3.6. Sp1 activates transcription from HIV-1 LTR in a manner dependent on the GC box

As Sp1 site appeared to be important for the E2-induced transcription from HIV-1 LTR, EMSA was performed with the GC box DNA derived from HIV-1 LTR. As shown in Fig. 6A, specific band was observed in the absence of stimulation, and the level was not affected in the presence of ER. However, E2 augmented the binding level in the presence of ERα. Supershift assay using antibodies against Sp1 or Sp3 revealed that the major bands are mostly comprised of Sp1 (Fig. 6B). Sp3 also appeared to be a component of the major band. Antibody against p65 used as a control did not induce supershift of the band. Supershift was not observed with antibodies against either ERα or ERβ, suggesting that Sp1 or Sp3 is not bound to either ERα or ERβ or their interaction is weak.

3.7. E2 induces transcriptional activity of Sp1

We then examined whether E2 induces the transcriptional activity of Sp1 through ERα. 293 cells were transfected with expression plasmid encoding GAL4-DBD (DNA-binding domain) or Sp1 fused with GAL4-DBD (GAL4-Sp1), together with a reporter plasmid containing five tandem repeats of GAL4-binding sites (Fig. 7A). E2 treatment did not induce transcriptional activity of GAL4-DBD in the presence or absence of ERα. In the presence of ERα, however, E2 could induce transcriptional activity of GAL4-Sp1 (Fig. 7B). These results indicate that E2 induces transcriptional activity of Sp1 in a manner dependent on ERα. We further examined whether Sp1 contributes to the E2-induced stimulation of transcription from HIV-1 LTR. As shown in Fig. 7C, E2 induced the transcription from HIV-1 LTR in a dose-dependent manner, and this E2 effect was further augmented by Sp1 overexpression. These results indicate that Sp1 is involved in the E2-induced activation of transcription from HIV-1 LTR.

4. Discussion

In this study, we observed that E2 stimulates HIV-1 transcription via ERα-dependent activation of DNA-

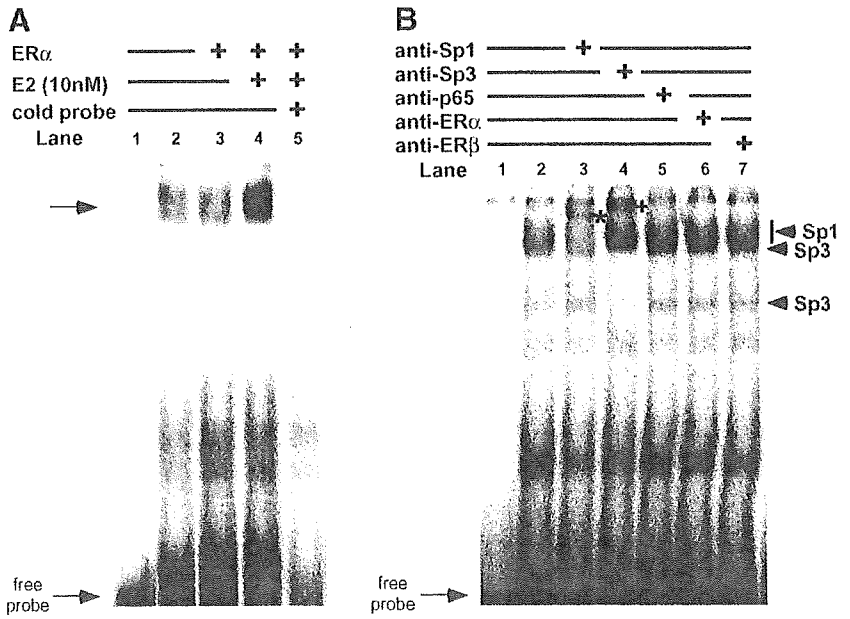

Fig. 6. E2 augments the binding of nuclear extracts from 293 cells to the GC rich region of HIV-1 LTR. (A) Effect of E2 on DNA-binding activity of nuclear extracts from 293 cells to the GC rich region of HIV-1 LTR. 293 cells were transfected with or without pcDNA3-ERα plasmid. After 16 h of transfection, cells were treated with or without 10 nM E2. Twenty-four hours after, cells were harvested and nuclear extracts were prepared and subjected to EMSA analysis with ^{32}P-labeled GC rich probe. Lane 1; free labeled probe. Lane 5; 250-fold excess of unlabeled GC rich probe was added to the labeled probe. (B) Binding of Sp1 and Sp3 to the GC rich region of HIV-1 LTR. 293 cells were transfected with pcDNA3-ERα plasmid. After 16 h of transfection, cells were treated with 10 nM E2. Twenty-four hours after that the cells were harvested and nuclear extracts were prepared and subjected to EMSA analysis with ^{32}P-labeled GC rich probe. Supershift analysis was performed using anti-Sp1 or anti-Sp3 antibodies. Supershifted bands are indicated with asterisk (*; Sp1) and plus (+; Sp3). Lane 1; free labeled probe. Representative data of more than three experiments are shown.

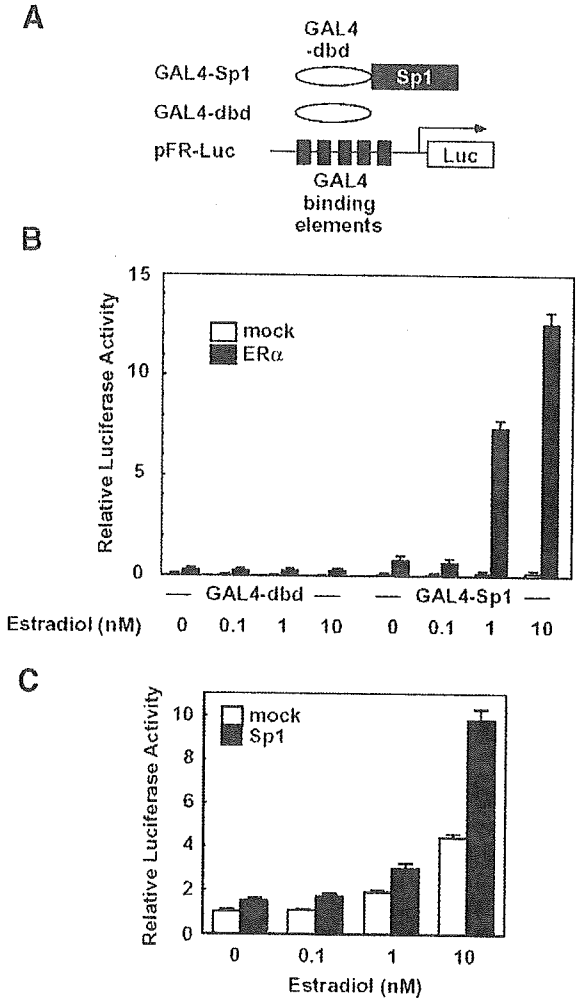

Fig. 7. Contribution of Sp1 to the E2-induced activation of HIV-1 LTR. (A) Schematic diagram of GAL4-Sp1 plasmids, which express Sp1 in fusion with GAL4-dbd and 5× GAL4-Luc, containing five tandem repeats of GAL4-binding sites. (B) Effect of E2 on transactivation activity of Sp1. 293 cells were cotransfected with 5× GAL4-Luc, GAL4-dbd or GAL4-Sp1 expression plasmids in the presence or absence of pcDNA3-ERα plasmid. After 16 h of transfection, cells were incubated with varying concentrations of E2. Cells were harvested 24 h after transfection, and luciferase activity was measured as described in Fig. 2. (C) Effect of E2 on transactivation activity of Sp1 for HIV-1 LTR. 293 cells were co-transfected with CD12-Luc and pcDNA3-ERα with or without pCIneo-Sp1. After 16 h of transfection, cells were incubated with indicated concentrations of E2 for 24 h and luciferase activity was measured. Representative data of three experiments are shown.

binding and transcriptional activity of Sp1. In an effort to analyze the effect of E2 on HIV-1 transcription, 293 cells, which do not express ERα but express ERβ, were transiently transfected with expression plasmid of ERα or ERβ together with CD12-Luc containing the full-length HIV-1 LTR, and examined the effect of E2. E2 activated the transcription from HIV-1 LTR in a manner dependent on ERα but not ERβ. Furthermore, the ERα-mediated E2 effect was verified by a specific antagonist to ER. E2 binds to both ERα and ERβ with a similar high affinity [37] and activates their functions. Indeed, in 293 cells E2 induced the transcriptional activity of both ERα and ERβ when it was assessed with ERE_3tk-Luc, a reporter plasmid containing three tandem repeats of ERE. Although 293 cells express ERβ, it did not appear to be functional. The endogenous ERβ may, therefore, be mutated. ERα and ERβ consist of three functional domains: an N-terminal domain involved in ligand-independent transcription (A/B), a central domain highly conserved in these two ERs and responsible for specific DNA-binding, and a C-terminal domain involved in ligand binding and ligand-dependent transcription. The N-terminal A/B domain of ERβ is short in its length as compared to ERα, consequently their functions are different depending on ligands, cell types and tissues [7,38,39].

As there is no ERE in the promoter region of HIV-1 LTR, we presumed that E2/ERα indirectly activates HIV-1 transcription through binding to other transcription factors. It is reported that ERα physically interacts with many transcription factors, including Sp1 [13–15], AP-1 [16], NF-κB [17], C/EBPβ and STAT5 [18], and modulates their transcriptional activity. By the experiments using deletion mutant reporter genes of HIV-1 LTR, E2 did not appear to require the transcription binding sites, including AP-1 (−247 to −222), NF-AT1 (−254 to −216), c-Myb (−304 to −299) and NF-κB for its augmenting effect. Reporter gene assay using the mutant containing mutations at two κB sites and EMSA also supported that NF-κB is not involved in the effect of E2. In contrast, the GC rich region corresponding to Sp1 sites appeared to be important for the effect of E2. Similar to these findings, we recently found that 3-methylcholanthrene activates HIV-1 replication via aryl hydrocarbon receptor and that Sp1, but not NF-κB, is involved [40].

We observed that Sp1 and Sp3 specifically bound to the nucleotide containing GC rich region in HIV-1 LTR, and that E2 in the presence of ERα augmented

Sp1 DNA-binding. The specific binding of Sp1 and Sp3 to the GC rich region was observed in the absence of ERα and E2 and the reporter gene assay showed that Sp1 site is critical to the basal transcription activity. These findings suggest that Sp1 and Sp3 binding to the GC rich region is important to the basal transcription activity of HIV-1 LTR. We also showed that in a luciferase reporter assay using GAL4-Sp1 fusion protein E2 could augment the transcriptional activity of Sp1 in a manner dependent on ERα. However, as far as we examined, ERα was not associated with the Sp1–DNA complex. Similar to our findings, it was previously reported that although ERα and Sp1 could physically interact and enhance Sp1 DNA-binding, ERα was not detected in the Sp1–DNA complex [13,14,41]. Therefore, Sp1/ERα complex may not be stable or ERα is released upon Sp1 binding to DNA. The function of Sp1 is regulated by posttranslational modifications and interaction with other protein molecules. The former type regulation includes phosphorylation [42], acetylation [15] and glycosylation [43]. The DNA-binding ability of Sp1 is regulated by phosphorylation of Ser located within the N terminal Glu-rich region, and Sp1 is phosphorylated by ERK1/2 or JNK upon stimulation with extracellular stimuli such as FGF, HGF and peroxide [44–46]. Therefore, it is possible that E2-activated ERα may induce such posttranslational modification of Sp1 to stimulate its transcriptional activity.

The reports that tamoxifen, an antiestrogenic reagent, inhibits the replication of HIV-1 in lymphocytes [47,48] are in agreement with our findings. We also observed that E2 augmented HIV-1 replication in OM10.1 cells (promyelocytic cell line latently infected with HIV-1) in a dose-dependent manner (data not shown). However, the E2 effect was not inhibited by ICI 182,780, rather ICI 182,780 augmented HIV-1 replication. However, it is reported that the effect of ICI 182,780 depends on cell types. In breast tumor cells MCF-7 and MDA-MB231 and prostate tumor cells LNcaP, ICI 182,780 works as an agonist for ERα-dependent gene activation [49]. Therefore, the E2 effect on OM10.1 cells does not rule out the involvement of ERα. Peripheral T cells, B cells and monocytes express ERα and ERβ mRNA [50]. Therefore, it is feasible that E2 augments HIV-1 replication in these cells.

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Induction of cell death in adult T-cell leukemia cells by a novel IκB kinase inhibitor

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Running title: Induction of ATL cell death by a novel IKK inhibitor

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Abstract

NF-κB is constitutively activated in adult T-cell leukemia (ATL) and is considered responsible for cell growth and prevention of cell death. In this study we demonstrate that NF-κB is constitutively activated in various HTLV-1 infected T-cell lines and ATL-derived cell lines irrespectively of Tax expression as evidenced by the phosphorylation of IκBα and p65 subunit of NF-κB, activation of NF-κB DNA binding, and upregulation of various target genes including *bcl-x_L, bcl-2, XIAP, c-IAP1, survivin, cyclinD1, ICAM-1* and *VCAM-1*. The effects of a novel IκB kinase (IKK) inhibitor, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl nicotinonitrile (ACHP), were examined on cell growth of these cell lines and fresh ATL leukemic cells. We found that ACHP could inhibit the phosphorylation of IκBα and p65, as well as NF-κB DNA-binding, associated with downregulation of the NF-κB target genes and induce cell growth arrest and apoptosis in these cells. When Tax-active and Tax-inactive cell lines were compared, ACHP could preferentially inhibit cell growth of Tax-active cells. Moreover, ACHP exhibited strong apoptosis-inducing activity in fresh ATL cells. These findings indicate that ACHP and its derivatives are effective in inducing ATL cell death and thus feasible candidates for the treatment of ATL.

Key words: ATL, NF-κB, IKK, chemotherapy, apoptosis

Introduction

Adult T-cell leukemia (ATL) is an aggressive lymphoproliferative disorder closely associated with the human T-cell leukemia virus type 1 (HTLV-1)[1,2]. ATL arises after a long latent period of over 50 years and is considered to involve multi-step mechanism of tumorigenesis[3]. ATL is characterized by diffuse lymphadenopathy, hepatosplenomegaly, and infiltration of malignant cells into skin and other organs[4]. Acute type of ATL is an aggressive form of T-cell leukemia with a median survival of only 6 months and a projected 4-year survival of about 5%. Conventional chemotherapies appeared to be ineffective in prolonging the life of patients with ATL, mostly due to the frequent acquisition of drug resistance and adverse effects. Although novel clinical trials using interferon and arsenic trioxide or zidovudine exhibited better

therapeutic responses, their efficacies were limited and only small percentages of patients achieved long-lasting remission[5]. Therefore, novel treatment modalities are desperately needed.

Recent studies have elucidated crucial roles of nuclear factor-κB (NF-κB) in tumor development and progression[6,7]. NF-κB is a transcription factor that regulates expression of various genes involved in cell cycle regulation and inhibition of apoptosis, such as *cyclinD1, bcl-2, bcl-x_L, XIAP, c-IAPs* and *survivin* as well as genes involved in inflammatory and immune responses[8]. In addition, intracellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1), which are not only important for cell adhesion but also for T-cell proliferation, are also under the control of NF-κB[9]. NF-κB is a hetero- or homo-dimer consisting of Rel family proteins, p65 (RelA), RelB, c-Rel, p105/p50 and p100/p52[10]. The p65/p50 heterodimer, a major form of NF-κB, is normally present in the cytoplasm in association with its inhibitor, IκBα. Stimulation by the inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-1β results in the activation of IκB kinase (IKK) complex through mitogen-activated protein kinase /extracellular signal-regulated kinase kinase 1,3 or NF-κB inducing kinase (NIK). IKKβ serves as catalytic subunit that phosphorylates IκBα on two serine residues (Ser32/S36) (called "canonical NF-κB activation pathway") [11]. On the other hand, IKKα is activated through NIK by alternative stimuli such as lymphotoxin β (LTβ), B-cell activating factor and CD40 ligand, and mediates the processing of p100 to p52 ("non-canonical NF-κB activation pathway"). In addition, we have previously reported that phosphorylation of p65 at Ser 536 is crucial for NF-κB transcriptional activity and is mediated by IKKα upon LTβ signaling[12]. Importantly, such non-canonical NF-κB activation cascade mediated by NIK-IKKα does not necessarily associate with phosphorylation of IκBα followed by its degradation. Thus, inhibitors for IκBα degradation may not be sufficient in inhibiting the NF-κB activity.

In ATL cells and HTLV-1 infected T-cells, a number of reports demonstrated the constitutive activation of NF-κB and its involvement in tumorigenesis[13-15]. This carcinogenic actions of HTLV-1 has been ascribed to Tax, a transactivator protein encoded by the virus, which is responsible for the activation of NF-κB[16-18]. For instance, Suzuki et al. reported that Tax directly binds to and activates NF-κB[16]. Tax is also known to activate NF-κB by stimulating IKK complex by interacting with IKKs[17]. Furthermore, Xiao et al demonstrated that Tax promotes the proteolytic processing of p100[18]. In fresh ATL cells, however, NF-κB was shown to be constitutively activated although Tax is not expressed[14,15]. Since p52 expression is elevated in these cells, the involvement of the non-canonical NF-κB activation pathway is suggested in ATL

cells. Thus, NF-κB plays a major role in ATL leukemogenesis and is considered to be a feasible target in the chemotherapy of ATL.

In this study, we confirmed the constitutive activation of NF-κB in HTLV-1 infected T-cell and ATL cell lines and examined the effect of a novel IKK inhibitor, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl nicotinonitrile (ACHP), on the growth and survival of HTLV-1 infected T-cell lines, ATL cell lines and the cells derived from ATL patients. ACHP exhibited a high selectivity for IKKβ and IKKα (The 50% of inhibitory concentration (IC_{50}) values for IKKβ and α are 8.5 nM and 250 nM, respectively, measured by *in vitro* kinase assays) over other kinases such as IKKγ, Syk and MKK4 (IC_{50} >20μM for these kinases)[19,20]. Here we demonstrate that ACHP could efficiently induce cell growth arrest and apoptotic cell death by blocking NF-κB in ATL cells.

Materials and Methods

Cells and Reagents

HTLV-1 infected T-cell lines, ATL-35T[21], 81-66/45[22], MJ[23] and MT-2[23] cells, human ATL cell lines established from ATL patients, ATL-102[24], ED-40515(-)[25] and TL-Om1[14] cells, and a HTLV-1-negative T-cell leukemia cell line Jurkat were used in this study. ED-40515 (-) and TL-Om1 cells were kind gifts from Drs. M. Maeda and M. Matsuoka (Kyoto University, Japan). Fresh leukemia cells derived from acute-type ATL patients were obtained with informed consent from each patient at Nagoya City University Hospital and Imamura Bun-in Hospital after approval by the institutional ethical committee. All samples from ATL patients contained more than 80 % leukemic cells. Peripheral blood mononuclear cells were derived from four healthy donors upon informed consent. All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 incubator. The novel IKK inhibitor, ACHP[19,20], was a kind gift from Drs. T. Murata and K. B. Bacon of Bayer Yakuhin Ltd. (Kyoto, Japan).

Immunoblot analysis and antibodies

Immunoblot analyses were performed as previously described[20]. Briefly, 1.0 x 10^6 cells were treated with or without ACHP. The cells were lysed in 50 μL of lysis buffer (20 mM HEPES-KOH (pH 7.9), 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 1 mM PMSF,

0.2% Triton X, protease inhibitor cocktail (Roche, Indianapolis, IN)) and the supernatant was collected. Equal amounts of the proteins were electrophoresed on 10 % SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were incubated with TBS-T (10 mM Tris-HCl (pH 8.0), 15 mM NaCl, 0.1 % Tween) with 5% non-fat milk containing 1:1,000 diluted primary antibodies against either phospho-IκB-α (Ser32), phospho-p65 (Ser536), phospho-p65 (Ser276), phospho-Akt (Ser473), Akt, phospho-IKKα (Ser181), phospho- p38 MAPK (Thr180/Tyr182), phospho-ATF-2 (Thr71) and phospho-JNK (Thr183/Tyr185) (Cell Signaling Technology, Beverly, MA), p65, p52/p100, IκB-α or α-tubulin (Santa Cruz, Santa Cruz, CA). After incubation, the membranes were rinsed and further incubated with HRP-conjugated secondary antibodies (Amersham Biosciences, Buckinghamshire, UK) in TBS-T with 5% non-fat milk. Each protein was detected by SuperSignal (PIERCE, Rockford, IL).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as described previously[20]. Nuclear extracts were prepared from 1.0 x 10^6 cells treated with or without ACHP. The cells were lysed with hypotonic lysis buffer (20 mM HEPES-KOH (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 1 mM PMSF, 0.2% Triton X, protease inhibitor cocktail), and after 20 min incubation on ice the samples were centrifuged to remove the cytoplasmic fraction and resuspended in hypertonic nuclear extraction buffer (50 mM HEPES-KOH (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 1 mM PMSF, 0.2% Triton X, protease inhibitor cocktail). After incubation at 4°C for 30 min, the samples were centrifuged and the supernatant was collected to be used as nuclear extract. EMSA was performed using nuclear extract and the double stranded oligonucleotide probes containing the NF-κB sequence in the promoter of *HIV1-LTR*. The sequence was as follows: 5'-TTT CTA GGG ACT TTC CGC CTG GGG ACT TTC CAG-3'. The DNA probe was 5'end-labeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP (Amersham Biosciences). DNA binding reactions were performed at 30°C for 15 min with labeled DNA probe and 15-30 μg protein of nuclear extract in 20 μL binding buffer (22 mM HEPES-KOH (pH7.9), 80 mM KCl, 5% glyceol, 0.1% NP-40, 1 mM dithiothreitol, 2 μg poly dI-dC, 2 μg tRNA and protease inhibitor). The samples were subjected to electrophoresis on 5% non-denaturing polyacrylamide gel with 0.5 x Tris-borate-EDTA buffer at 4°C, followed by autoradiography. For competition and supershift analyses, nuclear extracts were preincubated with unlabeled probe DNA and antibodies (4 μg) against p65, RelB, c-Rel, p52 or p50 (Santa Cruz), respectively, for 30 min at 30°C.

Reverse transcription-PCR (RT-PCR)

RT-PCR was performed as described previously[20]. Briefly, total RNA was prepared from approximately 1.0 x 10^6 cells. After incubation with DNase I (Invitrogen, Carlsbad, CA), total RNA was reverse transcribed using SuperScript First-Strand Synthesis System (Invitrogen). Each sample was subjected to PCR amplification for 30 or 35 cycles, and the products were analyzed by 1% agarose gel electrophoresis. The oligonucleotide primers for *bcl-2, cyclinD1, XIAP, cIAP-1* and *β-actin*, were used as described previously[20]. The primer for *bcl-x_L* and *survivin* were as follows: *bcl-x_L* sense, 5'-CAG AGC AAC CGG GAG CTG GT-3', antisense, 5'-GAT CCA AGG CTC TAG GTG GTC-3'; *survivin* sense, 5'-GGC ATG GGT GCC CCG ACG TTG-3', antisense, 5'-CAG AGG CCT CAA TCC ATG GCA-3'.

Growth inhibition assay

Approximately 1.5 x 10^4 cells were cultured in 96-well plate in triplicates at 37°C. Growth inhibitory effect of ACHP was determined using MTT assay as previously described[20]. Optical densities (OD) at 570nm and 630nm were measured with multi-plate reader. Cell viability (%) was calculated as follows: (OD_{630} - OD_{570} of the samples/OD_{630} - OD_{570} of the control) x 100 (%).

Flowcytometric analysis

The flowcytometric analyses were performed using FACSCAN (BD Bioscience, San Jone, CA) and CellQuest program as previously described[20]. For cell cycle analysis, cells were incubated with propiodium iodide (PI) (Sigma). For apoptosis analysis, cells were incubated with fluorescein isothiocynate (FITC)-conjugated annexinV (MBL, Nagoya, Japan). For ICAM-1 and VCAM-1 expression analysis, cells were incubated with phycoerythrin (PE)-conjugated mouse antibody against human ICAM-1 or VCAM-1, or isotype matched control IgG (BD Biosciences).

Results

Constitutive NF-κB activation in HTLV-1 infected T-cell lines and ATL cell lines

In order to analyze the NF-κB signaling in HTLV-1 infected T-cell lines and ATL cell lines, we examined the phosphorylation status of IκBα and p65. ATL-35T, 81-66/45, MJ, MT-2 and

ATL-102 cells are known to express Tax ("Tax-active"), whereas ED-40515(-) and TL-Om1 cells are known not to express Tax ("Tax-inactive")[14,21-25]. As shown in Fig.1A, the phosphorylation of IκBα at Ser32 and p65 at Ser536 were detected in all cell lines except Jurkat, a control T-cell line, in the absence of any stimuli. We also detected prominent band of p100/p52 in five Tax-active cell lines, but not in Tax-inactive and control cell lines. The phosphorylation of p65 at Ser 276, which is not mediated by IKK[26], was not correlated with IκBα phosphorylation. Constitutive phosphorylation of Akt at Ser 473 was observed in 5 Tax-active cell lines and Jurkat.

We then performed EMSA using specific probes containing NF-κB binding sequence in the HIV-1 LTR promoter. The results revealed that NF-κB was constitutively activated in these HTLV-1 infected T-cell lines and ATL-derived cell lines, but not in Jurkat cells (Fig.1B, Fig.2D). Representative results of MT-2 (Tax-active) and ED-40515 (-) (Tax-inactive) cells are shown in Fig.1B. Fig.1B also shows the result of supershift analysis using competitive antibody against each NF-κB subunit. In both cells, constitutive DNA binding of NF-κB was detected without any stimuli, and supershift bands were observed by addition of antibodies against p65, p52 or p50, indicating that activated NF-κB consisted of p65, p52 and p50 but not RelB or c-Rel.

Inhibitory effects of ACHP on NF-κB activation.

We then examined the inhibitory effect of ACHP, a specific inhibitor for IKKβ and IKKα, on the phosphorylation of IκBα and p65 in these cell lines. Representative results are shown in Fig.2A with MT-2 and ED-40515(-) cells. ACHP efficiently inhibited phosphorylation of IκBα and p65 (IC_{50} values in MT-2 cells were 0.4 μM and 0.2 μM, respectively. IC_{50} values in ED-40515 (-) cells were 10.2 μM and 29.5 μM, respectively). Inhibitory effect of ACHP was observed as early as 5 min after the treatment (Fig.2B). It is noted that ACHP treated MT-2 cells appeared to have an increasing net amount of IκBα concomitantly with the inhibition of IκBα phosphorylation. ACHP also exhibited similar effects with other Tax-active cell lines (data not shown). On the other hand, in ED-40515(-) cells, the amount of IκBα remained at the same level. In Fig. 2C, the inhibitory effect of ACHP on other kinases involved in NF-κB pathways and other major signal transduction pathways are shown. ACHP showed no effect on phosphorylation of IKKα at Ser 181, processing of p100/p52, or phosphorylation of p65 at Ser 276. Moreover, ACHP did not inhibit the phosphorylation of p38 MAPK, ATF-2 and JNK. Unexpectedly, ACHP inhibited the phosphorylation of Akt (IC_{50} values were 4.5 μM), which is presumably responsible for the cytotoxic effect of ACHP on Jurkat cell survival.

In Fig.2D, the inhibitory effect of ACHP on the DNA-binding activity of NF-κB was examined in MT-2, ED-40515(-) and Jurkat cells, using specific probes containing NF-κB binding sequence in the HIV-1 LTR promoter. In MT-2 cells, the NF-κB DNA binding was decreased by ACHP at greater than 1 μM. On the other hand, in ED-40515(-) cells, where the NF-κB DNA binding was weaker than MT-2, the significant inhibitory effect of ACHP was not observed until 50 μM.

Inhibitory effect of ACHP on expression of NF-κB target genes

In ATL, constitutive transcription of anti-apoptotic genes such as *bcl-x_L*, *XIAP*, *c-IAPs* and *survivin* has been reported and ascribed to the resistance against anti-cancer agents[27,28]. NF-κB is known to be involved in the proliferation of HTLV-1 infected cell lines and fresh ATL cells by upregulating growth-promoting genes such as *cyclin D1*[29]. In addition, NF-κB stimulates the expression of *ICAM-1* and *VCAM-1* genes, which mediate T-cell activation and proliferation[9,30].

As shown in Fig.3A, a distinct inhibition of *cyclin D1* gene expression was observed in MT-2 and ED-40515 (-) cells. Inhibition of *bcl-x_L*, *bcl-2*, *XIAP*, *c-IAP1* and *survivin* gene expressions were observed at high concentration of ACHP. These findings were reproducibly observed, although we do not currently know the reason of the different susceptibilities of individual genes to ACHP. In Jurkat cells, inhibition of these gene expressions was weaker than ATL cell lines even at high concentration of ACHP. Fig.3B and 3C demonstrate that ICAM-1 and VCAM-1are highly expressed in MT-2 and ATL-102 cells, and that significant inhibitory effect was evident even at 1 μM ACHP. Whereas ACHP downregulated the expression of VCAM-1 in ATL-102 and MT-2 cells, no significant inhibition was observed in ED-40515 (-) cells. ICAM-1 was not expressed in ED-40515 (-) cells. When expression of CD25, as a negative control, was examined, ACHP treatment did not change the level of CD25 (data not shown).

Suppression of cell cycle progression and induction of apoptosis by ACHP

As shown in Fig.4A, ACHP reduced the fraction of cells at S phase in MT-2 and ED-40515 (-) cells, whereas no effect was observed in control Jurkat cells. These findings indicate that ACHP inhibits the growth of HTLV-1 infected T-cell lines in which NF-κB is constitutively activated. In Fig.4B, the number of cells undergoing apoptosis (annexinV-positive) was measured. In all HTLV-1 infected T-cell cell lines, apoptosis induction was remarkably observed at 50 μM, after 8h treatment with ACHP. In addition, ACHP induced the cleavage of PARP in MT-2 and

ED-40515 (-) cells (data not shown). We then examined the effects of ACHP on the growth of 7 HTLV-1 infected T-cell lines and a control non-infected T-cell line (Jurkat). As shown in Fig.4C, ACHP inhibited the growth of these cells in a dose-dependent manner. Tax-active cell lines were more susceptible to ACHP than Tax-inactive cell lines and Jurkat (IC_{50} values in Tax-active cell lines, Tax-inactive cell lines or Jurkat were 3.1±1.3 μM, 10.7±1.7 μM and 23.6 μM, respectively), suggesting that the growth of Tax-active cells depends on NF-κB more than Tax-inactive cells. These observations are consistent with augmented NF-κB DNA binding and accelerated turn over of IκBα in Tax-active cells (Figs. 1 and 2).

Growth inhibition and apoptosis induction of fresh ATL cells by ACHP

We then evaluated the effects of ACHP on the growth of fresh ATL cells obtained from 4 independent acute-type ATL patients. Peripheral blood mononuclear cells (PBMCs) contained greater than 90% leukemic cells. Control PBMCs were similarly obtained from four healthy individuals. As shown in Fig.5A, ACHP inhibited growth of fresh ATL cells. Fresh ATL leukemic cells were more susceptible to the ACHP-induced cell growth inhibition than control PBMCs from healthy individuals with IC_{50} values of 8.6±1.1 and 55.7±7.5 μM, respectively ($p<0.01$). Compared with the data in Fig.4C with T-cell lines, the susceptibility of fresh ATL cells to ACHP was between Tax-active and inactive cells. However, healthy mononuclear cells were far more resistant to ACHP than Tax-inactive cells. These findings suggested that fresh ATL cells depend on NF-κB.

We then examined the levels of IκBα and p65 phosphorylation, as well as the NF-κB DNA binding, of fresh ATL cells and their susceptibility to ACHP-mediated apoptosis. As shown in Fig. 5B, constitutive phosphorylation of p65 at Ser 536 and NF-κB DNA binding were observed and inhibited by ACHP (IC_{50} value: 0.6 μM), however, phosphorylation of IκBα at Ser 32 was not detected in fresh ATL cells. Identical results of protein phosphorylation were observed with other fresh ATL cells, whereas no constitutive phosphorylation of p65 and IκBα was observed with control PBMCs (data not shown). The flowcytometric analysis of fresh ATL cells (Fig. 5C) revealed the appearance of prominent sub G_0/G_1 population suggesting the presence of cells undergoing apoptosis upon treatment with ACHP. Consistently, annexinV-positive cells were detected even with 10 μM of ACHP (Fig. 5D). Thus, ACHP was effective in blocking the growth and inducing the apoptosis of ATL cells as well as HTLV-1 infected T-cell lines.

Discussion

In this study, we demonstrated that a novel IKK inhibitor, ACHP, efficiently inhibited NF-κB that is constitutively activated in ATL cell lines and fresh ATL cells. We also observed that it could block their growth by inducing apoptosis and cell cycle arrest. The constitutive activation of NF-κB has been reported in other neoplasms such as colorectal cancer, hepatocellular carcinoma, multiple myeloma and various forms of malignant lymphoma[6,7,20]. In these neoplasms, NF-κB was shown to be involved in tumorigenesis and disease progression by upregulating anti-apoptotic proteins, cell cycle regulators, and cell adhesion molecules. In fact, inhibitors of NF-κB such as Bay11-7082[13] showed anti-tumor effects. In addition, we have previously reported the growth inhibitory effect of ACHP on multiple myeloma cell lines and the synergism with conventional chemotherapeutic agents[20].

We observed the growth inhibitory effect of ACHP especially in Tax-active HTLV-1 infected T-cell lines. In MT-2 cells, for example, the NF-κB DNA binding was prominent and was efficiently inhibited by ACHP (Fig. 2D). On the other hand, in Tax-inactive cell lines such as ED-40515(-), phosphorylation of IκBα/p65 and NF-κB DNA binding was not inhibited until higher concentration (greater than 10μM) of ACHP was used (Fig. 2A, D). It was noted that the effective ACHP concentrations in blocking the phosphorylation and NF-κB DNA binding in these cell lines correlated with the extents of growth inhibition by ACHP (Fig.4C). Similar observations were reported by Mori et al.[13] where Tax-active HTLV-1 infected T-cell lines were more susceptible to the growth inhibitory effect of Bay 11-7082, another inhibitor of IKK, than Tax-inactive ATL cell lines.

The functional interaction between Tax and NF-κB activation pathway has been well investigated in previous studies by others[16-18]. In addition, constitutive activation of NF-κB is also reported in Tax-independent ATL cells[14,15] although the mechanism is not established yet. In these cells, it is postulated that the overexpression of TNFα or LTβ, which is often found in ATL patients[22,31], could substitute the effects of Tax. We have demonstrated that LTβ receptor -mediated signaling specifically involves IKKα and induces p65 phosphorylation at Ser 536[12]. The constitutive phosphorylation of p65 at Ser 536 was evident in HTLV-1 infected T-cell lines irrespectively of the expression of functional Tax (Fig. 1). In support of this possibility, Hironaka et al. reported that dominant negative IKKα mutant, but not dominant negative IKKβ mutant,

could inhibit the NF-κB activity in ATL-derived cell lines[15]. Interestingly, we observed the NF-κB DNA binding associated with the constitutive p65 Ser 536 phosphorylation but not with the IκBα phosphorylation in fresh ATL samples (Fig.5B). In these cells, even lower concentrations of ACHP could induce apoptosis and cell growth arrest as compared with ATL cell lines (Fig.5C and D). These findings collectively suggest that the IKKα-mediated NF-κB activation pathway may play a crucial role in ATL.

In this study, we observed that various NF-κB target genes showed different susceptibility to the inhibitory effects of ACHP (Fig. 3). For example, whereas expression of ICAM-1 and VCAM-1 was inhibited by the low dose of ACHP (Fig. 3B,C), expression of anti-apoptotic genes, such as *bcl-XL, bcl-2, XIAP, c-IAP2* and *survivin*, required higher doses of ACHP (Fig. 3A). It is possible that various NF-κB target genes are regulated by distinct NF-κB activation pathways. In this context, we found that IκBα phosphorylation, primarily catalyzed by IKKβ, was inhibited by low dose ACHP in HTLV-1-infected T cell lines (Fig. 2). Thus, it appears that the ACHP-mediated inhibition of NF-κB target genes favors the canonical activation pathway. In addition, we found that ACHP could also induce apoptosis in Jurkat cells unexpectedly (Fig. 2C) because no constitutive NF-κB activation was detected in these cells (Figs.1, 2). In Jurkat cells the genetic defect of PTEN phosphatase is considered responsible for the cellular transformation[32]. The genetic defect of PTEN causes the constitutive Akt phosphorylation mediated by phosphatidylinositol-3 phosphate kinase (PI3K) and its inhibition by ACHP may have caused cell death in Jurkat. The constitutive Akt phosphorylation is also observed in the Tax-active cell lines (Fig. 1A). Thus, it is possible that net effects of ACHP in inhibiting the growth of ATL cell lines may be through IKK-NF-κB and PI3K-Akt kinase pathways.

In conclusion, our results indicate the therapeutic efficacy of ACHP and its derivatives in the treatment of ATL by blocking the signal transduction pathway leading to constitutive activation of NF-κB, as well as Akt phosphorylation. Additionally, we observed the apparent differences in the NF-κB activation pathways involved in Tax-active, Tax-inactive, and most notably, fresh ATL cells and highlighted the role of IKKα in ATL leukemogenesis as a candidate for novel therapy. Thus, ACHP and its derivatives could be feasible components of the novel anti-ATL chemotherapeutic regimen by sensitizing leukemic cells to the conventional cytotoxic agents.

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Figure Legends

Figure 1. Constitutive NF-κB activation in HTLV-1 infected T-cell lines and ATL-derived cell lines. A, Constitutive phosphorylation of IκBα, p65 and Akt. The whole cell extracts obtained from the HTLV-1 infected T-cell lines (ATL-35T (-), 81-66/45, MJ, MT-2) and 3 ATL-derived cell lines (ATL-102, ED-40515(-), TL-Om1). Immunoblotting analyses were performed with antibodies against IκBα, phospho-IκBα, p65, phospho-p65, phospho-p65, p100/p52, Akt, phospho-Akt and α-tubulin (internal control). B, Constitutive activation of NF-κB. The nuclear extracts were prepared from MT-2 and ED-40515(-) cell lines and EMSA was performed with specific DNA probe containing the NF-κB sequence. The results of supershift analysis using antibodies against each NF-κB subunits are shown. Closed and open arrowheads indicate positions of the specific DNA-NF-κB complex and the supershifted complex, respectively.

Figure 2. Inhibitory effects of ACHP. A. Dose-dependent inhibition of IκBα and p65 phosphorylation. MT-2 and ED-40515(-) cells were treated with ACHP (0-50 μM) for 20 min and were subjected to immunoblotting analyses with the indicated antibodies. B, Time-course of inhibition of IκBα and p65 phosphorylation. MT-2 cells were treated with ACHP (10 μM) and whole cell extracts were subjected to immunoblot analysis. C, Specificity of ACHP on IKK and other kinases. MT-2 cells were treated with ACHP (0-50 μM) for 20 min and were subjected to immunoblot analysis with the indicated antibodies. D, Inhibition of NF-κB DNA binding. Cells were treated with ACHP (0-50 μM) for 4 h. Equal amounts (15 μg protein) of nuclear extracts were analyzed for NF-κB binding activity by EMSA using specific probe containing the NF-κB. Closed arrowhead indicates the location of the DNA-NF-κB complex.

Figure 3. Inhibition of the NF-κB-dependent gene expression by ACHP. A, Inhibition of gene expression by ACHP. The mRNA levels of various NF-κB target genes were examined by RT-PCR. Cells were treated with ACHP (0-50 μM) for 4 h, and total RNA were prepared and subjected to the determination of mRNA levels of *cyclin D1*, *bcl-xL*, *bcl-2*, *XIAP*, *c-IAP1*,*survivin* and *β-actin*. Each sample was subjected to PCR amplification for 35 cycles (*cyclin D1* in Jurkat cells) or 30 cycles (other genes). B and C, Down-regulation of ICAM-1 and VCAM-1 expression by ACHP. Surface expression of ICAM-1 (B) and VCAM-1 (C) was examined with Jurkat, ATL-102, MT-2 and ED-40515(-) cells in the absence or presence of ACHP (1 μM) for 48 h by