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The specific anti-cancer activity of green tea (−)-epigallocatechin-3-gallate (EGCG)

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Summary. The effect of the green tea polyphenol-(−)epigallocatechin-3-gallate (EGCG) was tested in cultures of normal and transformed NIH-pATM*ras* fibroblasts. In this system transformation can be induced at will by the addition of dexamethasone, which induces the expression of H-*ras* by activating the mammary tumor virus long terminal repeat (MMTV-LTR) promoter. This facilitates a reliable comparison of the susceptibility of normal and transformed cells to EGCG. It has been shown that EGCG inhibited the growth of transformed but not of the normal fibroblasts. In an attempt to elucidate the mode of the preferential inhibitory activity of EGCG, its effect on growth promoting factors has been examined. The level of ornithine decarboxylase (ODC, EC 4.1.1.17), which is a signal for cellular proliferation, was reduced by EGCG in the transformed but not in the normal cells. EGCG also showed strong inhibition of tyrosine kinase and mitogen-activated protein kinase (MAPK) activities, without affecting the kinases in the normal cells. Similarly, EGCG also preferentially decreased the levels of the oncogenes Ras and Jun in transformed cell. EGCG preferentially induced apoptosis in the transformed fibroblasts. *In vitro* chemosensitivity tests demonstrated that EGCG inhibited the proliferation of leukemic cells. These findings suggest that EGCG has a therapeutic potential in the combat against cancer.

Keywords: Amino acids – (−)-Epigallocatechin-3-gallate (EGCG) – Ornithine decarboxylase (ODC) – Tyrosine kinase – Mitogen-activated protein kinase (MAPK) – Oncogene – Apoptosis

Introduction

The concept of treatment and prevention of cancer using naturally occurring substances that could be included in the diet is gaining increasing attention. Indeed, a wide array of phenolic substances, particularly those present in dietary and medicinal plants, have been reported to posses substantial anti-

carcinogenic and anti-inflammatory activities. These substances, which are included in the food and are consumed repeatedly, apparently posses limited toxicity. Green and black tea are two major types of tea manufactured from the leaves of the plant *Camelia sinesis*. It has been shown that 30–40% of the dry weight of the solids in brewed green tea is composed of polyphenols, of which EGCG is the most abundant (Balentine et al., 1997). This tea constituent, is the most extensively studied tea polyphenol (Yang and Wang, 1993), and has been shown to reduce cancer risk in a variety of animal tumor and/or proliferative bioassay systems (Katiyar and Mukhtar, 1996; Kohlmeier, 1997; Lin et al., 1999; Okabe et al., 1999; Lu et al., 1998). Epidemiological studies carried out in Shanghai, China, revealed that among drinkers of green tea, the risk of stomach cancer decreased significantly (Yu et al., 1995). Similarly, the incidence of prostate cancer in China, a population that consumes green tea on a regular basis, is the lowest in the world (Gupta et al., 1999). During the past decade, attempts have been made to elucidate the mechanism(s) of the anti-cancer activities of green tea extracts. In some of those studies cultured cells transformed chemically (Hu et al., 1995) or by oncogenes (Chung et al., 1999; Ahn et al., 1999) have been used.

Ras gene mutations, which perpetually turn on the growth signal transduction pathways, occurs frequently in many cancer types (Weijzen et al., 1999; Zachos and Spandidos, 1997). Cultured cells transfected with a mutant of H-*ras* gene, mimic carcinogenesis *in vitro*. EGCG was found to inhibit the growth and the signal transduction pathway in mouse epidermal JB6 cells, transfected with a mutant of H-*ras* gene (Chung et al., 1999). These studies thus confirmed the anti-cancer activity of tea extracts. Unfortunately, no data were available concerning the specific anti-cancer activity of EGCG and the effect of this compound on the properties of equivalent normal cells has not been compared. In the present study we used NIH 3T3 fibroblasts transfected with a construct (pATM*ras*) in which H-*ras* was under the transcriptional control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter (Tabib and Bachrach, 1998). Dexamethasone, which activates the promoter can induce transformation at will. Therefore, the specific anti-cancer effect of EGCG was studied by treating those cells with the tea extract in the presence or absence of dexamethasone. It will be shown that EGCG preferentially inhibits the growth of cultured transformed fibroblasts and of leukemic blast cells from an acute lymphoblastic leukemia (ALL) patient. These findings support the notion that EGCG can be used for the treatment and prevention of cancer.

Materials and methods

Cell culture and treatment

NIH 3T3 fibroblasts were transfected with a construct (pATM*ras*) in which Ha-*ras* was under the transcriptional control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter (Tabib and Bachrach, 1998). Cells (2×10^5) were grown on 10 cm plates for 48 h in Dulbecco's Modified Eagle's Medium (DMEM, Beit Haemek,

Israel) containing 5% fetal calf serum (FCS, GIBCO, Grand Island, NY), and then starved in the medium with 0.1% FCS for another 48 h, in a 5% CO_2 incubator in 95% air. Dexamethasone (Sigma) at 0.5 μM, was added to part of the cultures for 12 h to trigger the phenotypic transformation. Then both the normal and the transformed cells were exposed to EGCG (Sigma) for another 12 h at 1, 5, 10, 20, and 40 μM concentrations.

Western blots

Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with cell lysis buffer (New England Biolabs, Beverly, MA). Debris were removed by centrifugation for 10 min at 4°C and aliquots of 20 μl of the cell lysate were separated by SDS-PAGE (12% gel). Separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Bio Tracer, Gelman Sciences, Ann Arbor MI) and probed with a primary antibody, followed by a horseradish peroxidase-conjugated second antibody (Jackson ImmunoResearch, West Grove, PA). Anti-H-Ras, anti-c-Fos and anti-c-Jun antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ODC was produced in our laboratory (Wang et al., 1999). Anti-active MAPK antibody was purchased for Promega Corporation (Madison, WI). Antibody to phosphotyrosine was from Transduction Laboratories (Lexington KY). The bands on the blots were revealed by Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The blots were stripped and re-probed with anti-β-actin antibody (Santa Cruz) for internal protein standard. The protein levels were analysed by the NIH Image Analyst software.

Isolation and growth of leukemic blast cells

Heparinized fresh blood was taken from a 7 year-old boy suffering from acute lymphoblastic leukemia (ALL), treated in the Department of Bone Marrow Transplantation, Hadassah University Hospital, Jerusalem. The leukemic blast cells were isolated using Ficoll-paque (Pharmacia Biotech, Uppsala, Sweden) and suspended in RPMI 1640 medium containing 15% autologous plasma and 2.5 μg/ml phytohemagglutinin (PHA, Sigma). The PHA-stimulated cells were then incubated at 37°C in a 5% CO_2 incubator, with EGCG (20 μM). After 20 h, cells were washed with PBS, by centrifugation at 1200 r.p.m. for 10 min. Slides were prepared by dropping the cells on superfrost plus slides (Menzel-Glaser, Germany).

In vitro chemosensitivity assay

The assay was carried out as described elsewhere (Wang et al., 1999). Briefly, the slides were dried at room temperature and fixed for 20 min with a fresh mixture of methanol and acetic acid at a ratio of 3:1 (v/v). Slides were next blocked in 1% bovine serum albumin and probed with rabbit anti-human ODC antibodies. The level of ODC expression was revealed by Cy™2-conjugated affinipure goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA). Specimens were examined by a Confocal Laser Scanning Microscope, LSM 410 (Zeiss, Germany) and 8 images were randomly chosen and analyzed using Software Image-Pro Plus, version 4.0 (Media Cybernetics, Silver Spring, MD).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

For the demonstration and quantification of apoptosis, the TUNEL assay was performed using an ApopTag Floreacein *In Situ* Apoptosis Detection Kit (Intergen, Purchase, NY) according to the instructions of the manufacturers. Apoptotic nuclei were labeled with

FITC, and all nuclei were counter-stained with 8μg/ml propidium iodide (Sigma). Samples were examined by a Confocal Laser Scanning Microscope, LSM 410. For each slide 8 fields were randomly chosen. The index of apoptosis was determined by the percentage of apoptotic cells in the 8 fields.

Results

Effect of EGCG on the growth of normal and transformed cells

Figure 1 shows the effect of EGCG on the growth of NIH-pATM*ras* cells. It may be seen that EGCG at a 5μM concentration had a minimal effect on the growth of normal cells, whereas the growth of transformed cells (obtained by the induction of H-*ras* by dexamethasone) was inhibited by approximately 40%. The growth of normal cells was somewhat inhibited by 10μM of EGCG, whereas that of the transformed cells was reduced by approximately 55%. EGCG, at 20μM inhibited the growth of normal cells by 30–40% and that of the transformed counterpart by approximately 65%. Higher dosed of EGCG also inhibited the growth of normal cells, but transformed cells were significantly more sensitive (Fig. 1). It may be concluded that transformed cells are more sensitive to the effect of EGCG and that their growth is preferentially inhibited even at 5μM concentrations.

Effect of EGCG on Ras, Jun and ODC

The Ras protein, accumulates in transformed cells (Tabib and Bachrach, 1998). Indeed, the concentration of Ras in higher in dexamethasone-treated NIH-pATM*ras* cells, compared to the normal counterpart (Fig. 2). EGCG at 5μM concentrations, hardly affected the content of Ras in normal cells,

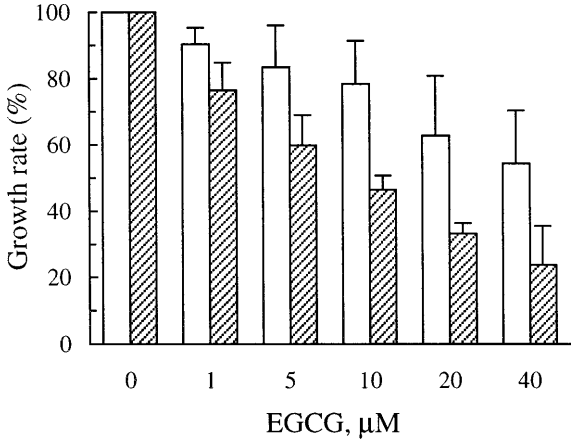

Fig. 1. Effect of EGCE on the growth rate of normal NIH-pATM*ras* cells and their transformed counterparts. Cells were starved and incubated for 12 h in the presence (striped columns) or the absence (open columns) of dexamethasone and then treated for 12 h with EGCG at various concentrations. Cells were trypsinized and counted using the trypan blue exclusion method. The values are the mean ± SD of six independent experiments

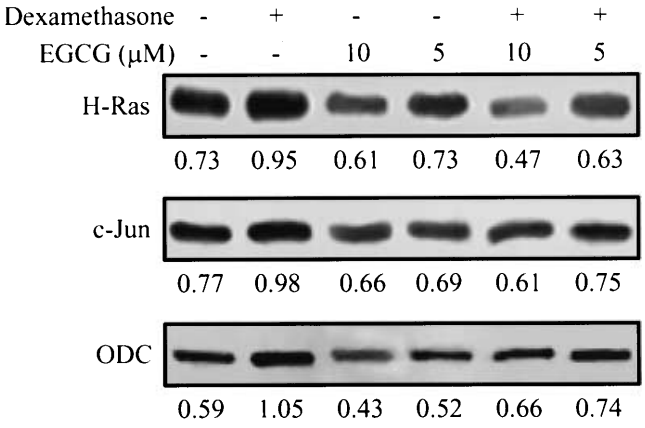

Fig. 2. Effects of EGCE on H-Ras, c-Jun and ODC in normal NIH-pATM*ras* cells and their transformed counterparts. Cells were treated for 12 h with EGCG at various concentrations and analyzed with H-Ras, c-Jun and ODC antibodies. The values below the protein bands are relative densities of the bands corrected by those of β-actin as the internal standard. Similar results were obtained in four independent experiments

while the concentration of this protein in transformed cells was reduced by approximately 35% (Fig. 2). At higher concentrations of EGCG (10μM), the Ras content of normal cells was reduced by 17% and that of the transformed cells by approximately 50%. These findings also suggest that EGCG exert a preferential inhibitory effect on Ras synthesis, similar to the effect on cellular growth (cf. Fig. 1).

Jun is a nuclear proto-oncogene, which constitutes a part of the transcription factor AP-1 (Karin et al., 1997) and is therefore involved in growth processes (Angel and Karin, 1991). It is evident from Fig. 2 that EGCG had a minimal effect on cellular Jun content of normal cells, while that of the transformed cells was reduced by 10μM EGCG by approximately 40%.

Ornithine decarboxylase (ODC) has been closely linked with cellular proliferation (Russell, 1985) and has also been regarded as an oncogene (Auvinen et al., 1992). It is therefore not surprising that the ODC protein concentration was almost twofold higher in the transformed cells, in comparison to the normal cultured cells (Fig. 2). EGCG (5 and 10μM) caused a progressive decrease of ODC protein in the transformed cells (an approximately 40% inhibition in the presence of 10μM EGCG, Fig. 2). These findings thus support the notion that EGCG exerts a preferential growth inhibitory effect.

Effect of EGCG on protein phosphorylation by tyrosine kinase

Tyrosine kinase plays an important role in signal transduction and in malignant transformation processes. Increased tyrosine phosphorylation of a 130-kD protein has been implicated in cell transformation by Src-family tyrosine kinases (Auvinen et al., 1992; Reynolds et al., 1989) and by c-H-*ras* (Auvinen et al., 1992). It was therefore of interest to find out whether EGCG inhibits

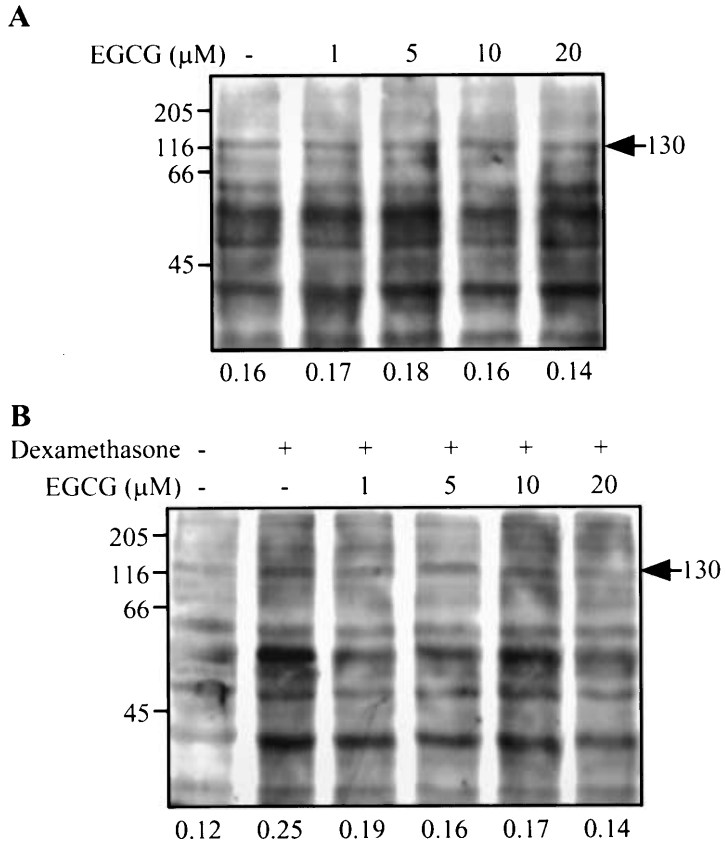

Fig. 3. Effect of EGCG on tyrosine phosphorylated proteins. Cells were treated for 12 h with EGCG at various concentrations and analyzed with monoclonal antibodies to phosphotyrosine. (**A**) Normal NIH-pATM*ras* cells. (**B**) Transformed cells. The relative positions of molecular weight standards (in thousands) are indicated on the left. The values below the protein bands are relative densities of the bands Kd-130 (indicated by arrows) corrected by those of β-actin as the internal standard. Similar results were obtained in four independent experiments

tyrosine phosphorylation, with special attention to the 130-kD protein. It may be seen (Fig. 3A) that EGCG, hardly affected the phosphorylation of the 130-kD in normal cells (grown in the absence of dexamethasone). As expected, the addition of dexamethasone to cultured NIH-pATM*ras* cells, resulted in the twofold increase in the phosphorylation of the 130-kD protein (Fig. 3B). Moreover, EGCG caused a 30–50% inhibition of tyrosine phosphorylation in that protein (Fig. 3B). These findings suggest that EGCG elicits an anti-cancer effect.

Effect of EGCG on MAPK activity

The activation of a signal transduction pathway, stimulated by either growth factors, stress, or an oncogene such as *ras* has been shown to involve MAPKs (Denhardt, 1996). These MAPKs are activated upon phosphoryla-

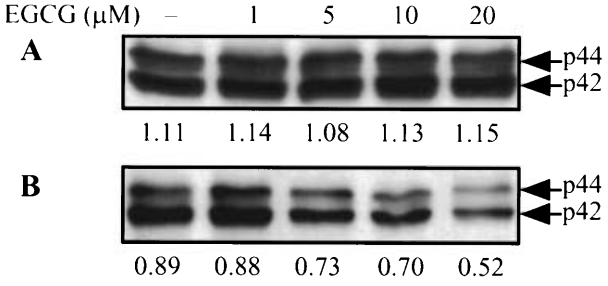

Fig. 4. Effect of EGCG on the activation of MAP kinases. Cells were treated for 12 h with EGCG at various concentrations and analyzed with antibodies to active form of MAP kinases. (**A**) Normal NIH-pATM*ras* cells. (**B**) Transformed cells. The values below the protein bands are relative densities of the bands (both p44 and p42 phospho-ERK) corrected by those of β-actin as the internal standard. Similar results were obtained in four independent experiments

tion, which then allows them to activate transcription factors such as AP-1 (Franklin and Kraft, 1995).

The phosphorylation of ERK1 and ERK2 in normal NIH-pATM*ras* cells was not affected by EGCG (Fig. 4A). The addition of EGCG to the transformed cells resulted in a significant decrease in the phosphorylation of ERK1 and ERK2 (Fig. 4B). These findings are in line with the assumption that EGCG suppresses transformation and neoplastic growth.

Effect of EGCG on apoptosis

Programmed cell death (apoptosis) is characterized by chromatin condensation and its fragmentation into multiple nucleosome-sized units owing to the action of an endogenase (Wyllie et al., 1984). This process can be examined by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick and labeling (TUNEL) method (Gavrieli et al., 1992). To find out whether EGCG triggers a preferential apoptosis, the TUNEL method was used. It may be seen (Fig. 5) that EGCG caused the occurrence of TUNEL-positive cells, predominantly in the transformed and not in the normal cultures. Thus after exposing transformed cells to 5μM EGCG for 12 hours, 32% of the transformed cells, showed apoptotic nuclear changes and only 12% in the normal controls. EGCG at 20μM concentrations, induced nuclear changes of 50% in the transformed cells and only in 14% of the normal controls (Fig. 5). All these finding strongly suggest that the growth inhibitory effect of EGCG is preferential and that more apoptosis is induced in transformed cells than in the normal controls by EGCG.

In vitro chemosensitivity test of lymphocytes from a leukemia patient

We have recently described an immunohistochemical method to determine the sensitivity to anticancer drugs of lymphocytes of hematological cancer patients (Wang et al., 1999). In the aforementioned experiments the effect

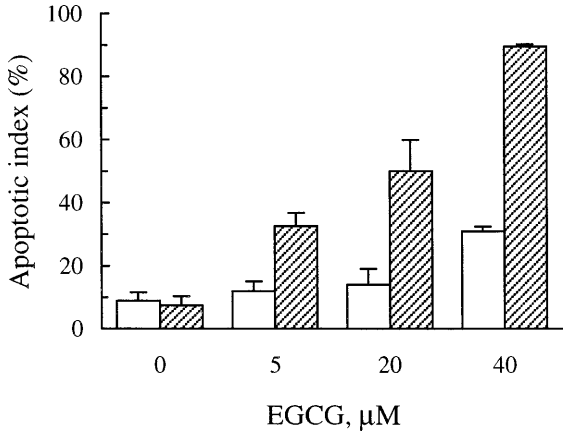

Fig. 5. Induction of apoptosis by EGCG in normal NIH-pATM*ras* cells (open columns) and their transformed counterparts (striped columns). Cells were treated for 12 h with EGCG at various concentrations. Apoptosis was detected by TUNEL assay. The apoptotic index was determined by the percentage of apoptotic cells. The values are the mean ± SD of three independent experiments

of EGCG on cultured cells was studied. It was of great interest to find out whether leukemic cells from human leukemia patients would also be affected by EGCG. Blood was taken from a 7 year old boy suffering from acute lymphoblastic leukemia (ALL). The *in vitro* chemosensitivity assay was based on the immunohistochemical detection of the growth promoting protein-ODC (Wang et al., 1999). Results described in Fig. 6 show that the leukemic blast cells contained considerable amounts of ODC and that phytohemagglutinin (which usually stimulates growth) enhanced ODC production by approximately 25% (Fig. 6a,b). EGCG (20μM) demonstrated therapeutic activity and the amount of ODC was reduced by approximately 50% (Fig. 6c). These results strongly suggest that EGCG has a therapeutic potential.

Discussion

Recently, considerable attention has been focused on identifying naturally occurring substances capable of inhibiting, retarding and preventing cancer. A wide array of phenolic substances, particularly those present in dietary and medicinal plants, have been reported to possess these activities. Among them green tea polyphenols were studied extensively. It has been demonstrated that green tea polyphenols inhibit the proliferation of cultured mammalian cells, including colon carcinoma, hepatoma (Yu et al., 1997), vascular smooth muscle cells (Ahn et al., 1999), lung carcinoma (Suganuma et al., 1999), breast carcinoma (Chen et al., 1998), mouse epidermal cells (Chung et al., 1999), melanoma, prostate (Paschka et al., 1998) and leukemic cells (Lea et al., 1993; Asano et al., 1997).

Green tea polyphenols differentially modulate nuclear factor kappaB in cancer cells versus normal human epidermal keratinocytes (Ahmad et al.,

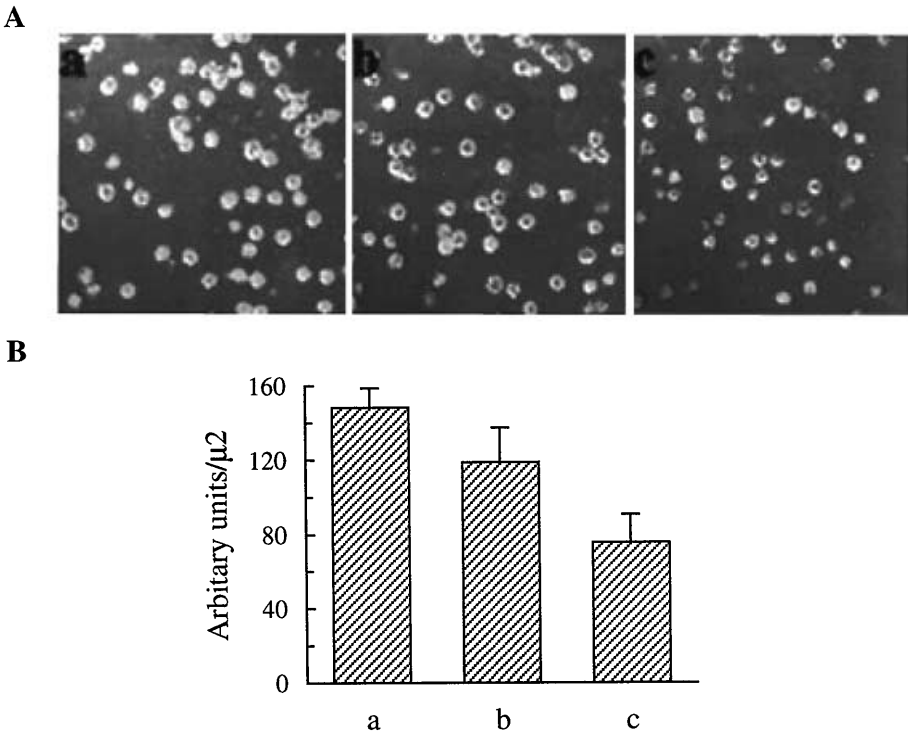

Fig. 6. *In vitro* chemosensitivity test of ALL cells to EGCG in comparison with that to cyclophosphophamide. The lymphoblasts were cultured for 20 h with (**a**) 2.5 μg/ml PHA, (**b**) non-stimulation, (**c**) 2.5 μg/ml PHA and 20 μM EGCG. Immunohistochemical detection of ODC was performed using the ODC antibody and Cy™2-conjugated anti-rabbit IgG antibody. (**A**) Immunohistochemical staining of ODC in ALL cells. (**B**) Densitometric analysis of ODC of the specimens. Data are the means ± SD of all the cells in the 8 images of each pecimen

2000). EGCG (50–200 μM) also induced apoptosis in SV40 virally-transformed human fibroblasts, but not in the normal cells (Chen et al., 1998). In those studies different cell lines, derived from the same parent line have been used. During the passages the growth rates and requirements for nutrients could vary from one line to the other and therefore the physiological properties of the normal and transformed cells may differ significantly. To circumvent this difficulty we used the same parent cell, which grew under identical conditions and the induction of transformation was initiated at will (within 8 h) by the addition of dexamethasone to the NIH-pATM*ras* cells. This permitted a reliable comparison between normal and cancerous cells and their response to EGCG treatment.

EGCG at relatively low concentrations, preferentially inhibited the growth of the transformed cells, while the growth of the normal cells was not markedly affected (Fig. 1). These findings are in line with published data indicating the inhibition of growth of cancer cells (Mukhtar and Ahmad, 2000).

In the present study, we tried to clarify the mechanism of growth inhibition by EGCG and tested its effect on the various steps of the signal transduction pathway. They included kinases such as tyrosine kinase, MAPK and oncogenes such as c-*jun* and H-*ras*.

As expected, transformation of NIH-pATM*ras* cells resulted in an increase in cellular Ras concentrations (Fig. 2). EGCG at 5μM concentrations reduced Ras levels in the transformed cells by approximately 35%, but did not affect Ras in the normal counterpart (Fig. 2). A similar decrease in the expression of K-*ras* by EGCG was also reported for cultured prostate cancer cells (Lyn-Cook et al., 1999). A preferential inhibitory effect of EGCG on Jun synthesis in transformed NIH-pATM*ras* cells was also observed (Fig. 2). The inhibition of Jun (Lu et al., 1998) and AP-1 syntheses (Okabe et al., 1999; Barthelman et al., 1998; Nomura et al., 2000) by EGCG has also been reported. EGCG has been found to inhibit tyrosine kinase activity in Ehrlich ascites (Kennedy et al., 1998) and in human glioblastoma cells (Sachinidis et al., 2000). In the present study, tyrosine kinase activity (as measured by the phosphorylation of a 130-kD protein) was inhibited by EGCG in transformed cells (Fig. 3B), but not in the normal counterpart (Fig. 3A).

The effect of EGCG on MAPK is more complex; the effect of 1–20μM of EGCG on normal cells was minimal (Fig. 4), but at higher concentrations EGCG stimulated MAPK activity (unpublished results and Ahn et al., 1999; Yu et al., 1997). On the other hand, EGCG inhibited MAPK activity in H-*ras*-transformed mouse epidermal JB6 cells (Chung et al., 1999) and transformed NIH-pATM*ras* fibroblasts (Fig. 4).

ODC, a rate-limiting enzyme of polyamine biosynthesis, was inhibited in transformed NIH-pATM*ras* cells, but not in the normal controls (Fig. 2). The synthesis and activity of ODC also decreased after incubating various cells with EGCG (Kennedy et al., 1998; Achiwa et al., 1997; Steele et al., 2000; Katiyar and Mukhtar, 1997; Conney et al., 1999).

Fragmentation of DNA by EGCG could be the result of changes in the signal transduction pathway. Indeed, numerous studies indicated that EGCG can induce apoptosis in cultured cancer cells (Suganuma et al., 1999; Chen et al., 1998; Paschka et al., 1998; Islam et al., 2000; Ahmad et al., 1997; Hibasami et al., 1998; Li et al., 2000; Otsuka et al., 1998; Yang et al., 1998). In the present study we studied apoptotic DNA fragmentation using TUNEL method. It is evident from Figure 5 that a significant DNA fragmentation was observed in EGCG-treated transformed cells. On the other hand normal NIH-pATM*ras* fibroblasts were not affected by EGCG at 5 and 20μM concentrations (Fig. 5).

Experiments described in this paper strongly suggest that the anti-cancer activity of EGCG is selective and therefore may have a therapeutic value. It has already been shown that EGCG preferentially induced apoptosis in cultured T lymphocytes of leukemia patients, whereas normal cultured peripheral blood lymphocytes were not affected (Li et al., 2000). Similarly, EGCG inhibited the proliferation of leukemic blast cells (Asano et al., 1997; Otsuka et al., 1998) from patients with acute myeloblastic leukemia (Asano et al., 1997). An *in vitro* chemosensitivity assay revealed that lymphocytes from an ALL patient were inhibited by 20μM EGCG (Fig. 6).

The above described well-controlled study confirms published data that green tea extracts selectively inhibit the growth of cancer. This may open new possibilities for prevention and cure of neoplastic diseases.

Acknowledgement

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