Tumor Biol.
DOI 10.1007/s13277-015-3649-y

RESEARCH ARTICLE

Targeting increased copper levels in diethylnitrosamine induced hepatocellular carcinoma cells in rats by epigallocatechin-3-gallate

Mohd Farhan[1] · **Asim Rizvi**[1,2] · **Imrana Naseem**[1] · **S. M. Hadi**[1] · **Aamir Ahmad**[3]

Received: 22 April 2015 / Accepted: 4 June 2015

Abstract We have earlier elucidated a pathway for the anticancer action of plant polyphenolic compounds against malignant cells involving mobilisation of endogenous copper ions and the consequent prooxidant action. To further confirm our hypothesis in vivo, we induced hepatocellular carcinoma (HCC) in rats by diethylnitrosamine (DEN). We show that in such carcinoma cells, there is a progressive elevation in copper levels at various intervals after DEN administration. Concurrently with increasing copper levels, epigallocatechin-3-gallate (EGCG; a potent anticancer plant polyphenol found in green tea) mediated DNA breakage in malignant cells is also increased. The cell membrane permeable copper chelator neocuproine inhibited the EGCG-mediated cellular DNA degradation, whereas the membrane impermeable chelator bathocuproine was ineffective. Iron and zinc specific chelators desferoxamine mesylate and histidine, respectively, were also ineffective in inhibiting EGCG mediated DNA breakage. Through the use of specific scavengers, the mechanism of DNA breakage was determined to be mediated by reactive oxygen species. In summary, we provide an in vivo evidence of accumulating copper in hepatocellular carcinoma that is targeted by EGCG, leading to its anticancer role in a prooxidant manner. Our findings confirm a novel mechanism of anticancer activity of EGCG in particular and plant derived nutraceuticals in general.

Keywords Copper · Diethylnitrosamine · EGCG · Hepatocellular carcinoma · Liver

Mohd Farhan and Asim Rizvi contributed equally to this work.

✉ Aamir Ahmad
ahmada@karmanos.org

[1] Department of Biochemistry, Faculty of Life Sciences, AMU, Aligarh, India

[2] Section of Radiobiology, Department of Radiology, The Biomedical Research Tower, The Ohio State University, 460W 12th Avenue, Columbus, OH, USA

[3] Karmanos Cancer Institute, Wayne State University, 707 HWCRC, 4100 John R St., Detroit 48201, MI, USA

Introduction

Elevation of copper levels in the serum and blood of patients with malignancies is well documented [1–4]. Studies with plant-derived polyphenols such as epigallocatechin-3-gallate (EGCG) [5, 6] show that they are potent inducers of apoptosis in malignant cells, whereas normal cells are refractory to this effect. About a decade and a half back, we had proposed that an important mechanism for anticancer and cell death inducing properties of plant derived polyphenols involves mobilisation of endogenous copper and the consequent prooxidant action [7].

Among the metal ions, the concentration of iron is the highest in cells, whereas in the nucleus, copper and zinc are the major metals present [8, 9]. Among the transition metals, copper possesses the highest redox activity [10], facilitating rapid recycling in the presence of molecular oxygen and compounds such as EGCG, leading to the formation of reactive oxygen species (ROS). Such a mechanism would be independent of receptor and mitochondria-mediated programmed cell death [11].

Over the years, we have validated our hypothesis successfully [10, 12–19]. Specifically, we have shown that oxidative cellular breakage by polyphenols involves mobilisation of nuclear copper [14], and copper overload in normal lymphocytes leads to increased cellular DNA degradation by polyphenols [17]. Finally, we have demonstrated that polyphenols induce

Published online: 12 June 2015

growth retardation in various cancer cell lines and this effect is inhibited significantly by copper specific chelators, whereas iron and zinc chelators are relatively ineffective [18–20].

In the present report, we have induced hepatocellular carcinoma in rats by diethylnitrosamine (DEN). As further confirmation of our hypothesis, we show that during cancer induction, copper levels in liver are progressively increased, and consequently epigallocatechin-3-gallate (EGCG; a potent apoptosis-inducing agent present in green tea) mediated DNA breakage in malignant liver cells is also enhanced. These studies take the confirmation of our hypothesis a step further and emphasise the importance of plant polyphenols as lead compounds in designing novel anticancer drugs.

Experimental procedures

Materials

Diethylnitrosamine, epigallocatechin-3-gallate, agarose, low melting point agarose, bathocuproine, neocuproine, desferoxamine mesylate, superoxide dismutase, histidine, thiourea, catalase, RPMI 1640, trypan blue, histopaque 1077 and phosphate buffered saline were purchased from Sigma-Aldrich (St. Louis, USA).

Induction of hepatocellular carcinoma in rats by DEN

Animal experimentations were permitted by Ministry of Environment and Forests, Government of India, under registration no. 714/02/a/CPCSEA issued by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) dated 16 November 2002 and approved by the institutional ethical committee of Department of Biochemistry, Aligarh Muslim University, Aligarh India (order number: DNo1). Male albino rats (100±25 g) were purchased from a local animal vendor. The animals were housed in polypropylene cages and fed standard rat chow and water provided ad libitum and acclimatised for 2 weeks before beginning the experiment.

Animals were divided into two groups of 25 each. Group one served as control and group two was administered 100 mg/kg body weight DEN dissolved in 1 ml normal saline (pH 7.0, 37 °C) as a single bolus intraperitoneal injection. The animals were further provided DEN in drinking water (0.05 % *v*/*v*) placed in the cages. The animals were sacrificed on days 15, 30, 45 and 60, and blood was collected in heparinised tubes for assessment of liver function markers. The liver was isolated and washed several times with normal saline (pH 7.0, 37 °C). When the livers were visually examined at all stages including day 15, they exhibited an abnormal appearance. However, at day 60, the livers showed a clear development of tumours when compared to untreated animals (Fig. 1a, b).

Liver function tests

Liver function tests were carried out by a commercial laboratory (Lalpath Labs, New Delhi), using standard kits, as per the manufacturer's instructions.

Isolation and treatment of hepatocytes

Hepatocytes were isolated by the method of Hassan et al. [21] and their viability was assessed by the trypan blue test and was found to be >93 %. Hepatocytes were suspended in PBS, and aliquots of 0.5 ml (approximately 10^6 cells) were used in reaction mixtures. Hepatocytes were exposed to different concentrations of EGCG in a total volume of 3 ml and incubated at 37 °C for 1 h. The reactions were also performed in the presence of specific metal ion chelators. Desferoxamine (50 μM) was used for chelation of Fe (II) ions, histidine (50 μM) was used for Zn (II) and bathocuproine and neocuproine (50 μM each) were used for chelation of Cu (II) ions. Free radical scavengers (catalase 100 μg/ml, superoxide dismutase (SOD) 100 μg/ml and thiourea 1 mM) were used in a separate set of reactions to examine the role of ROS in Cu (II) mediated oxidative cellular DNA breakage. At the end of the reaction, cell viability was again determined and was found to be not less than 88 %.

Estimation of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were estimated in hepatocytes by the method of Ramanathan et al. [22]. To 1.5 ml reaction mixture, 0.5 ml of 10 % trichloroacetic acid (TCA) and 0.5 ml of 0.6 M TBA (2-thiobarbituric acid) were added, and the mixture was incubated in a boiling water bath for 20 min. The absorbance was read at λ=532 nm and converted to nanomoles of TBARS using the molar extinction coefficient.

Comet assay (single cell alkaline gel electrophoresis)

Comet assay was performed using the method employed by Singh et al. [23] with minor modifications. Fully frosted slides precoated with 1.0 % normal melting agarose were used. About 10,000 hepatocytes after treatment were mixed with 90 μl of 1.0 % low melting point agarose to form a cell suspension and pipetted over the first layer and covered with a cover slip. The slides were placed on ice pack for 10 min to solidify the agarose. The coverslips were gently removed, and a third layer of 0.5 % low melting point agarose was pipetted. The slides were covered with cover slips and were placed on ice packs to solidify. The cover slips were then removed, and the slides were immersed in ice cold lysis buffer for an hour. After lysis, the DNA was allowed to unwind in alkaline electrophoretic solution (300 mM NaOH, 1 mM EDTA, pH>13). Electrophoresis was performed at 4 °C in a field strength of 0.7 V/cm and 300 mA current. The slides were neutralised with ice cold

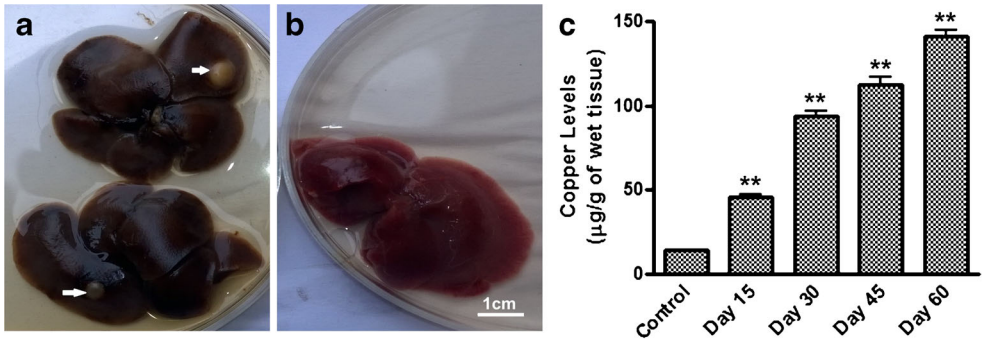

Fig. 1 Gross pictures of **a** malignant livers (*arrows* indicate malignant lesions) and **b** control liver. **c** Copper levels in liver tissue at different intervals after DEN administration. Rats were sacrificed at intervals indicated (post 100 mg/kg body weight intraperitoneal injection of DEN), and the liver tissue was isolated to estimate the level of copper (see 'Experimental procedures'). Values reported are mean±SEM of three independent experiments. $^{**}p<0.01$ (relative to control)

0.4 M Tris pH 7.5 and stained with 75 µl ethidium bromide (20 mg/ml) and covered with a cover slip. Slides were scored using an image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, UK) attached to an Olympus (CX41) fluorescent microscope (Olympus Optical Co., Tokyo, Japan) and a COHU 4910 integrated CC camera (equipped with 510–560 nm excitation and 590 nm barrier filters) (COHU, San Diego, CA, USA). Images of 25 cells were analysed from each triplicate slide. Tail length (migration of DNA from nucleus in micrometres) was the parameter used to asses DNA breakage.

Measurements of copper levels

To 0.1 g fresh tissue, 0.5 ml concentrated nitric acid was added, and the sample was incubated at 90 °C for 2 h in a glass stoppered test tube. The sample was then cooled and 0.5 ml 30 % hydrogen peroxide was added. The sample was incubated at 50 °C overnight and was filtered and analysed using GBC 932 Plus atomic absorption spectrophotometer (JT Baker, Philipsburg, NJ, USA). The results were analysed using GBC Avanta Ver 1.33.

Statistical analysis

Values are expressed as mean±SEM from three independent experiments. Data was analysed by unpaired *t* test using GraphPad Prism. A p value<0.05 was considered to be statistically significant.

Results

DEN induced hepatocellular carcinogenesis proceeds with a progressive rise in copper level

DEN administration led to a rise in copper levels (Fig. 1c) and observable malignant nodules (Fig. 1a, b) at day 60, post administration. The rise in copper levels positively correlated with a rise in serum level of alpha fetoprotein (Table 1), a specific diagnostic blood marker for hepatocellular carcinoma [24, 25]. It is worth noting that in this study, a tenfold increase in copper levels was observed in the malignant liver as compared to the controls (Fig. 1c). To the best of our knowledge, this study is the first observation of such a dramatic rise in tissue copper levels during experimental induction of hepatocellular carcinoma. The malignancy progressed with a classical rise in blood markers of liver damage such as bilirubin, serum glutamic oxaloacetic transaminase (EC 2.6.1.1) (sGOT), serum glutamic pyruvic transaminase (EC 2.6.1.2) (sGPT), gamma glutamyl transpeptidase (EC 2.3.2.2) (GGTP) and alkaline phosphatase (EC 3.1.3.1) (ALP) (Table 1). As expected, serum albumin levels declined consistently [26].

EGCG induces oxidative DNA breakage in malignant hepatocytes

We have previously shown that the treatment of various cancer cell lines [18, 19] by plant polyphenols at micromolar concentrations leads to inhibition of cell proliferation and induction of apoptosis. This property was attributed to mobilisation of endogenous copper ions leading to reduction of Cu (II) to Cu (I) which in the presence of molecular oxygen leads to the formation of ROS, which in turn act as proximal DNA cleaving agents [27]. Since EGCG is a potent inducer of apoptosis in cancer cell lines, we have used this polyphenol to examine cellular DNA breakage in hepatocytes at different intervals after DEN administration. EGCG treatment led to significant DNA breakage at all four time points (Fig. 2), and the DNA breakage by EGCG treatment positively correlated with the linear increase of copper levels during the establishment of hepatocellular carcinoma (Fig. 1c). This is consistent with our previous studies [17], where we show that EGCG induced DNA breakage in lymphocytes is a function of the concentration of nuclear copper. The increase in tail length of control (day 0) with increasing EGCG concentration (25–75 µM) is possibly due to the toxicity of copper itself as

Table 1 Liver function markers during induction of hepatocellular carcinoma in rats by DEN

	Control	Day 15	Day 30	Day 45	Day 60
sGOT (U/l)	422±6	456±8	479±5	501±13	548±9
sGPT (U/l)	93±4	102±12	128±11	135±10	148±9
GGTP (U/l)	28±8	30±6	35±9	44±2	50±5
ALP (U/l)	158±11	170±7	182±18	191±10	200±12
Bilirubin (mg/dl)	1.10±0.04	1.31±0.02	1.53±0.02	1.87±0.09	2.00±0.4
Alpha-fetoprotein (ng/ml)	2±3	187±8	300±7	430±6	500±6
Serum albumin (g/dl)	3.50	3.18	2.01	1.22	0.83

Rats were sacrificed at intervals indicated (post 100 mg/kg body weight intraperitoneal injection of DEN), and blood was isolated in heparinised tubes by cardiac puncture. Liver function markers were estimated using standard kits as per manufacturer's instructions. Values reported are a mean±SEM of three independent experiments. *sGOT* serum glutamic oxaloacetic transaminase (EC 2.6.1.1), *sGPT* serum glutamic pyruvic transaminase (EC 2.6.1.2), *GGTP* gamma glytamyl transpeptidase (EC 2.3.2.2), *ALP* alkaline phosphatase (EC 3.1.3.1), *U/l* international units per litre

copper is the most redox active metal ion which leads to the formation of ROS causing cytotoxicity. Since ROS induced DNA damage gives rise to thiobarbituric acid reactive substances (TBARS) [28, 29], we estimated TBARS in malignant hepatocytes (a measure of oxidative stress) as a function of increasing concentrations of EGCG. It was observed that TBARS level increased linearly with an increase in EGCG concentration, and this effect was observable at all four time points tested (Fig. 3).

Effect of metal specific sequestering agents and ROS scavengers on EGCG induced DNA breakage in malignant hepatocytes

Since the DNA breakage induced by EGCG is a consequence of its interaction with nuclear copper, we utilised copper chelators, neocuproine (a membrane permeable copper chelator) and bathocuproine (a membrane impermeable copper chelator) to test if unavailability of copper in the cells would lead to a decrease in DNA damage. Further, to rule out the role of other redox active cellular metals such as iron and zinc, specific chelators desferoxamine mesylate (iron chelator) and histidine (zinc chelator) were used. It was observed that removal of copper by the membrane permeable chelator neocuproine leads to a marked decrease in DNA damage, while chelation and hence unavailability of iron and zinc by desferoxamine mesylate and histidine did not inhibit DNA degradation (Fig. 4a). This clearly elucidates the role of intracellular copper as a participant in EGCG mediated DNA damage in malignant cells.

To prove if reactive oxygen species (ROS) were the terminal DNA cleaving agents, we added ROS scavengers (superoxide dismutase, catalase and thiourea) to malignant cells

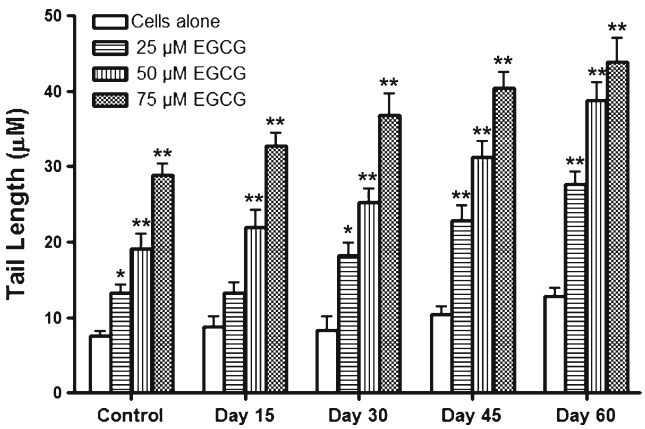

Fig. 2 Cellular DNA breakage induced by EGCG in DEN induced hepatocellular carcinoma cells, as analysed by comet assay. Rats were sacrificed at intervals indicated (post 100 mg/kg body weight intraperitoneal injection of DEN), and the hepatocytes were isolated and subjected to comet assay with increasing concentration of EGCG (see 'Experimental procedures'). Comet tail length values are reported in micrometres as mean±SEM of three independent experiments. *$p<0.05$; **$p<0.01$, relative to respective controls with cells without EGCG treatment

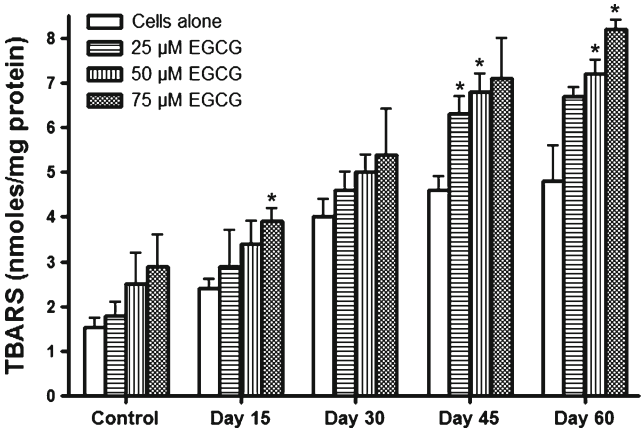

Fig. 3 TBARS generation by increasing concentrations of EGCG at various stages of hepatocellular carcinoma induction in rats by DEN. Rats were sacrificed at intervals indicated (post 100 mg/kg body weight intraperitoneal injection of DEN), and the hepatocytes were isolated to estimate the levels of TBARS. Reaction mixture contained 10^6 cells in 1 ml PBS alone or with increasing concentration of EGCG (see 'Experimental procedures'). TBARS are reported in nanomoles per milligramme protein as mean±SEM of three independent experiments. *$p<0.05$, relative to respective controls with cells without EGCG treatment

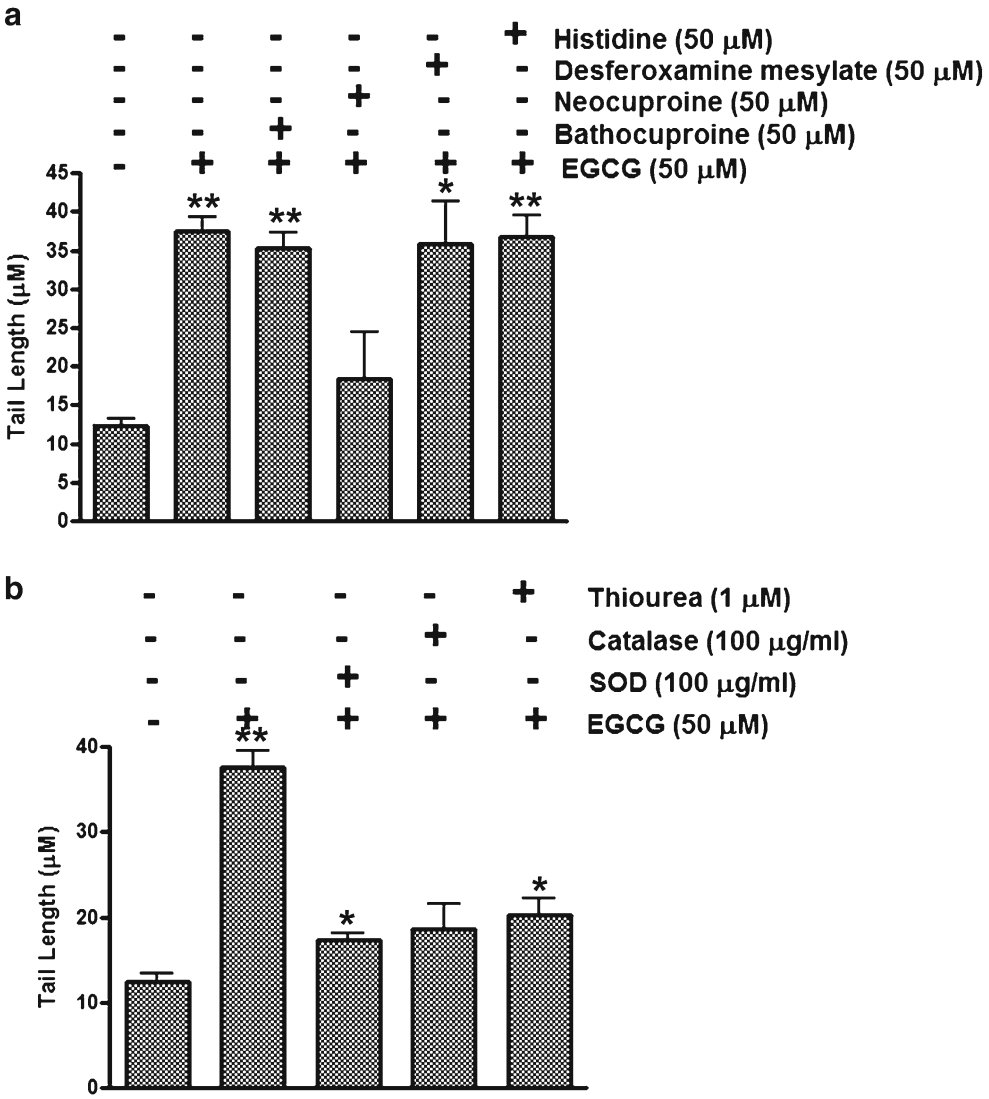

Fig. 4 **a** Effect of metal chelators and **b** ROS scavengers on EGCG induced cellular DNA breakage in DEN induced hepatocellular carcinoma cells. Rats were sacrificed at day 60 (post 100 mg/kg body weight intraperitoneal injection of DEN), and the hepatocytes were isolated and subjected to comet assay with/without 50 μM EGCG and/or scavengers and chelators (see 'Experimental procedures' for treatment of hepatocytes). Comet tail length values are reported in micrometres as mean±SEM of three independent experiments. *$p<0.05$; **$p<0.01$, relative to control cells without EGCG treatment

treated with EGCG. It was observed that ROS scavengers significantly inhibited DNA damage, thereby confirming that EGCG copper interaction within malignant cells leads to ROS production which leads to subsequent DNA breakage (Fig. 4b).

Discussion

The fact that copper levels, both in serum and tissue, are elevated in various malignancies has been known for several decades [1, 2, 4]. There is a considerable body of evidence to indicate that copper is required for tumour angiogenesis [30]. Ceruloplasmin, the major copper transport protein is elevated in tumours [31, 32]. In view of its role in tumorigenesis, copper has attracted attention as a putative target for anticancer therapeutic agents. The importance of elevated copper as a target for anticancer agents lies in the fact that it is one of the properties that is unique to cancer cells. This property lends cancer cells to preferential cytotoxicity to anticancer agents such as EGCG. The results presented above demonstrate that during chemically induced carcinogenesis, copper levels progressively increase and that such increased level of copper can be targeted by plant-derived polyphenols for tumour cell toxicity.

In the last 20 years, it has been recognised that plant derived polyphenols, such as resveratrol, tea catechins (particularly EGCG) and curcuminoids (such as curcumin) possess anticancer properties. This anticancer property is primarily attributed to their antioxidant behaviour and to their inducing cell death via apoptosis, suppressing anti apoptotic pathways and modulating a number of proteins implicated in sustaining the growth of cancer cells [33]. Nevertheless, workers in the field are yet to identify the main mechanism underlying the preferential cancer selectivity elicited by these agents which spare normal cells. The hypothesis proposed by us explains

such selectivity by suggesting that increased copper levels in cancer cells facilitate electron transfer between copper and plant polyphenols, leading to the generation of superoxide and hydroxyl radicals in the vicinity of cellular DNA [10, 16]. It therefore follows, that at appropriately high concentrations, plant polyphenols should also be toxic to normal cells. This is indeed the case, as we have shown in several of our previous publications [14]. In this connection, it may also be noted that EGCG has been shown to inhibit the growth of SV40 virally transformed W138 cells but not their normal counterparts. The IC_{50} value of EGCG was estimated to be 120 and 10 μM for normal versus the transformed cells, respectively [34].

Thus, we propose that it is the prooxidant activity of plant polyphenols rather than the antioxidant property which is more important for their anticancer action. In recent years, a number of laboratories have confirmed our result which has resulted in increasing acceptance of the prooxidant behaviour of these compounds [35, 36]. These and other studies unequivocally prove that in addition to their antioxidant behaviour, natural dietary and diet derived polyphenolic agents can elicit prooxidant behaviour, and this property cannot be ignored when drawing up a design for a clinical trial investigating the anticancer activity of natural agents.

However, it must be taken into consideration that plant polyphenols have a relatively low bioavailability due to their biotransformation and rapid elimination. For example, it has been shown that highest concentration of resveratrol in plasma was reached within the first 5 min (2.6±1 μM) after receiving 20 mg res/kg b.w. orally [37]. Previous studies by others have shown that green tea polyphenols, such as EGCG, also inhibit various receptor tyrosine kinases and some pathways of signal transduction. These include MAP kinases, NF-kB, insulin-like growth factor (IGF-1) and epidermal growth factor receptor (EGFR) mediated pathways. Some of these, such as IGF-1, are overexpressed in neoplastic transformation [38]. We propose our mechanism is one of the several pathways utilised by EGCG to exert its anticancer effects.

Copper is an essential constituent of chromatin and is bound to GC base pairs in cellular DNA [39]. This facilitates the generation of hydroxyl radical in the vicinity of DNA leading to its cleavage. It is to be noted that the diffusion radius of hydroxyl radical is small, and it will react with any macromolecule or metabolite it encounters [16, 40]. Thus, the redox cycling of DNA bound copper and consequent generation of hydroxyl radical confers an advantage to such a mechanism over various other mechanisms mentioned above. In addition to these other mechanisms, mobilisation of elevated copper levels in cancer cells by plant polyphenols in general [41] and EGCG in particular could be an important anticancer mechanism of such naturally occurring anticancer agent.

It is understood that inherited genetic variation and acquired genomic aberrations contribute to cancer initiation and progression. In a recent paper, at least ten subgroups of breast cancer were identified. In each subgroup, a different set of genes were mutated in terms of copy number variants and single nucleotide polymorphisms [42]. Watson has recently recognised that a vast majority of agents used to directly kill cancer cells, such as ionising radiation and chemotherapeutic agents, work through generating ROS that inhibit the cell cycle [43]. Thus, it is highly unlikely that the search for a single bullet cure for various cancers directed towards their genetic origin would be successful. Therefore, the interest in anticancer research has moved to curing of cancer on the biochemistry/metabolism of cancer cells. In this context, our studies assume considerable significance as they provide a basis for designing novel anticancer compounds based on targeting copper levels in cancer cells [44–46].

Conflicts of interest None

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