Fludarabine induces apoptosis of human T-cell leukemia virus type 1-infected T-cells via inhibition of the nuclear factor-κB signal pathway

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The abbreviations are: ATL, adult T-cell leukemia; HTLV-1, human T-cell lymphotropic virus type I; NF-κB, nuclear factor kappa B; XIAP, X-linked inhibitor of apoptosis protein.

Abstract

Adult T cell leukemia/lymphoma (ATL) is a highly aggressive disease in which the human T-cell lymphotropic virus type I (HTLV-I) has been recognized as the etiologic agent. Fludarabine is a purine analogue that has demonstrated significant activity in B-cell malignancies, including chronic lymphocytic leukemia and indolent non-Hodgkin's lymphoma. This study explored the effects of fludarabine on HTLV-1-infected T-cells (MT-1, -2, -4 and HUT102). Fludarabine induced growth arrest and apoptosis of these cells, as measured by 3-(4,5-dimethylithiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, cell cycle analysis and annexin V staining. Moreover, exposure of HTLV-1-infected T cells to fludarabine decreased the levels of X-inhibitor of apoptosis protein in conjunction with inhibition of nuclear factor κB (NF-κB)/DNA binding activity, as measured by Western blot analysis and electrophoretic mobility shift and reporter gene assays, respectively. Further studies found that fludarabine accumulated NF-κB and inhibitory subunit of NF-κB in cytosole in conjunction with down-regulation of NF-κB in nucleus, suggesting that fludarabine blocked nuclear translocation of NF-κB. Taken together, fludarabine may be useful for treatment of individuals with ATL and other types of cancer in which NF-κB plays a role.

Introduction

Adult T-cell leukaemia (ATL) is an aggressive malignancy of $CD4^+$ T lymphocytes, in which the human T-cell lymphotropic virus type I (HTLV-I) has been recognized as the etiologic agent (1,2). Despite the development of intensive combination chemotherapy regimens supported by granulocyte colony-stimulating factor, median survival time of individuals with ATL is less than 13 months (3,4). Thus, it is urgent to develop new treatment strategies.

Fludarabine is a purine analogue that has demonstrated significant activity in B-cell malignancies, including chronic lymphocytic leukemia and indolent non-Hodgkin's lymphoma (5). Fludarabine is converted intracellularly into its active metabolite F-ara-ATP, which inhibits DNA as well as RNA synthesis, resulting in induction of growth arrest and apoptosis of tumor cells (6). Also, fludarabine has been included in conditioning regimens for stem cell transplantation because of its immnunosuppressive effect (7), although the mechanisms by which fludarabine inhibits T-cell function remain to be fully elucidated.

Nuclear factor κB (NF-κB) is a transcription factor that regulates expression of various genes involved in cell cycle regulation and inhibition of apoptosis, such as cyclinD1 and Bcl-2 family members and X-linked inhibitor of apoptosis protein (XIAP)

as well as genes involved in inflammatory and immune responses (8-10). NF-κB acts as survival factors and is activated in various types of tumors including hematological malignancies (8-10). Previous studies found that NF-κB was activated in ATL cells and inhibitors of NF-κB such as PS11248, bortesomib, or oridonin effectively inhibited ATL cells (11-13).

XIAP is a member of inhibitor of apoptosis proteins (IAPs), which selectively bind and inhibit caspases-3, -7, and -9, resulting in blockade of downstream portion of apoptosis pathway (14). XIAP is aberrantly expressed in variety types of solid tumor as well as leukemia cells and thought to be an attractive molecular target for cancer therapy (14).

This study demonstrated new biological function of fludarabine; it downregulated levels of XIAP via inhibition of NF-κB in HTLV-1-infected T cells and induced growth arrest and apoptosis of these cells.

Materials and Methods

Cell culture. HTLV-1-infected T-cell lines, MT-1, MT-2 and MT-4 were kind gifts of I. Miyoshi (Kochi Medical School, Kochi, Japan). HUT102 cells were generously provided by Y. Maeda (Kinki University School of Medicine, Osaka, Japan). Cells were suspended in standard RPMI 1640 medium (Sigma, St. Louis, Missouri) supplemented with 10% heat inactivated fetal bovine serum.

Reagents. Fludarabine phosphate was provided by Schering AG (Berlin, Germany).

MTT Assays. HTLV-1-infected cells ($5x10^5$/ml) were cultured with various concentrations of fludarabine for 2 days in 96-well plates (Flow Laboratories, Irvine, CA). After culture, cell number and viability were evaluated by measuring the mitochondrial-dependent conversion of the 3-(4,5-dimethylithiazol-2-yl)-2,5-diphenyl tetrazolium salt (MTT) (Sigma), to a colored formazan product.

Immunoassays for the soluble form of CD30. The levels of soluble form of CD30 (sCD30) in culture media were determined by a sandwich enzyme-linked immunosorbent assay (human sCD30 ELISA; Bender MedSystems, Vienna, Austria), as previously described (14).

Cell Cycle analysis by Flow Cytometry. Cell cycle analysis was performed on HTLV-1-infected cells incubated with fludarabine (2.5 or 5 μM) for 2 days at $5x10^5$

cells/ml in 12-well plates (Flow Laboratories, Irvine, CA). After incubation, cells were collected, fixed in chilled methanol and suspended in solution containing RNase A (100 units/ml, Sigma) before staining with 50 μg/ml propidium iodide. A minimum of 10000 cells were measured using FACSCalibur apparatus (Beckton Dickinson) and was analyzed using the CellQuest software package (Beckton Dickinson).

Assessment of apoptosis. Cells were plated at a density of $1x10^5$ cells/ml and incubated with fludarabine (2.5, 5 μM) for 2 days in 12-well plates (Flow Laboratories, Irvine, CA). The ability of fludarabine to induce apoptosis was measured by annexin V-FITC apoptosis detection kit according to the manufacturer's instruction (Pharmingen, Inc., San Diego, CA).

Electrophoretic Mobility Shift Assay (EMSA). Electrophoretic mobility shift assay (EMSA) was done as previously described (15). Briefly, 4 μg unclear extracts were incubated with 16 fmol ^{32}P-end labeled, NF-κB binding probe. The DNA-protein complex was separated from the free oligonucleotide on a 5% polyacrylamide gel. Gels were dried and exposed to Kodak XAR film (Eastman Kodak, New Haven, CT).

Transfections and Reporter Assay. Transfections and reporter assay was performed as previously described (13). Briefly, the NF-κB reporter construct (pGL3-NF-κB) containing three copies of NF-κB site cloned into pGL3-basic plasmid (Promega,

Meison, WI) was a generous gift form Dr. Moshe Arditi (Cedars-Sinai Medical Center, University of California at Los Angeles School of Medicine, Los Angeles, CA). MT-1 cells cells ($1x10^7$) were transfected with pGL3-NF-κB (10 μg) by electroporation (150V). After 18 h, cells were exposed to fludarabine (2.5 μM) for 48 h. Luciferase activity in cell lysates was measured by the Dual Luciferase assay system (Promega, Madison, WI) which was normalized by Renilla activities.

Western Blot Analysis. Western blot analysis was performed as described previously (13). Protein concentrations were quantitated using a Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved on a 10% SDS polyacrylamide gel, transferred to an immobilon polyvinylidene difluoride membrane (Amersham Corp., Arlington Heights, IL), and robed sequentially with antibodies. Anti-IκBα (Imgenex, San Diego, CA), anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-XIAP (Cell Signaling Technology Inc., Beverly, MA), anti-Bcl-2 (Santa Cruz), anti-Bcl-xL (Santa Cruz) and anti-α-tubulin (Santa Cruz Biotechnology) antibodies were used.

Immunocytochemistry. HUT102 cells were cultured either with or without fludarabine (5 μM). After 6 hrs, cells were harvested and cytocentrifuge slides were prepared. Anti-p65 (Santa Cruz) and anti-rabbit secondary antibodies (Amersham Corp.) were used for immunocytochemistry. Immune complexes were visualized using the LSAB2

system (Dako, Corp., Carpinteria, CA). Sections were counterstained with hematoxylin and mounted.

Statistical analysis. All statistical analyses were carried out using the SPSS software (SPSS Japan, Tokyo, Japan) and the results were considered to be significant when the P-value was < 0.05.

Results

Effect of fludarabine on proliferation of HTLV-1-infected T-cells.

To examine the effects of fludarabine on the proliferation of HTLV-1-infected T-cells, we cultured MT-1, -2, -4 and HUT102 cells in the presence of various concentrations of fludarabine (0.1-5 μM) for 48 h. Their viability was determined by the MTT assay. Fludarabine inhibited the growth of MT-1, -4 and HUT102 cells with IC50 ranging from 2.5 to 5 μM (Fig. 1A). The proliferation of MT-2 cells was also inhibited by fludarabine in a dose-dependent manner, although IC50 was not reached (Fig. 1A).

We have recently shown that ATL cells produced soluble form of CD30 (sCD30) and serum levels of sCD30 correlated with disease activity (15). We therefore measured levels of sCD30 in culture media after exposure of HTLV-1-infected T-cells to fludarabine (Fig 1B). Control MT-1 cells produced 449 ± 55 U/ml sCD30 during 48 h culture; when these cells were exposed to fludarabine (2.5 or 5 μM), levels of sCD30 decreased to 368 ±77 or 300 ± 43 U/ml, respectively (Fig. 1B). Levels of sCD30 in MT-4 and HUT102 were 94 ± 8 and 160 ± 6 U/ml, respectively. Exposure of these cells to fludarabine (2.5 μM, 48 h) significantly decreased levels of sCD30 by approximately 70 or 50%, respectively (Fig. 1B).

Effect of fludarabine on cell cycle distribution of HTLV-1-infected T-cells.

To investigate the mechanisms by which fludarabine inhibited the growth of HTLV-1-infected T-cells, we explored the effects of fludarabine on cell cycle distribution of these cells by flow cytometry (Fig. 2). Exposure of HTLV-1-infected T-cells to fludarabine (2.5 or 5 μM, 48h) prominently induced accumulation of these cells in the pre-G1 phase of the cell cycle, a feature characteristic of apoptosis with a concomitant decrease in the proportion of cells in the S phase (Fig. 2).

Fludarabine induces apoptosis of HTLV-1-infected T-cells.

To further confirm the ability of fludarabine to induce apoptosis of HTLV-1-infected T-cells, annexin V staining was utilized (Fig. 3). Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic in cells entering apoptosis. Exposure of HTLV-1-infected cells to fludarabine (2.5 μM, 48h) profoundly increased the population of cells positive for Annexin V (Fig. 3).

Effect of fludarabine on levels of antiapoptotic proteins in HTLV-1-infected T-cells.

We next examined whether fludarabine modulated the levels of antiapoptotic proteins, including XIAP, Mcl-1, Bcl-xl, and Bcl-2 by Western blot analysis. HTLV-1-infected T cells constitutively expressed XIAP, Mcl-1, and Bcl-xl proteins, which was consistent with the findings of previous studies (Fig. 4)(17). Exposure of

these cells to fludarabine (2.5 or 5 μM) for 48 h dramatically decreased levels of these proteins (Fig. 4). Likewise, HUT102 cells constitutively expressed Bcl-2 and exposure to fludarabine downregulated levels of Bcl-2 in these cells (Fig. 4). Levels of Bcl-2 were not modulated in MT-4 cells after exposure to fludarabine (Fig. 4). Bcl-2 proteins were barely detectable in MT-1 and -2 cells; exposure of these cells to fludarabine (2.5 or 5 μM) for 48 h induced levels of Bcl-2 (Fig. 4).

Effect of fludarabine on NF-κB activity.

The *XIAP* is one of the NF-κB target genes (14). We therefore examined whether fludrabine affected NF-κB activity in HTLV-1-infected cells (Fig. 5). We first utilized EMSA to explore the effect of fludarabine on NF-κB/DNA binding activity (Fig. 5A). Fludarabine (5 μM, 48 h) effectively disrupted the formation of NF-κB/DNA binding complex in MT-1, -2, and -4 cells (Fig. 5A). The NF-κB/DNA complex was tested in competition with a 100-times molar excess of unlabeled oligonucleotides, but not with the same molar excess of mutated oligonucleotides confirming the specificity of the NF-κB band (Fig. 5A).

Moreover, we performed an NF-κB reporter assay to confirm the effect of fludarabine on NF-κB activity in HTLV-1-infected cells. We temporarily transfected MT-1 cells with an NF-κB reporter construct, and cultured these cells with either

fludarabine or control diluent. MT-1 cells possessed measurable NF-κB transcriptional activity, and fludarabine (5 μM, 48 h) inhibited this activity by approximately 80% (Fig. 5B).

Activation of NF-κB involves two important steps: (*i*) phosphorylation and subsequent degradation of IκBα caused by IκB kinase resulting in the release of NF-κB, and (*ii*) the nuclear translocation of the activated NF-κB. To elucidate the effect of fludarabine on these steps, we measured the levels of NF-κB proteins in the cytoplasm and nucleus of HTLV-1-infected T-cells after exposure to fludarabine (Fig. 6). Fludarabine markedly decreased the levels of NF-κB in the nucleus, whereas the proteins accumulated in cytosol with the concomitant accumulation of IκBα in HTLV-1-infected cells, suggesting that fludarabine blocked the translocation of NF-κB from cytoplasm to nucleus (Fig. 6). Immunocytochemistry also showed that NF-κB accumulated in the nucleus in control HUT102 cells; however, exposure to fludarabine induced accumulation of NF-κB in cytoplasm (Fig. 6C), which further augmented our hypothesis that fludarabine inhibited nuclear translocation of NF-kB in HTLV-1-infected cells.

Discussion

This study demonstrated a new biological action of fludarabine; it blocked NF-κB activity via inhibition of the degradation of IκBα and blockade of the nuclear translocation of NF-κB in HTLV-1-infected T-cells. In addition, we found that fludarabine downregulated the levels of NF-κB target protein including XIAP that functions as an anti-apoptotic molecule. This may be the molecular mechanism by which fludarabine induced apoptosis of HTLV-1-infected T-cells. Previous study has shown that the blockade of NF-κB by either diterpenoid oridonin (18), the proteasome inhibitor Velcade (Bortezomib) (19), or the IκB kinase inhibitor Bay 11-7082 (20) effectively induces apoptosis of ATL cells. Thus, NF-kB intimately involved in the regulation of pro-survival signals in ATL cells and can hence act as an attractive molecular target of treatment of this lethal disease.

Fludarabine possesses potent activity in B-cell malignancies and is widely utilized for treatment of individuals with B-cell chronic lymphocytic leukemia (CLL) as a first line therapy (21). NF-κB is activated in CLL and new treatment strategy targeting this transcription factor has been undergoing testing in preclinical as well as clinical studies with favorable results; the proteasome inhibitor bortezomib which inhibits NF-κB showed biological activity in CLL cells (22-24). Fludarabine may inhibit the

growth of CLL cells at least in part via inhibition of NF-κB signaling.

Recently, fludarabine was also shown to be effective for a subset of T-cell malignancies such as cutaneous T-cell lymphoma, although the number of patients was limited (25,26). This study for the first time demonstrated the efficacy of fludarbine in HTLV-1-infected T-cells in vitro. Larger clinical study is warranted to explore the effect of fludarabine against T-cell malignancies.

Fludarabine possesses an immunosuppressive effect and is included in conditioning regimens for stem cell transplantation, although the precise mechanism whereby fludarabine suppresses immune functions remains unknown. Other investigators have found that fludarabine inhibits signal transducers and activator proteins 1 (STAT1) but not other STAT family members in peripheral blood mononuclear cells (PBMNCs) (27). Fludarabine inhibited the PHA-stimulated proliferation of PBMNCs and the production of interferon gamma (INF-γ) in these cells (27). The authors concluded that the immunosuppressive effect of fluarabine may be mediated by blockade of STAT1 (27). PHA is known to activate NF-κB in PBMNCs (28). Fludarabine probably blocks PHA-induced activation of NF-κB in PBMNCs, resulting in inhibition of production of inflammatory cytokines. This could also explain the immunosuppressive effects of fludarabine. Further studies will be required to

confirm our hypothesis.

Taken together, fludarabine may be useful for the treatment of T-cell malignancies as well as inflammatory diseases such as graft versus host disease.

References

1. Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, Kinoshita KI, et al. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. Proc Natl Acad Sci U S A 1981;78:6476-6480.
2. Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc Natl Acad Sci U S A 1982;79:2031-2035.
3. Taguchi H, Kinoshita KI, Takatsuki K, Tomonaga M, Araki K, Arima N, et al. An intensive chemotherapy of adult T-cell leukemia/lymphoma: CHOP followed by etoposide, vindesine, ranimustine, and mitoxantrone with granulocyte colony-stimulating factor support. J Acquir Immune Defic Syndr Hum Retrovirol 1996;12:182-186.
4. Yamada Y, Tomonaga M, Fukuda H, Hanada S, Utsunomiya A, Tara M, et al. A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukaemia-lymphoma: Japan Clinical Oncology Group Study 9303. Br J Haematol 2001;113:375-382.
5. Keating MJ, Kantarjian H, Talpaz M, Redman J, Koller C, Barlogie B, et al. Fludarabine: a new agent with major activity against chronic lymphocytic leukemia.

Blood 1989;74:19-25.

6. Plunkett W, Gandhi V, Huang P, Robertson LE, Yang LY, Gregoire V, et al. Fludarabine: pharmacokinetics, mechanisms of action, and rationales for combination therapies. Semin Oncol 1993;20:2-12.
7. Dasgupta RK, Rule S, Johnson P, Davies J, Burnett A, Poynton C, et al. Fludarabine phosphate and melphalan: a reduced intensity conditioning regimen suitable for allogeneic transplantation that maintains the graft versus malignancy effect. Bone Marrow Transplant 2006;37:455-61.
8. Aggarwal BB. Nuclear factor-kappaB: the enemy within. Cancer Cell 2004;6:203-208.
9. Pikarsky E, Porat RM, Stein I, Avramovitch R, Amit S, Kasem S et al. NF-kappaB functions as tumor promoter in inflammation associated cancer. Nature 2004;431:461-466.
10. Baldwin AS Jr. The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649-683.
11. Mori N, Yamada Y, Ikeda S, Yamasaki Y, Tsukasaki K, Tanaka Y, et al. Bay 11-7082 inhibits transcription factor NF-kappaB and induces apoptosis of HTLV-1-infected T-cell lines and primary adult T-cell leukemia cells. Blood 2002;100:1828-1834.

12. Satou Y, Nosaka K, Koya Y, Yasunaga JI, Toyokuni S, Matsuoka M. Proteasome inhibitor, bortezomib, potently inhibits the growth of adult T-cell leukemia cells both in vivo and in vitro. Leukemia 2004;18:1357-63.

13. Ikezoe T, Yang Y, Bandobashi K, Saito, T, Takemoto S, Machida H, et al. Oridonin, a diterpenoid purified from *Rabdosia rubescens*, inhibits the proliferation of cells from lymphoid malignancies in association with blockade of the NF-kappa B signal pathways. Mol Cancer Ther 2005;4:578-86.

14. Schimmer AD, Dalili S, Batey RA, Riedl SJ. Targeting XIAP for the treatment of malignancy. Cell Death Differ 2006;13:179-88.

15. Nishioka C, Takemoto S, Kataoka S, Yamanaka S, Moriki T, Shoda M, et al. Serum level of soluble CD30 correlates with the aggressiveness of adult T-cell leukemia/lymphoma. Cancer Sci 2005;96:810-5.

16. Ikezoe T, Yang Y, Heber D, Taguchi H, Koeffler HP. PC-SPES: potent inhibitor of nuclear factor-kappa B rescues mice from lipopolysaccharide-induced septic shock. Mol Pharmacol 2003;64:1521-1529.

17. Okudaira T, Tomita M, Uchihara JN, Matsuda T, Ishikawa C, Kawakami H, et al. NIK-333 inhibits growth of human T-cell leukemia virus type I-infected T-cell lines and adult T-cell leukemia cells in association with blockade of nuclear

factor-kappaB signal pathway. Mol Cancer Ther 2006;5:704-12.

18. Ikezoe T, Yang Y, Bandobashi K, Saito T, Takemoto S, Machida H, et al. Oridonin, a diterpenoid purified from Rabdosia rubescens, inhibits the proliferation of cells from lymphoid malignancies in association with blockade of the NF-kappa B signal pathways. Mol Cancer Ther 2005;4:578-86.
19. Satou Y, Nosaka K, Koya Y, Yasunaga JI, Toyokuni S, Matsuoka M. Proteasome inhibitor, bortezomib, potently inhibits the growth of adult T-cell leukemia cells both in vivo and in vitro. Leukemia 2004;18:1357-63.
20. Mori N, Yamada Y, Ikeda S, Yamasaki Y, Tsukasaki K, Tanaka Y, et al. Bay 11-7082 inhibits transcription factor NF-kappaB and induces apoptosis of HTLV-I-infected T-cell lines and primary adult T-cell leukemia cells. Blood 2002;100:1828-34.
21. Johnson SA. Use of fludarabine in the treatment of mantle cell lymphoma, Waldenstrom's macroglobulinemia and other uncommon B- and T-cell lymphoid malignancies. Hematol J 2004;5 Suppl 1:S50-61.
22. Pahler JC, Ruiz S, Niemer I, Calvert LR, Andreeff M, Keating M,et al. Effects of the proteasome inhibitor, bortezomib, on apoptosis in isolated lymphocytes obtained from patients with chronic lymphocytic leukemia. Clin Cancer Res 20031;9:4570-7.

23. Kelley TW, Alkan S, Srkalovic G, Hsi ED. Treatment of human chronic lymphocytic leukemia cells with the proteasome inhibitor bortezomib promotes apoptosis. Leuk Res 2004;28:845-50.

24. Faderl S, Rai K, Gribben J, Byrd JC, Flinn IW, O'brien S, et al. Phase II study of single-agent bortezomib for the treatment of patients with fludarabine-refractory B-cell chronic lymphocytic leukemia. Cancer 2006;107:916-24.

25. Quaglino P, Fierro MT, Rossotto GL, Savoia P, Bernengo MG. Treatment of advanced mycosis fungoides/Sezary syndrome with fludarabine and potential adjunctive benefit to subsequent extracorporeal photochemotherapy. Br J Dermatol 2004;150:327-36.

26. Go RS, Gazelka H, Hogan JD, Wester SM. Subcutaneous panniculitis-like T-cell lymphoma: complete remission with fludarabine. Ann Hematol 2003;82:247-50.

27. Frank DA, Mahajan S, Ritz J. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. Nat Med 1999;5:444-7.

28. Klotz L, Schmidt M, Giese T, Sastre M, Knolle P, Klockgether T, et al. Proinflammatory stimulation and pioglitazone treatment regulate peroxisome proliferator-activated receptor gamma levels in peripheral blood mononuclear cells from healthy controls and multiple sclerosis patients. J Immunol 2005;175:4948-55.

Figure legends

Fig. 1. Inhibition of growth of HTLV-1-infected T-cells by fludarabine. (A), MTT assay. HTLV-1-infected T-cells (MT-1, -2, -4 and HUT102) were plated in 96-well plates and cultured with various concentrations of fludarabine (0.1-5 μM). After 48h, the cells were treated with MTT for 30min, and absorbance was measured. Results represent the mean±SD of 3 experiments performed in triplicate. **(B), Immunoassay for sCD30.** HTLV-1-infected T-cells (MT-1, -4 and HUT102) were plated in 6-well plates and cultured with fludarabine (2.5 or 5 μM) for 48 h. Cell culture supernatant was analyzed for levels of sCD30 by ELISA. Results represent the mean ± SD of three experiments with duplicate dishes per experimental point. Flu, fludarabine.

Fig. 2. Cell cycle analysis. MT-1, -2, -4 and HUT102 cells were cultured with fludarabine (2.5 or 5 μM). After 48 h, cell cycle of these cells was analyzed. The statistical significance was determined by paired *t*-test. Results represent the mean ± SD of 3 experiments performed in duplicate. Flu, fludarabine.

Fig. 3. Fludarabine increased the proportion of cells positive for Annexin V in HTLV-1-infected T-cells. MT-1, -2, -4 and HUT102 cells were cultured with fludarabine (2.5 μM). After 48h, cells were stained with annexin-V/ propidium iodide, and analyzed by flow cytometry. early apoptosis represents annexin-positive/propidium

iodide-negative cells; late apoptosis represents annexin-positive/propidium iodide-positive cells.

Fig. 4. Effect of fludarabine on the expression of Bcl-2 family proteins. HTLV-1-infected MT-1, -2, -4, and HUT102 cells were treated with fludarabine (2.5 or 5 μM) for 48h. The levels of Bcl-2 family proteins were determined by Western blot analysis. The membrane was sequentially probed with anti-XIAP, -Mcl-1, -Bcl-xl, Bcl-2, and -α-tubulin antibodies.

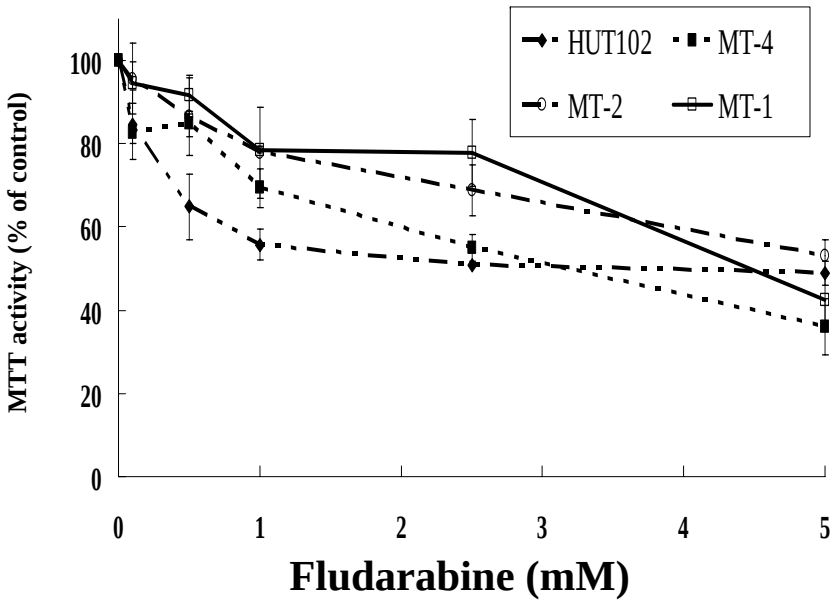
Fig. 5. Effect of fludarabine on NF-κB activity in HTLV-1-infected T-cells. (A), EMSA. MT-1, -2, -4 and HUT102 cells were cultured either with or without fludarabine (2.5 or 5 μM). After 48 h, nuclear protein was extracted and subjected to EMSA. The arrow indicates the gel location of NF-κB bound to DNA. **(B), NF-κB luciferase reporter assay.** Shown at the *top* is the construct (pGL3-NF-κB) containing three copies of the NF-κB binding site cloned into pGL3-basic plasmid. MT-1 cells were transfected with pGL3-NF-κB. After 18 h, cells were exposed to fludarabine (2.5 μM, 48 h). pRL-SV40-Luciferase (Renilla luciferase) vector was cotransfected for normalization. Results represent the mean ± SD of three experiments done in triplicate.

Fig. 6. Fludarabine inhibited degradation of IκBα in cytosol and decreased levels of NF-κB in nucleus in HTLV-1-infected T-cells. MT-1, -2, -4 and HUT102 cells were

exposed to fludarabine (2.5 or 5 μM). After 48 h, cytoplasmic (A) and nuclear extracts (B) of these cells were prepared and subjected to Western blot analysis to measure the level of IκBα and p65 of NF-κB, respectively. Flu, fludarabine. **(C), immunocytochemistry.** HUT102 cells were cultured either with or without fludarabine (5 μM) for 6 hrs. Cells were harvested and cytocentrifuge slides were prepared. Anti-p65 and anti-rabbit secondary antibodies were used and immune complexes were visualized using the LSAB2 system. Sections were counterstained with hematoxylin.

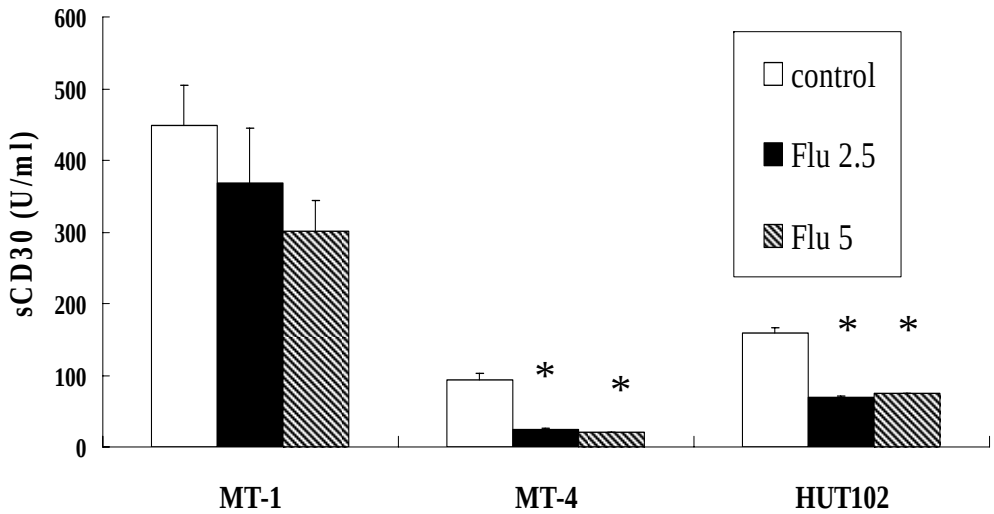
Fig 1

A


HUT102
MT-4
MT-2
MT-1
MTT activity (% of control)
100
80
60
40
20
0
0
1
2
3
4
5
Fludarabine (mM)

B

* P<0.01


sCD30 (U/ml)
600
500
400
300
200
100
0
control
Flu 2.5
Flu 5
*
*
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*
MT-1
MT-4
HUT102

Fig2

* P<0.01
** P<0.05

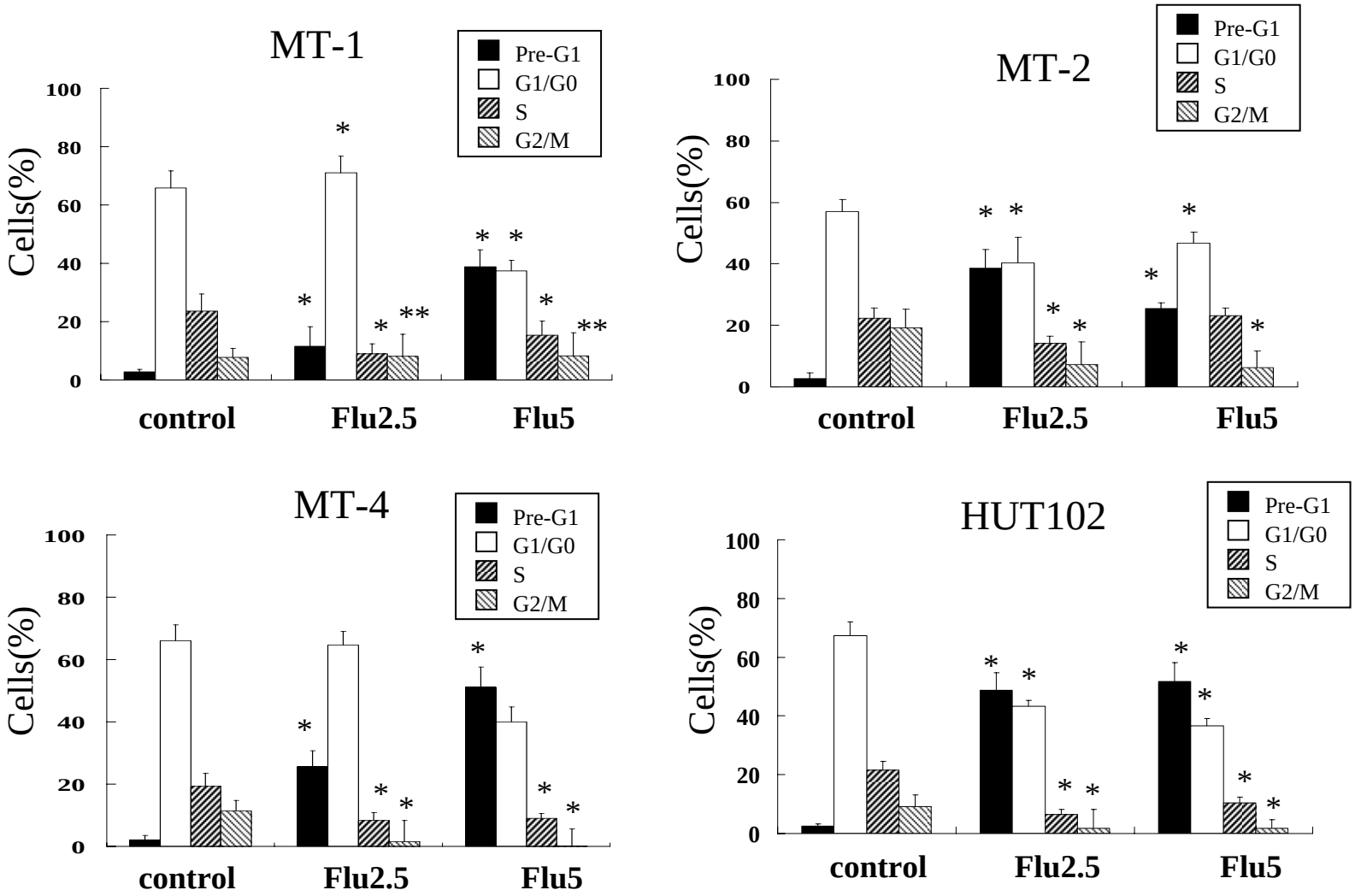
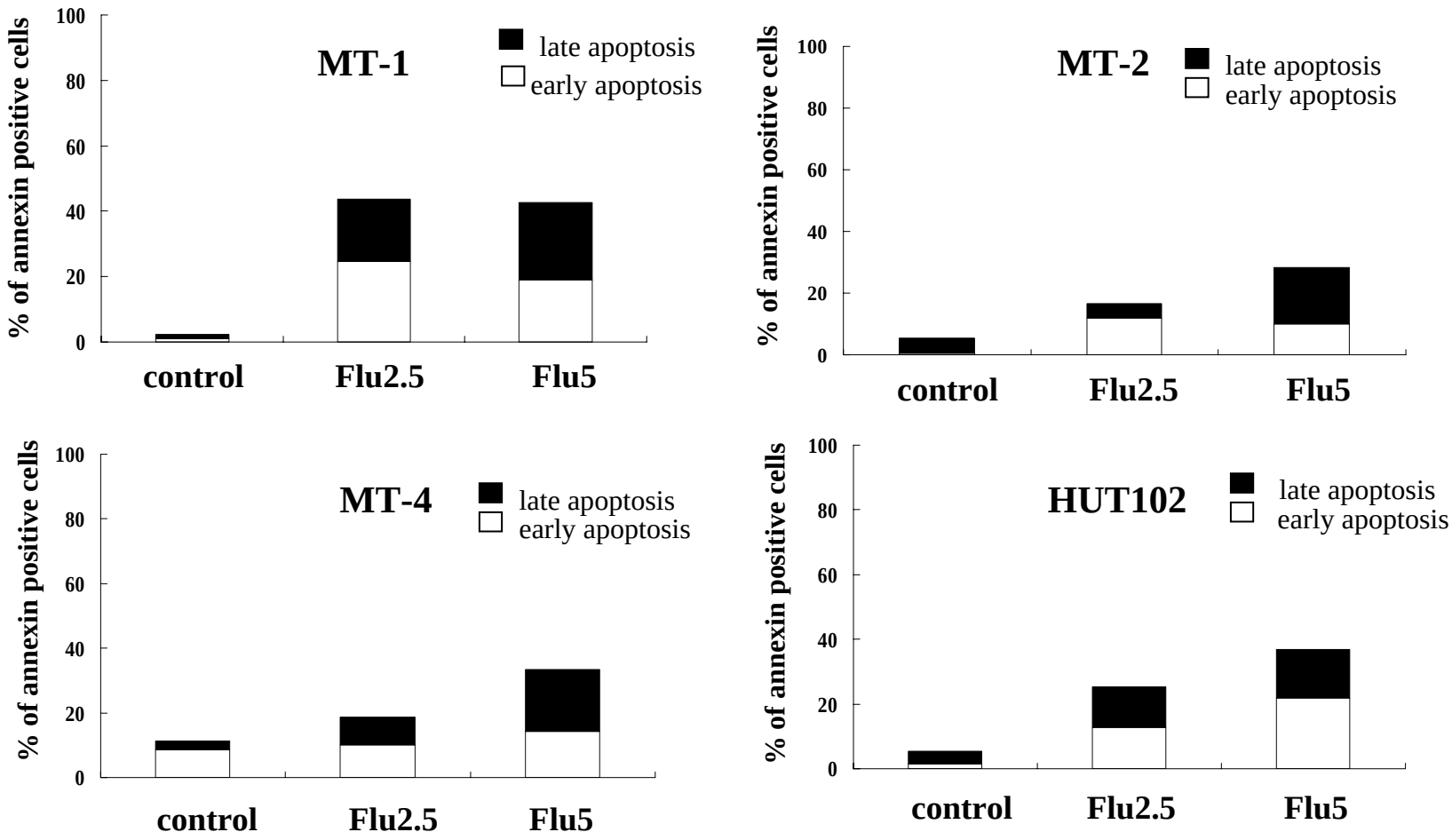


Fig 3


MT-1
late apoptosis
early apoptosis
% of annexin positive cells
100
80
60
40
20
0
control
Flu2.5
Flu5
MT-2
late apoptosis
early apoptosis
% of annexin positive cells
100
80
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control
Flu2.5
Flu5
MT-4
late apoptosis
early apoptosis
% of annexin positive cells
100
80
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0
control
Flu2.5
Flu5
HUT102
late apoptosis
early apoptosis
% of annexin positive cells
100
80
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20
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control
Flu2.5
Flu5

Fig 4

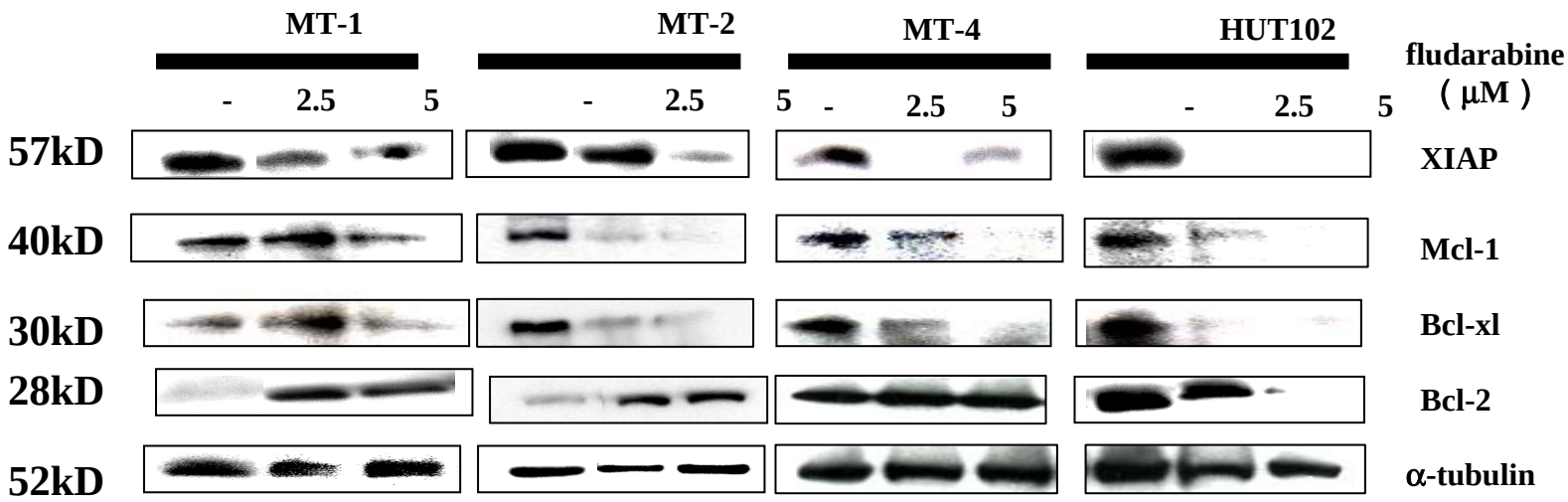
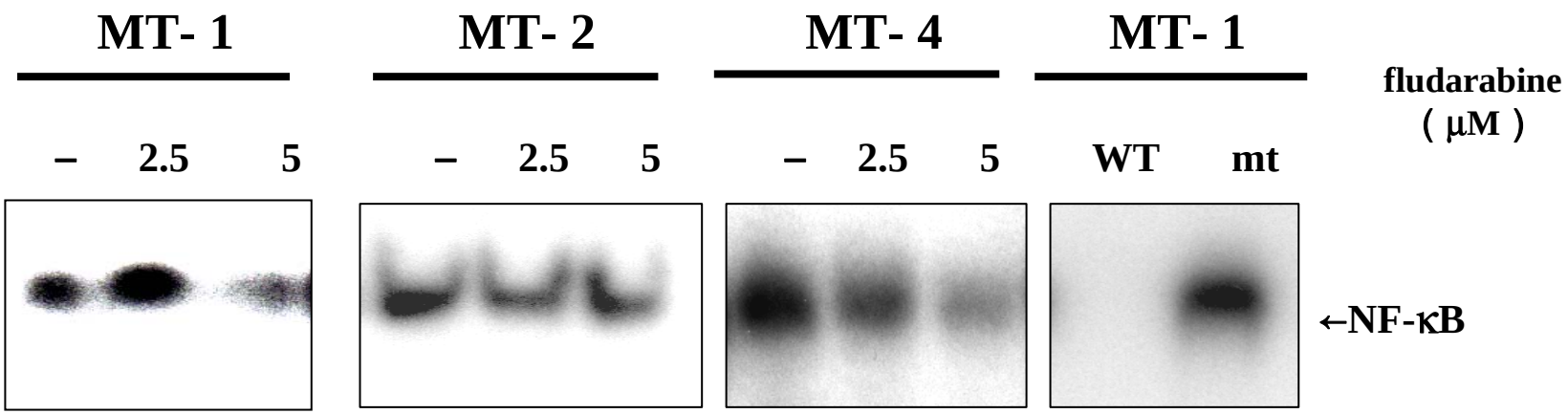

MT-1
MT-2
MT-4
HUT102
fludarabine
（μM）
- 2.5 5
- 2.5 5
- 2.5 5
- 2.5 5
57kD
40kD
30kD
28kD
52kD
XIAP
Mcl-1
Bcl-xl
Bcl-2
α-tubulin

Fig 5 A



B

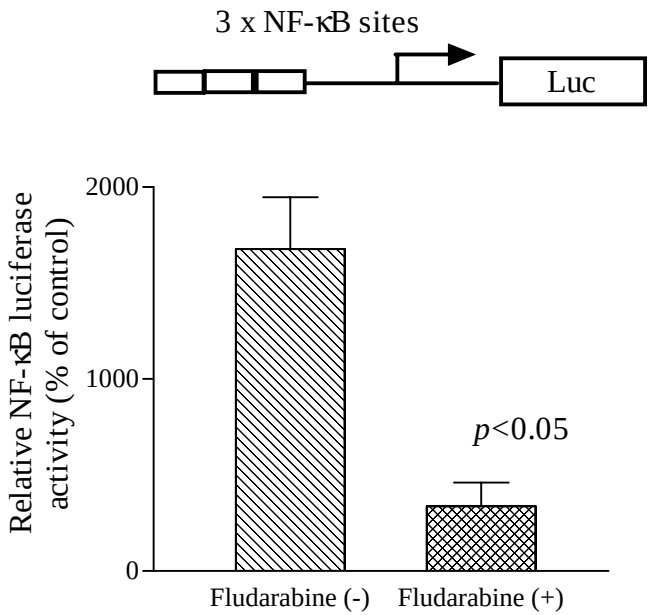
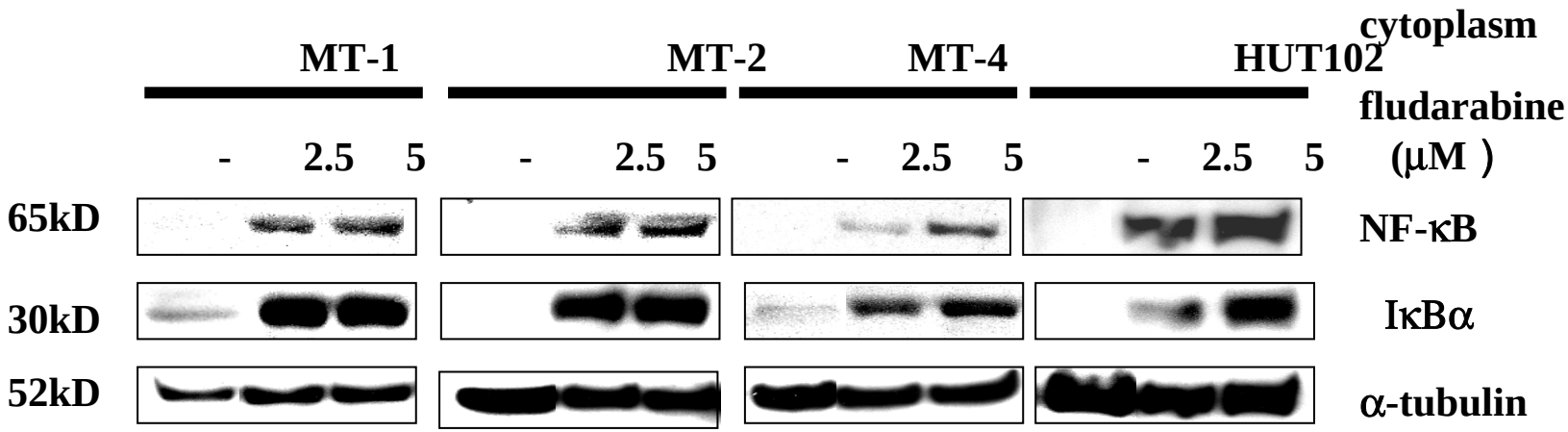
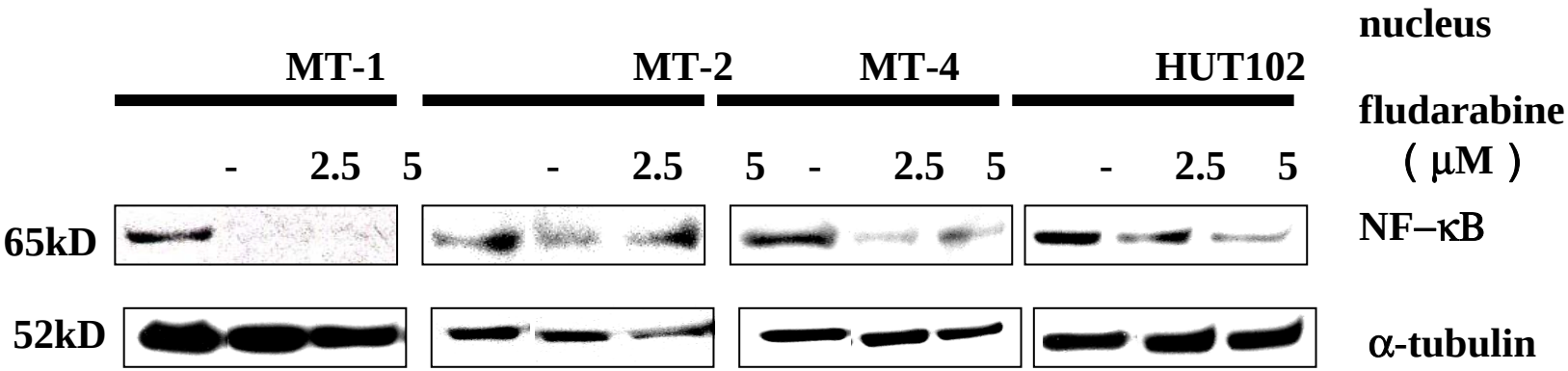

3 x NF-κB sites
Luc
Relative NF-κB luciferase activity (% of control)
2000
1000
0
p<0.05
Fludarabine (-)
Fludarabine (+)

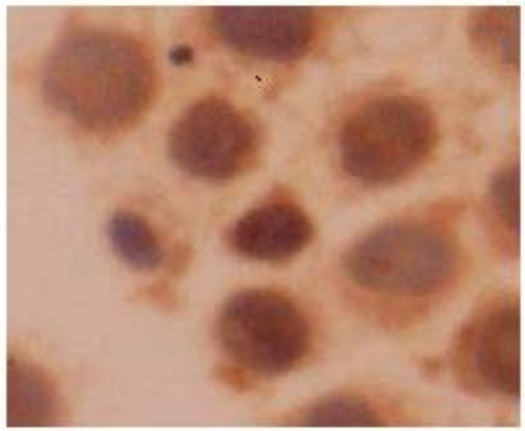
Fig 6A


cytoplasm
MT-1
MT-2
MT-4
HUT102
fludarabine
- 2.5 5 - 2.5 5 - 2.5 5 - 2.5 5
(μM)
65kD
NF-κB
30kD
IκBα
52kD
α-tubulin

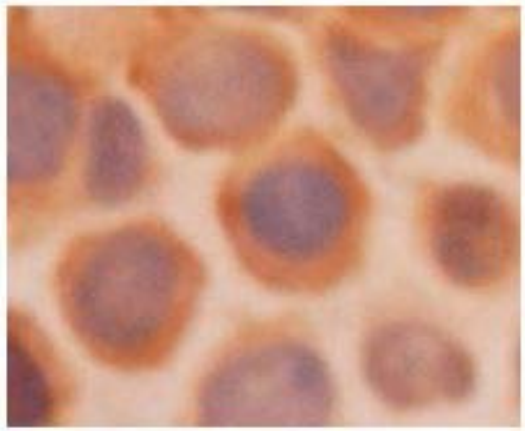
B


nucleus
MT-1
MT-2
MT-4
HUT102
fludarabine
（μM）
- 2.5 5 - 2.5 5 - 2.5 5 - 2.5 5
65kD
NF–κB
52kD
α-tubulin

C



Fludarabine (-)



Fludarabine (+)