anti-cancer mechanism of plant polyphenols involves mobilization of endogenous copper, possibly chromatin-bound copper and the consequent pro-oxidant action.

Materials and methods

Materials

Resveratrol, EGCG, gallic acid, caffeic acid, daidzein, neocuproine, bathocuproine disulphonate, superoxide dismutase (SOD), agarose, low melting point agarose (LMPA), RPMI 1640, Triton X-100, Trypan blue, Histopaque1077 and phosphate buffered saline (PBS) Ca^{++} and Mg^{++} free were purchased from Sigma (St. Louis, MO). Delphinidin was purchased from Extra Synthese (Genay, France). All other chemicals were of analytical grade. Resveratrol and Delphinidin were dissolved in 3 mM NaOH before use as a stock of 1 mM solution. All other polyphenols were dissolved in water. Upon addition to reaction mixtures, in the presence of buffers mentioned and at concentrations used, all the polyphenols used remained in solution. The volumes of stock solution added did not lead to any appreciable change in the pH of reaction mixtures.

Isolation of lymphocytes

Heparinized blood samples (2 ml) from a single healthy donor was obtained by venepuncture and diluted suitably in Ca^{++} and Mg^{++} free PBS. Lymphocytes were isolated from blood using Histopaque 1077 (Sigma) and the cells were finally suspended in RPMI 1640. A single donor donated blood for all experiments (first author).

Viability assessment of lymphocytes

The lymphocytes were checked for their viability before the start and after the end of the reaction using Trypan Blue Exclusion Test [18].

Treatment of lymphocyte nuclei with polyphenols and evaluation of DNA breakage by using lysed version of comet assay

Treatment of nuclei by polyphenols was carried out by lysing the lymphocytes embedded in agarose on slides and subsequently treating the nuclei in such slides by polyphenols and other agents. Such a lysed version of the comet assay has been used to study the direct interaction of various agents with cell nuclei as it eliminates the effect of the cell membrane as a barrier and the intracellular environment [19]. A lysed version of comet assay was performed as described by Kasamatsu et al. [20] with some modifications. Lymphocytes isolated from 2 ml blood were diluted to the count of 2×10^5 cells/2 ml and

suspended in RPMI 1640. Approximately 10 000 of these cells were mixed with 75 µl of pre-warmed LMPA in PBS and immediately applied to a frosted microscopic slide layered with 75 µl of 1% standard agarose in PBS. The slides were allowed to gel at 4°C for 10 min. Lysis of cells was then performed by submerging the slides in a tank containing lysis solution in the absence of light for 1 h at 4°C. The use of a tank instead of a coplin jar allowed simultaneous processing of a number of slides. The lysis solution (pH 10) consisted of 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris and 1% Triton X-100 added just prior to use. After lysis, slides were transferred to another tank containing 0.4 M Phosphate buffer (pH 7.5) for 10 min. Each slide was then transferred to a rectangular dish (8 cm × 3 cm × 5 mm) which contained a reaction mixture of polyphenols and other additions as mentioned in various legends to figures and tables. The slides with the reaction mixture was incubated at 37°C. The slides were then washed twice by placing in 0.4 M phosphate buffer pH 7.5 for 5 min at room temperature. DNA unwinding and expression of alkali labile sites was done by leaving the slides in the high pH electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH > 13 prepared in PBS) at 4°C for 30 min. Subsequently, the electrophoresis, neutralization and staining of the slides was carried out as described earlier [16]. Comet images were observed at 100 × magnification with a fluorescence microscope (Olympus CX41) and COHU 4910 (equipped with a 510–560 nm excitation and 590 nm barrier filters) integrated CC camera. Fifty images were randomly selected from each sample and their lengths (diameter of the nucleus plus migrated DNA) and % DNA in tail were measured on the screen as automatically generated by Komet 5.5 image analysis system of Kinetic Imaging (Liverpool, UK).

Treatment of whole lymphocytes with polyphenols and evaluation using the standard comet assay

Treatment of whole lymphocytes with polyphenols and the subsequent Comet assay was performed essentially as described earlier [16]. However, since the DNA breakage had to be compared with that in lymphocyte nuclei, the treatment of cells with polyphenols was done on slides rather than in eppendorf tubes. Further, the lysis of cells was carried out after the polyphenol treatment. The other conditions remained the same as described above.

Detection of H_2O_2 generation by various polyphenols in the incubation medium of nuclei.

The ferrous oxidation-xylenol orange (FOX) assay [21] was adapted to detect and quantify the generation of H_2O_2 in the incubation medium (phosphate buffer 0.4 M, pH 7.5) by various polyphenols. The simplified reaction sequence involves the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) ions by H_2O_2 with the subsequent binding of the Fe^{3+} ion to the ferric sensitive dye xylenol orange, yielding an orange to purple complex, which is measured at 560 nm. The reaction mixture contained the polyphenol and phosphate buffer (incubation medium used in the treatment of nuclei). After incubation for 2 h at 37°C, an aliquot of 200 µl was analysed for H_2O_2 formation.

Isolation of nuclei from lymphocytes and determination of TBARS

Nuclei were isolated from lymphocytes by following the procedure given in Qiagen Genomic DNA Handbook (2001). Three millilitres of lymphocyte suspension (2×10^7 cells) was added to an equal volume of ice cold cell lysis buffer (1.28 M Sucrose, 40 mM Tris-Cl pH 7.5, 20 mM $Mgcl_2$, 4%Triton X-100) and three volumes of ice cold distilled water. The suspension was mixed by inverting the tubes several times and incubated for 10 min on ice. The buffer lyses the cells but stabilizes and preserves the nuclei. The lysed cells were then centrifuged at 4°C for 15 min at 1300 xg. The nuclear pellet thus obtained was resuspended in 1.5 ml of nuclei suspension buffer consisting of 50 mM Tris-Hcl pH 7.5, 20% Glycerol, 5 mM $Mgcl_2$ and 0.1 mM EDTA [22].

Thiobarbituric-acid-reactive substance (TBARS) was determined according to the method of Ramanathan et al. [23]; 200 µl of the nuclei suspension was incubated with EGCG (0–200 µM) at 37°C in a total volume of 3 ml for 1 h. The reaction was stopped by the addition of 30 µl of 5 N NaOH. In some experiments nuclei were pre-incubated with a fixed concentration of neocuproine and thiourea. To 1.5 ml of the reaction mixture was added 0.5 ml of 10% TCA and 0.5 ml of 0.6 M TBA (2-thiobarbituric acid) and the mixture incubated in a boiling water bath for 10 min. The absorbance was read at 532 nm and converted into nmoles of TBA reactive substance using the molar extinction coefficient.

Statistics

The statistical analysis was performed as described by Tice et al. [24] and is expressed as ± SEM of three independent experiments. A student's *t*-test was used to examine statistically significant differences. Analysis of variance was performed using ANOVA. *P*-values < 0.05 were considered statistically significant.

Results

DNA breakage by H_2O_2 and EGCG in whole lymphocytes and lymphocyte nuclei as measured by comet assay

Since in the lysed version of the Comet assay membrane and cytoplasmic barrier is eliminated it

would be reasonable to assume that the polyphenol is able to directly interact with the cell nuclei. Thus, considerably greater DNA breakage should be observed in the lysed version as compared to the standard version where intact lymphocytes are used. H_2O_2 is a well known DNA damaging agent and is membrane permeable [25]. Increasing concentrations of H_2O_2 (5, 10, 20 μM) and EGCG (25, 50, 100 μM) were tested for DNA breakage in intact lymphocytes (standard version of Comet assay) and compared with that observed with lymphocyte nuclei (lysed version). Photographs of comets obtained with 20 μM H_2O_2 and 50 μM EGCG in the standard and lysed version of Comet assay are shown in Figure 1. In Figure 2 the data is plotted as % DNA in tail of comets with increasing concentration of H_2O_2 and EGCG. In both cases, it is seen that the rate of tail formation is considerably greater in the case of the lysed version, suggesting that H_2O_2 and the polyphenol EGCG are able to directly interact with the nuclei when the lysed version of comet assay is used. Similar results have also been reported by Kasamatsu et al. [20] using H_2O_2 and bleomycin.

Effect of pre-incubation of lymphocyte nuclei with polyphenols on polyphenol-Cu(II) mediated DNA breakage

It is well known that polyphenols auto-oxidize in cell culture media to generate H_2O_2 and quinones that can enter cells/nuclei-causing damage to various macromolecules [21,25,26]. This may lead to extraneous production of reactive oxygen species that could account for cellular DNA breakage. In order to exclude this possibility, nuclear DNA breakage was studied by pre-incubating the nuclei with polyphenols (resveratrol and EGCG) after which the nuclei were washed with PBS and incubated further in the presence of 5 μM $CuCl_2$ (Figure 3). It may be noted that the concentrations of polyphenols when used alone (2, 5, 10 μM) give rise to only a limited degree of nuclear DNA breakage (2–5 μM). With either resveratrol or with EGCG, a progressive and significant DNA breakage could only be seen after incubating the pre-treated nuclei further in the presence Cu (II). It is well established that the nuclear pore complex is permeable to small molecules [27]. Thus, the nuclear DNA breakage observed in these experiments is presumably the result of direct interaction of polyphenols with chromatin. For example, it is considered that the polyphenol resveratrol affects several aspects of DNA metabolism, i.e. DNA replication, recombination, repair, relaxation and telomere maintenance through its binding to cellular DNA [28]. In Table I, we give an experiment which further supports this idea. Nuclear DNA tail length formation was determined for a number of polyphenols at a fixed concentration of 50 μM. The least efficient among these was found to be tannic acid and the most effective was diadzin. The differential rate of

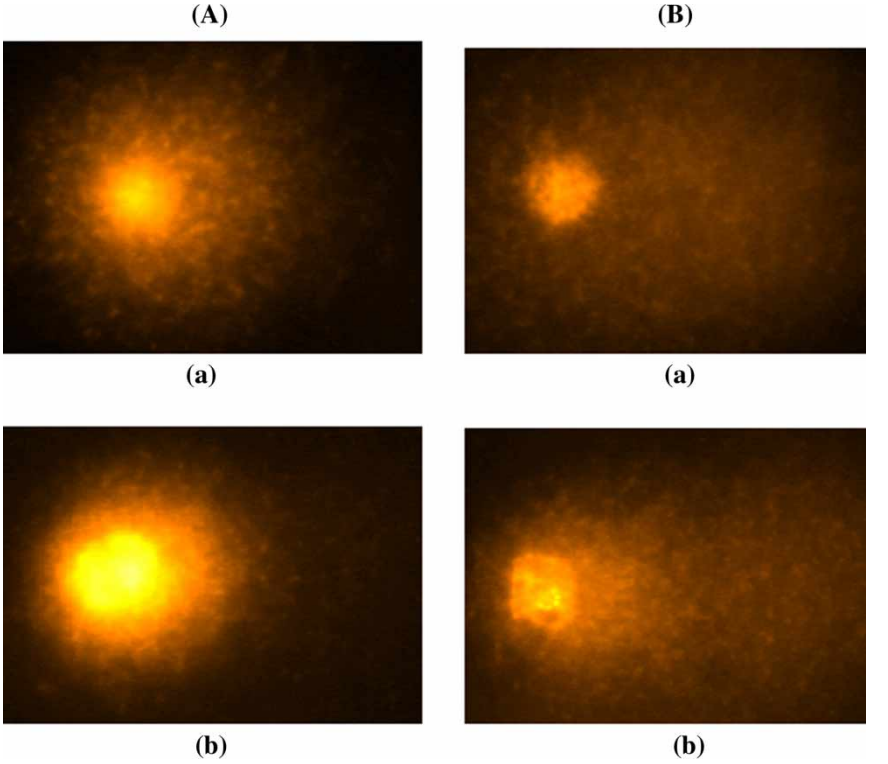

Figure 1. Single cell gel electrophoresis of human peripheral lymphocytes (A) and lymphocyte nuclei (B) showing comets (100 ×) after treatment with H_2O_2 (20 μM) (a) and with EGCG (50 μM) (b). The incubation of lymphocytes and lymphocyte nuclei with both H_2O_2 and EGCG was for 1 h at 37°C.

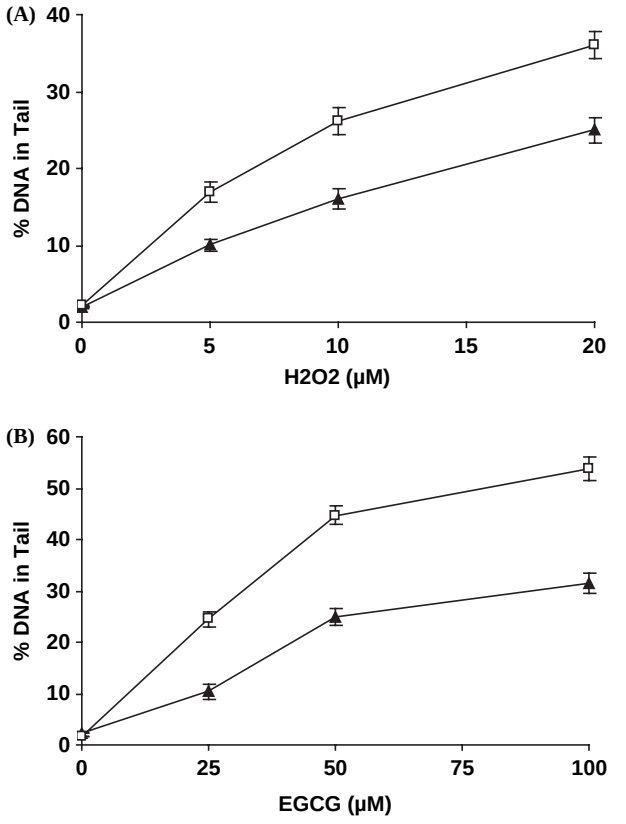

Figure 2. A comparison of DNA breakage in intact lymphocytes (▲) and lymphocyte nuclei (□) using increasing concentrations of H_2O_2 (A) and EGCG (B). The data obtained in the experiment given in Figure 1 is plotted. Values reported are ±SEM of three independent experiments.

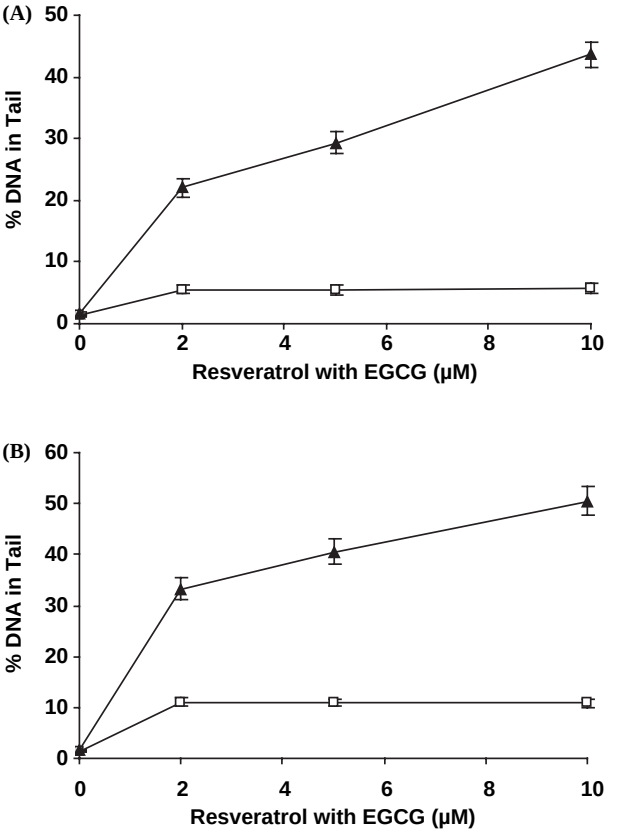

Figure 3. Effect of pre-incubating the lymphocyte nuclei with increasing concentrations of resveratrol (A) and EGCG (B) on DNA breakage. The lymphocyte nuclei layered on slides (two sets) were pre-incubated with the indicated concentrations of resveratrol (A) and EGCG (B) for 15 min at 37°C. The slides were then washed twice for 5 min each with PBS and further incubated for 30 min in the presence of 5 μM Cu(II) (▲). The other set which served as control was further incubated in PBS alone (□). Values reported are ±SEM of three independent experiments.

nuclear DNA degradation by the polyphenols tested is possibly the result of differential copper binding and copper reducing efficiency of various polyphenols [7] as well as their affinity for DNA binding [29]. H_2O_2 production by the polyphenols (in the absence of nuclei, in 0.4 M phosphate buffer, the suspension medium of nuclei) at the same concentration was also determined. As can be seen, tannic acid is the most efficient generator of H_2O_2 but is least effective as a DNA degrading agent and no correlation exists between the relative H_2O_2 production and the DNA tail length formation by the various polyphenols.

Effect of active oxygen scavengers on resveratrol-induced DNA breakage in lymphocyte nuclei

We have previously shown [17] that resveratrol-induced DNA breakage in intact lymphocytes is inhibited to significant degrees by various scavengers of reactive oxygen species. Table II gives the results of an experiment where three scavengers (superoxide dismutase, catalase and thiourea) were tested for their effect on resveratrol-induced DNA breakage in lymphocyte nuclei using the lysed version of Comet assay. All three caused significant inhibition of DNA breakage as evidenced by decreased % DNA in tail of comets. It may be mentioned that due to the site-specific nature of the reaction of hydroxyl radicals with DNA it is difficult for any trapping molecules to intercept them completely [30]. We conclude that superoxide anion and H_2O_2 are essential components in the pathway that leads to the formation of hydroxyl radical and other species which would be the

Table I. Percentage DNA in the tail and relative H_2O_2 production by various polyphenols in lymphocyte nuclei.

Polyphenol production (50 μM)	% DNA in tail	H_2O_2 (nmoles)
Untreated	2.41±0.26#	0.00
Daidzein	61.52±5.00*	1.46
Gallic acid	54.32±4.03*	1.97
Delphinidin	47.65±4.55*	0.19
EGCG	40.67±3.82*	10.81
Caffeic acid	39.17±3.42*	0.69
Resveratrol	32.86±2.78*	1.65
Tannic acid	20.00±1.51*	13.35

The incubation period for the reaction mixture (lymphocyte nuclei and polyphenols) was 1 h for 37°C.
* $p < 0.05$ by comparison with control (#). data represent ±SEM of three independent experiments.

Table II. Effect of scavengers of active oxygen species on resveratrol-induced nuclear DNA breakage.

Dose	% DNA in the tail	% Inhibition
Untreated	2.27±0.04	—
Resveratrol (50 μM)	34.95±2.68#	—
+SOD (100 μg/ml)	9.11±0.84⋆	73%
+Catalase (100 μg/ml)	10.59±0.92⋆	69%
+Thiourea (1 mM)	12.24±1.19⋆	64%

The incubation period of the reaction mixture (lymphocyte nuclei, resveratrol and scavengers) was 1 h at 37°C.
All values represent ±SEM of three independent experiments.
⋆ p-values <0.05 when compared to control (#).

proximal DNA cleaving agents. Since the results are similar to those seen with whole lymphocytes, it is suggested that the same mechanism involving reactive oxygen species is responsible for DNA breakage, irrespective of whether intact lymphocytes or lymphocyte nuclei are treated with resveratrol.

Mobilization of nuclear copper by plant polyphenols

In a previous study [17], we have shown that resveratrol mediated DNA degradation of lymphocyte DNA is inhibited by neocuproine, which is a Cu(I) specific chelating agent and is membrane permeable [31,32]. In the experiment shown in Figure 4 we have also used bathocuproine disulphonate, the water soluble membrane impermeable analogue of neocuproine, to show that whereas neocuproine inhibits EGCG-induced DNA breakage in intact lymphocytes, bathocuproine as expected is ineffective in causing such inhibition (Figure 4A). However, when these two copper-specific chelators were tested for DNA breakage inhibition in lymphocyte nuclei, both were found to inhibit DNA breakage in a dose-dependent manner (Figure 4B). Similar results were obtained when resveratrol instead of EGCG was used in this experiment. In the lysed version of Comet assay, membrane and cytoplasmic barrier is eliminated. Further, the nuclear pore complex is permeable to small molecules. Therefore, we take these results to indicate that, as proposed by us, plant polyphenols mobilize chromatin-bound copper, leading to oxidative DNA breakage.

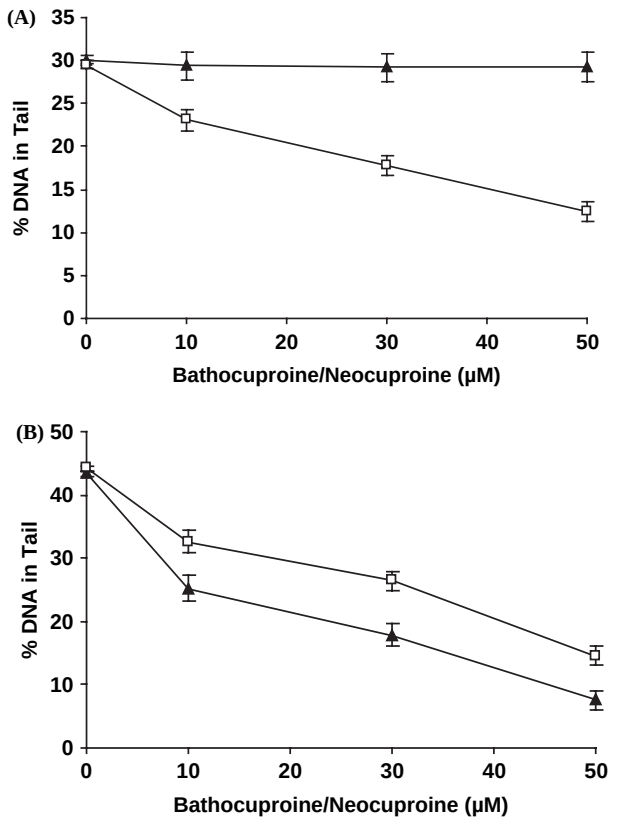

Figure 4. Effect of increasing concentrations of bathocuproine (▲)/neocuproine (□) on EGCG-induced DNA breakage in lymphocytes (A) and lymphocyte nuclei (B). The reaction mixture contained lymphocyte/lymphocyte nuclei, EGCG and neocuproine/bathocuproine. The incubation period was 1 h at 37°C. Values reported are ±SEM of three independent experiments.

Determination of TBARS as a measure of oxidative stress in nuclei by EGCG in the presence of neocuproine and thiourea

According to our hypothesis, the DNA breakage observed in lymphocyte nuclei is the result of the generation of hydroxyl radicals and other reactive oxygen species *in situ*. Oxygen radical damage to deoxyribose or DNA is considered to give rise to TBA reactive material [33,34]. We have therefore determined the formation of TBA reactive substance (TBARS) as a measure of oxidative stress in lymphocyte nuclei with increasing concentrations of EGCG. The effect of pre-incubating the nuclei with neocuproine and thiourea was also studied. Results given in Figure 5 show a dose-dependent increase in the

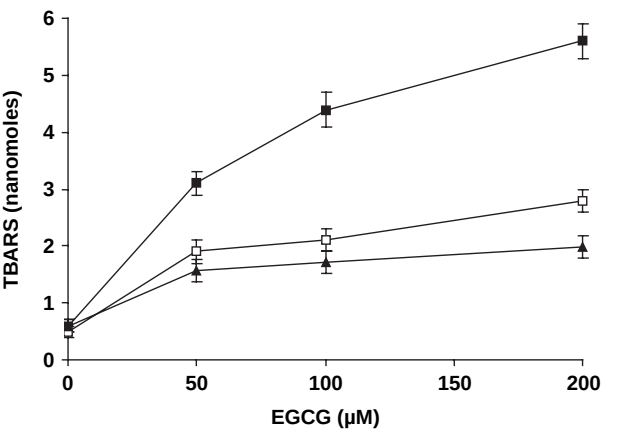

Figure 5. Effect of pre-incubation of lymphocyte nuclei with neocuproine and thiourea on TBARS generated by increasing concentrations of EGCG. EGCG alone (■), EGCG+neocuproine (1 mM) (□), EGCG+thiourea (1 mM) (▲). The nuclei suspension was pre-incubated with fixed concentration of neocuproine and thiourea for 30 min at 37°C after which it was further incubated for 1 h in the presence of increasing EGCG concentration. Values reported are ±SEM of three independent experiments.

formation of TBA reactive substance in lymphocyte nuclei by EGCG. However, a considerable decrease in the rate of formation of TBARS was observed in the presence of neocuproine and thiourea. The results indicate that DNA breakage in nuclei is inhibited by Cu (I) chelation and scavenging of reactive oxygen. Thus it may be concluded that the oxidative stress induced by polyphenols in lymphocyte nuclei is at least in part mediated by chromatin-bound copper.

Discussion

We have earlier proposed that an important mechanism for the cytotoxic action of plant-derived polyphenolic compounds against cancer cells could be the mobilization of endogenous copper ions and the consequent pro-oxidant action. This is based on several lines of indirect evidence in literature and our own studies where we have observed that apoptotic DNA fragmentation properties of a number of plant polyphenols correlate with the ability to cause oxidative DNA breakage *in vitro* in the presence of copper ions [35]. We have also shown that the polyphenol-Cu (II) system is able to cause cellular DNA degradation in isolated lymphocytes and thus is physiologically feasible and could be of biological significance [16]. At a relatively higher concentration the polyphenols alone are also capable of causing DNA breakage in cells and such DNA breakage possibly involves mobilization of endogenous copper ions. Further, we have suggested that the preferential cytotoxicity of plant polyphenols towards cancer cells is explained by the observation made several decades earlier which showed that serum [36,37], tissue [38] and intracellular copper levels in cancer cells [39] are significantly increased in various malignancies. Since cancer cells contain elevated levels of copper, they may be more subject to electron transfer with polyphenols [40] to generate reactive oxygen species (ROS). Thus, because of higher intracellular copper levels in cancer cells it may be predicted that the cytotoxic concentrations of polyphenols required would be lower in these cells as compared to normal cells. Such lower cytotoxic concentrations of polyphenols against cancer cells have been demonstrated [41,42]. Indeed, plant polyphenols have been shown to cause regression of tumours in animal models [43–46].

Several other mechanisms have been proposed to account for the anti-cancer properties of plant polyphenols. Among the various classes of polyphenols, resveratrol has attracted the maximum attention. It is considered that resveratrol is able to block each step in the carcinogenesis process by inhibiting several molecular targets such as kinases, cyclooxygenases, ribonucleotide reductase and DNA polymerases [47]. Further, several plant polyphenols have also been shown to induce G_1 phase arrest and to trigger mitochondrial-dependent, p53-dependent, ROS-dependent, bcl-2 sensitive apoptotic response in tumour cells [47,48]. Resveratrol has been shown to induce activation of p53 accumulation and inhibition of NFκB. Other studies have emphasized the role of polyphenols as topoisomerase II poisons causing enhanced cellular DNA cleavage [49,50]. A further evaluation of the literature would indicate that the subject of anti-cancer mechanisms of plant polyphenols is rather complicated and controversial. However, it does appear that the anti-carcinogenic activities of plant polyphenols may be related but not due entirely to their anti-oxidative and the above mentioned properties. As already mentioned, a pro-oxidant action may be important in anti-cancer and apoptosis-inducing properties of these compounds.

According to our hypothesis, plant polyphenols possessing anti-cancer and apoptosis-inducing properties are able to mobilize endogenous copper ions, possibly the copper bound to chromatin, leading to the formation of ROS such as the hydroxyl radical close to the proximity of the site of DNA cleavage [51,52]. Essentially, this would be an alternative, non-enzymatic and copper-dependent pathway for the cytotoxic action of certain anti-cancer agents that are capable of mobilizing and reducing endogenous copper. Indeed such a common mechanism better explains the anti-cancer effects of polyphenols with diverse chemical structures as also the preferential cytotoxicity towards cancer cells. As such this would be independent of Fas and mitochondria mediated programmed cell death. Several studies have indicated that apoptosis induction by several polyphenols and other anti-cancer agents is independent of caspases and mitochondria [53,54] and is accompanied by an increase in the intracellular levels of ROS [55–57]. An important component of our hypothesis is that plant polyphenols mobilize chromatin-bound copper which is redox cycled and which in turn leads to the formation of ROS. This is also in concurrence with the idea that because of its extreme reactivity the hydroxyl radical must be produced in the vicinity of DNA [51] in order to cause its cleavage. In the present results we have shown that polyphenols are able to mobilize endogenous copper ions from lymphocyte nuclei and thus are in agreement with this requirement. Thus, our results are an important step in further validation of our hypothesis.

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References

[1] Hanasaki Y, Ogawa S, Fukui S. The correlation between active oxygen scavenging and antioxidative effects of flavonoids. Free Radic Biol Med 1994;16:845–850.

[2] Mukhtar H, Das M, Khan WA, Wang ZY, Bik DP, Bickers DR. Exceptional activity of tannic acid among naturally occurring plant phenols in protecting against 7,12-dimethyl benz(a)anthracene-, benzo(a)pyrene-, 3-methylcholanthrene- and N-methyl-N-nitrosourea-induced skin tumorigenesis in mice. Cancer Res 1988;48:2361–2365.

[3] Inoue M, Suzuki R, Koide T, Sakaguchi N, Ogihara Y, Yabu Y. Antioxidant, gallic acid, induces apoptosis in HL60RG cells. Biochem Biophys Res Commun 1994;204: 898–904.

[4] Ahmed N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3-gallate, and induction of cell cycle arrest in human carcinoma cells. J Natl Cancer Inst 1997;89:1881–1886.

[5] Kuo ML, Huang TS, Lin JK. Curcumin, an antioxidant and antitumor promoter, induces apoptosis in human leukemia cells. Biochim Biophys Acta 1996;1317:95–100.

[6] Clement HV, Hirpara JL, Chawdhury SH, Pervaiz S. Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signalling-dependent apoptosis in human tumor cells. Blood 1998;92:996–1002.

[7] Ahmad MS, Fazal S, Rahman A, Hadi SM, Parish JH. Activities of flavonoids for the cleavage of DNA in the presence of Cu(II): correlation with the generation of active oxygen species. Carcinogenesis 1992;13:605–608.

[8] Khan NS, Hadi SM. Structural features of tannic acid important for DNA degradation in the presence of Cu(II). Mutagenesis 1998;13:271–274.

[9] Ahsan H, Hadi SM. Strand scission in DNA induced by curcumin in the presence of Cu(II). Cancer Lett 1998; 124:23–30.

[10] Malik A, Azam S, Hadi N, Hadi SM. DNA degradation by water extract of green tea in the presence of copper ions: implications for anticancer properties. Phytother Res 2003;17:358–363.

[11] Ahmad A, Asad SF, Singh S, Hadi SM. DNA breakage by resveratrol and Cu(II): reaction mechanism and bacteriophage inactivation. Cancer Lett 2000;154:54–57.

[12] Kagawa TF, Geierstanger BH, Wang AHJ, Ho PS. Covalent modification of guanine bases in double stranded DNA: the 1:2-AZ-DNA structure of dc(CACACG) in the presence of $CuCl_2$. J Biol Chem 1991;266:20175–20184.

[13] Hadi SM, Asad SF, Singh S, Ahmad A. A putative mechanism for anticancer and apoptosis including properties of plant-derived polyphenolic compounds. IUBMB Life 2000; 50:1–5.

[14] Schumacker PT. Reactive oxygen species in cancer cells: live by the sword, die by the sword. Cancer Cell 2006;10: 175–176.

[15] Trachootham D, Zhou Y, Zhang H, Demizu Y, Chen Z, Pelicano H. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. Cancer Cell 2006;10:241–252.

[16] Azmi AS, Bhat SH, Hadi SM. Resveratrol-Cu(II) induced DNA breakage in human peripheral lymphocytes: implications for anticancer properties. FEBS Lett 2005;579: 3131–3135.

[17] Azmi AS, Bhat SH, Hanif S, Hadi SM. Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: a putative mechanism for anticancer properties. FEBS Lett 2006;580: 533–538.

[18] Renner HW, Schmezer P. Assesment of genotoxic effects of lindane. Food Chem Toxicol 1993;31:184–191.

[19] Szeto YT, Collins AR, Benzie IFF. Effects of dietary antioxidants on DNA damage in lysed cells using a modified comet assay procedure. Mutat Res 2002;500:31–38.

[20] Kasamatsu T, Kohda K, Kawazoe Y. Comparison of chemically induced DNA breakage in cellular and subcellular systems using the comet assay. Mutat Res 1996;369: 1–6.

[21] Long LH, Clement MV, Halliwell B. Artifacts in cell culture: rapid generation of hydrogenperoxide on addition of (−)-epigallocatechin, (−)-epigallocatechingallate, (+)-catechin and quercitin to commonly used cell culture media. Biochem Biophys Res Commun 2000;273:50–53.

[22] Bauer AL, Paulding WR, Striet JB, Schnell PO, Czyzyk-Krzeska MF. Endogenous von Hippel-Lindau tumor suppressor protein regulates catecholaminergic phenotype in PC12 cells. Cancer Res 2002;62:1682–1687.

[23] Ramanathan R, Das NP, Tan CH. Effect of γ-Linolenic acid, flavonoids and vitamins on cytotoxicity and lipid peroxidation. Free Radic Biol Med 1994;16:43–48.

[24] Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Riyu JC, Sasaki YF. Single cell gel electrophoresis/Comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen 2000;35:206–21.

[25] Halliwell B. Oxidative stress in cell culture: an underappreciated problem? FEBS Lett 2003;540:3–6.

[26] Clement MV, Long LH, Ramalingam J, Halliwell B. The cytotoxicity of dopamine may be an artifact of cell culture. J Neurochem 2002;81:414–421.

[27] Mazzanti M. Ion permeability of the nuclear envelope. News Physiol Sci 1998;13:44–50.

[28] Gatz SU, Wiesmuller L. Take a break-resveratrol in action on DNA. Carcinogenesis 2008;29:321–332.

[29] Rahman A, Shahabuddin S, Hadi SM, Parish JH. Complexes involving quercetin DNA and Cu(II). Carcinogenesis 1990; 11:2001–2003.

[30] Czene S, Tiback M, Harms-Ringdahl M. pH-dependent DNA cleavage in permeabilized human fibroblasts. Biochem J 1997;323:337–341.

[31] Barbouti A, Doulias PE, Zhu BZ, Galaris D. Intracellular iron, but not copper plays a critical role in hydrogen peroxide-induced DNA damage. Free Radic Biol Med 2001;31: 490–498.

[32] Stefan C, Nobel I, Kimland M, Lind B, Orrenius S, Slater AFG. Dithiocarbamates induce apoptosis in thymocytes by raising the intracellular level of redox-active copper. J Biol Chem 1995;270:26202–26208.

[33] Smith C, Halliwell B, Aruoma OI. Protection by albumin against the prooxidant action of phenolic dietary components. Food Chem Toxicol 1992;30:483–489.

[34] Quinlan GJ, Gutteridge MC. Oxygen radical damage to DNA by Rifamycin SV and copper ions. Biochem Pharmacol 1987;36:3629–3633.

[35] Hadi SM, Showket HB, Azmi SA, Hanif S, Shamim U, Ullah MF. Oxidative breakage of cellular DNA by plant polyphenols: a putative mechanism for anticancer properties. Semin Cancer Biol 2007;17:370–376.

[36] Ebadi M, Swanson S. The status of zinc, copper and metallothionien in cancer patients. Prog Clin Biol Res 1988;259:61–175.

[37] Margalioth EJ, Udassin R, Cohen C, Maor J, Anteby SO, Schenker JG. Serum copper level in gynaecologic malignancies. Am J Obstet Gynaecol 1987;157:93–96.
[38] Yoshida D, Ikeda Y, Nakazawa S. Quantitative analysis of copper, zinc and copper/zinc ratio in selective human brain tumors. J Neurooncol 1993;16:109–115.
[39] Ebara M, Fukuda H, Hanato R, Saisho H, Nagato Y, Suzuki K, Nakajima K, Yukawa M, Kondo F, Nakayama A, Sakurai H. Relationship between zinc, copper and metallothionein in hepatocellular carcinoma and its surrounding liver parenchyma. J Hepatol 2000;33:415–422.
[40] Zheng LF, Wei QY, Cai YJ, Fang JG, Zhou B, Yang L, Liu ZL. DNA damage induced by resveratrol and its synthetic analogs in the presence of Cu(II) ions: mechanism and structure-activity relationship. Free Radic Biol Med 2006;41: 1807–1816.
[41] Chen ZP, Schell JB, Ho CT, Chen KY. Green tea epigallocatechin galate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. Cancer Lett 1998;129:73–179.
[42] Lu J, Ho CT, Ghai G, Chen KY. Differential effects of theaflavin monogallates on cell growth, apoptosis, and Cox-2 gene expression in cancerous versus normal cells. Cancer Res 2000;60:6465–6471.
[43] Orsolic N, Knezevic AH, Sver L, Terzic S, Basic I. Immunomodulatory and antimetastatic action of propolis and related polyphenolic compounds. J Ethnopharmacol 2004;94:307–315.
[44] Orsolic N, Terzic S, Sver L, Basic I. Honey-bee products in the prevention and therapy of murine transplantable tumors. J Sci Food Agric 2005;85:363–370.
[45] Mukhtar H, Katiyar SK, Agarwal R. Green tea and Skin-anticarcinogenic effects. J Invest Dermatol 1994;102:3–7.
[46] Gupta S, Hastak K, Ahmad N, Lewin JS, Mukhtar H. Inhibition of prostate carcinogenesis in TRAMP mice by oral infusion of green tea polyphenols. Proc Natl Acad Sci 2001;98:10350–10355.
[47] Saiko P, Szakmary A, Jaeger W, Szekeres T. Reseveratrol and its analogs: defence against cancer, coronary disease and neurodegenerative maladies or just a fad? Mutat Res 2008;658:68–94.
[48] Surh YJ. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal polyphenolic substances. Mutat Res 1999;428:305–327.
[49] Bandele OJ, Clawson SJ, Osheroff N. Dietary polyphenols as Topoisomerase II poisons: B ring and C ring substituents determine the mechanism of enzyme-mediated DNA cleavage. Chem Res Toxicol 2008;21:1253–1260.
[50] Austin CA, Patel S, Ono K, Nakane H, Fischer LM. Site specific DNA cleavage by mammalian DNA topoisomerase II induced by novel flavone and catechin derivatives. Biochem J 1992;282:883–889.
[51] Pryor WA. Why is hydroxyl radical the only radical that commonly adds to DNA? Hypothesis: it has rare combination of high electrophilicity, thermochemical reactivity and a mode of production near DNA. Free Radic Biol Med 1988;4: 219–233.
[52] Chevion M. Site-specific mechanism for free radical induced biological damage. The essential role of redox-active transition metals. Free Radic Biol Med 1988;5:27–37.
[53] Piwocka K, Zablocki K, Weickowski MR, Skierski J, Feiga I, Szopa J, Drela N, Wajtczak L, Sikora E. A novel apoptosis-like pathway, independent of mitochondria and caspases, induced by curcumin in human lymphoblastoid T (Jurkat) cells. Exp Cell Res 1999;249:299–307.
[54] Leist M, Jaattela M. Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2001; 2:589–598.
[55] Yoshino M, Haneda M, Naruse M, Htay HH, Tsuboushi R, Qiao SL, Li WH, Murakami K, Yokochi T. Prooxidant activity of curcumin: copper-dependent formation of 8-hydroxy-2′-deoxyguanosine in DNA and induction of apoptotic cell death. Toxicol In Vitro 2004;18:783–788.
[56] Noda C, He J, Takano T, Tanaka C, Kondo T, Tohyama K, Yamamura H, Tohyama Y. Induction of apoptosis by epigallocatechin-3-gallate in human lymphoblastoid B cells. Biochem Biophys Res Commun 2007;362:951–957.
[57] Heiss EH, Schilder YDC, Dirsch VM. Chronic treatment with resveratrol induces redox stress- and ATM-dependent senescence in p53-positive cancer cells. J Biol Chem 2007;282:26759–26766.

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