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Capsaicin inhibits growth of adult T-cell leukemia cells

Jie Zhang [a,*], Makoto Nagasaki [a], Yuetsu Tanaka [b], Shigeru Morikawa [a]

[a] Department of Pathology First Unit, Shimane Medical University, 89-1 Enya-cho, Izumo, 693-8501 Shimane, Japan

[b] Department of Infection Disease and Immunology, Okinawa-Asia Research Center of Medical Science, University of the Ryukyus, Nakagami, 903-0215 Okinawa, Japan

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Abstract

Human T-cell leukemia virus type 1 (HTLV-1)-associated adult T-cell leukemia (ATL) is resistant to conventional chemotherapy. We examined the in vitro effects of capsaicin, the principal ingredients of red pepper, on three ATL cell lines. Capsaicin treatment inhibited the growth of ATL cells both in dose- and time-dependent manner. The inhibitory effect was mainly due to the induction of cell cycle arrest and apoptosis. Capsaicin treatment also induced the degradation of Tax and up-regulation of Iκ-Bα, resulting in the decrease of nuclear factor (NF)-κB/p65 DNA binding activity. In addition, the Bcl-2 level was found to be decreased. Based on these findings, capsaicin may be considered for chemoprevention of ATL.

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Keywords: Capsaicin; ATL cells; Growth inhibition; Cell cycle arrest; Apoptosis; Tax; Iκ-Bα/NF-κB; Bcl-2

1. Introduction

Adult T-cell leukemia (ATL) is an aggressive form of human T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1) [1–3]. Tax, a HTLV-1 transcriptional transactivator protein, can interact with various cellular proteins, such as nuclear factor (NF)-κB, cAMP response element binding (CREB) protein and serum response factor (SRF), thus activates the transcription of proto-oncogenes (c-*fos*, c-*jun*, *fra-1* and c-*myc*), cytokines (IL-2, IL-6, TGF-β and GM-CSF) and cytokine receptor (IL-2R) [4,5]. Tax can also repress the transcription of cellular genes, such as DNA polymerase β and *bax* [6,7]. So Tax is considered to play a crucial role in several pathways on the transformation of T cells by HTLV-1.

Capsaicin, a homovanillic acid derivative (8-methyl-*N*-vanillyl-6-nonenamide), is an active component of the red pepper of the genus *Capsicum* and has been found to be protective against experimentally induced mutagenesis and tumorigenesis [8,9]. In vitro, capsaicin has been found to inhibit the growth of various immortalized and malignant cells [10,11] and induce apoptosis in transformed cells [12,13]. In addition, capsaicin is also found to be a potent inhibitor of NF-κB activation [14].

NF-κB is a ubiquitous transcription factor that binds to a specific DNA sequence as a dimeric complex composed of various combinations of members of the Rel/NF-κB family, homodimers or heterodimers of RelA (p65), p50, c-Rel, p52 and RelB [15]. In resting lymphocyte, NF-κB dimers are sequestered in the cytoplasm in an inactive form by association with an inhibitory IκB subunit, mainly Iκ-Bα. Following cellular activation, multiple kinase lead to phosphorylation of Iκ-Bα and proteosome-mediated degradation, resulting in the release of an active NF-κB complex that translocates to the nucleus. In the nucleus, NF-κB binds its response elements and activates various genes involved in the inflammation, immune response and cellular growth control [16,17]. The activation of NF-κB is also essential for the inhibition of apoptosis [18]. As activation of NF-κB by Tax protein plays one of the major roles in the pathogenesis of ATL [19], and capsaicin can inhibit the activation of NF-κB, we hypothesized that capsaicin may have an inhibitory potential against HTLV-1 induced-ATL.

Multiple chemotherapy combinations have been tried for the treatment of ATL, however, the results have been disappointing with a median survival time of 8 months [20]. With an objective to find out newer chemotherapeutic agents for ATL, this study was designed to investigate the growth-inhibitory potentials of capsaicin on several ATL cell lines and the possible mechanisms involved in such in vitro growth-inhibitory effect.

* Corresponding author. Tel.: +81-853-20-2139; fax: +81-853-20-2135.
E-mail address: zhangjie@shimane.med.ac.jp (J. Zhang).

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2. Materials and methods

2.1. Cell lines and cell culture

Two ATL cell lines (HPB-ATL-T and HPB-CTL-I) were established in our laboratory from peripheral blood leukocytes (PBL) of two ATL patients [21]. ATL-T cells were derived from an acute type patient and CTL-I cells were derived from chronic type patient. Both ATL-T and CTL-I had been long-term cultured, established and maintained without IL-2. We utilized another HTLV-1 producing cell line, HUT-102 [22], and HTLV-1 negative leukemia T-cell line, Jurkat. Both of them were provided by Dr. Y. Matsuo (Fujisaki Cell Center, Hayashibara Biochemical Labs, Okayama, Japan). Another HTLV-1 negative leukemia cell line, HPB-ALL (ALL) [23], was also used in this study. All the cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Resting peripheral blood T-lymphocytes and stimulated PBMCs

Heparinized peripheral blood cells were obtained from healthy donors and mononuclear cells (PBMCs) were isolated by Ficoll–Conray density gradient centrifugation. Rosetted T-lymphocytes were separated over Ficoll-gradient as previously described [24] after 2-aminoethylisothisothiouronium bromide Conray hydromid treated sheep erythrocytes were added to the PBMCs. Isolated peripheral blood T (PB T) cells were grown in RPMI 1640 in the presence of IL-2 1 ng/ml. To obtain proliferating PBMCs, we stimulated PBMCs with phytohemaggultinin (PHA) (1/500) as well as IL-2 1 ng/ml (Sigma) for 72 h and T cells rich population (>90% $CD3^+$) was acquired.

2.3. Experimental drug

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) (molecular weight 305.42) was purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). The drug was prepared freshly before use and dissolved in ethanol, then mixed into complete media to obtain the desired concentration and applied to the growing cells. Ethanol concentration in the medium was maintained 0.1 vol.% and this concentration of ethanol was not found to influence the cell growth.

2.4. Viable cell counting

Effect on cell growth was evaluated by direct cell counting using a haemocytometer. Cells (1×10^5) in a 24-well plate were cultured with or without 100 μM capsaicin, after 24, 48 or 72 h, cells were harvested and viable cells were counted by trypan blue dye exclusion. Tests were performed in triplicate and repeated three times for each cell line.

2.5. ^{3}H-Thymidine incorporation assay

DNA synthesis was assessed by ^{3}H-thymidine incorporation. An amount of 1×10^5 cells were cultured in 24-well plates. Fresh medium with experimental drug in various concentrations was added to the growing cells. After 24 h treatment with drug, 1 μCi [methyl-^{3}H]-thymidine (ICN Pharmaceuticals Inc., USA) was added to each well and incubated for additional 4 h. To examine the Tax-overexpressed cell lines, the cells were pre-treated with 1.0 μM sodium butyrate (Wako Pure Chemical Co. Ltd.) for 48 h, washed twice and treated with capsaicin in various concentrations. Cells were then harvested onto fiberglass filters by a cell harvester, radioactive counts were quantified by using 1450 Microbeta^{TM} scintillation counter (Wallac Oy, Turku, Finland). ^{3}H-Thymidine incorporation rate was expressed as percentage of corresponding control. Each experiment was performed in quadruplicate and repeated for three times.

2.6. Cell cycle analysis

The proportion of cells in G_0/G_1, S and G_2/M was determined by flow cytometry (EPICS Elite, Coulter Electronics, FL, USA). Cell cycle distributions in three cell lines were measured after 24 h treatment with 0, 100 or 200 μM capsaicin. Cells were harvested and approximately 2.5×10^6 cells were washed twice with phosphate buffered saline (PBS). The cells were re-suspended with 5 ml of cold (−20 °C) 70% ethanol, fixed overnight at 4 °C. Then the cells were washed twice in PBS and incubated with RNase (Sigma) at room temperature in dark. Cellular DNA histograms were analyzed using Multicycle AV software (Phoenix, San Diego, CA, USA).

2.7. Detection of apoptosis

2.7.1. DNA fragmentation assay

Cells were grown in a flat-bottomed six-well plate with 0, 100 or 200 μM capsaicin for 24 h. Then the cells were collected by centrifugation (800 × *g*, 5 min), washed twice in cold PBS and re-suspended at a density of 1×10^6 cells/100 μl in ice-cold lysis buffer (10 mM Tris–HCl buffer, 10 mM EDTA, pH 7.4) containing 0.5% Triton X-100 for 10 min at 4 °C. The lysates were centrifuged at 20,000 × *g* for 20 min at 4 °C. The pellet was treated with 0.4 mg/ml RNase A at 37 °C for 1 h, and then treated with 0.4 mg/ml Proteinase K (Roche Molecular Biochemicals, USA) at 37 °C for 1 h. DNA was precipitated with 0.5 M NaCl and 50% isopropanol at −80 °C for 20 min, and centrifuged at 20,000 × *g* for 30 min. The pattern of DNA fragmentation was visualized by electrophoresis on 2% agarose gel containing ethidium bromide and photographed under UV light.

2.7.2. Morphological studies

Cells were incubated in a six-well plate with or without 100 μM capsaicin for 24 h. After washing with PBS, nuclei

were stained with 50 μg/ml Hoechst 33342 (Nacalai Tesque, Kyoto, Japan) for 5 min at room temperature. Morphological changes in the nuclear chromatin of apoptotic cells were then observed under fluorescence microscopy.

2.7.3. Annexin V/FITC binding assay

CTL-I and ATL-T cells were incubated with various concentration of capsaicin for 24 h at 37 °C. Cells were analyzed by an Annexin V/FITC kit (Bender MedSystems, Austria) according to manufactures' protocol. Briefly, approximately 2×10^6 cells were washed twice in PBS and re-suspended in 195 μl 1× binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), added 5 μl Annexin V/FITC and incubated for 10 min at room temperature in dark. Cells were then stained with 10 μl of 20 μg/ml propidium iodide and analyzed by flow cytometry.

2.8. Western blot analysis

ATL-T cells were treated with 0, 50, 100 and 200 μM capsaicin for 24 h. Cell lysates were prepared by treating the cells for 30 min in RIPA lysis buffer: 1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride, 30 μg/ml aprotinin (all chemicals were from Sigma). The lysates were centrifuged at 20,000 × *g* for 30 min and the protein concentration in the supernatant was determined with a Coomassie plus protein assay reagent kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by SDS–polyacrylamide gel electrophoresis on a 12.5% gel and then were transferred to nitrocellulose membranes by semidry blotting apparatus (Bio-Rad, Hercules, CA, USA). The membranes were incubated with mouse monoclonal antibodies directed to Tax (Lt-4) [25], Bcl-2 (C-2; Santa Cruz, CA, USA), Bax (5B7), β-actin (AC-74; Sigma) and Iκ-Bα (1037; IBL, Gunnma, Japan). The membranes were subsequently incubated in a peroxidase-labeled polymer conjugated to anti-mouse and anti-rabbit immunoglobulins (Dako Corporation, Carpinteria, CA, USA) and developed by chemiluminescence using the ECL Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.9. Electrophoretic mobility shift assay

Cells were treated with different concentrations of capsaicin for 24 h and nuclear extracts (NEs) were prepared as described [26]. Briefly, 1×10^7 cells were washed with cold PBS containing 1 mM Na_3VO_4 and 5 mM NaF. The cells were then treated with 0.2% Nonidet P-40 in lysis buffer containing 20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml aprotinin. After centrifugation, the pellets were further treated at 4 °C for 30 min with lysis buffer supplemented with 420 mM NaCl and 20% glycerol and then subjected to centrifugation. The resulting supernatant was used as the NE. Binding assay of NF-κB proteins to NF-κB elements was examined by NF-κB/p50 and NF-κB/p65 electrophoretic mobility shift assay (EMSA) kit (Geneka Biotechnology Inc., Montreal, Que., Canada) according to the manufacture's protocol. Briefly, 10 μg of NEs were diluted with the same volume of dilution buffer and then incubated with a binding buffer and stabilizing solution for 20 min at 4 °C. They were further incubated with approximately 1×10^5 cpm of ^{32}P-labeled oligonucleotide probes containing NF-κB responsive elements for 20 min at 4 °C. To examine the specificity of the NF-κB like element of the probe, unlabeled competitor oligonucleotides (wild and mutant) were pre-incubated with NEs for 20 min at 4 °C prior to incubation with probes. The DNA–protein complex was separated on a 5% non-denaturating polyacrylamide gel and visualized by autoradiography.

2.10. Statistical analysis

All of the experiments were performed at least three times. Values were expressed as mean ± S.E. Statistical comparisons were made by ANOVA followed by a Schffe test and P-values <0.05 were considered significant.

3. Results

3.1. Capsaicin inhibited growth of ATL cells

Trypan blue staining and direct counting of viable cells were performed after 24, 48 and 72 h with 0 μM (control) or 100 μM capsaicin treatment, respectively. Results showed a significant decrease in viable cell counts of both HTLV-1 positive and HTLV-1 negative leukemia cells, and a little decrease in stimulated PBMCs, but almost no change in resting PB T cells (Fig. 1A). Capsaicin also inhibited the time-dependent increment in cell count of ATL cell lines as well as HTLV-1 negative leukemia cells (Fig. 1B).

3.2. Growth inhibition was associated with inhibition of DNA synthesis

Cells DNA synthesis was assessed by the ability of cells to incorporate ^{3}H-thymidine into their DNA in three ATL cell lines and Jurkat as well as ALL cells after 24 h capsaicin treatment. There was a significant dose-dependent decrease in ^{3}H-thymidine incorporation with all concentrations of capsaicin. More than 90% inhibition was observed in all cell lines at 200 μM capsaicin, about 80% at 100 μM and nearly 50% at 60 μM, respectively, when compared with cells in medium supplemented with ethanol only. No major difference was observed in ^{3}H-thymidine incorporation between HTLV-1 positive ATL cell lines and HTLV-1 negative leukemia cells. We also examined the effect of capsaicin on PBMCs pre-stimulated with PHA and IL-2 for 72 h. Compare to the cancer cell lines, capsaicin treatment induced a

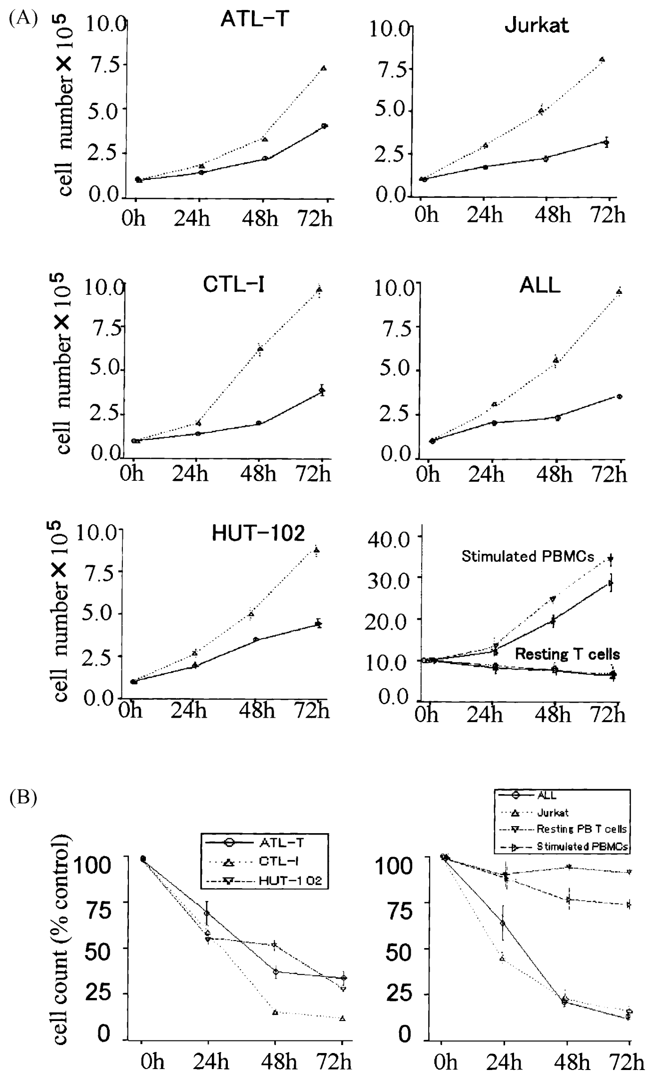

Fig. 1. Cells were incubated in presence (100 μM, solid line) or absence (control, dotted line) of capsaicin for 24, 48 and 72 h, respectively. (A) Total number of viable cells was counted by trypan blue dye exclusion. (B) The count rates were expressed as percentage of corresponding control (left figure included HTLV-1 positive cells, and right figure included HTLV-1 negative cells). Each value indicates the mean ± S.E. of three experiments in triplicate.

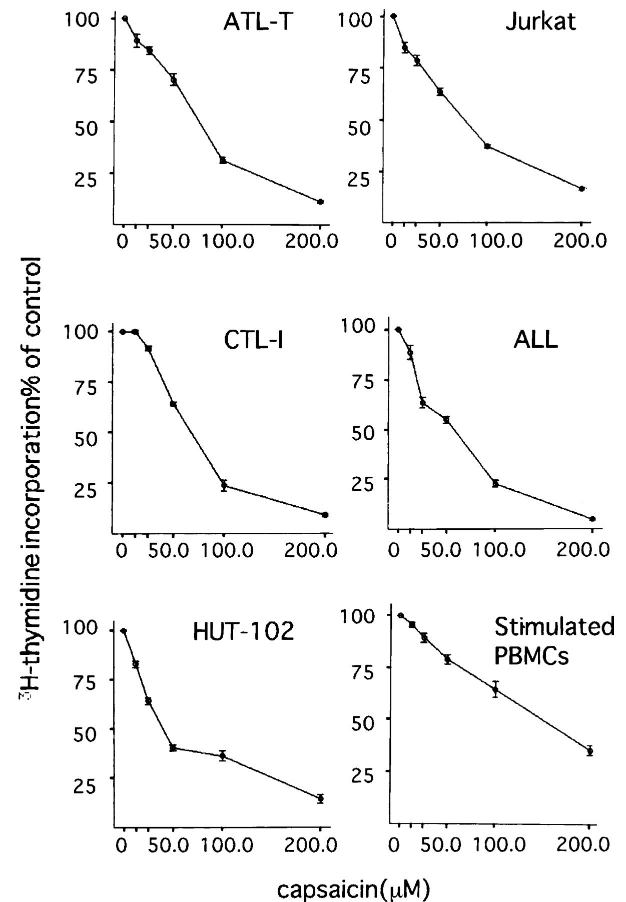

Fig. 2. Cells were treated without (control) or with capsaicin at the indicated dose for 24 h. ^{3}H-Thymidine incorporation assay was performed and the incorporation rates were expressed as percentage of corresponding control. Data represent mean ± S.E. values from three assays in quadruplicate wells.

little inhibition in ^{3}H-thymidine incorporation at doses up to 100 μM (Fig. 2).

3.3. Capsaicin altered cell cycle progression

The cell cycle distribution changes were found after exposure of ATL cells to capsaicin (Fig. 3). With increasing dose of capsaicin, cell cycle distribution significantly increased in G_1 phase and decreased in S phase.

3.4. Induction of apoptosis by capsaicin in ATL cells

The effect of capsaicin on apoptotic cell death of ATL cells was examined using three different methods. Capsaicin-induced DNA fragmentation in a concentration-dependent manner visualized on agarose gel electrophoresis with ethidium bromide staining (Fig. 4A). To further confirm apoptotic cell death, we examined nuclear morphologic changes using a permeation DNA-staining fluorescence dye, Hoechst 33342. After treatment with 100 μM capsaicin for 24 h, apoptotic cells exhibited condensed and fragmented nuclei, indicative of apoptotic cell morphology. However, the nuclei of capsaicin treated PB T cells showed no significant changes (Fig. 4B). Furthermore, the proportion of apoptotic cells was determined by Annexin V/FITC kit. As shown in Fig. 4C, after 24 h treatment with 100 and 200 μM capsaicin, approximately 5.4 and 32.2% of CTL-I cells, and 8.8 and 19.8% of ATL-T cells displayed positive Annexin V/FITC and PI staining, respectively, indicating that these tumor cells underwent apoptosis and secondary necrosis.

3.5. Capsaicin treatment down-regulated Tax expression and Bcl-2 expression

In an attempt to unveil the molecular mechanisms underlying the capsaicin-induced apoptosis of ATL cells, acute type

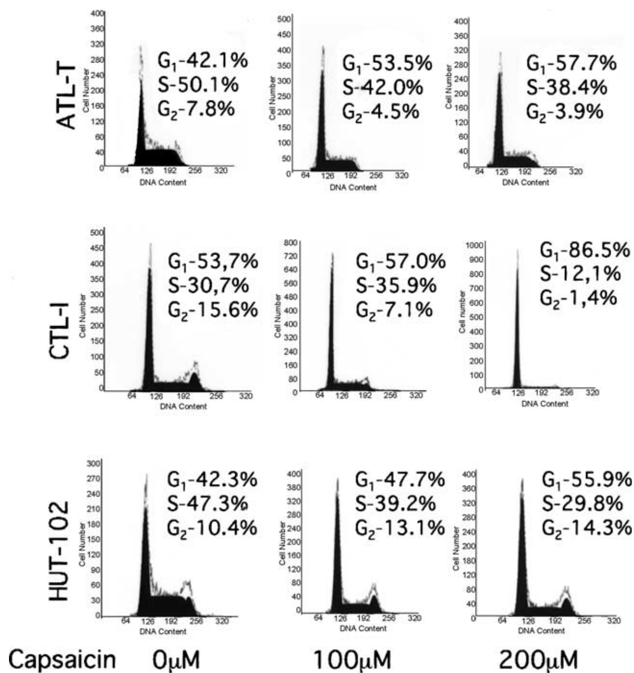

Fig. 3. Capsaicin-induced inhibition of cell cycle progression. Cell cycle analysis was performed with propidium iodide staining after 24 h treatment with 0, 100 and 200 μM capsaicin. A markedly increased G_1 phase and decreased S phase were observed in all cell lines.

ATL-T cell line was used as the representative cell line. We examined the level of HTLV-1 transactivator protein (Tax) and two key apoptosis-linked gene products, anti-apoptosis oncoprotein Bcl-2 and death-promoting Bax by Western blot analysis as shown in Fig. 5. The expression of Tax and Bcl-2 were decreased dose-dependently, with a little increase in the expression of Bax.

3.6. *Capsaicin increased Iκ-Bα expression and down-regulated nuclear NF-κB/p65 binding activity*

Tax has been shown to target Iκ-Bα for phosphorylation and degradation [27]. Tax degradation would therefore be expected to diminish Iκ-Bα proteolysis. Indeed, capsaicin treatment resulted in the decrease of phosphorylated Iκ-Bα (Iκ-Bα-P; Figs. 6A and 7) accompanied by accumulation of the 36 kDa non-phosphorylated Iκ-Bα species, consistent with its effect on Tax degradation (Fig. 5).

We then examined the binding activity of NF-κB in the NE to the specific oligonucleotide probe after capsaicin treatment by EMSA (Fig. 6B). The results indicated by the signal of slow migrating complex on EMSA were quite specific since the band disappeared when unlabeled wide type oligonucleotide was added (w), but not the oligonucleotide with mutated binding sites (m). Among NF-κB subunits, p65 binding activity, but not p50 (open arrow), was inhibited after capsaicin treatment.

3.7. *Tax overexpression can partially overcome the inhibitory effect of capsaicin*

Sodium butyrate, a differentiating agent that stimulates expression of cellular and viral genes through the inhibition of histone deacetylases [28], resulting in hyperacetylation of histones, which has been shown to be associated with transcriptionally active chromatin [29]. It has been reported that sodium butyrate can induce Tax overexpression in HTLV-1 transformed cell lines [30]. ATL-T cells were pre-treated with 0.5 and 1.0 μM sodium butyrate for 48 h, and then examined for the expression of the Tax and Bcl-2 by Western blot analysis. The Tax level was found to be increased, but the Bcl-2 level was not changed. Then DNA synthesis of ATL-T cells with Tax overexpression was examined by ^{3}H-thymidine incorporation assays after capsaicin treatment. DNA synthesis was inhibited with an IC_{50} value of approximately 120 μM after sodium butyrate pre-treatment, compared to an IC_{50} value of 60 μM without pre-treatment.

4. Discussion

Capsaicin has been initially used in humans for topical treatment of cluster headache and vasomotor rhinitis due to its specific action on the sensory neurons [31,32]. However, recent studies have shown the growth-inhibitory effect of capsaicin on several cancer cells, such as melanoma, mammary adenocarcinoma and hepatocellular carcinoma [10,11,33]. In hematopoitic malegnancy, ATL cells, which are commonly refractory to conventional chemotherapeutics, were investigated in this study to evaluate the growth-inhibitory potential of capsaicin.

Capsaicin inhibited DNA synthesis in ATL cells with an IC_{50} value of approximately 60 μM as evidenced by ^{3}H-thymidine incorporation. The inhibition of proliferation was also observed in all three ATL cell lines by direct cell counting. The growth-inhibitory effect of capsaicin was found to be dose-dependent as well as time-dependent and the inhibitory effect of capsaicin on three cell lines was similar. Capsaicin inhibited the proliferation of these ATL cell lines as effectively as that of HTLV-1 negative leukemia cell lines Jurkat and ALL.

We also examined the effect of capsaicin on resting T cells as well as proliferating T-cell rich population derived from PHA-stimulated PBMCs. In viable cell count assays, capsaicin induced a little decrease in stimulated PBMCs, but almost no change in resting PB T cells. In ^{3}H-thymidine incorporation assays, we observed also a little inhibition especially at lower doses in stimulated PBMCs. However, capsaicin induced neither apoptosis nor necrosis in normal PB resting T cells which were treated with up to 100 μM for 72 h. A recent study also reported that capsaicin did not induce any growth inhibition in non-cancerous, spontaneously immortalized human breast epithelial cell line MCF10A [34], which

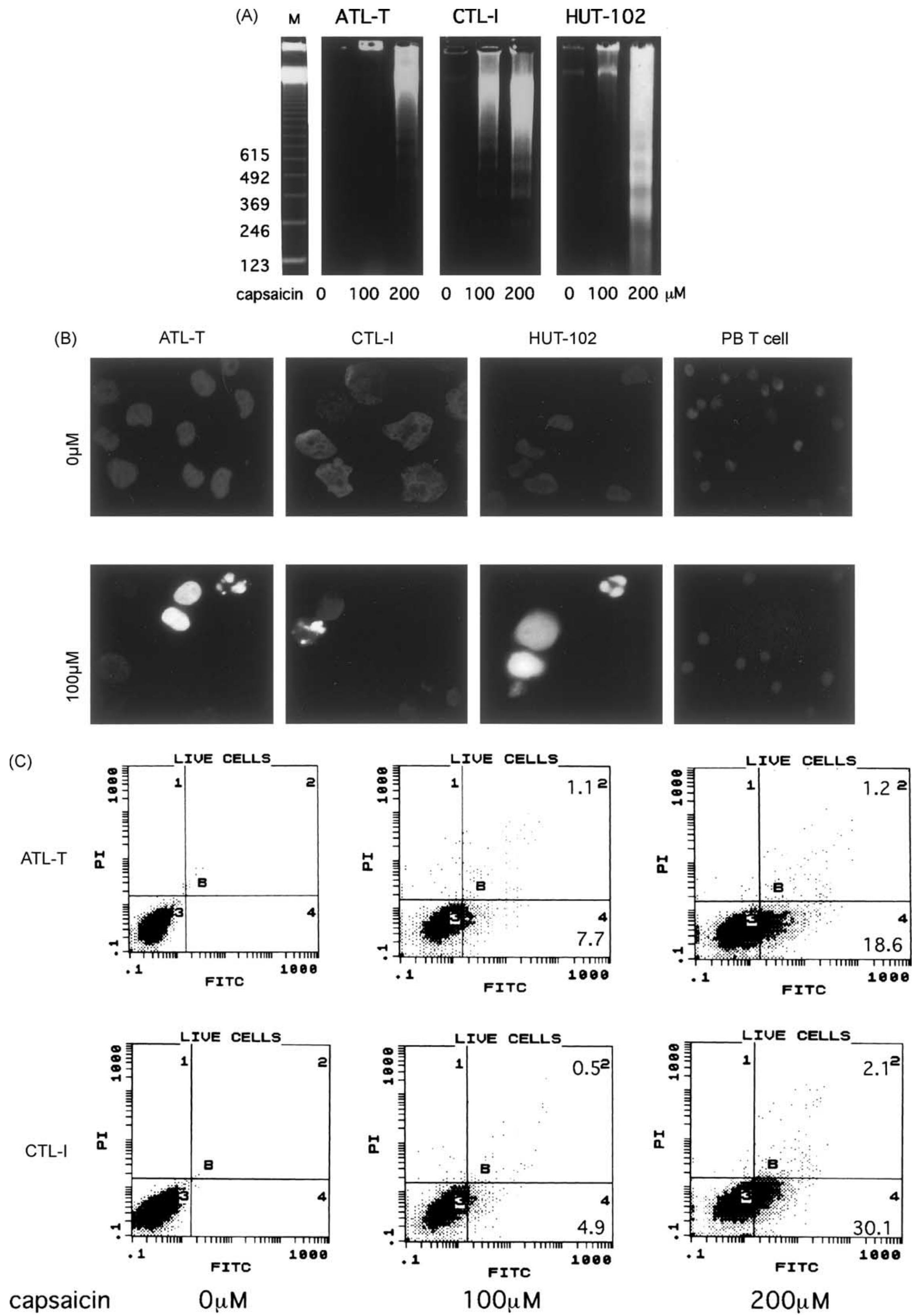

Fig. 4. Capsaicin-induced apoptosis in ATL cell lines. (A) Internucleosomal DNA fragmentation. DNA was extracted from cells treated with 0, 100 and 200 μM capsaicin for 24 h and analyzed by 2% agarose gel electrophoresis. (B) Nuclear morphological analysis. ATL and PB T cells were treated with 0 or 100 μM capsaicin for 24 h and stained with Hoechst 33342. Stained cells were examined by fluorescence microscope. (C) Annexin V/FITC and PI staining. Percentage of apoptotic cells were analyzed by flow cytometry after 24 h treatment in ATL-T and CTL-I cells. Quadrants 2–4 show secondary necrotic, viable and apoptotic cells, respectively.

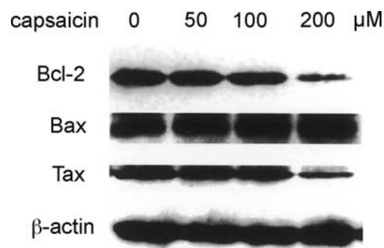

Fig. 5. Effect of capsaicin on the expression of Tax, Bcl-2 and Bax in ATL-T cells. Cells were treated with indicated dose of drug, then total cellular proteins were extracted and Western blot analysis was performed. The expression of Tax and Bcl-2 were decreased after capsaicin treatment. As internal control, β-actin (42 kDa) expression was detected.

suggests capsaicin inhibitory effect only on undifferentiated cells.

With topical application, capsaicin has been used to rheumatoid arthritis, osteoarthritis, diabetic neuropathy and post-herpetic neuralgia [35]. Capsaicin has been also reported to show protective role against aspirin-induced gastroduodenal mucosal injury with oral administration [36]. A study has indicated about the serum level that could be attained after topical application of capsaicin. After topical application of capsaicin (Genderm Co., Zostrix, USA) in a normal therapeutic doses (four to five applications per day), the serum concentration could be achieved approximately to 20 μM [37], which suggest that appropriate dosing in alternative route (i.e. oral or parenteral) can result a serum level necessary for the growth-inhibitory effect.

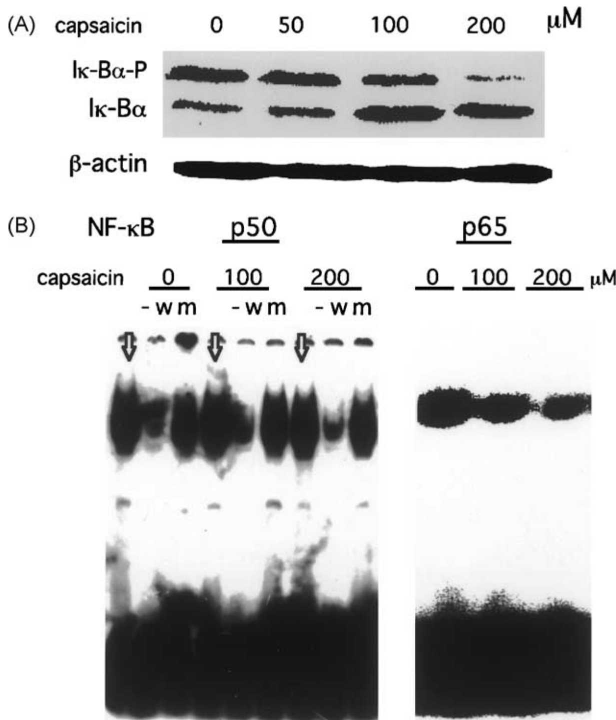

Fig. 6. Effect of capsaicin on the level of Iκ-Bα by Western blot analysis (A) and the DNA binding activity of transcription factor NF-κB assessed by EMSA (B). (A) The lower bands (Iκ-Bα) of 36 kDa major Iκ-Bα protein were up-regulated, and the upper bands (Iκ-Bα-P) of about 38 kDa presumably phosphorylated Iκ-Bα were down-regulated by capsaicin treatment. Detection of β-actin expression was used as internal control. (B) ATL-T cells were treated with 0, 100 or 200 μM capsaicin for 24 h. After NEs were prepared, NF-κB/p50 DNA binding activity was analyzed by EMSA using a ^{32}P-radiolabeled p50 binding specific oligonucleotide probe (see Section 2). No significant change was found in NF-κB/p50 DNA binding activity after capsaicin treatment (lane labeled with open arrow). For lane labeled as (m) and (w), mutant and wild oligonucleotides were used to determine the binding specificity. NF-κB/p65 DNA binding activity using p65 binding specific probe was inhibited after capsaicin treatment.

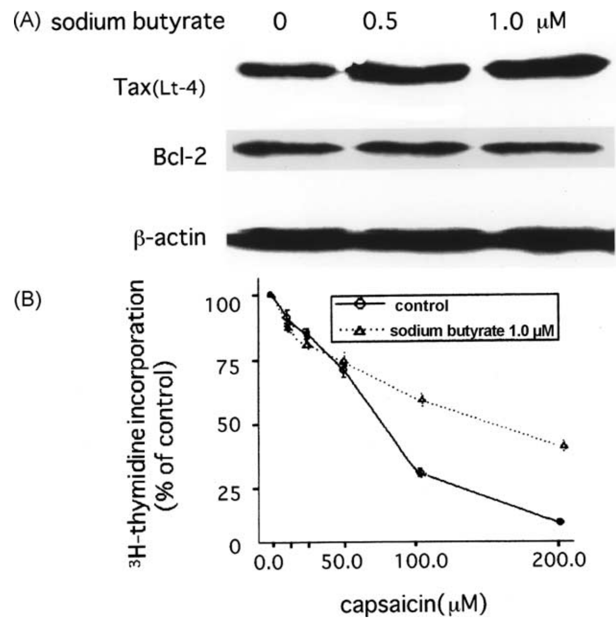

Fig. 7. (A) ATL-T cells were pre-treated with 0, 0.5 and 1.0 μM sodium butyrate for 48 h, then total cellular proteins were extracted and Western blot analysis was performed. The expression of Tax, but not Bcl-2, was increased after sodium butyrate treatment. (B) Sodium butyrate pre-treated cells were washed and re-seeded to culture plate with capsaicin at the indicated dose for 24 h. ^{3}H-Thymidine incorporation assay was performed and the incorporation rates were expressed as percentage of corresponding control. Sodium butyrate pre-treatment decreased the sensitivity of ATL-T cells to capsaicin.

The growth-inhibitory potential of capsaicin on ATL cells was found mainly due to the induction of cell cycle arrest and apoptosis, because a significant population of cells remained in the G_1 phase of cell cycle and underwent to apoptosis after treatment of capsaicin dose-dependently. G_1 cell cycle arrest has been found to be a common feature of many anticancer drugs [38,39]. Resent studies support another hypothesis that in ATL cells, Tax may induce cell cycle progression through protein–protein interaction [40,41]. Cell cycle arrest in G_1 phase by capsaicin treatment may be associated with the down-regulation of Tax expression. However, we can not exclude the other possible mechanism of G_1 cell cycle arrest, like induction of CDK inhibitors.

Activation of NF-κB plays an important role in prevention of apoptosis due to elevated expression levels of several NF-κB-inducible cytokines [18]. In this study, we have

observed that capsaicin treatment induced the decrease in phosphorylated Iκ-Bα accompanied by accumulation of the non-phosphorylated Iκ-Bα by Western blot analysis. We also detected by EMSA that capsaicin treatment decreased the NF-κB binding activity of p65, but not p50, indicating selective inhibition of p65 subunit in NF-κB family by capsaicin. Decrease in the NF-κB activity, at least in part, may be responsible for induction of apoptosis in ATL cells.

In addition to the decreased NF-κB activity, down-regulation of Bcl-2 may be also responsible for the capsaicin-induced apoptosis. Bcl-2 and Bcl-xL are well known to protect cells against apoptosis, whereas Bax is known as an accelerator of apoptosis [42]. In general, the Bcl-2/Bax ratio is thought to be important determinants of apoptosis [43]. In this study, the expression of Bcl-2 as well as the ratio of Bcl-2/Bax was found decreased after capsaicin treatment. Similar findings were also found in a hepatocellular carcinoma cell line [33].

Capsaicin-induced cell cycle arrest and apoptosis in ATL cell lines, which was associated with decreased NF-κB activity and altered Bcl-2/Bax ratio. In addition, capsaicin also inhibited the level of Tax expression in ATL cells, which might have attributed to the growth-inhibitory effect of capsaicin on ATL cells. This was also supported by our observation that there was a partial alleviation of the growth-inhibitory potential of capsaicin on ATL-T cells which had sodium butyrate induced Tax overexpression.

Primary ATL cells are known to have very low or no expression of Tax and therefore, growth of ATL cells in vivo are believed to be Tax-independent [44], although Tax expression is induced within hours of in vitro culture. Some reports indicated that Tax might not remain silent in vivo. Expression of *tax1*/*rex1* mRNA could be detected in primary ATL cells [45] and a correlation was found between induction of NF-κB and Tax expression in primary acute type ATL cells [46], which suggest the involvement of Tax induced NF-κB in proliferation and activation of primary ATL cells in vivo. Also, in vitro studies, Tax is a well-known activator of NF-κB [4,27,47] and inducer of Bcl-xL expression [48], resulting in increased resistance to apoptosis in HTLV-1 transformed cells. Therefore, the growth inhibition of ATL cell lines induced by capsaicin appears to be mediated by both Tax-dependent and -independent pathways. We conclude that capsaicin might be used as a chemotherapeutic or chemopreventive agent in ATL, but further clinical studies will be necessary to assess its potential to primary fresh ATL cells.

Acknowledgements

J. Zhang provided the concept, design, assembled and analyzed the data and drafted the manuscript. M. Nagasaki contributed to the concept, design, analysis of the data, provided statistical expertise as well as logistical support. S. Morikawa contributed to the study design, provided critical input to the revision, obtained the necessary funding and gave final approval. Y. Tanaka provided study materials.

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