

Green Tea Polyphenols Induce Apoptosis *in vitro* in Peripheral Blood T Lymphocytes of Adult T-Cell Leukemia Patients

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Green tea polyphenols (TEA) are known to exhibit antioxidative activity as well as tumor-suppressing activity. In order to examine the tumor-suppressing activity of TEA against adult T-cell leukemia (ATL), we cultivated peripheral blood T lymphocytes of ATL patients (ATL PBLs), an HTLV-I-infected T-cell line (KODV) and healthy controls (normal PBLs) for 3 days in the presence of TEA and its main constituent, epigallocatechin-3-gallate (EGCg), to measure cell proliferation and apoptosis, and to quantitate mRNAs of *HTLV-I pX* and β -actin genes of the cultured cells. Growth of ATL PBLs was significantly inhibited by 9–27 μ g/ml of TEA and EGCg, in contrast to minimal growth inhibition of T cells of normal PBLs. Inhibition of KODV was intermediate between ATL PBLs and normal PBLs. The ATL PBLs and KODV treated with 27 μ g/ml of either TEA or EGCg induced apoptotic DNA fragmentation, producing terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells, while the normal PBLs treated with the same concentration of TEA or EGCg produced a negligibly small number of TUNEL-positive cells, in which apoptotic DNA fragmentation was not detectable. Expression of *HTLV-I pX* mRNA was suppressed more than 90% in ATL PBLs by treatment with 3–27 μ g/ml of either TEA or EGCg, while expression of β -actin mRNA was much less suppressed by treatment with the same concentration of TEA or EGCg. These results indicate that TEA and EGCg inhibit growth of ATL PBLs, as well as HTLV-I-infected T-cells, by suppressing *HTLV-I pX* gene expression and inducing apoptotic cell death.

Key words: EGCg — HTLV-I — ATL — Apoptosis — Green tea

The green tea polyphenols (TEA) effectively inhibit mouse skin tumorigenesis,¹⁾ the proliferation of acute myeloblastic leukemia cells²⁾ and the growth of human tumor cell lines,³⁾ as well as inducing apoptosis in gastric cancer cells⁴⁾ and other tumor cell lines.^{5–8)} Apoptosis is programmed cell death in which tumor cells commit suicide, resulting in structural changes of the plasma membrane and DNA fragmentation in nuclei.⁹⁾ EGCg, a major constituent of TEA suppresses gene expression of tumor necrosis factor (TNF)- α ,¹⁰⁾ c-Jun,¹¹⁾ iNOS¹²⁾ and epidermal interleukin-1 α .¹³⁾ Thus, TEA may be useful as antimutagenic substances for cancer chemoprevention¹⁴⁾ and as antioxidants against multistage carcinogenesis.¹⁵⁾ The chemopreventive effects of TEA were confirmed by epidemiological studies on breast cancers¹⁶⁾ and stomach cancers.^{17, 18)}

Adult T-cell leukemia (ATL) is an aggressive malignancy of mature CD4⁺ T lymphocytes^{19, 20)} which is caused by human T-cell leukemia virus type I (HTLV-I).^{21–23)} ATL

is endemic in Southwestern Japan, South America, the Caribbean Basin, West-Central Africa, North Iran, South India and other isolated tropical regions.^{24, 25)}

The HTLV-I encodes an oncoprotein Tax (p40tax) from its *pX* gene, which plays a central role in leukomogenesis of ATL.^{23, 26)} Therefore, a search for an appropriate agent to suppress the function of Tax oncoprotein might provide a means for the chemoprevention of ATL. However, no study has yet been reported regarding the anti-leukemic effects of TEA on ATL patients.

Here we present new evidence that TEA inhibit *in vitro* growth of ATL PBLs and HTLV-I-infected T-cells by suppressing HTLV-I *pX* mRNA expression and inducing apoptotic cell death.

MATERIALS AND METHODS

Green tea extract The green tea product used was Yabukita-Cha, cultivated in the fields of Chiran, Southern Kyushu. Ten grams of dried green tea leaves was immersed in 100 ml of hot water at 95°C for 30 min with

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gentle stirring. The hot water extract of green tea was mixed with an equal volume of ethyl acetate and the ethyl acetate fraction was evaporated to produce a dry powder. The powder was dissolved in 0.5% methanol to make a stock solution containing 1 mg TEA/ml, as reported by other investigators to make a stock solution of EGCg.²⁷⁾ The EGCg content was measured by an HPLC-UV method.²⁸⁾

Epigallocatechin-3-gallate was purchased from Kurita Chemical Co. (Tokyo) and used as a standard TEA.

Study subjects Three ATL patients, three HTLV-I seronegative healthy adult controls and one HTLV-I-infected T-cell line established from HTLV-I-associated myelopathy (KODV) were the subjects of this study (Table I). All patients and controls came from Kagoshima Prefecture. We drew 30 ml of peripheral blood with informed consent and separated peripheral blood lymphocyte-rich mononuclear cells (PBLs) to cryopreserve them in liquid nitrogen until tested.³⁰⁾ The defrozen ATL PBLs and normal PBLs as well as the KODV were used for *in vitro* assays for proliferation and apoptosis, as well as quantification of HTLV-I pX mRNA after cultivation with TEA and EGCg as described below.

***In vitro* assays for proliferation and apoptosis** For proliferation assay, 5×10^4 ATL PBLs, KODV or normal PBLs were cultivated with or without TEA and EGCg in a humidified 5% CO₂ incubator for 3 days in 100 μ l of RPMI-1640 medium supplemented with 50 units/ml rIL-2 (TGP-3; Takeda Pharmaceutical Industry Co., Ltd., Osaka) and heat-inactivated 10% fetal calf serum using a 96-well flat-bottomed microtray (Falcon #3072, Becton Dickinson Co., New Jersey). *In vitro* growth of the cultured ATL PBLs and normal PBLs was monitored by daily examination of cell numbers using the WST-8 assay (Cell Counting Kit-8; Wako Pure Chemical Industries, Ltd., Osaka) according to the manufacturer's protocol.³¹⁾ The relative cell growth was estimated as the ratio of cell counts before and 3 days after cultivation.

For the apoptosis assay, the cultured cells were divided into two parts, one for electrophoretic analysis of DNA fragmentation,^{32,33)} and the other for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL).³⁴⁾ In brief, the cultured cells were incubated with digoxigenin-dUTP terminal deoxynucleotidyl transferase mixture and then stained with peroxidase-conjugated antibody to digoxigenin (Apoptosis In Situ Detection Kit; Wako Pure Chemical Industries, Ltd.), counter-stained with 1% methyl green in 100 mM sodium acetate buffer (pH 4.0) and mounted. Specimens were examined and photographed under a microscope at 400 \times magnification.

Quantification of HTLV-I pX mRNA Total cellular RNAs of the ATL PBLs and normal PBLs treated with TEA or EGCg as described above, were isolated using an High Pure RNA Isolation Kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's protocol and were cryopreserved at -80°C until tested.

HTLV-I pX mRNAs expressed in the ATL PBLs and KODV were quantified by reverse transcriptase-polymerase chain reaction (RT-PCR) using a GeneAmp EZ rTth RNA PCR Kit (Perkin-Elmer Corp., Branchburg, NJ) and the GeneAmp PCR System 9600 (Perkin-Elmer Corp.). In brief, 300 ng of total cellular RNA was used to produce HTLV-I pX cDNA by RT-PCR amplification in the presence of the specific primer set (RPX-11; 5'-TAATAGC-CGCCAGTGGAAAG-3', positioned at 5001–5023 and PX9; 5'-TGATCTGATGCTCTGGACAG-3', positioned at 7460–7441) to amplify 340 base pairs of HTLV-I pX RNA.³⁵⁾ The mixture was incubated at 60°C for 35 min followed by 94°C for 2 min in a final volume of 50 μ l, then the cDNA products were amplified by 40 cycles of denaturation at 94°C for 30 s and annealing at 59°C for 45 s, followed by a final step of 10 min at 60°C.

In order to quantitate HTLV-I pX mRNA with increased sensitivity and specificity, 2 μ l aliquots of the first RT-

Table I. Characteristics of Subjects

Subjects	Age	Sex	Diagnosis	HTLV-I antibody ^{a)}	WBC/mm ³	% atypical lymphocytes	
ATL patients	Case 1	65	M	Acute type	4096	53400	89.0
	Case 2	62	M	Acute type	256	19800	14.5
	Case 3	54	M	Acute type	4096	31700	82.0
Normal donors	Case 4	49	M	Healthy	<16	9200	0
	Case 5	31	M	Healthy	<16	6500	0
	Case 6	35	M	Healthy	<16	8300	0
HAM patient	KOD	65	F	Grade 7–8 HAM ^{b)}	8192	HTLV-I-infected T-cell line (KODV) ^{c)}	

a) Anti-HTLV-I antibody titer was determined by the particle agglutination method (SERODIA HTLV-I; Fujirebio, Tokyo).

b) Osame *et al.*²⁹⁾

c) Established from a HAM patient (KOD) by 60-day cultivation. More than 60% of cells were positive for HTLV-I group specific antigen (gag) and envelope antigens by immunofluorescent antibody staining.

PCR product were subjected to nested PCR using the second primer set (RPX-3; 5'-ATCCCGTGGAGACTCCTCAA-3', positioned at 5096-5115 and RPX4; 5'-AACACGTAGACTGGGTATCC-3', positioned at 7357-7338) to amplify 145 base pairs of HTLV-I pX DNA.³⁶⁾ The reaction was done in the presence of 2.5 units of AmpliTaq Gold polymerase (Perkin-Elmer Corp.) in a final volume of 50 μ l, at 95°C for 9 min, followed by 30 cycles of denaturation at 95°C for 30 s and annealing at 59°C for 45 s, and a final step of 10 min at 60°C. After the reaction, 10 μ l of the amplified products was electrophoresed in a 2% agarose gel (Type II: Medium EEO, Sigma-Aldrich Chemical GmbH, Steinheim, Germany) and visualized by ethidium bromide staining.

For the detection of β -actin mRNA expressed in the ATL cells, the specific primer set (BA2; 5'-TACATGGCTGGGGTGTGAA-3', positioned at 439-420 and BA3; 5'-AAGAGAGGCATCCTCACCT-3', positioned at 222-241) was used as described elsewhere.³⁷⁾

For quantification of mRNAs of HTLV-I pX and β -actin, we made 10-fold serial dilutions of the cellular RNAs extracted from the treated ATL cells and subjected them to nested RT-PCR amplification with the primer sets described above. All the experiments included a set of control samples incubated without RT to exclude contamination with cellular DNAs during laboratory procedures of mRNA extraction and RT-PCR amplification.

RESULTS

Inhibitory effect of TEA and EGCg on growth of ATL PBLs The ATL PBLs, normal PBLs and HTLV-I-infected T-cell line (KODV) cells were cultured *in vitro* for 3 days in the presence of various concentrations of TEA or EGCg. Growth of ATL PBLs was inhibited by TEA in the range of 3-27 μ g/ml of TEA; cases 1 and 3 ATL PBLs showed a sharp decrease in relative growth but case 2 ATL PBLs showed a different relative growth curve (Fig. 1A, ATL PBLs). KODV showed an intermediate growth curve between ATL and normal PBLs (Fig. 1A, KODV). Growth of normal PBLs was only slightly inhibited by TEA (Fig. 1A, normal PBLs). EGCg was more inhibitory on the growth of ATL PBLs in the range of 3-27 μ g/ml and the inhibition pattern was similar for all ATL PBLs (cases 1, 2, 3). KODV again showed an intermediate inhibition curve between ATL PBLs and normal PBLs. Growth of normal PBLs was definitely inhibited at 27 μ g/ml of EGCg, while negligible inhibition was observed in the range of 3-9 μ g/ml (Fig. 1B).

Induction of apoptosis in ATL cells by TEA and EGCg To investigate the mechanism of growth inhibition by TEA and EGCg, we examined the apoptotic response of ATL PBLs, normal PBLs and KODV to TEA and EGCg treatments. TUNEL-positive cells were observed in the

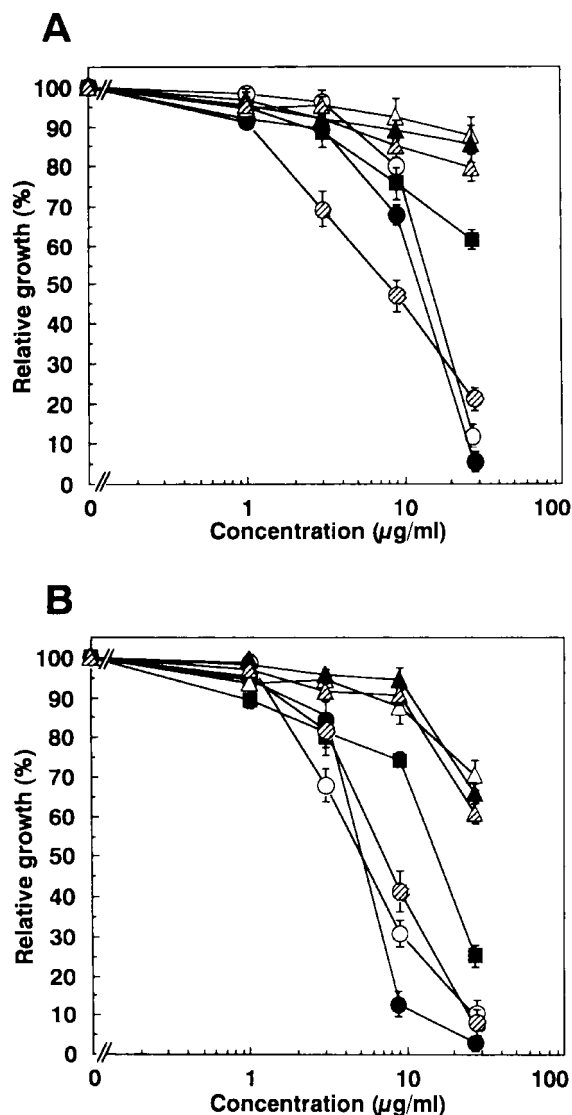


Fig. 1. Inhibitory effect of TEA (A) and EGCg (B) on growth of ATL PBLs, KODV and normal PBLs. Cells were incubated in the presence of various concentrations of either TEA or EGCg (1, 3, 9 and 27 μ g/ml) for 3 days and *in vitro* growth of the cultured cells was measured by WST-8 assay. Average content of EGCg in the TEA samples was 156-189 μ g EGCg/mg TEA. Relative growth of the cultured cells is presented as the mean \pm SEM determined on ATL PBLs (\circ case 1, \otimes case 2, \bullet case 3), HTLV-I-infected T-cell line (\blacksquare KODV) and normal PBLs (\triangle case 4, Δ case 5, \blacktriangle case 6). One hundred percent relative growth was designated as the total number of ATL cells grown in the 3-day cultures without TEA or EGCg.

ATL PBLs and KODV treated with 27 μ g/ml TEA or EGCg, while very few TUNEL-positive cells were observed in the treated normal PBLs (Fig. 2A, b and c). Untreated ATL PBLs, KODV and normal PBLs were neg-

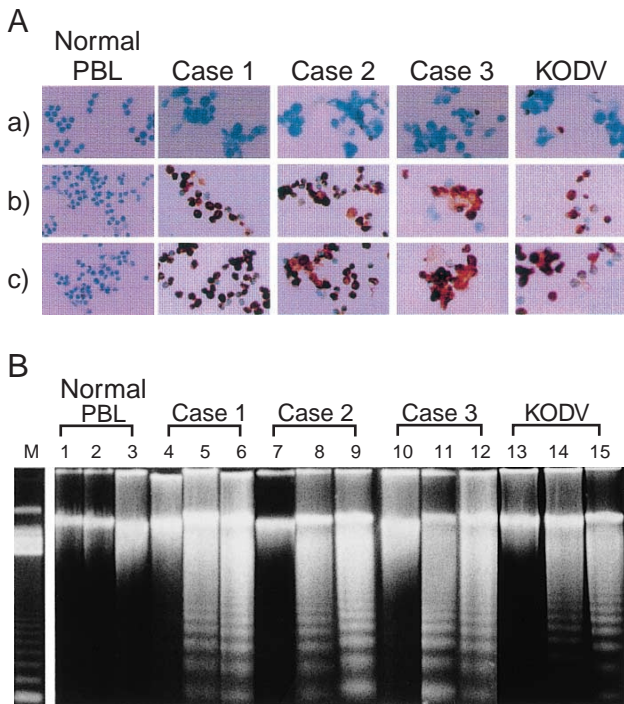


Fig. 2. Induction of apoptosis by TEA and EGCg in ATL PBLs, KODV and normal PBLs. (A) TUNEL staining of TEA or EGCg-treated cells. ATL PBLs (case 1, case 2, case 3), KODV and normal PBLs (case 4) were cultured for 3 days with either TEA or EGCg as described in Fig. 1: a) untreated controls, b) treated with 27 $\mu\text{g/ml}$ of TEA, c) treated with 27 $\mu\text{g/ml}$ of EGCg. (B) DNA fragmentation of TEA or EGCg-treated cells. DNA fragmentation of the treated cells was detected on 1.5% agarose gel electrophoresis. DNA ladder of the untreated controls (lanes 1, 4, 7, 10, 13), DNA ladder of the cells treated with 27 $\mu\text{g/ml}$ of TEA (lanes 2, 5, 8, 11, 14) and with 27 $\mu\text{g/ml}$ of EGCg (lanes 3, 6, 9, 12, 15). M: 123 bp DNA ladder (123–3075 bp).

ative for TUNEL staining (Fig. 2A, a). Trypan blue exclusion testing revealed that all the TUNEL-negative cells were viable (data not shown). Electrophoretic analysis revealed that the treated ATL PBLs and KODV showed prominent DNA fragmentation after culture with either TEA or EGCg, but no significant DNA fragmentation was seen in the normal PBLs (Fig. 2B).

Suppression of *HTLV-I pX* gene expression by TEA and EGCg To investigate whether apoptosis of ATL PBLs and KODV by TEA and EGCg is associated with the expression of *HTLV-I pX* gene, we measured mRNAs of *HTLV-I pX* and β -actin isolated from the treated ATL PBLs and KODV by a semiquantitative RT-PCR method. We carried out electrophoretic detection of RT-PCR products after dilution of total RNA samples by 1:10¹–10⁷ to quantitate *HTLV-I pX* mRNA and β -actin mRNA. A typi-

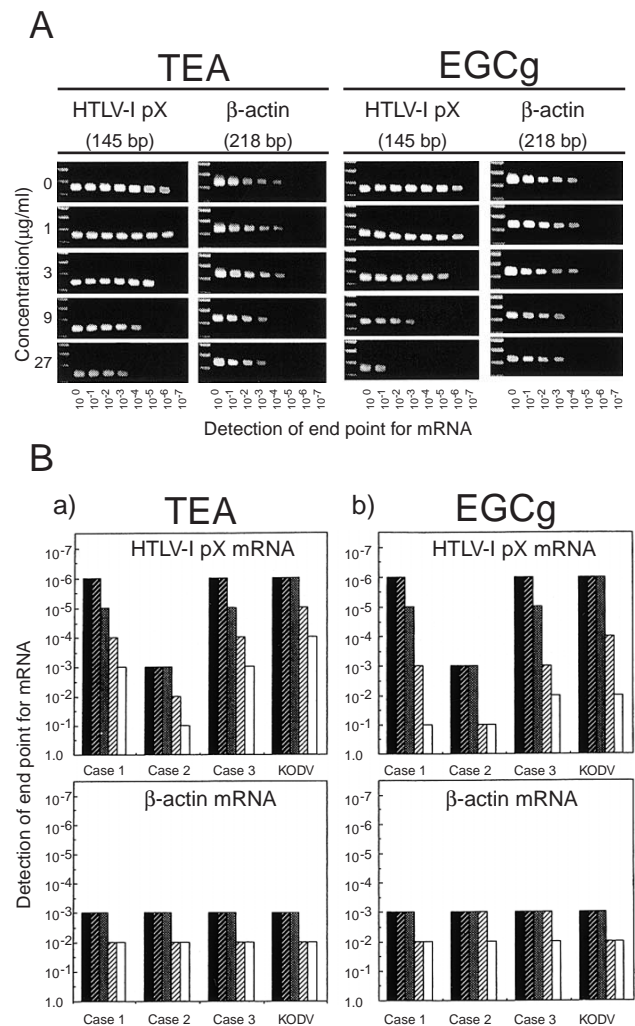


Fig. 3. Expression of *HTLV-I pX* mRNA and β -actin mRNA after treatment with TEA and EGCg. (A) ATL PBLs and KODV were incubated with TEA or EGCg as described in the legend to Fig. 1. The resulting RT-PCR products were analyzed on 2% agarose gel with ethidium bromide staining. The nested RT-PCR product (145 bp) of *HTLV-I pX* mRNA and the β -actin RT-PCR product (218 bp) are apparent in the electrophoregram. (B) The suppression rate of *HTLV-I pX* mRNA and β -actin mRNA from ATL PBLs and KODV induced by various concentrations of TEA (a) and EGCg (b). 0 $\mu\text{g/ml}$ (■), 1 $\mu\text{g/ml}$ (▨), 3 $\mu\text{g/ml}$ (▩), 9 $\mu\text{g/ml}$ (▧), 27 $\mu\text{g/ml}$ (□).

cal result for ATL PBLs (case 1) showed electrophoretic bands of RT-PCR products; *HTLV-I pX* mRNA was suppressed from 1:10 to 1:100 000 by 3–27 $\mu\text{g/ml}$ of TEA or EGCg, whereas β -actin mRNA was suppressed 1:10 by 9–27 $\mu\text{g/ml}$ of TEA or EGCg (Fig. 3A). Suppression patterns of *HTLV-I pX* mRNA by TEA and EGCg were very similar in case 1 and case 3 ATL PBLs, but case 2 ATL

PBLs and KODV showed different patterns (Fig. 3B, HTLV-I pX mRNA).

DISCUSSION

Tea (*Camellia sinensis*) is the most commonly used beverage worldwide. It is consumed in three main forms: black (78%), green (20%) and oolong(2%). Polyphenols are the major components of tea, including EGCg, epigallocatechin (EGC), epicatechin-3-gallate (ECg) and epicatechin (EC), although the EGCg content of black tea is one-tenth that of green tea as a result of the oxidation during black tea processing.³⁸⁾ Polyphenols of green tea are cytotoxic to various tumor cell lines,^{3, 8, 18)} but few studies of the anti-leukemic activity of tea polyphenols have been reported.²⁾

The subjects of this study were 3 acute-type ATL patients, 1 HTLV-I-infected T-cell line established from a HTLV-I-associated myelopathy (HAM) patient and 3 HTLV-I seronegative healthy controls. PBLs of ATL patients and normal donors were cultured *in vitro* with IL-2 in 10% fetal calf serum (FCS)-RPMI-1640 medium, in which T-cells are known to grow preferentially.^{39, 40)}

Growth of ATL PBLs was inhibited by either TEA or EGCg more than that of normal PBLs, suggesting that atypical T lymphocytes of ATL PBLs may be relatively sensitive to TEA and EGCg, especially the case 1 and case 3 ATL PBLs (Fig. 1, A and B). The difference in dose-response curves among ATL PBLs may be relevant to the hematological features of the case 2 ATL patient, whose WBC count, % atypical lymphocytes and HTLV-I antibody titer were different from those of the other two ATL patients (Table I). HTLV-I-infected T-cells (KODV) showed an intermediate growth inhibition between ATL and normal PBLs, suggesting that leukemic transformation of T-cells after HTLV-I infection may determine the increased sensitivity to TEA and EGCg. It has been estimated⁴¹⁻⁴³⁾ that one cup of green tea (1 g dried tea leaf/100 ml hot water) contains 120 mg of EGCg, and that the absorption rate of EGCg into human plasma is 0.3–0.5%. Thus, it is possible that 50% growth inhibition of ATL cells by 5–9 µg/ml TEA or EGCg can be achieved by drinking 10 cups of green tea.

The growth inhibition of ATL PBLs and KODV by TEA and EGCg was associated with apoptotic cell death. We observed TUNEL-positive cells and DNA fragmentation in both ATL PBLs and KODV treated with TEA and EGCg (Fig. 2, A and B). An apoptotic response to tea

polyphenols has been reported in a variety of cancer cells.^{6, 7)}

Suppression of HTLV-I pX mRNA in ATL PBLs and KODV by TEA and EGCg was first demonstrated in this study (Fig. 3, A and B). Case 1 and case 3 ATL PBLs were more sensitive than case 2 ATL PBLs to suppression of HTLV-I pX mRNAs by TEA and EGCg. This finding is compatible with the growth inhibition patterns of ATL PBLs as depicted in Fig. 1. Intermediate suppression of HTLV-I pX mRNAs in KODV suggests that the increased sensitivity of T-cells to TEA and EGCg is determined by not only HTLV-I infection, but also some additional factor(s) involved in the leukemic changes of ATL PBLs.

The molecular mechanism of the TEA-induced apoptosis is unknown, although it may be mediated by a protein receptor on T-cells, as shown in the cases of protein kinase C,⁴⁴⁾ DNA topoisomerase II,⁴⁵⁾ urokinase,⁴⁶⁾ activator protein 1 (AP-1) binding protein⁴⁷⁾ and vascular endothelial growth factor.⁴⁸⁾

Based on our evidence that TEA and EGCg inhibit T-cell proliferation, suppress HTLV-I pX mRNA and induce apoptosis in ATL PBLs, we speculate that the anti-leukemic effect of tea polyphenols is primarily due to TEA, which suppresses HTLV-I pX mRNA expression and secondarily induces apoptosis leading to cell cycle arrest of ATL PBLs. Further study is necessary to clarify the molecular interactions between tea polyphenols and the putative trans-signal receptor(s) of ATL PBLs resulting in HTLV-I pX suppression and apoptosis induction.

In conclusion, TEA appear to be effective to inhibit the growth of ATL PBLs by suppressing *HTLV-I pX* gene expression and by inducing apoptotic cell death.

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