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Research paper

Involvement of pRb-E2F pathway in green tea extract-induced growth inhibition of human myeloid leukemia cells

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ABSTRACT

Both inhibitory and stimulatory effect of EGCG on cancer cells have been reported, which often is linked to receptor tyrosine kinase signaling. In this study, we present evidence that green tea extract and its chemical component, Epigallocatechin-3-gallate (EGCG), inhibit growth of human myeloid leukemia cells through the regulation of pRb synthesis and formation of pRb-E2F complexes. Addition of green tea extract to the culture of TF-1a and MV4-11 myeloid leukemia cells significantly inhibited their proliferation with a substantial portion of cell death being observed. The green tea extract and EGCG had no significant effect on the expression of G1 CDKs and the CDK inhibitors but downregulated the formation of pRb-CDKs. Surprisingly, the expression of pRb was markedly upregulated while the phosphorylation of pRb downregulated. The upregulation of pRb was blocked by pre-treatment with cycloheximide, a protein synthesis inhibitor, suggesting a requirement of protein synthesis. In agreement with these results, pRb-E2F complexes were upregulated and E2F DNA binding activity decreased. Since both TF-1a and MV4-11 are factor-independent cell lines, the upregulation of pRb-E2F complexes and inhibition of DNA binding activity by green tea extract is most likely through a receptor tyrosine kinase-independent pathway. We also found that the stem/progenitor cells derived from these two leukemia cell lines are more sensitive to the inhibitory effect of green tea extract. Our result suggests that concentrated green tea extract and EGCG may have potential for clinical investigation as an inducer of cancer cell death.

1. Introduction

Green tea is believed to have many health benefits including the prevention of several types of cancers [1–5]. The health-promoting effects of green tea are mainly attributing to its antioxidant polyphenol including (−)-Epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epigallate catechin (EGC), and (−-epicatechin (EC) [6]. Currently, most of the studies on the biological activity of green tea has focused on the EGCG [7–11]. By using growth factor-dependent cancer cell lines as research models, EGCG has been shown to inhibit cell growth via downregulating multiple components in the receptor tyrosine kinase induced-growth signal molecules, such as p44/42 ERK, p38 MAPK, the c-Jun NH2-terminal kinase (JNK), and PI3K/Akt [7,9,12]. Conversely, several labs observed that EGCG upregulated cell proliferation signals in multi-type normal and cancer cells. EGCG has been found to stimulate proliferation of mice neural stem cell (NSC) [13]. In neuroblastoma cells, one group observed that the biological effect of EGCG is related to its concentrations: low concentration of EGCG induced anti-apoptosis response in the models of Parkinson's disease [14]. Another group reported that EGCG attenuated SH-SY5Y neuroblastoma cell death induced by 6-hydroxydopamine, which was linked to the activation of protein kinase C [15]. In rat hepatoma cells, EGCG reduced glucose production accompanied with activation of PI3K, a proliferation signal molecule, and IRS-1, an upstream molecule of Ras-MAPK pathway [16]. The effect of EGCG on the glucose metabolism also linked to the enhanced expression of phosphorylated AMP-activated protein kinase α and acetyl-CoA carboxylase [17]. In normal human keratinocytes, EGCG stimulated several proliferating signals: Ras, MEKK1, MEK3, and p38 [18]. Surprisingly, high expression of p67-LR (EGCG receptor) is responsible for the proliferation of leukemia cells and increased expression of growth factor (GM-CSF) receptor [19]. Moreover, in a chemotherapy study, green tea polyphenols are not synergist but block the anticancer effects of boronic acid-based proteasome inhibitors [20]. Recently, EGCG has been found to stimulate proliferation of human bone marrow mesenchymal stem cells [21]. These inconsistent studies suggest that the biological activity and action mechanisms of EGCG on cancer cells are not well established. There is little information on the biological effect of green tea extract and EGCG in factor-independent cells.

pRb is a nuclear phosphoprotein, which is expressed throughout the

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cell cycle. Its phosphorylation level, however, oscillates in a cell cycle-dependent manner and connects CDKs and transcription factor E2F DNA binding activity. Binding of dephosphorylated pRb to E2F transcription factors inhibits activation of S-phase transcriptional target genes, arresting cells in G1 phase. Nevertheless, whether and how pRb-CDKs and pRb-E2F complexes regulates, if any, biological activity of green tea extract and EGCG in factor-independent human myeloid leukemia cells is largely unknown. To mimic the biological activity of drinking tea, in this study we applied two type green tea extracts, one (powder) was purchased from Bulksupplents and the other was freshly prepared extract from the green tea leaves in our lab. The results were compared with EGCG-treated cells. The primary aim of this study was to determine whether green tea extract stimulates or inhibits the growth of two factor-independent human myeloid leukemia cell lines and their progenitors and assess the involvement of pRb and pRb-E2F pathway in response to green tea extract treatment.

2. Materials and methods

2.1. Green tea extract (powder), EGCG, antibodies, and reagents

The Bulk-green tea extract powder (B-GTE) containing 80% catechins were purchased from Bulksupplents.com (Henderson, NY). The B-GTE powder was dissolved in distilled water (ddH_2O) at a concentration of 10% (gram/ml) as a stock solution. EGCG (powder) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies used, and their sources were as follows: pRb, CDK2, CDK4, CDK6, p21, p27, E2F-1, -2, -3, -4, and -5 (Santa Cruz Biotechnology, Dallas, TX); phosphorylated pRb, GAPDH, β-actin, and HRP-conjugated anti-rabbit and -mouse IgG (Cell Signaling, Danvers, MA). XTT and BrdU proliferation assay kits were purchased from ATCC (Manassas, VA) and Abcam (Cambridge, MA), respectively. Protein G-Agarose were obtained from ThermoFisher, Invitrogen (Grand Island, NY). E2F-1 EMSA Assay Kit was purchased from Signosis (Santa Clara, CA).

2.2. Preparation of green tea extract from tea leaves

Two types of Chinese green tea (leaves), Xihu Longjing (X-GT) and Yellow Mountain (Y-GT), were purchased and the tea leaves were soaked in hot ddH_2O for 48 h at a concentration of 8% (gram/ml) followed by filtration, after which the extracts (X-GTE and Y-GTE) as a stock solution were stored in the refrigerator.

2.3. Maintenance of cell lines

Human TF-1a and MV4-11 leukemia cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). These cell lines were routinely maintained in RPMI 1640 (for TF-1a) and IMDM (for MV4-11) supplemented with 10% fetal bovine serum (FBS), and 1% of Streptomycin-Penicillin-Glutamine (SPG) at 37 °C in humidified air containing 5% CO_2. The culture media and FBS were purchased from Life Technology (Grand Island, NY) and ATCC (Manassas, VA), respectively.

2.4. Assays of cell proliferation

Cell proliferation was measured by indirect colorimetric immunoassays, XTT and BrdU. For XTT assay, cells were grown in microtiter plates in a final volume of 100 µl RPMI or IMDM in the presence or absence of green tea extract or EGCG. After 72 h incubation, the XTT labeling reagents (50 µl) were added to the cells, and the cells were incubated for 4 h at 37 °C in a humidified atmosphere air containing 5% CO_2. After solubilizing, the formazan dyes were quantitated using a microtiter plate (ELISA) reader at a wavelength of 450 nm, as recommended by the manufacturer (ATCC, Manassas, VA). For BrdU assay, the cells were treated with green tea extract or EGCG in the same way as for the XTT assay. After 72 h, 1x BrdU were added to the cells and incubated for 24 h. The cells then were denatured and fixed, after which an anti-BrdU detector antibody were added followed by addition of a secondary peroxidase conjugated antibody following the procedure recommended by the manufacturer (Abcam, Cambridge, MA). After wash with wash buffer and TMB peroxidase substrate reaction, the signal from the reaction was measured at 450 nm in a spectrophotometer.

2.5. Semi-solid clonal cultures

Semi-solid agar was used for growth of leukemia stem/progenitor cells. Exponentially growing cells were harvested by centrifugation. The supernatants were discarded, and cell pellets resuspended in complete culture medium. A total of 4000 cells were plated in 12-well culture plate (Falcon, Cockeysville, MD) in a final volume of 2 ml containing 0.4% agar (Difco Laboratories, Detroit, MI) and 15% fetal bovine serum in the presence or absence of green tea extract or EGCG at 37 °C in humid air containing 5% CO_2 for 2–3 weeks until colonies visible. For HEP-G2 stem/progenitor cell culture, a 0.4% of agarose solution was added to the plate before added 0.4% agar solution. Visible colonies were enumerated with eyes and inspected with an inverted microscope.

2.6. Trypan blue viability assay

Cells were diluted in 0.4% Trypan Blue solution in 1:1 ratio and incubated at room temperature for 1–2 min, after which the cells were loaded into a hemocytometer chamber. The blue and non-blue cells were counted under a light microscope.

2.7. Cytology analysis

Cells were cultured in the medium at the condition described under “Maintenance of Cell Lines”. During log-phase growth, green tea extract or EGCG was added to the cells and incubated for 24–48 h. Slides were prepared and stained with methylene blue/eosin stain as described previously [22]. The cell morphology and nucleus/cytoplasm ratio were observed under a light microscope at 10 × 100.

2.8. Preparation of whole cell lysates and nuclear extracts

For preparation of whole cell lysates, cell pellets were lysed in 1x RIPA buffer (Santa Cruz, Biotechnology, Dallas, TX) supplemented with PMSF, sodium orthovanadate and protease inhibitor cocktail, and incubated on ice for 30 min. Subsequently, the lysates were sonicated and centrifuged at 14,000 g at 4 °C for 15 min. The supernatants were carefully transferred to a fresh microfuge tube and storied at −20 °C. To prepare cytosol and nuclear extracts, TF-1a and MV4-11 cells were washed twice with 1x PBS and once with the PBS containing 1 mM sodium orthovanadate (Na3VO4) and 5 mM NaF. Subsequently, the cells were washed with 2 ml of 16 hypotonic buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA), and lysed in 1x hypotonic buffer supplemented with 0.2% NP40. Thereafter, the supernatants (cytoplasmic extracts) were transferred to a fresh tube and the nuclear pellets were resuspended in 50 ± 100 ml of 16 high salt buffer (420 mM NaCl, 20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, 20% Glycerol), after which they were incubated at 4 °C for 30 min under constant rotation. Subsequently, the nuclear extracts were collected by centrifugation and stored at −80 °C.

2.9. Western blot analysis

Cells were harvested and lysed in the RIPA lysis buffer. Aliquots of proteins in sample buffer were heated at 100 °C for 4 min before applied to a SDS-polyacrylamide gel. The proteins were separated in the gel and

electrophoretically transferred to a nitrocellulose membrane (BIO-RAD, Hercules, CA) and blotted in blocking buffer (5% dry milk in 1xTBST). The proteins in the membrane then were immunoblotted with a corresponding primary antibody at 4 °C overnight, after which the primary antibody was removed, and the blots were washed three times in TBST buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH7.5), each 5 min. The blots were then incubated for 1 h with an HRP-conjugated anti-rabbit or anti-mouse secondary antibody at room temperature. Subsequently, the antibody reactions were detected by chemiluminescence reaction.

2.10. Immunoprecipitation

Cells were lysed in RIPA lysis buffer and immunoprecipitation was performed using cell lysates containing 300 µg of total proteins. The lysates were incubated with an appropriate antibody for 2 h or overnight with agitation at 4 °C. Subsequently, 30 µl of protein A-Sepharose or protein G-agarose beads was added to the lysates and the incubation continued for another 1 h at 4 °C. Immune complexes were washed three times with 1x lysis buffer and then resuspended in 2x SDS sample buffer. Lysates in the sample buffer were subjected to Western blot analysis and detected by the chemiluminescence reaction as described in the section of "Western blot analysis" above.

2.11. E2F-1 EMSA assay

E2F-1 DNA binding activity was carried out by the protocol provided by the manufacturer with minor modifications. Briefly, the cells in log-phase were harvested and responded in the culture medium containing 20% FBS for 24 h. Then these cells were treated with green tea extract, PMA, or vehicle (DMSO) for 36 h, after which their nuclear extract was prepared, and the nuclear extract was incubated with E2F-1 DNA probe (5′-ATTTAAGTTTCGCGCCCTTTCTCAA-3′) at 22 °C for 30 min in a PCR machine. After the reaction the protein/DNA complexes were separated on a 6% non-denaturing polyacrylamide gel and subsequently transferred to a nylon membrane for addition of Streptavidin-HRP conjugate followed by substrate reaction for detection of shifted E2F-1-DNA complexes.

2.12. Statistical analysis

All results were expressed as mean ± SD of data obtained from three or more independent experiments. The statistical significance of differences between group means was determined using the Student's *t*-test.

3. Results

3.1. Green tea extracts and EGCG inhibited the growth of human myeloid leukemia cells

In recent years, XTT or MTT assay has been a common and convenient mean to measure cell proliferation. To study the role of green tea extract in regulation of cell growth and signaling pathways, green tea extract was added to myeloid leukemia cells in log-phase growth, after three days the cells were harvested, and the cell proliferation was assessed with an XTT assay. The addition of B-GTE, variably, but significantly, inhibited the growth of MV4-11 and TF-1a cells. The inhibitory effect of B-GTE was dose-dependent, with a maximal effect being observed at the concentrations of 0.2% (v/v) or more. Less than 0.04% B-GTE could not induce significant growth inhibition. The growth inhibition of MV4-11 and TF-1a cells by B-GTE at concentration of 0.2% was 39% and 44%, respectively (Fig. 1A). Y-GTE (the green tea extract obtained from Yellow Mountain tea leaves) and X-GTE (the green tea extract obtained from Xihu Lonjing leaves), at relatively higher concentrations (≥2%), also inhibited the cell proliferation (Fig. 1B and C). By comparison, when the Y-GTE and X-GTE extracts were added to the cells at final concentrations of 4–6% (v/v), they reached to a similar inhibitory level on the cells caused by 0.2% B-GTE. Since XTT is a colorimetric assay, the color intensity induced by reductases in cells is proportional to the numbers of functional or viable cells present. This suggests that at least a portion of the inhibitory effect is a result of cell death caused by the green tea extract (B-GTE, Y-GTE, and X-GTE). To confirm this result, we next performed Trypan **blue** stain of the cells. The death rate in TF-1a cells caused by 4%Y-GTE, 4% X-GTE, and 0.2% B-GTE is 30%, 22%, and 20%, (all $p < 0.01$ as compared with the control), respectively, and the death rate in MV4-11 cells 32%, 28%, and 22% (all $p < 0.01$ as compared with the control), respectively. However, we did not observe significant cell death when 50 µM EGCG were applied to TF-1a and MV4-11 cells. To determine in what degree the green tea extracts inhibit cell division, we next used 5-bromo-2′-deoxyuridine (BrdU) assay to analyze growth status of both MV4-11 and TF-1a cells in the absence or presence of B-GTE. BrdU assay is based on the incorporation of BrdU into newly made DNA: the more dividing cells, the more BrdU incorporation. The cell proliferation measured by BrdU assays showed remarkable inhibitory effect of green tea extract on the cell division. (Fig. 1D). TF-1a was more sensitive than MV4-11 cells to the inhibitory effect of B-GTE, with as little as 0.008% of B-GTE inhibiting TF-1a cells by 25%. The same concentration of B-GTE did not affect the proliferation of MV4-11 cells. At a concentration of 0.2%, B-GTE inhibited as much as 75% and 56% of TF-1a and MV4-11 cells, respectively (Fig. 1D).

EGCG has been reported to be the most abundant catechin in green tea, we next examined whether EGCG alone could induce similar growth inhibition in these leukemia cells as induced by B-GTE, Y-GTE, and X-GTE. As shown in Fig. 1E, EGCG at concentrations of 25–50 µM caused marked proliferation inhibition of TF-1a cell measured by BrdU assay. The inhibition is dose-dependent with maximal inhibition (48%) being observed when 50 µM EGCG were applied to the cell culture. Although EGCG has been reported to be functional at a concentration of 10 µM in several types of cancer cells, the same concentration of EGCG had no effect on the growth of the human leukemia cells we tested. A little surprising to us, neither trypan blue staining nor XTT assay could detect significant growth inhibition in the cells treated with up to 50 µM EGCG as compared with the control cells treated with DMSO (data not shown), suggesting that EGCG had no cytotoxic effect on the cells. To confirm this result, we next performed cell staining with methylene blue/eosin and observed cell morphology under light microscope. As shown in Fig. 2. TF-1a cells show relatively homogeneous population of medium-size cells with the appearance of blasts. They have a smooth cytoplasmic border with big nucleus/cytoplasm ratio. MV4-11 cells are a little smaller (about 1/3 to 1/4 smaller in diameter) than TF-1a cells in size. Addition of high concentrations of B-GTE decreases the cell numbers and causes cell morphology changes indicated by decreased cell sizes, shrinkage of cell body, and the formation of cell blebbing. However, addition of EGCG did not cause significant cell morphology changes or cell death.

3.2. Green tea inhibited growth of human myeloid leukemias stem/progenitor cells in clonal culture

Many studies have demonstrated that cancer has a stem cell origin and is a stem cell disease. Therefore, we carried out soft-agar culture to find out the alterations of the colony-forming cells derived from TF-1a and MV4-11 cell lines in clonal culture in the presence of Y-GTE, B-GTE, and EGCG. These colony-forming cells eventually represent stem/progenitor cells. Y-GTE inhibited dose-dependently the colony-forming cells with a maximal inhibition being observed when 2% Y-GTE was added to the culture. At this concentration Y-GTE abolished 88% (control: 73 ± 10.2; Y-GTE: 9.0 ± 1.9, $p < 0.01$) of TF-1a and 85% (control: 35 ± 4.1, Y-GTE: 5.0 ± 1.0, $p < 0.01$) of MV4-11 colony-forming cells. The morphology examination under inverted microscope

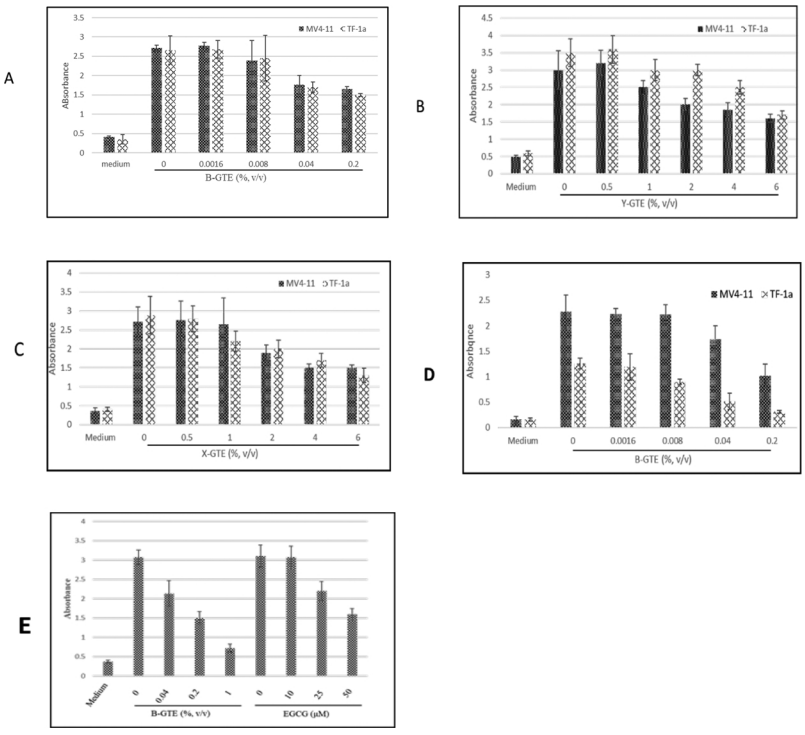

Fig. 1. Green tea extract inhibits growth of MV4-11 and TF-1a cells. Cells (2×10^5) were seeded in 96-well microplates in the presence or absence of green tea extract or EGCG. After 3 days of culture at 37 °C, the cells were labeled with XTT (A, B, C) or BrdU (D and E) labeling reagent for 4 h, and cell proliferation was determined by a color reaction using an ELISA plate reader at 450 nM. Results are given as Mean ± SD from 3 to 5 separate experiments. The concentrations of the green tea extracts in the figure are the final concentrations in the cell culture after addition of their stock solutions.

show that there are three different type colonies: compact, dispersed, and mixed, with compact dominant in the control cells treated with vehicle. Most of the colonies derived from green tea extract-treated cells are dispersed and mixed. Similarly, B-GTE caused a large decrease in the colony forming cells with 92 and 88% reduction being observed when 0.2% of B-GTE was added to TF-1a and MV4-11 agar cell culture, respectively (Table 1). EGCG alone also inhibited the stem/progenitor cells derived from TF-1a and MV4-11 cells. At a concentration of 5 μM EGCG decreased the TF-1a and MV4-11 colony numbers by 88% and 85%, respectively. The inhibition is dose-dependent and less than 1 μM EGCG did not cause significant reduction in the colony-forming cells (Table 1). EGCG-treated cells displayed as small numbers of dispersed individual cells without cell colonies being observed in the semi-solid agar.

3.3. Expression of pRb and phosphorylated pRb in response to green tea extract

To begin to understand the nature of the inhibitory effects of the green tea extracts on the leukemia cells, we exposed the cells to Y-GTE and B-GTE, then measured the changes in the expression of pRb, a nuclear protein that has been shown to function as a tumor suppressor and cell cycle regulator. Non-phosphorylated pRb is the active form of the protein and is capable of binding to transcription factors, E2Fs, thereby inhibited E2F-dependent gene transcription and the cell cycle, whereas the phosphorylated pRb promotes cell cycle progression due to the loss of its E2F binding activity [23]. In an exponentially growing phase, pRb protein in TF-1a cells has a molecular size of approximately 110 kDa (Fig. 3A, lane 1). On day 2 (48 h) after the addition of Y-GTE and B-GTE to the cells, the expression of pRb was significant upregulated. The upregulation of pRb is dose-dependent with a maximal increase being observed when 4% Y-GTE and 0.2% B-GTE was added to TF-1a cells (Fig.3A, Lanes 2 and 4). The lower concentrations (0.8% Y-GTE and 0.04% B-GTE) of the green tea extracts have less enhancing effect on pRb levels. Similar enhancing effect of Y-GTE and B-GTE on pRb was observed in MV4-11 cells. The enhanced pRb was blocked by Cycloheximide, an inhibitor of mRNA translation, demonstrating a requirement of protein synthesis (Fig. 3B, lanes 4, 5, and 6). In contrast, the phosphorylation of pRb was dramatically reduced, starting from 6 h after the initiating the green tea extract treatment (data not shown). By 48 h, Y-GTE and B-GTE inhibited the phosphorylation of pRb by more than 80% in both TF-1a and MV4-11 cells (Fig. 3C, lanes 2 and 4). Short incubation (less than 3 h) did not significantly affect phosphorylation of pRb.

3.4. The alterations of pRb-CDK and pRb-E2F complexes in green tea extract-treated cells

Although EGCG-induced downregulation of G1 CDKs and upregulation of CDK inhibitors have been observed in human skin, breast, epidermoid and pancreatic cancer cells [24–28], it is unclear whether the green tea extract-induced growth inhibition of the human myeloid leukemia cells we observed is a result of the alterations of G1 CDKs and the CDK inhibitors. To clarify this question, we examined the expression of G1 CDKs, CDK4, CDK6, and G1 CDK inhibitors, p21, and p27 in the absence or presence of green tea extract and EGCG. In proliferating TF-

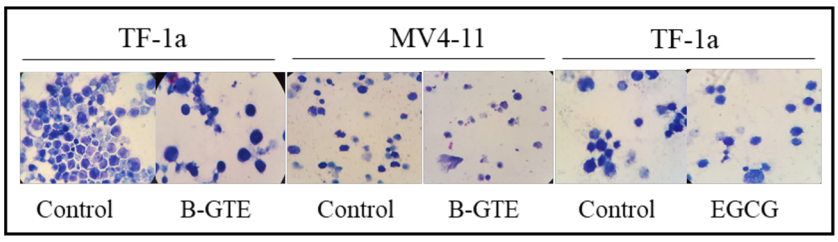

Fig. 2. Green tea extract causes cell death. Cells treated with vehicle (ddH_2O for B-GTE, DMSO for EGCG), B-GTE (0.2%), or EGCG (50 μM) for 3 days, after which the cells were collected, and slides prepared. The slides were stained with methylene blue/eosin and the morphology was examined under a light microscope (x1000). The images show significant decreased cell numbers and the changes in cell morphology in the cells treated with B-GTE but not EGCG.

Table 1
Effect of B-GTE and EGCG on the growth of colony-forming cells.

Cell line/ *p* value	Control	B-GTE (%, v/v)		EGCG (μM)	
		0.04	0.2	10	50
TF-1a	125±11.2	41.5 ± 6.2 (-67%)	10 ± 5.7 (-92%)	99 ± 11 (-21%)	15 ± 6.4 (-88%)
p value		< 0.01	< 0.01	< 0.01	< 0.01
MV4-11	78± 7.1	30.0 ± 3.9 (-61%)	9.4 ± 3.1 (-88%)	65 ± 4.0 (-17%)	12 ± 5.0 (-85%)
p value		< 0.01	< 0.01	< 0.01	< 0.01

Cells were cultured in semi-solid agar in the presence or absence of B-GTE or EGCG at 37 °C in humid air containing 5% CO_2 for 3 weeks, after which the colonies were enumerated. Results are given as mean ± SD from 2 separate experiments. Controls (0 group) were treated with vehicle (DMSO). The percentage inhibition (as compared with the control) are given in the brackets.

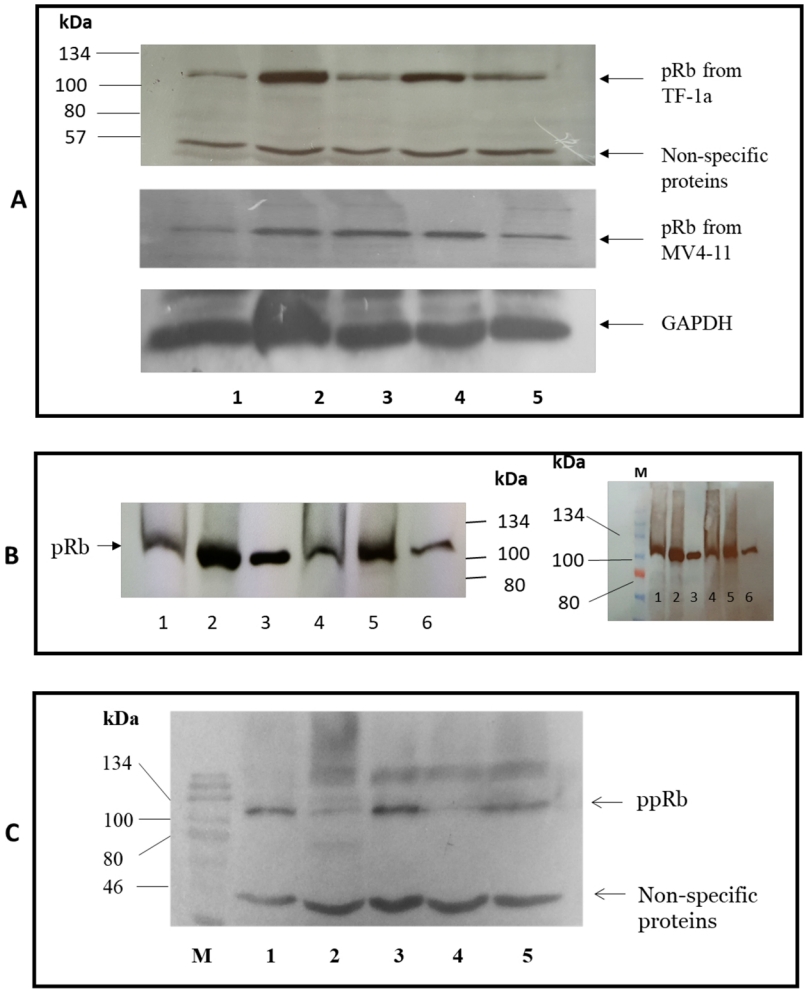

Fig. 3. Effect of green tea extract on the expression of pRb and phosphorylated pRb. Cells treated with Y-GTE or B-GTE for 24 h were harvested and proteins extracted. The expression of pRb was immunoblotted by anti-pRb (A and B) or anti-phospho-pRb antibody (C) and analyzed by Western blotting. M. protein marker, A. Lanes (1) Control, (2) 4% Y-GTE, (3) 0.8% Y-GTE, (4) 0.2% B-GTE, and (5) 0.04% B-GTE. B. Lanes (1) Control, (2) 4% Y-GTE, (3) 0.2% B-GTE (4) CY (10 mM), (5) CY + Y-GTE, (6) CY + B-GTE. CY: cycloheximide (10 mM). **C**. Lanes (1) Control, (2) 4% Y-GTE, (3) 0.8% Y-GTE, (4) 0.2% B-GTE, and (5) 0.04% B-GTE. All the percent concentrations are v/v.

1a cells CDK4- and CDK6-polypeptides showed a strong 32–36-kD band that had no significantly changes following addition of Y-GTE and B-GTE at the concentrations that caused upregulation of pRb (Fig. 4A). Similarly, these two extracts also did not significantly affect the expression of p21 and p27 (data not shown). CDKs are assumed to modify pRb by phosphorylation through direct contact, thereby promoting cell cycle progression toward DNA replication. To determine whether green tea extract affects the formation of pRb-CDK complexes, we next examined the expression of pRb-CDK complexes in proliferating cells and the cells inhibited by B-GTE and Y-GTE. As shown in Fig. 4A (bottom panels), pRb-associated CDK4 and CDK6 are clearly existed in proliferating cells (the cells treated with vehicle). After the cells treated with Y-GTE and B-GTE for 24 h, the formation of pRb-CDK4 and pRb-CDK6 was dramatically decreased (lanes 2 and 4 on the bottom panels). Subsequently, we analyzed the expression of pRb-E2F complexes in the cells treated with or without green tea extract. In contrast to the formation of pRb-CDK complexes, pRb-E2F1, -E2F2, and -E2F3 complexes were significantly upregulated by B-GTE and Y-GTE (Fig.4B). The upregulation of the E2Fs are clearly dose-dependent with the significant increase of the E2Fs, especially E2F-1 being observed when 4% Y-GTE and 0.2% B-GTE were added to the cells for 24 h.

3.5. E2F-1 DNA binding activity in green tea-treated cells

To determine whether upregulation of pRb-E2F complexes affects E2F-DNA binding activity, electromobility shift assay (EMSA) was

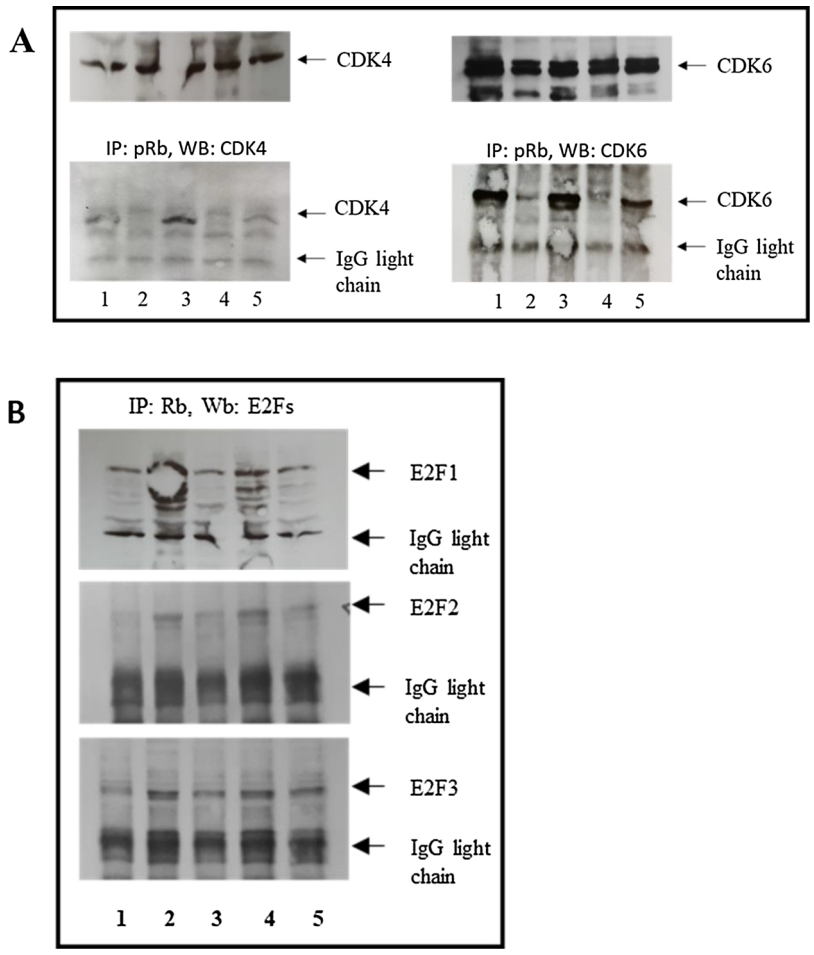

Fig. 4. Green-tea downregulates pRb-CDK and upregulates pRb-E2F complexes. TF-1a cells treated with or without green-tea extract for 24 h at 37 °C, 5% CO_2 were collected and lysed. Aliquot of the extracts were subjected to Western blot (A, top panel) or immunoprecipitation analysis (A, bottom panel and B). IP: Rb; WB: CDK4, CDK6, E2F1, E2F2, E2F3, E2F5). Lanes (1) Control, (2) 4% Y-GTE, (3) 0.8% Y-GTE, (4) 0.2% B-GTE, and (5). 0.04% B-GTE. All the percent concentrations are v/v.

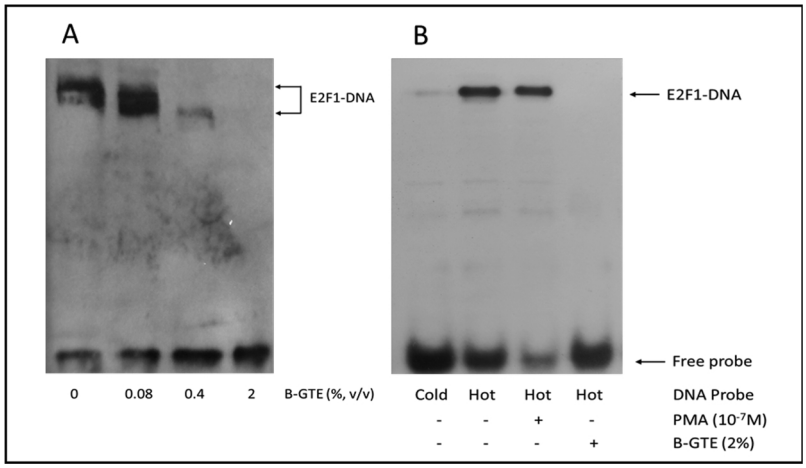

Fig. 5. Inhibitory effect of green tea extract on specific E2F–DNA binding activity. TF-1a cells supplemented with 20% of FBS treated with green tea extract, PMA, or vehicle (DMSO) for 24 h were harvested and their nuclear extract was prepared. Subsequently, the nuclear extract was incubated with the DNA probe containing E2F-1 binding site or cold probe and the protein–DNA complexes were resolved on a 6% non-denatured polyacrylamide gel in TBE buffer. After transfer and immobilization of the complexes under UV light, the membrane was incubated with Streptavidin-HRP Conjugate for 1 h, after which the specific E2F-1-DNA complexes were detected after addition of chemiluminescent substrate solution.

performed. For this experiment, we focused on E2F-1 DNA binding activity, because pRb-E2F1 complex was mostly upregulated by green tea extract (Fig. 4). In this study, we used E2F-1 EMSA kit (Signosis, Sana Clara, CA) with slightly modifications. This kit provides a biotin-labeled DNA probe specific for E2F-1 binding. As seen from Fig. 5A, serum-stimulated cells (lane 0) express clear E2F-1-DNA complex as a strong band in the gel. Addition of B-GTE inhibited dose-dependently the formation of E2F-1-DNA complex with a complete abolishment of the complex being observed when 2% of B-GTE were added to TF-1a cells for 36 h. At concentration of 0.08% (v/v) or less, B-GTE had no significant effect on the shifted E2F-1 DNA complex. To confirm the specificity of the green tea extract-induce inhibition of E2F-1 DNA binding activity, in parallel experiment, a non-E2F DNA probe (cold) and PMA (10^{-7}M), a cell growth inhibitor and differentiation inducer, were added to TF-1a cells for 36 h, respectively. Fig. 5B illustrates the expression of E2F-1-DNA complex in the absence or presence of cold probe, hot probe, PMA and B-GTE. The cells treated with the cold probe in the presence of 20% FBS show very little background band with

strong free probe cumulated on the button of the gel. In contrast, the cells treated with E2F-1 probe caused a significant shift in response to 20% FBS. In our previous study, PMA at 10^{-7}M was found to inhibit TF-1a cell growth and stimulate microphage-like differentiation with prolonged activation of MAPK [29]. However, the same concentration of PMA failed to inhibit E2F-1 DNA binding activity. Similarly, we also could not detect any inhibition of E2F-1 DNA activity in the cells treated with Nocodazole (data not shown), a cytoskeleton inhibitor, although this chemical compound significantly inhibited TF-1a cell growth [30]. These data demonstrated the specificity of the green tea extract-induced abolishment of E2F-1 DNA binding activity by high concentration of fetal bovine serum.

4. Discussion

It has long believed that green tea has many health benefits including the prevention of cancers. However, the clinical research has not provided conclusive evidence on these benefits especially on cancer prevention and treatment [31,32]. In basic research, there are many inconsistent reports with both inhibitory and stimulatory effect of EGCG on cancer cells being observed. Thus, further clarification of the biological effect and acting mechanisms of green tea on the growth of different type cells are needed, especially in factor-independent leukemia cells. For this study, we focused on two factor-independent human myeloid leukemia cell lines, TF-1a and MV4-11. The TF-1a was derived from the human factor-dependent erythroid leukemia TF-1 cell line and established by corresponding author of this article in 1998 [33]. TF-1a cells are CD38-/CD34+ and is one of the earliest hematopoietic cell lines. TF-1a contains the most powerful stem cells with the strongest proliferation ability as compared with most hematopoietic and non-hematopoietic cell lines we have tested. MV4-11 is CD34 negative and is one of the latest progenitor cell line. By selecting these two cell lines as research models, we can examine effect of green tea or EGCG on both early and later leukemia stem/progenitor cells. In this study, we have found that the green tea extract remarkably inhibited the growth of the human myeloid leukemia cells, evidenced by following observations: (*a*) addition of B-GTE, Y-GTE, and EGCG to the cell culture inhibited the growth of both TF-1a and MV4-11 cells, measured by XTT and BrdU assays; (*b*) B-GTE and Y-GTE greatly decreased the colony-forming cells derived from TF-1a and MV4-11 cells in soft-agar culture; (c) B-GTE and Y-GTE also caused cell death in a portion of the cells. Although EGCG had similar inhibitory effect on the leukemia cells, EGCG (10–50 μM) did not cause significant cell death, which suggests that non-EGCG chemical components in the green tea extract may play a role in the green tea extract-induced cell death. Unexpectedly, we found that the stem/progenitor cells in these two cell lines appear more sensitive to green tea extract-induced growth inhibition compared with the inhibition of the whole cell population in liquid culture by the green tea extract. It is unclear whether the catechins in the green tea extract preferentially act on stem/progenitor cells due to the differences of the cell surface markers in the stem/progenitor cells and more matured cell population.

pRb acts as a signal transducer collecting signals from cell cycle regulatory molecules to transcriptional machinery. Active pRb prevents excessive cell growth by inhibiting the cell cycle progression, which is through binding and inhibiting the transcription factors, E2Fs. When the cell is ready to divide, pRb is phosphorylated by CDKs, becomes inactive and allows cell cycle progression [23]. By using several approaches, we have demonstrated that pRb-E2F pathway involves in the green tea extract-induced growth inhibition of human myeloid leukemia cells. First, in the absence of B-GTE and X-GTE Exponentially growing TF-1a and MV4-11 cells express relatively a low-level pRb and phosphorylated pRb. Addition of B-GTE and Y-GTE upregulated pRb but downregulated phosphorylated pRb. Second, the upregulated pRb was blocked by protein synthesis inhibitor, cycloheximide. This is the first demonstration that green tea extract upregulates synthesis of pRb in cell culture. Third, the green tea differentially regulated the formation of pRb-CDKs and pRb-E2Fs complexes. As a result, E2F-DNA binding activity was greatly decreased. It has been reported that CDK-cyclin promote the cell cycle progress via modify phosphorylation of pRb. Downregulation of CDKs and upregulation of CDK inhibitors were often observed in Transforming Growth Factor-beta (TGFβ)-induced growth inhibition of hematopoietic leukemia cells [34]. However, green tea extract appeared having no effect on the expression of G1 CDKs, CDK4 and CDK6 but downregulated CDK-pRB complexes, suggesting that green tea extract and EGCG blocks the formation of CDK-pRB complexes. Much evidence suggests that dephosphorylated pRb suppresses growth partly by affecting the transcription of genes required for proliferation. Several cellular pRb-binding proteins have been identified and transcription factor E2F is a particularly strong candidate to be a physiologically relevant target of pRb action. When overproduced in cultured cells, E2F can activate E2F-dependent transcription, drive G1-arrested cells into S phase, and act as an oncogene. Conversely, inhibition or absence of E2F activity blocks cells in G1 [35,36]. Consistent with the upregulation of pRb-E2F complexes induced by B-GTE, the E2F-1 DNA binding activity also dramatically decreased.

Activation and inactivation of pRb is often linked to the presence or absence of a mitogen. Mitogens act through a classic tyrosine kinase pathway. Receptors for mitogens become active by phosphorylation when bound to mitogens. The phosphorylated domains are then recognized by a guanine nucleotide exchange factor for Ras, in turn activates MEK and ERK. However, the green tea-induced growth inhibition of TF-1a and MV4-11 cells reported here appears not linked to the inhibition of receptor tyrosine kinase pathway, because these two cell lines are factor-independent without addition of any growth factor in this study. In addition, there were no detectable changes in the expression of MEK or ERK in the presence of green tea extract or EGCG (our unpublished preliminary data). It is most likely that the biological activity and signals-induced by the green tea extract and EGCG appear not to be trigged by inhibition of tyrosine receptors.

EGCG at the concentrations of 10–50 μM did not have significant cytotoxic effect on the cells tested. However, the green tea extract, Y-GTE and B-GTE at the higher concentrations caused similar inhibitory effect on the cells as EGCG did also induced a portion of cell death. The cell death was evaluated by XTT assay, trypan blue staining, and cytology examination. However, these assays cannot distinguish whether the dead cells are apoptotic or necrotic. Since EGCG did not cause cell death, our results suggest that some chemical components in the green tea extract cause cell death, although high concentration of EGCG can also induce apoptosis reported by others [37]. We will identify the chemical components in the green tea extract, which caused cell death and identify apoptotic signal molecules if there are any.

5. Conclusion

This study reveals that green tea extract and EGCG inhibited growth and caused partial cell death of two human myeloid leukemia cell lines via regulating pRb synthesis and pRb-E2F complexes, and E2F DNA binding activity. All these activities appeared through the receptor tyrosine kinase-independent manner. Surprisingly, the stem/progenitor cells derived from the cell lines were more sensitive to the inhibitory effect of green tea extract and EGCG. Our data suggests as a beverage one should not consume high amounts of green tea (extract) due to the possible cytotoxic or apoptotic effect, especially its significant inhibitory effect on stem/progenitor cells, although the biological effect or activity of green tea extract *in vivo* could quite different from *in vitro*. However, the concentrated green tea extract and EGCG may have potential for clinical investigation as an inducer of cancer cell death.

Declaration of interest

None

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