Anti-adult T-cell leukemia effects of *Bidens pilosa*

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Abstract. We evaluated the effects of *Bidens pilosa*, a plant found in tropical and subtropical regions, and investigated the molecular pathways responsible for the anti-adult T-cell leukemia (ATL) effect. Water extracts of *B. pilosa* had growth suppressive effects on human T-cell leukemia virus type 1 (HTLV-1)-infected T-cell lines and ATL cells. *B. pilosa* extracts arrested cells in G_1 cell cycle and induced apoptosis of HTLV-1-infected T-cell lines. *B. pilosa* extracts inhibited also the phosphorylation of IκB kinase β and IκBα, and NF-κB-DNA binding, in conjunction with reduction of expression of proteins involved in G_1/S cell cycle transition and suppression of apoptosis. Reactive oxygen species played a role in *B. pilosa*-mediated suppression of NF-κB activity. *B. pilosa* extracts also inhibited the expression of JunB and JunD, resulting in suppression of AP-1-DNA binding. In animals harboring tumors of HTLV-1-infected T-cell origin, treatment with *B. pilosa* extracts suppressed tumor growth. Our results suggest that *B. pilosa* is a potentially useful medicinal plant for treatment of ATL.

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) infection causes adult T-cell leukemia (ATL), a malignancy of peripheral $CD4^+$ T cells (1-3). Despite the availability of various combination chemotherapy regimens, the median survival time of individuals with aggressive ATL such as acute and lymphoma types, is <13 months (4). This extremely grave outcome is mainly due to intrinsic resistance of leukemic cells to conventional or even high doses of chemotherapy and also to severe immunosuppression. Therefore, it is important to find appropriate therapeutic methods to prevent the development of ATL or to prolong survival after its occurrence. Three decades of research in this field has resulted in accumulation of a wealth of knowledge on the molecular pathways underlying the proliferation of ATL cells. Nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) are induced by a unique HTLV-1 gene, Tax, and are related to proliferation of ATL cells (5). Therefore, targeting these molecules may provide a new approach to the treatment of ATL.

Researchers have been looking for anti-tumor agents in natural products to develop novel therapeutic agents for cancer. Herbal medicine has attracted a great deal of recent attention as an alternative cancer therapy because of its low toxicity and low cost. *Bidens pilosa Linn. var. radiata* is a tropical weed widely present in tropical and subtropical regions. This plant was originally found in tropical America and later introduced into the Pacific region and parts of Asia including Miyako Island, Okinawa, Japan. The whole plant or its aerial parts are used in various folk medicines and as a popular ingredient in herbal tea for its anti-inflammatory, anti-septic, liver-protective, blood-pressure lowering and hypoglycemic effects (6-10). Although diverse bioactivities have been identified in *B. pilosa*, its effect on human leukemia has not attracted attention so far. Ethyl caffeate isolated from *B. pilosa* has been reported to suppress NF-κB activation and its downstream inflammatory mediators (11). With the objective of finding newer agents for the treatment of ATL, the present study was designed to investigate whether *B. pilosa* is a pharmacologically safe and effective inhibitor of cell growth of HTLV-1-infected T cells. We report here the anti-proliferative effects and molecular mechanisms of the apoptotic effects induced by *B. pilosa* in HTLV-1-infected T cells.

Materials and methods

Reagents. The *B. pilosa* powder produced from the aerial parts of *B. pilosa Linn. var. radiata* cultured on Miyako Island was provided by the Musashino Research Institute for Immunity (Tokyo, Japan). Antibodies to cyclin D2, c-IAP2, IκBα, JunB and JunD, and NF-κB subunits p65, p50, c-Rel, p52 and RelB, and AP-1 subunits c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD for super shift assay were purchased from Santa Cruz

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Biotechnology (Santa Cruz, CA). Antibodies to Bax, Bcl-2, CDK4, CDK6, c-FLIP, p21, p53 and actin were purchased from NeoMarkers (Fremont, CA). Antibodies to XIAP and cyclin D1 were purchased from Medical and Biological Laboratories (Nagoya, Japan). Antibodies to IκB kinase (IKK) β, phospho-IKKβ (Ser181), phospho-IκBα (Ser32 and Ser36), cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, cleaved poly(ADP-ribose) polymerase (PARP), survivin and Bcl-x_L were purchased from Cell Signaling Technology (Beverly, MA). Antibody to Tax, Lt-4, was described previously (12). *N*-acetylcysteine (NAC) and caffeic acid were from Wako Pure Chemical Industries, Osaka, Japan.

Cells. The HTLV-1-infected T-cell lines, MT-2 (13), MT-4 (14), HUT-102 (1) and ED-40515(-) (15) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. MT-2 and MT-4 are HTLV-1-transformed T-cell lines established by an *in vitro* coculture protocol. ED-40515(-) is a T-cell line of leukemic cell origin established from a patient with ATL. HUT-102 was also established from a patient with ATL and constitutively expresses viral genes, but its clonal origin is not clear. Peripheral blood mononuclear cells (PBMC) from healthy volunteers and patients with acute type ATL were also analyzed. Activated PBMC were grown in RPMI-1640 medium supplemented with phytohemagglutinin (PHA) (10 μg/ml) or interleukin-2 (IL-2) (10 ng/ml) for 48 h. All samples were obtained after informed consent.

Cell viability and apoptosis assays. Cell lines ($1x10^5$/ml) or PBMC ($1x10^6$/ml) were cultured with various concentrations of *B. pilosa* in 96-well plates. After 24 h, cell viability was evaluated by measuring the mitochondrial-dependent conversion of the water-soluble tetrazolium (WST)-8 (Nacalai Tesque, Kyoto, Japan) to a colored formazan product. Apoptotic events in cells were detected by staining with phycoerythrin-conjugated Apo2.7 monoclonal antibody (Beckman Coulter, Marseille, France) (16), and analyzed by flow cytometry (Epics XL, Beckman Coulter, Fullerton, CA).

Cell cycle analysis. Cell cycle analysis was performed with the CycleTest Plus DNA reagent kit (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Cell suspensions were analyzed on a Coulter EPICS XL using EXPO32 software. The population of cells in each cell cycle phase was determined with the MultiCycle software.

In vitro measurement of caspase activity. Caspase activity was measured using the colorimetric caspase assay kits (Medical and Biological Laboratories). Cell extracts were recovered using the cell lysis buffer and assessed for caspase-3, -8 and -9 activities using colorimetric probes. The colorimetric caspase assay kits are based on detection of chromophore ρ-nitroanilide after cleavage from caspase-specific-labeled substrates. Colorimetric readings were performed in an automated microplate reader.

Measurement of intracellular ROS levels. Generation of reactive oxygen species (ROS) was measured by the total ROS detection kit (Enzo Life Sciences, Plymouth Meeting, PA). After treatment of cells with *B. pilosa*, HUT-102 cells were washed with wash buffer and then loaded for 30 min with 500 μl of the ROS detection solution. The cells were then analyzed using a Coulter EPICS XL.

Western blot analysis. Cells were lysed in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate, 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (20 μg) were subjected to electrophoresis on sodium dodecyl sulphate-polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing with the specific antibodies. The bands were visualized with the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were obtained as described by Antalis and Goldbolt (17) with modifications, and EMSA was performed as described previously (18). Briefly, 5 μg of nuclear extract was incubated with ^{32}P-labeled probes. The DNA-protein complex was separated from the free oligonucleotides on a 4% polyacrylamide gel. The probes used were prepared by annealing the sense and antisense synthetic oligonucleotides; a typical NF-κB element from the IL-2 receptor α chain (IL-2Rα) gene (5'-gatcCGGCAGGGGAATCTCCCTCTC-3') and an AP-1 element of the IL-8 gene (5'-gatcGTGATGACTCAGGTT-3'). The oligonucleotide 5'-gatcTGTCGAATGCAAATCACTAGAA-3', containing the consensus sequence of the octamer binding motif, was used to identify specific binding of the transcription factor Oct-1. This transcription factor regulates the transcription of a number of so-called housekeeping genes. The above underlined sequences represent the NF-κB, AP-1 or Oct-1 binding site.

Measurement of p19 antigen of HTLV-1. MT-2 cells were treated with *B. pilosa* (500 μg/ml) for 6 h. Samples of the culture supernatant were collected and used to measure the p19 antigen of HTLV-1 (ZeptoMetrix, Buffalo, NY) by enzyme-linked immunosorbent assay.

In vivo therapeutic effect of B. pilosa. Five-week-old female C.B-17/Icr-scid mice were obtained from Ryukyu Biotec Co. (Urasoe, Japan). They were engrafted with $1x10^7$ HUT-102 cells by subcutaneous injection in the postauricular region and then randomly placed into two groups of five mice each, one received vehicle only, while the other was treated with *B. pilosa*. Treatment was initiated on the next day of cell inoculation. *B. pilosa* was dissolved in distilled water at a concentration of 150 mg/ml, and 3.75 g/kg body weight of *B. pilosa* was administered by oral gavage every day for 28 days. Control mice received the same volume of the vehicle only for 28 days. Tumor size was monitored once a week. All mice were sacrificed on day 28, and then the tumors were dissected out and their weight was measured. This study was performed according to the Guidelines for the Animal Experimentation of the University of the Ryukyus and was approved by the Animal Care and Use Committee of the University of the Ryukyus.

Statistical analysis. Data are expressed as the mean ± SD. Differences between groups were assessed for statistical

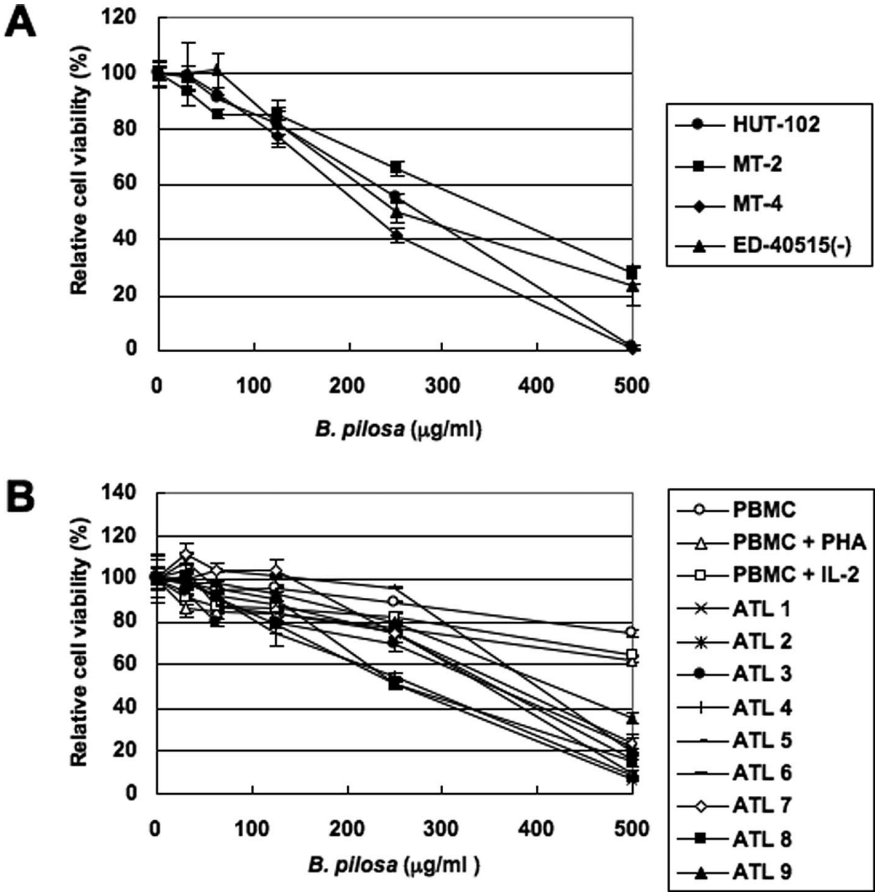

Figure 1. Water extracts of *B. pilosa* inhibit cell viability of HTLV-1-infected T-cell lines and PBMC from ATL patients. HTLV-1-infected T-cell lines (A), PBMC from ATL patients and a healthy control, and PHA- (10 μg/ml) or IL-2- (10 ng/ml) activated PBMC (B) were cultured with the indicated concentrations of *B. pilosa* for 24 h, and cell viability was determined in triplicate cultures by WST-8 assay. Data are mean ± SD values expressed as percentage of the control.

significance by the Mann-Whitney U-test. $P<0.05$ denoted the presence of a statistically significant difference.

Results

B. pilosa inhibits cell viability of HTLV-1-infected T-cell lines and primary ATL cells. First, we determined the effects of *B. pilosa* on cell viability of HTLV-1-infected T-cell lines and primary ATL cells. Tax protein was detected by immunoblot analysis in the three HTLV-1-infected T-cell lines (MT-2, MT-4 and HUT-102) but not in the ATL-derived T-cell line [ED-40515(-)] (data not shown). Cell viability was assessed by the WST-8 assay. Treatment with *B. pilosa* for 24 h resulted in a dose-dependent inhibition of cell viability of all tested HTLV-1-infected T-cell lines (Fig. 1A). The concentrations of *B. pilosa* required to inhibit cell viability of HTLV-1-infected T-cell lines by 50% (IC_{50} values) ranged from 220 to 355 μg/ml. Importantly, resting and PHA- or IL-2-activated PBMC were resistant to *B. pilosa*, but activated PBMC were sensitive to *B. pilosa* compared with resting PBMC (Fig. 1B). We also examined the effects of *B. pilosa* on ATL cells freshly isolated from nine patients with the acute type ATL. ATL cells treated with *B. pilosa* showed reduced cell survival compared with resting and PHA- or IL-2-activated PBMC from normal healthy volunteers (Fig. 1B).

B. pilosa induces apoptosis of HTLV-1-infected T-cell lines. To investigate the mechanisms by which *B. pilosa* inhibited the cell viability of HTLV-1-infected T-cell lines, we analyzed apoptosis by immunostaining with Apo2.7, which specifically detects the 38-kDa mitochondrial membrane antigen 7A6 expressed on the mitochondrial outer membrane during apoptosis (16). A significant increase in the proportion of cells positive for 7A6 was detected in all HTLV-1-infected T-cell lines after treatment with *B. pilosa* for 24 h, and an increase in the apoptotic population was detected in HUT-102 cells in a *B. pilosa* dose-dependent manner (Fig. 2A).

B. pilosa-induced apoptosis is caspase-dependent. We then investigated the mechanism of the observed apoptosis. Cell extracts were obtained after various treatments and processed for Western blot analysis. Indeed, in HUT-102 and MT-2 cells, *B. pilosa*-induced apoptosis was associated with caspase activation, as shown by PARP cleavage (Fig. 2B). Furthermore, *B. pilosa* treatment resulted in cleavage of procaspase-3, -8 and -9. In addition, we assessed caspase-3, -8 and -9 activities using colorimetric probes. *B. pilosa* resulted in activation of caspases-3, -8 and -9 in HUT-102 cells (Fig. 2C). These results indicate that *B. pilosa*-induced apoptosis of HTLV-1-infected T-cell lines is mediated through caspase activation.

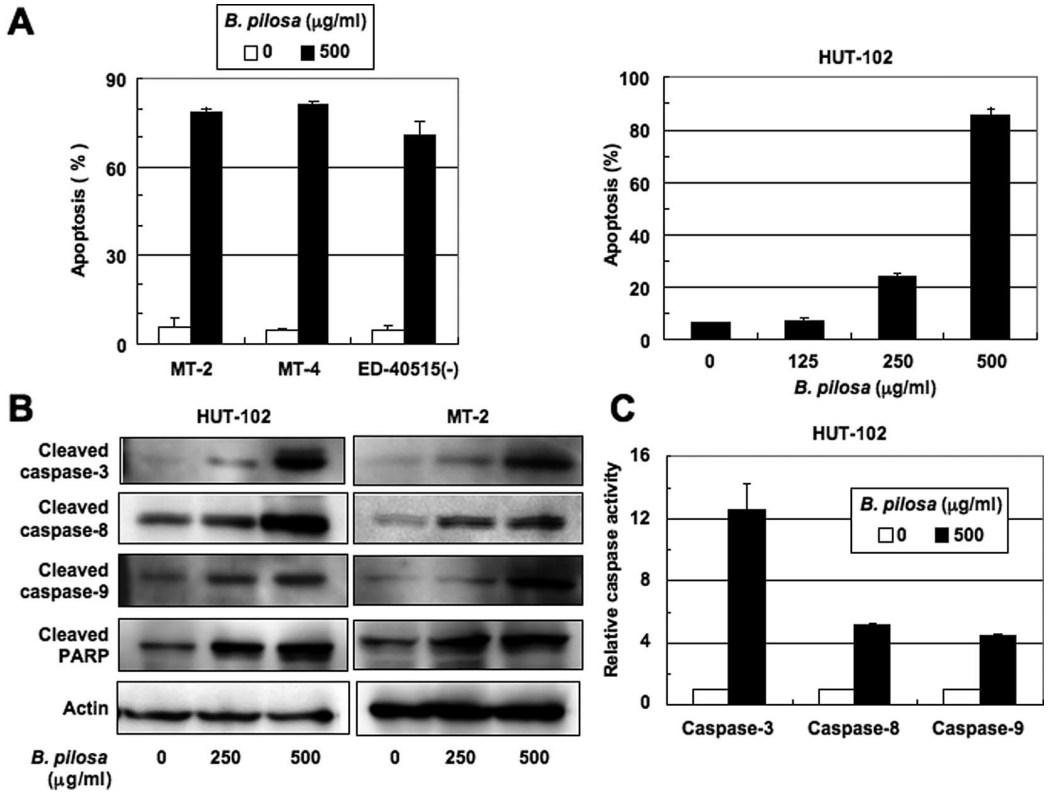

Figure 2. *B. pilosa* induces apoptosis of HTLV-1-infected T-cell lines. (A) HTLV-1-infected T-cell lines were cultured in the absence or presence of *B. pilosa* (500 μg/ml) (left panel). After 24 h, Apo2.7 staining was analyzed by flow cytometry. Data are the mean ± SD percentages of apoptotic cells of both untreated (open bars) and *B. pilosa*-treated (solid bars) cells. HUT-102 cells were also incubated with various concentrations of *B. pilosa* for 24 h (right panel). The pro-apoptotic activity of *B. pilosa* was assessed by Apo2.7 staining. Data are the mean ± SD (n=3). (B) Cells were treated with the indicated concentrations of *B. pilosa* for 24 h and subjected to immunoblot analyses with the indicated antibodies. (C) HUT-102 cells were treated with or without *B. pilosa* (500 μg/ml) for 24 h. Caspase activity was assayed as described in Materials and methods and expressed relative to untreated cells, which were assigned a value of 1. Values represent the mean ± SD (n=3).

B. pilosa causes G_1 cell cycle arrest. We also examined the cellular DNA contents distribution by flow cytometric analysis on cell treatment. HUT-102 and MT-2 cells were incubated with 500 μg/ml *B. pilosa* for 24 h (Fig. 3A). Cultivation with *B. pilosa* for 6-12 h increased the population of the cells in the G_1 phase, with a marked reduction of the cells in the S phase, relative to untreated cells. This G_1 cell cycle arrest was dose-dependent (Fig. 3B). At 12 h after treatment, the percentage of HUT-102 cells in sub-G_1 markedly increased from 4.1 in untreated cells to 52.6% (Fig. 3C), suggesting that cell cycle arrest is the cause of apoptosis. These results indicate that, together with induction of apoptosis, *B. pilosa* treatment induces a G_1 cell cycle arrest in HTLV-1-infected T-cell lines, which likely contributes to the growth inhibitory effects.

Effects of B. pilosa on cell cycle and apoptosis regulatory proteins. To clarify the molecular mechanism of *B. pilosa*-induced inhibition of cell growth and apoptosis of HTLV-1-infected T-cell lines, we investigated the effect of *B. pilosa* on the expression of several intracellular regulators of cell cycle and apoptosis including cyclin D1, cyclin D2, CDK4, CDK6, p53, p21, Bcl-2, Bcl-x_L, c-IAP2, XIAP, survivin, Bax and c-FLIP by Western blot analysis. As shown in Fig. 4, *B. pilosa* did not alter CDK4 and p53 expression levels. In contrast, *B. pilosa* significantly decreased the expression of cyclin D1, cyclin D2, CDK6, Bcl-2, Bcl-x_L, c-IAP2, XIAP, survivin and c-FLIP in HUT-102 and MT-2 cells in a dose-dependent manner. In addition, *B. pilosa* increased the expression of p21 and Bax. Comparable loading of protein was confirmed with a specific antibody for the housekeeping gene product actin (Fig. 4). Because cyclin D1, cyclin D2, CDK6, Bcl-x_L, c-IAP2, XIAP and c-FLIP are Tax-responsive genes (19-25), we also examined the expression level of Tax. *B. pilosa* did not change the Tax protein level in HUT-102 and MT-2 cells (Fig. 4A). These results indicate that the altered expression levels of cyclin D1, cyclin D2, CDK6, Bcl-x_L, c-IAP2, XIAP and c-FLIP proteins did not result from Tax down-regulation.

Inhibitory effects of B. pilosa on NF-κB activation. Several reports have suggested that NF-κB can prevent apoptosis and caspase activation as a survival factor and is required for the proliferation of various tumor cell types (26). Because NF-κB is constitutively active in Tax-expressing and HTLV-1-infected T-cell lines as well as primary ATL cells (27), and Tax stimulates the expression of cyclin D2, CDK6, Bcl-x_L, c-IAP2, XIAP and c-FLIP through the NF-κB pathway (20-25), we examined whether *B. pilosa* inhibits the NF-κB pathway. To study the DNA-binding activity of NF-κB, we performed EMSA with radiolabeled double-stranded NF-κB oligonucleotides and nuclear extracts from untreated or *B. pilosa*-treated HTLV-1-infected T-cell lines. NF-κB oligonucleotide probe

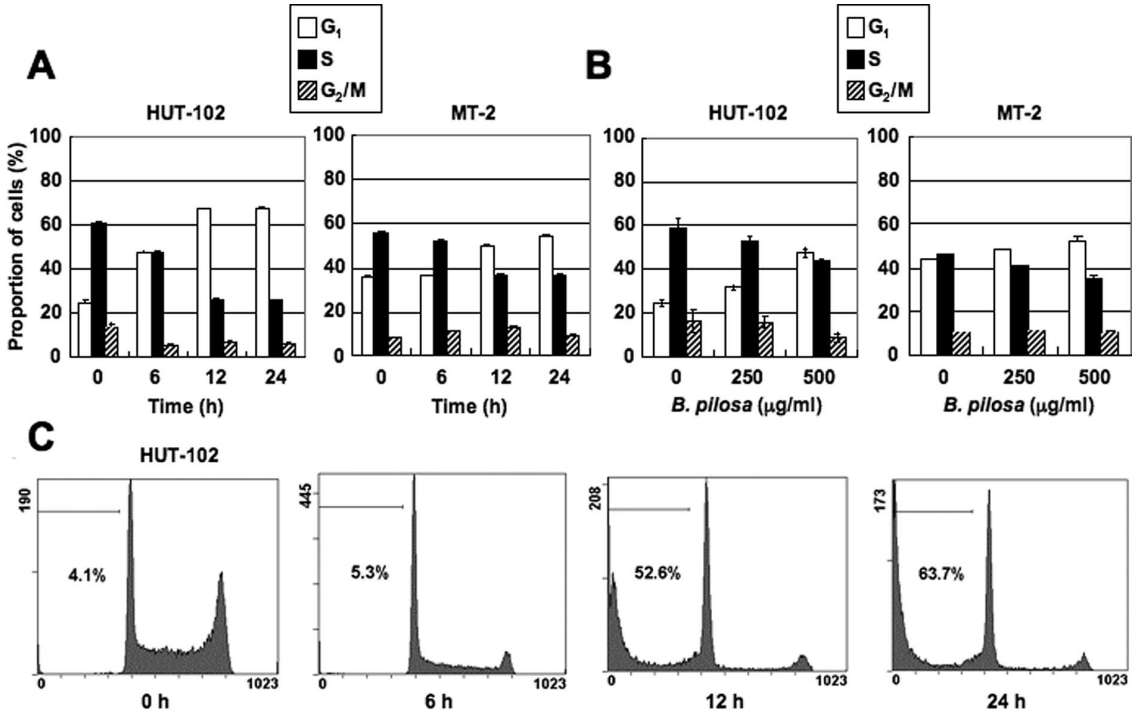

Figure 3. *B. pilosa* induces G_1 cell cycle arrest in HTLV-1-infected T-cell lines. (A) HUT-102 and MT-2 cells were incubated with *B. pilosa* (500 μg/ml) for the indicated time periods. (B) HUT-102 and MT-2 cells were incubated with the indicated concentrations of *B. pilosa* for 12 h. (C) HUT-102 cells were incubated with *B. pilosa* (500 μg/ml) for the indicated time periods. Cell cycle distribution was analyzed by flow cytometry by staining with propidium iodide. Data are the mean ± SD percentages of cells at various phases of the cell cycle (n=3).

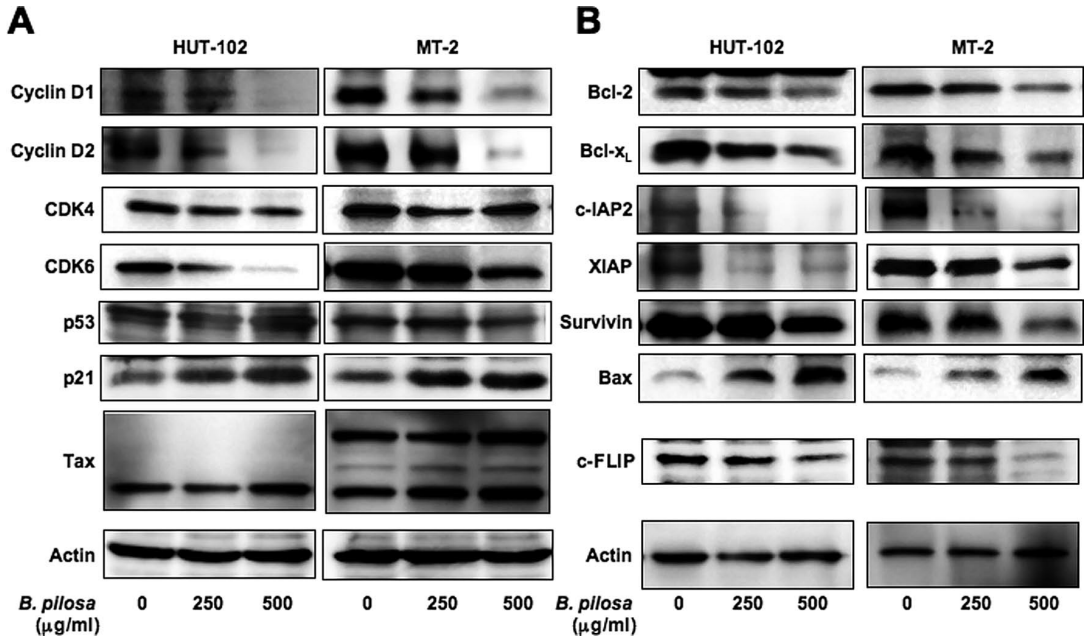

Figure 4. Effects of *B. pilosa* on the cell cycle and apoptosis regulatory proteins, and viral protein Tax. Cells were treated with various concentrations of *B. pilosa* for 24 h. Whole cell extracts were prepared and immunoblotted with specific antibodies against cell cycle and Tax (A), and apoptosis regulatory proteins (B).

with nuclear extracts from untreated HTLV-1-infected T-cell lines generated DNA-protein gel shift complexes (Fig. 5A, left panels). These complexes were due to specific bindings of nuclear proteins to the NF-κB sequences because the binding activities diminished following the addition of cold probe but not by an irrespective sequence (Fig. 5A, left panels, lanes 2 and 3). We also showed that NF-κB complexes contain p50, p65, c-Rel and RelB in HUT-102, and p50, p65,

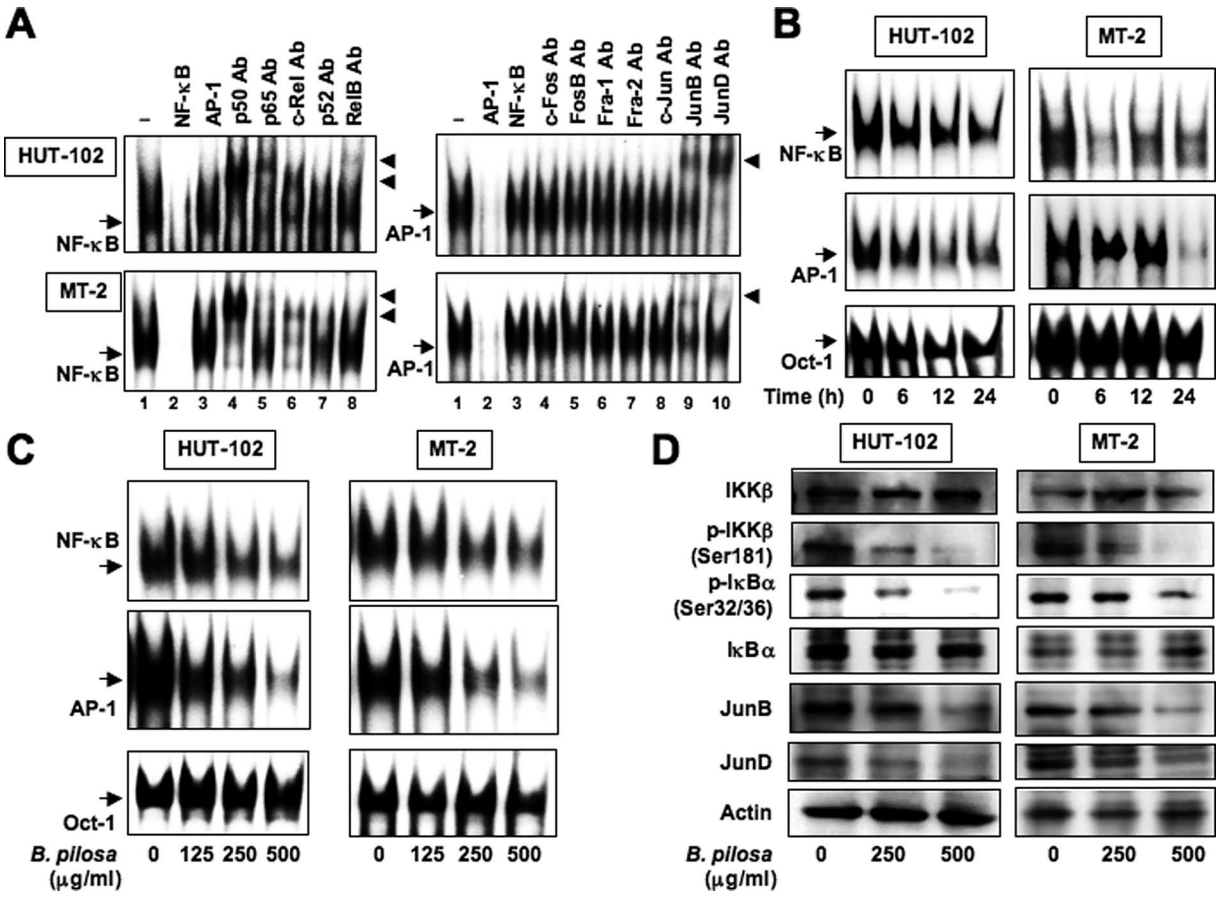

Figure 5. *B. pilosa* inhibits NF-κB and AP-1 activities in HTLV-1-infected T-cell lines. (A) EMSA using untreated HUT-102 and MT-2 nuclear extracts and radiolabeled NF-κB and AP-1 probes generated DNA-protein complexes, which were eliminated by 100-fold molar excess of self-competitors but not by the same molar excess of the irrespective oligonucleotides. Supershift assays using the radiolabeled NF-κB and AP-1 probes, untreated nuclear extracts, and the indicated antibodies (Ab) to NF-κB and AP-1 components. Arrows: the specific complexes, arrowheads: the DNA binding complexes supershifted by antibodies. (B and C) Effects of *B. pilosa* on activation of NF-κB, AP-1 and Oct-1 in HTLV-1-infected T-cell lines assessed by EMSA using oligonucleotide probes for NF-κB, AP-1 and Oct-1, respectively. Cells were treated with *B. pilosa* (500 μg/ml) for the indicated time periods (B) or with the indicated concentrations of *B. pilosa* for 24 h (C). (D) Effects of *B. pilosa* on the levels of IKKβ, IκBα, JunD, JunB and phosphorylated IκBα and IKKβ by Western blot analysis. Cells were treated with various concentrations of *B. pilosa* for 24 h, followed by protein extraction. Whole cell extracts of treated cells were immunoblotted with specific antibodies.

c-Rel and p52 in MT-2 cells (Fig. 5A, left panels, lanes 4-8). As shown in Fig. 5B and C, nuclear extracts prepared from HTLV-1-infected T-cell lines treated with *B. pilosa* exhibited a decrease in the intensity of the NF-κB-containing gel shift complexes in a time- and dose-dependent manner, suggesting that *B. pilosa* down-regulates the DNA-binding activities of NF-κB. Inhibition appeared specific to NF-κB and was not due to cell death because no significant change in binding activity of Oct-1 was observed after treatment of cells with *B. pilosa* (Fig. 5B and C).

Degradation of IκBα and subsequent release of NF-κB require prior phosphorylation at Ser32 and Ser36 residues (28). To investigate whether the inhibitory effects of *B. pilosa* are mediated through alteration of phosphorylation of IκBα, HUT-102 and MT-2 cells were treated with *B. pilosa* and their protein extracts were checked for phospho-IκBα expression. Untreated cells constitutively expressed Ser32- and Ser36-phosphorylated IκBα (Fig. 5D), while *B. pilosa* decreased the phosphorylated IκBα in a dose-dependent manner, suggesting that *B. pilosa* inhibited phosphorylation of IκBα. IKK is part of a multiprotein complex that contains IKKα and IKKβ subunits, and IKKβ is critical in mediating IκBα phosphorylation (28). Active IKKβ is phosphorylated on two Ser177 and Ser181, within the activation loop of the kinase domain. *B. pilosa* suppressed IKKβ phosphorylation in a dose-dependent manner, suggesting that *B. pilosa* suppresses NF-κB activation by inhibiting IKKβ activity (Fig. 5D).

ROS play a role in B. pilosa-mediated growth inhibition and NF-κB suppression. The protein extract of *Bidens alba*, a *Bidens* spp. plant, has been reported to induce apoptosis related to the production of ROS in human colon cancer (29). In addition, ROS play a role in suppression of NF-κB activity (30). We measured the capacity of *B. pilosa* to cause intracellular oxidation in HTLV-1-infected T cells. When HUT-102 cells were treated with *B. pilosa*, intracellular ROS concentration was increased within 15 min as observed in Fig. 6A. NAC is a widely used thiol-containing anti-oxidant that scavenges ROS in cells by interacting with OH and H_2O_2, thus affecting ROS-mediated signaling pathways. To address whether ROS plays a role in *B. pilosa*-induced inhibition of cell viability, we pretreated HUT-102 cells with NAC for 2 h then with

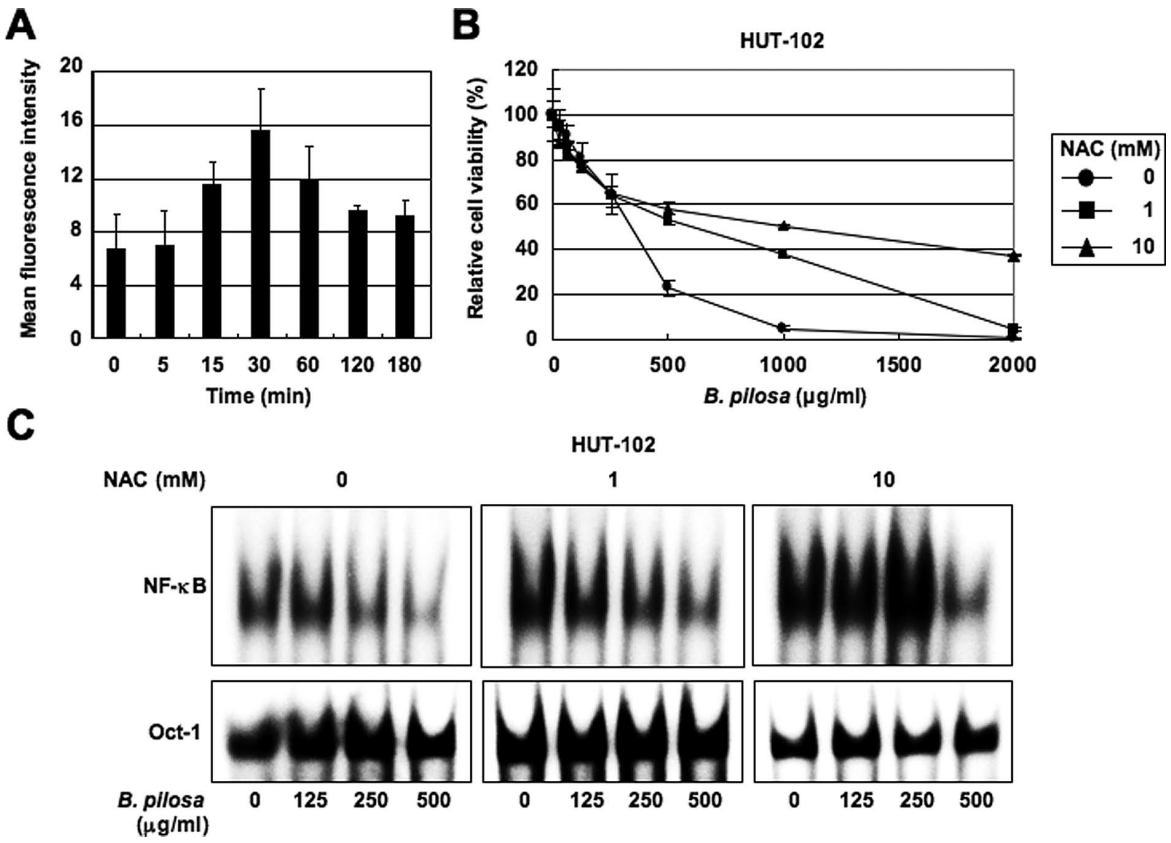

Figure 6. NAC attenuated *B. pilosa*-induced suppression of cell viability and constitutively active NF-κB. (A) Effect of *B. pilosa* treatment on ROS generation. Intracellular ROS concentration was determined by treating HUT-102 cells with *B. pilosa* (500 μg/ml) for 5-180 min, the cells were loaded with total ROS detection reagent, and the fluorescence was measured by flow cytometry. (B and C) HUT-102 cells were preincubated with 1 or 10 mM NAC for 2 h before exposure to various concentrations of *B. pilosa* for 24 h. (B) Cell viability was determined in triplicate cultures by WST-8 assay. Data are the mean ± SD values expressed as percentage of the control. (C) Nuclear extracts were prepared and EMSA was performed using oligonucleotide probes for NF-κB and Oct-1, respectively.

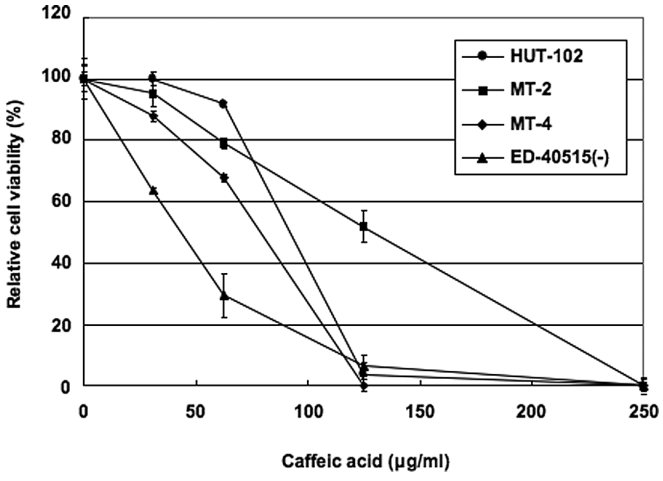

Figure 7. Effects of caffeic acid on the viability of HTLV-1-infected T-cell lines. Cells were incubated with various concentrations of caffeic acid for 24 h, and cell growth was determined in triplicate cultures by WST-8 assay. Data are the mean ± SD values expressed as percentage of the control.

B. pilosa for 24 h. As shown in Fig. 6B, pretreatment with NAC partially suppressed *B. pilosa*-induced inhibition of cell viability in a dose-dependent manner, suggesting that *B. pilosa*-induced inhibition of cell viability is in part mediated by ROS. We next sought to determine whether ROS inhibit the NF-κB activity in HTLV-1-infected T-cell lines. HUT-102 cells were pretreated with NAC and subsequently treated with *B. pilosa*. EMSA showed that pretreatment of cells with NAC partially suppressed *B. pilosa*-induced inhibition of active NF-κB (Fig. 6C), suggesting that ROS play a role in *B. pilosa*-mediated suppression of NF-κB activity.

Inhibitory effects of B. pilosa on AP-1 activation. AP-1 is also a crucial mediator of both cell cycle promoting and cell-death inhibiting pathways in HTLV-1-infected T cells (31). Therefore, we examined the effect of *B. pilosa* treatment on AP-1. High constitutive AP-1 DNA-binding activities were detected in HUT-102 and MT-2 cells (Fig. 5A, right panels). Supershift analysis with antibodies indicated that the AP-1 complex in both cell lines contained JunB and JunD. As shown in Fig. 5B and C, AP-1 DNA-binding activity diminished in the presence of *B. pilosa* in a time- and dose-dependent manner. In addition, *B. pilosa* also dose-dependently decreased the expression of JunB and JunD (Fig. 5D). These findings suggest that *B. pilosa* depletes JunB and JunD, resulting in inactivation of AP-1.

Inhibition of viral production. MT-2 cells normally produce infectious HTLV-1 virions that could be detected in the super-

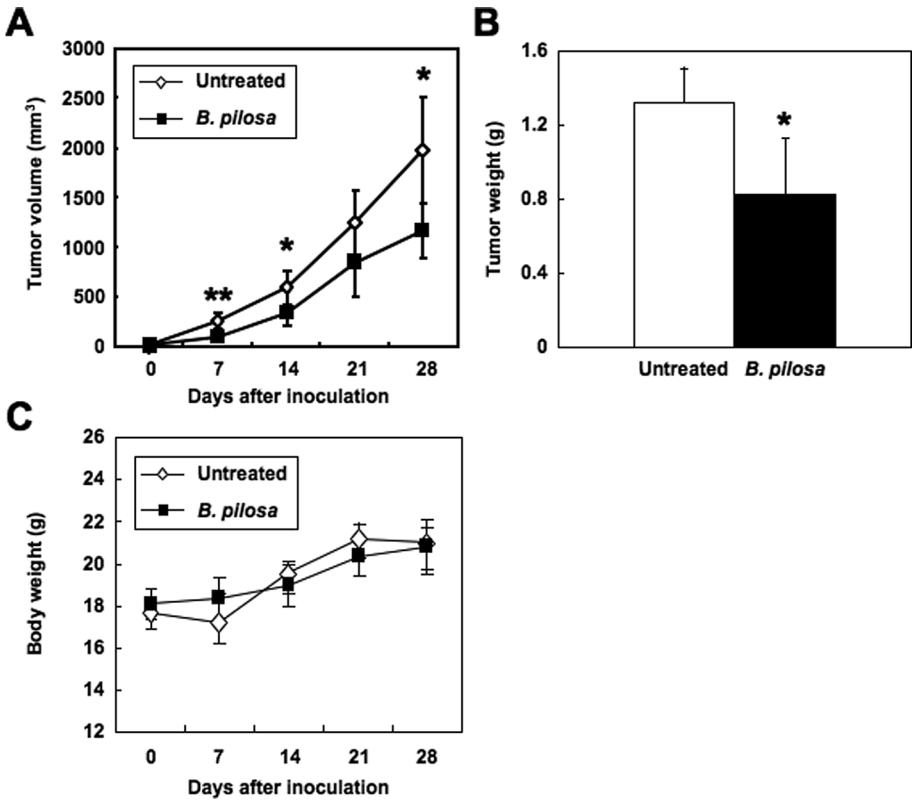

Figure 8. Inhibition of growth of HUT-102 cells in SCID mice. (A) Growth of the tumors after inoculation of HUT-102 cells subcutaneously. Note the growth-suppressive effect of *B. pilosa*. (B) Weight of tumors removed from *B. pilosa*-treated mice and untreated mice on day 28 after cell inoculation. (C) Body weight of mice measured weekly for four weeks. Data are the mean ± SD of five mice in each group. $^{*}P<0.05$, $^{**}P<0.01$, compared with the control.

natant using p19 gag enzyme-linked immunosorbent assay. We therefore treated MT-2 cells with *B. pilosa* (500 μg/ml) for 6 h. Compared to untreated cells (1000 pg/ml), *B. pilosa* treatment reduced p19 levels (687 pg/ml). These results imply that short-time incubation of *B. pilosa* that does not cause cell death effectively inhibits viral production.

Inhibition of cell viability induced by caffeic acid. Six caffeic acid derivatives (neochlorogenic acid, chlorogenic acid, 4-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid) and 7 flavonoids (rutin, quercetin, quercetin derivative, hyperin, isoquercitrin, centaurein and jacein) have been isolated from *B. pilosa* (32). To determine the role of the active compounds on *B. pilosa*-induced cytotoxicity, we examined the effect of caffeic acid on the viability of HTLV-1-infected T-cells. Caffeic acid suppressed cell viability in a dose-dependent manner (Fig. 7).

In vivo effects of B. pilosa in SCID mice inoculated with HTLV-1-infected T-cells. Because the *B. pilosa*-induced inhibition of ATL cell viability suggests that this compound is a potentially effective agent in the treatment of ATL, we examined the *in vivo* effects of *B. pilosa* in a SCID mouse model. After 28-day treatment, the mean tumor volume (Fig. 8A) and weight (Fig. 8B) were significantly lower than those of vehicle-treated mice. There was no significant difference in body weight gain during the period from day 0 to day 28 between the vehicle and *B. pilosa*-treated groups (Fig. 8C). During this period, mice treated with *B. pilosa* appeared generally healthy. These results suggest that *B. pilosa* also has *in vivo* anti-ATL effect.

Discussion

Several constituents of *B. pilosa* have a variety of clinical effects, but whether it has anti-leukemia effect remains to be determined. In the present study, we investigated the anti-ATL effect of extracts of *B. pilosa*. Our data indicated that *B. pilosa* is cytotoxic against all tested HTLV-1-transformed and ATL-derived T-cell lines as well as primary ATL cells. This cytotoxic effect appears to be HTLV-1-infected T-cell-specific, because *B. pilosa* had negligible effect on normal resting and activated PBMC.

How does *B. pilosa* induce inhibition of cell growth? Two mechanisms are proposed here: i) *B. pilosa* had an impact on cell cycle phase. In the present study, flow cytometry of HUT-102 and MT-2 cells treated with *B. pilosa* indicated the accumulation of cells at the G_1 peak, and reduction of cells in the S phase. Therefore, it is conceivable that *B. pilosa* inhibited the proliferation of HTLV-1-infected T-cell lines by arresting cell cycle; ii) *B. pilosa* induced apoptosis of HTLV-1-infected T-cell lines. Apo2.7 staining confirmed that *B. pilosa* induced apoptosis of HTLV-1-infected T-cell lines.

Targeting the key regulators of the G_1/S transition such as cyclins, CDKs and CDK inhibitors may be one of the mecha-

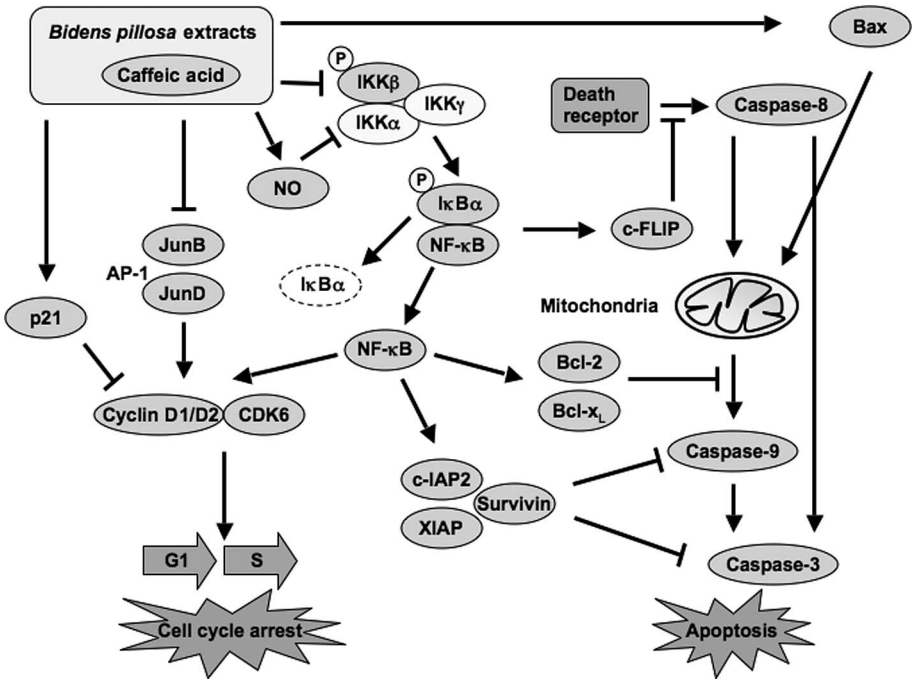

Figure 9. The proposed model to delineate the actions of *B. pilosa*.

nisms by which *B. pilosa* induces cell cycle arrest. We found that cyclin D1, cyclin D2 and CDK6 are down-regulated and p21 is up-regulated in a p53-independent manner in parallel with cell cycle G_1 arrest in HTLV-1-infected T-cell lines exposed to *B. pilosa* (Fig. 9).

Analysis of the apoptotic pathway revealed activation of both membrane and mitochondrial pathways, as evidenced by detection of cleaved products and activation of caspase-8 and -9. *B. pilosa* down-regulated the expression of the anti-apoptotic proteins, Bcl-2, Bcl-x_L, c-IAP2, XIAP and survivin. Bcl-2 and Bcl-x_L are the principal regulators of the mitochondrial-dependent pathway for apoptosis (33), and their down-regulation is involved in caspase-9-dependent apoptosis. Because c-IAP2, XIAP and survivin are associated with caspase-3 and caspase-9, and inhibit their activities (34,35), it appears that *B. pilosa* stimulates caspase-3- and caspase-9-dependent apoptosis by down-regulating the expression of these IAP family proteins. The anti-apoptotic protein c-FLIP suppresses the activation of caspase-8 (36). Decrease in expression of c-FLIP may be responsible for the *B. pilosa*-induced caspase-8 activation. On the other hand, the pro-apoptotic protein Bax was up-regulated by *B. pilosa* treatment, suggesting that Bax also plays a role in *B. pilosa*-induced apoptosis (Fig. 9).

NF-κB is a transcription factor that plays crucial roles in cell proliferation and apoptosis (26). Many studies have suggested that NF-κB-mediated cell proliferation and survival may be closely related to its downstream genes involved in the cell cycle machinery and suppression of apoptosis (26). In HTLV-1-infected T cells, NF-κB activation serves as a proliferative and survival signal (5). Our studies indicate that treatment with *B. pilosa* inhibited activation of NF-κB as indicated by EMSA. We confirmed that the inhibitory action of *B. pilosa* was based on its effect on phosphorylation of IKKβ and IκBα. Treatment of HTLV-1-infected T-cell lines with *B. pilosa* down-regulated the expression of cyclin D2, CDK6, Bcl-x_L, c-IAP2, XIAP, survivin and c-FLIP genes regulated by NF-κB (20-25,37) (Fig. 9).

Our results also showed that abrogation of ROS by NAC partially prevented *B. pilosa*-induced events in the NF-κB pathway. Furthermore, a scavenger of ROS attenuated *B. pilosa*-mediated cytotoxicity. Therefore, accumulation of ROS partially contributed to the cytotoxicity of *B. pilosa* and may be a mechanism parallel to NF-κB inhibition. However, NAC could not completely block the *B. pilosa*-induced cytotoxicity and NF-κB inhibition, implying that some other pathways are involved in the *B. pilosa* protein-extract treatment (Fig. 9).

AP-1 is also known to regulate cell proliferation (38), and required for proliferation of HTLV-1-infected T cells (31). Our results also showed that *B. pilosa* inhibited JunB and JunD expression, resulting in the suppression of AP-1 DNA-binding in HTLV-1-infected T-cell lines. AP-1 regulates the expression and function of cell cycle regulators such as cyclin D1 (39). In addition, the cyclin D2 promoter contains NF-κB and AP-1 sites (40). JunB is also controlled by NF-κB (41). It is therefore likely that NF-κB and AP-1, in concert, support the proliferation of HTLV-1-infected T cells by activating cyclin D1 and cyclin D2. We speculate that *B. pilosa* inhibits the expression of cyclin D1 and cyclin D2 through the suppression of both NF-κB and AP-1, resulting in the induction of G_1 cell cycle arrest (Fig. 9).

We performed experiments in MT-2 cells that can produce viruses (13). Interestingly, *B. pilosa* inhibited viral production without having any toxic effects. Thus, *B. pilosa* seems to be a viable option in inhibiting infection. Our results showed that the active compound in *B. pilosa*, caffeic acid, suppressed cell viability in HTLV-1-infected T-cell lines. Although the IC_{50}

values for caffeic acid and *B. pilosa* against HTLV-1-infected T-cell lines were found to range from 44 to 131 μg/ml and from 220 to 355 μg/ml, respectively, the content of caffeic acid was 12 mg/g of extract. Therefore, the roles of the synergistic activities of caffeic acid and other compounds in the cytotoxic activity of *B. pilosa* remain to be investigated.

Notably, *B. pilosa* showed potent anti-ATL activity against xenografted tumors in SCID mice. *Ex vivo* treatment with *B. pilosa* was effective against primary ATL cells. Our report is the first to show that *B. pilosa* is effective both *in vitro* and *in vivo* against ATL cells. The cytotoxic effects of *B. pilosa* on ATL cells in conjunction with its minimal cytotoxicity against normal cells could make it a potentially chemopreventive and/or therapeutic agent for the treatment of ATL.

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