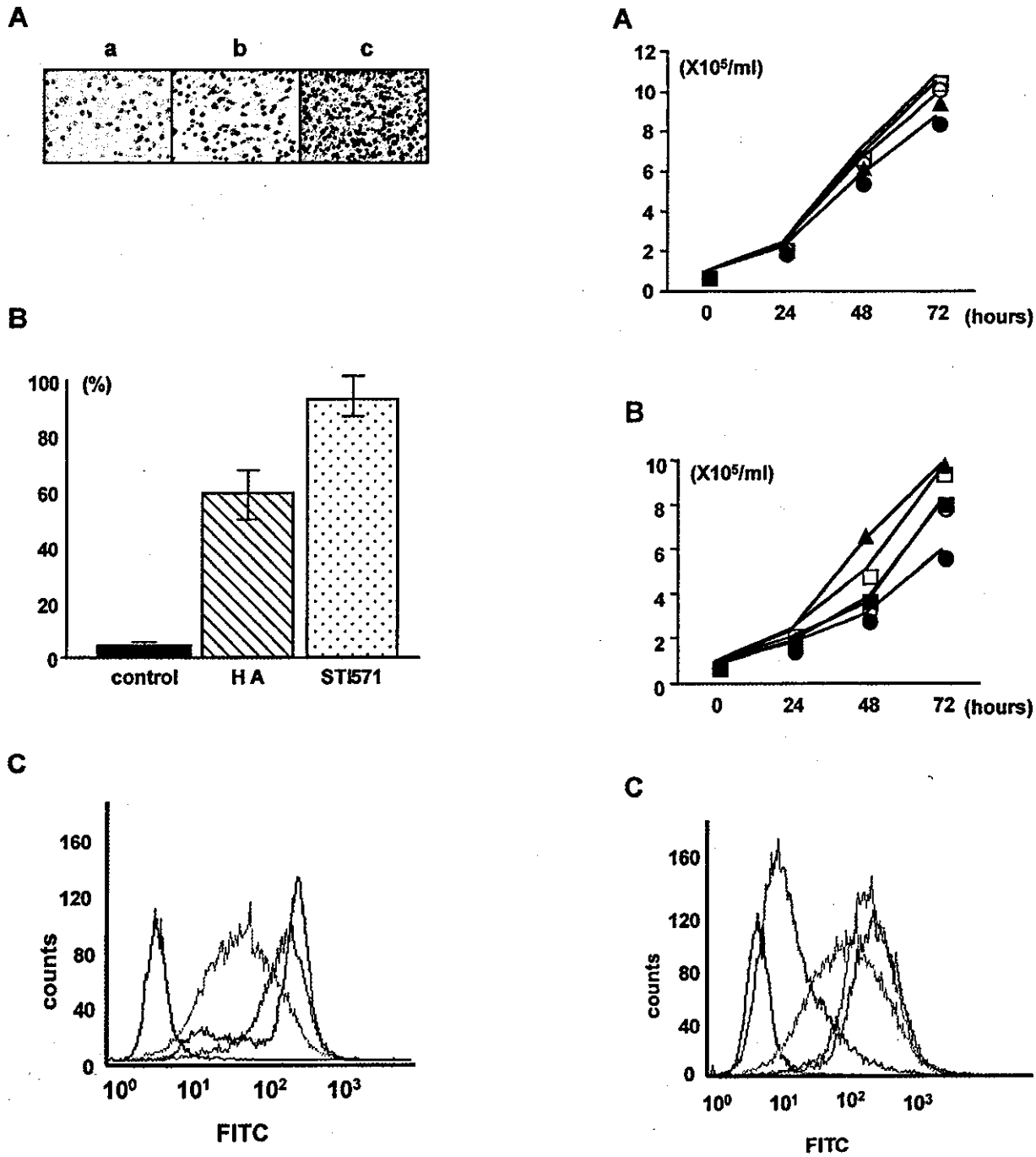


*Fig. 1.* Herbimycin A and STI571 induce erythroid differentiation of K562 cells. (A) Cytochemical analysis of benzidine-positive cells. K562 cells were treated with 800 nM herbimycin A (HA) or 250 nM STI571 for 48 h, and stained with benzidine. a: control, b: herbimycin A, c: STI571. (B) Percentage of benzidine-positive cells deduced from (A). Experiments were done 3 times, and the mean and SD are shown. (C) Flowcytometric analysis of glycophorin A. K562 cells were treated with 800 nM herbimycin A or 250 nM STI571 for 48 h. Expression of glycophorin A was analyzed by FACScan. The tentative mean value after each treatment is shown in parentheses. Red line: STI571-treated cells (mean: 250.3), pink line: herbimycin A-treated cells (mean: 200.0), blue line: untreated K562 cells (mean: 37.9), black line: IgG-k (mean: 4.0).




*Fig. 3.* Characterization of K562/MEK1 and K562/CL100 cells. (A) Growth curve of K562/MEK1 cells. Solid triangles: K562, open circles: K562/MEK1 (#5), open squares: K562/MEK1 (#25), Solid circles: mock transfected. (B) Growth curve of K562/CL100 cells. Solid triangles: K562, Open squares: K562/CL100 (#10) without IPTG, Solid squares: K562/CL100 (#10) with IPTG, open circles: K562/CL100 (#12) without IPTG, solid circles: K562/CL100 (#12) with IPTG. (C) Surface expression of glycophorin A shown by flow cytometry. Tentative mean values are given in parentheses. Black line: negative control of anti-immunoglobulin kappa (mean 4.0), blue line: K562 (mean 37.9), dark-blue line: K562/MEK1 (#5) (mean 8.6), pink line: K562/CL100 (#10) without IPTG (mean 98.2), red line; K562/CL100 (#10) with IPTG (mean 118.6).

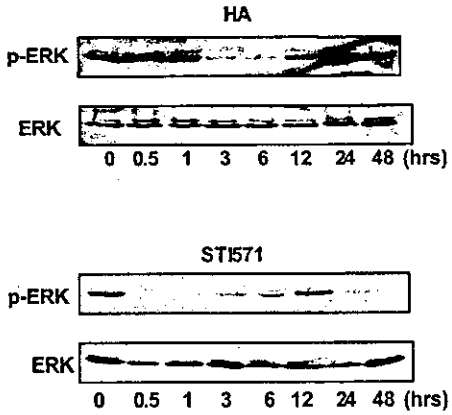


*Fig. 2.* Changes of ERK activity with herbimycin A or STI571 treatment. Cells were sequentially harvested at the designated times, and activation of ERK was studied by immunobloting using a phospho-ERK specific antibody.

of these cells in response to herbimycin A or STI571. K562/MEK1 cells were highly resistant to herbimycin A-induced erythroid differentiation (Fig. 4A). It was found that 56.7% of parental cells and 52.9% of mock-transfected cells showed erythroid differentiation, while two clones (#5 and #25) of K562/MEK1 cells showed only 27.4 and 22.8% erythroid differentiation by benzidine staining after herbimycin A treatment. Analysis of glycophorin A also demonstrated that K562/MEK1 cells were resistant to herbimycin A-induced differentiation (Fig. 4B). The mean level of glycophorin A expression after treatment of parental cells and mock-transfected cells was 200.0 and 148.6, respectively. In contrast, K562/MEK1 clones (#5) and (#25) showed levels of 27.6 and 33.9, respectively. In K562/MEK1 cells, down-regulation of ERK activity was barely observed after herbimycin A treatment, confirming the ectopic expression of MEK1 (Fig. 4C).

We then studied the erythroid differentiation by STI571 (Fig. 5). 86.3% of mock transfected cells became benzidine-positive after 250 nM of STI571 treatment for 48 h, while K562/MEK1 cells (#5 and #25) underwent erythroid-differentiation in 43.3 and 54.0%, respectively (Fig. 5A). The resistance of these two clones to erythroid differentiation by STI571 was also proved by reduced expression of glycophorin A. The mean intensity of glycophorin A after treatment of parental cells and mock transfected cells was 250.3 and 322.0, respectively. In contrast, that of K562/MEK1 (#5 and #25) cells was 30.8 and 35.6, respectively (Fig. 5B). We also studied the activity of ERK during STI571 treatment (Fig. 5C). K562/MEK1 cells were resistant for STI571 induced down-regulation of ERK activity, but it was not so obvious as observed in herbimycin A treatment. In the early phase of treatment from 0.5–1 h, and in the 24–48 h late phase treatment, a little downregulation of ERK was observed in K562/MEK1 cells, but phosphorylated ERK was barely observed in mock transfected cells after STI571 treatment.

*Potentiation of erythroid differentiation by induction of ERK-specific phosphatase*

Next, we studied the effect of ERK activity on erythroid differentiation by the modulation of ERK-specific phosphatase. When K562/CL100 cells were treated by 800 nM herbimycin A for 48 h, 43% of the cells underwent erythroid differentiation by benzidine staining, while it increased to more than 60% in the presence of IPTG to induce CL100 (Figs 6A and 6B). Although, the extent of erythroid differentiation was slightly different, the similar effect of CL100 was also observed in another clone (K562/CL100-#12). Expression of glycophorin A by these cells was also studied after 800 nM herbimycin A treatment. The mean intensity of glycophorin A in parental cells, K562/CL100 (#10) cells without IPTG, and K562/CL100 (#10) cells with IPTG was 200.0, 278.8 and 346.0, respectively (Fig. 6C). Same results were also observed using K562/CL100 (#12) cells (data not shown). To confirm the changes of ERK activity in the presence or absence of phosphatase induction, phospho-ERK specific immunoblot analysis was done (Fig. 7). Treatment with IPTG down-regulated basal ERK activity, and the herbimycin A-induced transient inactivation of ERK was enhanced by IPTG.

## Discussion

We explored the role of the MEK1/ERK system along with erythroid differentiation induced by herbimycin A or STI571. Both drugs are well-characterized Bcr-Abl tyrosine kinase inhibitors [4, 16], and one of the signals from Bcr-Abl is mediated through the Ras/Raf/MAPK pathway [7]. Thus, the erythroid differentiation induced by these two compounds is mostly caused by altering this pathway. But, the involvement of MEK/ERK pathway for erythroid differentiation is controversial. Kang *et al.* demonstrated that constitutive activation of Ras in K562 cells resulted in the activation of ERK, and that the cells showed erythroid differentiation with a retarded growth rate [12]. Interestingly, herbimycin A treatment of cells transfected with mutant Ras led to enhanced erythroid differentiation, while inhibition of the MEK1/ERK system by a specific MEK1 inhibitor (PD098059) showed a biphasic pattern depending on the concentration. A low to moderate concentration of PD098059 weakened the commitment to erythroid differentiation, while a high concentration enhanced erythroid differentiation after herbimycin A treatment [12]. Woessmann *et al.* studied using constitutive ERK- and dominant negative ERK-transfected cells. They defined that the activation of ERK was necessary for the erythroid

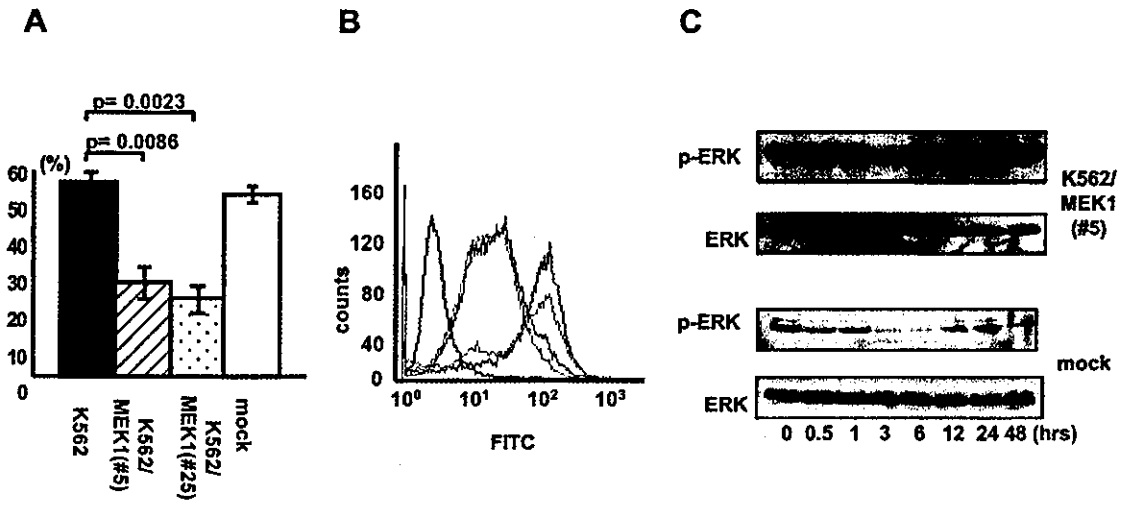


*Fig. 4.*

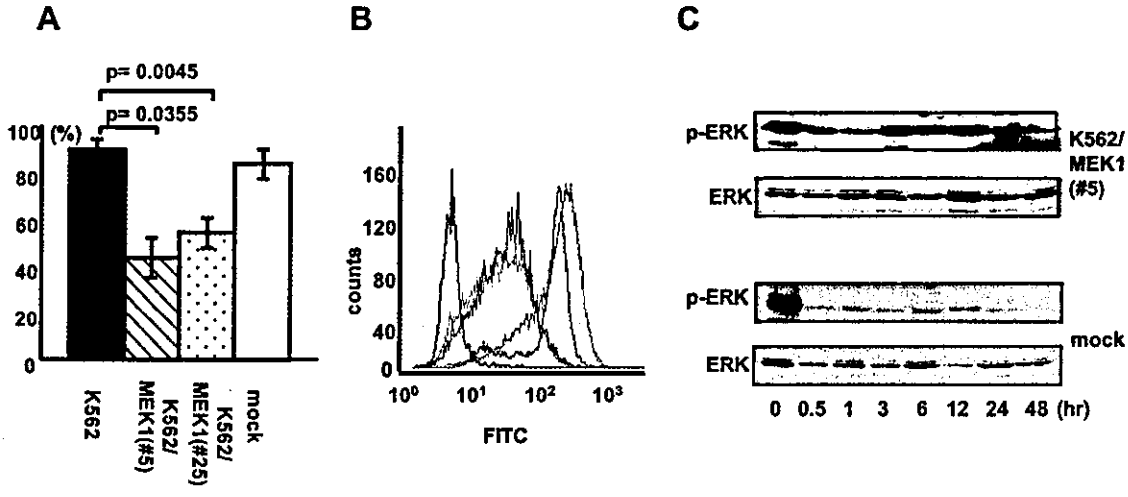


*Fig. 5.*

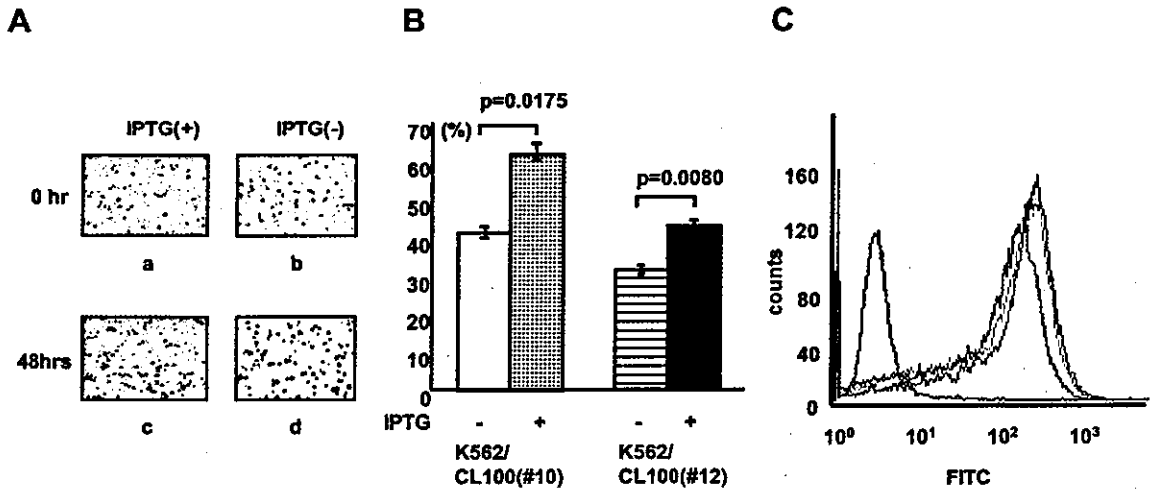


*Fig. 6.*

differentiation induced by hemin [13]. In our experiments, K562/MEK1 cells showed reduced expression of glycophorin A without any treatment when compared with parental cells (Fig. 3C), suggesting that these cells had reverted from their intrinsic commitment to the erythroid lineage. K562/MEK1 cells were also resistant to herbimycin A- or STI571-induced erythroid differentiation (Figs 4 and 5). We also assessed the function of ERK by direct inactivation experiments (Fig. 6). Activation of ERK-specific phosphatase resulted in the enhancement of erythroid differentiation, while the proliferation of K562/CL100 cells was slightly impaired. The basal expression of glycophorin A by K562/CL100 cells without induction by IPTG was higher than that of K562 cells. Because the ERK activity of control cells was lower than that of parental cells, the basal level of ERK-specific phosphatase in K562/CL100 cells might have been a little higher than that in the parental cells. Taken together these data, the commitment of K562 cells to erythroid differentiation by modulating Bcr-Abl activity may mainly depend on the down-regulation of MEK/ERK pathway. But, the erythroid differentiation by other inducers may be determined through each inducer specific pathways. One of them, a stress-activated MAPK system, has been demonstrated to be involved in erythroid differentiation by butyrate. Witt *et al.* showed that erythroid differentiation induced by butyrate was inhibited by SB203580, a specific p38MAPK inhibitor [6], suggesting that the p38-MAPK pathway could modulate the erythroid differentiation of K562 cells. However, p38MAPK was not activated during herbimycin A-induced differentiation in our study (data not shown ), demonstrating another instance that various inducers may select different pathways to erythroid differentiation.

Yu *et al.* have recently demonstrated that MEK inhibition concomitant with STI571 treatment synergistically enhance apoptosis of Bcr-Abl possessing cells [21]. STI571 single treatment induced the down-regulation of ERKs in early phase of treatment and ERKs were reactivated at 24 h. Our study is basically consisted with their results but the reactivation was weak, and no phosphorylated ERK was observed at 48 h. This difference might be dependent on the difference of STI571 concentration. We used 250 nM instead of 200 nM STI571, thus, K562 cells were biologically affected more severely in this study. We have not studied such a co-treatment experiment, but, Kano *et al.* showed the fundamental results about the combinatory use of anticancer agents with STI571 [22]. Thus the combination with not only signal modulators, but also anticancer agents may be promising strategy for new differentiation therapy.

Another important aspect of differentiation via the MAPK pathway is the extent of activation. Several studies have revealed that activation of MEK leads to the induction of megakaryocytic differentiation with decreased proliferation [3, 11]. The surface expression of CD41 by K562/MEK1 cells was slightly higher than that of parental K562 cells in our experiment (data not shown), but the proliferation rate was exactly same as that of the parental cells. As Whalen demonstrated, the kinase activity of each mutant MEK1 was different, and the level of activity affected the lineage [11]. Therefore, the level of MEK1 activity that we achieved might not have been sufficient to cause megakaryocytic differentiation.

As mentioned above, activation of the MEK/ERK pathway prevents the erythroid differentiation of K562 cells, while its inactivation accelerates erythroid differentiation. The downstream targets of MEK1/ERKs in erythroid differentiation have not yet been clarified, but c-myc and cyclin D1 are two major candidate genes. Several lines of evidence have shown that c-myc inhibits erythroid differentiation by K562 cells [23]. Since the stability of c-myc is maintained through its phosphorylation by ERKs [24], transient down-regulation of the MEK1/ERKs pathway might have contributed to the inhibition of c-myc. Cyclin D1 may also modulate erythroid

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*Fig. 4.* K562/MEK1 cells were resistant to erythroid differentiation induced by herbimycin A. (A) Statistical analysis of benzidine-positive cells. Cells were treated with herbimycin A for 48 h. Experiments were done 3 times and the mean and S.D. is demonstrated. Closed column: K562, hatched column: K562/MEK1 (#5), dotted column: K562/MEK1 (#25), open column: mock K562. (B) Surface expression of glycophorin A by flow cytometry. Tentative mean values are shown in parentheses. Blue line: K562/MEK1 (#5) (mean 27.6), dark-blue line: K562/MEK1 (#25) (mean 33.9), red line: K562 (mean 200.0), green line: mock transfected (mean 148.6), black line: IgG-κ (mean: 4.0). (C) Changes of ERK activity during herbimycin A-induced erythroid differentiation. In K562/MEK1(#5) cells, the early ERK down-regulation seen in parental cells was not observed, while ERK was down-regulated in mock-transfected cells similarly to parental cells.

*Fig. 5.* K562/MEK1 cells were resistant to erythroid differentiation induced by STI571. (A) Statistical analysis of benzidine-positive cells. Cells were treated with 250 nM STI571 for 48 h. Experiments were done 3 times and the mean and S.D. is demonstrated. Closed column: K562, hatched column: K562/MEK1 (#5), dotted column: K562/MEK1 (#25), open column: mock K562. (B) Surface expression of glycophorin A by flow cytometry. Tentative mean values are shown in parentheses. Blue line: K562/MEK1 (#5) (mean 30.8), dark-blue line: K562/MEK1 (#25) (mean 35.6), red line: K562 (mean 250.1), green line: mock transfected (mean 322.0), black line: IgG-κ (mean: 4.0). (C) Changes of ERK activity during STI571-induced erythroid differentiation. In K562/MEK1 (#5) cells, the early ERK down-regulation seen in parental cells was not observed, while ERK was down-regulated in mock-transfected cells.

*Fig. 6.* Inactivation of ERK potentiated herbimycin A-induced erythroid differentiation. Cells were treated with 800 nM herbimycin A for 48 h with or without IPTG. (A) Benzidine staining of K562/CL100 cells. (B) Statistical analysis of benzidine-positive cells. Experiments were done 3 times and the mean and S.D. is demonstrated. (C) Surface expression of glycophorin A by flow cytometry. Tentative mean values are shown in parentheses. Blue line: K562/CL100 without IPTG (mean 278.8), dark-blue line: K562/CL100 with IPTG (mean 346.0), red line: K562 ( mean 200.0), black line: IgG-κ (mean: 4.0).

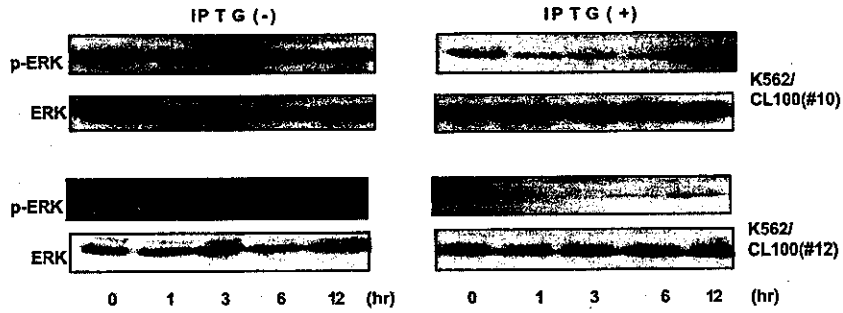


*Fig. 7.* Changes of phosphorylated ERK by herbimycin A under the induction of CL100. K562/CL100 cells were treated with 800 nM herbimycin A with or without IPTG pre-treatment. Under the CL100 induced condition, basal ERK activity was suppressed in both clones and the phosphorylated ERK was scarcely observed in the early phase of treatment.

differentiation. Cyclin D1 is transcriptionally regulated by ERKs [25], so direct suppression of ERKs may alter the expression of cyclin D1 and result in the induction of erythroid differentiation. Our previous studies partly demonstrated that c-myc disturbed herbimycin A-induced G1 accumulation, and cyclin D1 also partially perturbed G1 accumulation [14]. Moreover, our preliminary data have demonstrated that ectopic expression of cyclin D1 suppressed herbimycin A-induced erythroid differentiation.

We investigated the fundamental relationship between inhibition of Bcr-Abl tyrosine kinase and modulation of the MEK1/ERK pathway during erythroid differentiation. Molecular targeting for the modulation of various pathways may lead to safe and effective therapeutic strategies for CML in the near future.

## Acknowledgements

This work was supported in part by a 'Bio-Venture Research Fund Project Aid' from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and also supported by a grant from the Vehicle Racing Commemorative Foundation.

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JCP-04-0089(20235)

JOURNAL OF CELLULAR PHYSIOLOGY 9999:1–11 (2004)



# Involvement of the Tumor Necrosis Factor (TNF)/TNF Receptor System in Leukemic Cell Apoptosis Induced by Histone Deacetylase Inhibitor Depsipeptide (FK228)

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Inhibition of histone deacetylase (HDAC) is a novel strategy for the treatment of leukemias via restoration of aberrantly silenced genes. In this study, we conducted a detailed analysis of anti-leukemic effects of an HDAC inhibitor (HDI), depsipeptide (FK228), using myeloid leukemia cell lines HL-60 and K562. DNA chip analysis revealed upregulation of TNF-α mRNA and a number of molecules involved in TNF-signaling such as TRAF-6, caspases-10, and -7 in depsipeptide-treated HL-60 cells, which prompted us to examine the involvement of the TNF/TNF receptor system in the anti-leukemic effects of the drug. Upregulation of TNF-α was induced by depsipeptide in HL-60 and K562 cells, which expressed type I TNF receptors (TNF-RI). Depsipeptide activated caspases-8 and -10, which in turn cleave caspases-3 and -7, leading to apoptotic cell death in both cell lines. Anti-TNF-α neutralizing antibody and short interfering RNA (siRNA) against TNF-RI alleviated the activation of the caspase cascade and the induction of apoptosis, indicating the presence of an autocrine loop. Finally, we demonstrated that the enhanced production of TNF-α by depsipeptide was due to transcriptional activation of the TNF-α gene through hyperacetylation of histones H3 and H4 in its promoter region (−208 to +35). These results suggest that autocrine production of TNF-α plays a role in the cytotoxicity of depsipeptide against a subset of leukemias. J. Cell. Physiol. 9999: 1–11, 2004. © 2004 Wiley-Liss, Inc.

Modifications of core histone tails are implicated in the regulation of gene transcription. Accumulating evidence suggests that acetylation and deacetylation are particularly important among them, and the balance between the two processes defines the status of transcription of most eukaryotic genes (Jenuwein and Allis, 2001). Histone acetylation triggers the initiation of gene transcription by recruiting chromatin remodeling factors and the general transcription machinery to promoter regions (Agalioti et al., 2002). In contrast, histone deacetylation acts in favor of gene silencing and contributes to the formation of transcriptionally inactive heterochromatin in concert with histone methylation (Nakayama et al., 2001).

Histone deacetylation is mediated by a group of enzymes collectively known as histone deacetylases (HDACs) (Khochbin et al., 2001). Recently, it has been shown that HDACs are involved in leukemogenesis. Various leukemic fusion proteins, including PML/RARα, PLZF/RARα, AML-1/ETO, and CBFβ/MYH11, form a complex with HDACs with higher affinities than their normal counterparts, which aberrantly suppresses the expression of genes required for cell differentiation and growth control, leading to the transformation of primitive hematopoietic cells (Hong et al., 1997; Lin et al., 1998).

Given the role of HDACs in leukemogenesis, the use of HDAC inhibitors (HDIs) is expected to set a novel strategy for the treatment of leukemia called "transcription therapy" (Minucci et al., 2001; Melnick and Licht, 2002; Johnstone and Licht, 2003). HDIs can restore the expression of genes aberrantly suppressed in leukemic cells, which may result in cell cycle arrest, differentiation, and apoptosis. Indeed, HDIs had cytotoxic effects on leukemic cell lines (Murata et al., 2000) and primary cells from patients with chronic lymphocytic leukemia in vitro (Byrd et al., 1999). Furthermore, Ueda et al. (1994) reported that HDIs could prolong the life of mice-bearing transplanted tumors including P388 and L1210 leukemias. Currently, phase I and II clinical trials are ongoing for four different types of HDIs, sodium phenylbutyrate, depsipeptide (FK228), suberoylanilide hydroxamic acid (SAHA), and MS-275, in hematologic malignancies and various solid tumors (Gore et al., 2002; Sandor et al., 2002). Among these compounds, depsipeptide (FK228) is especially promising in the field of clinical hematology, because this agent is reported to exhibit significant therapeutic effects in patients with T-cell lymphoma with minimal toxicity (Piekarz et al., 2001). For safe and effective clinical applications, however, it is essential to clarify the molecular basis of the cytotoxic activity of this drug. Unfortunately, relatively little is known about the mechanisms of the cytotoxic effects of HDIs on leukemias compared with solid tumors. In this study, with this background in mind, we investigated the mechanisms of

Contract grant sponsor: Ministry of Education, Science and Culture of Japan; Contract grant sponsor: Japan Leukemia Research Fund.

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Received 5 March 2004; Accepted 2 September 2004

Published online in Wiley InterScience (www.interscience.wiley.com.), 00 Month 2004. DOI: 10.1002/jcp.20235

the anti-leukemic effects of depsipeptide (FK228) using HL-60 and K562 leukemia cell lines.

## MATERIALS AND METHODS

### Reagents

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Depsipeptide (FK228) was provided by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan), dissolved in dimethylsulfoxide at 2 mM, and stored at −20°C until use. We obtained short interfering RNA (siRNA) against the type I TNF receptor (TNF-RI) and its control from Santa Cruz Biotechnology (Santa Cruz, CA), and used according to the manufacturer's protocol.

### Cells and cell culture

We purchased human myeloid leukemia cell lines, HL-60 and K562, from American Type Culture Collection (ATCC; Manassas, VA). These cell lines were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum.

### Flow cytometry

The cell cycle profile was obtained by staining DNA with propidium iodide in preparation for flow cytometry with the FACScan/CellQuest system (Becton-Dickinson, San Jose, CA). The size of the sub-G1, G0/G1, and S+G2/M fractions was calculated as a percentage by analyzing DNA histograms with the ModFitLT 2.0 program (Verity Software, Topsham, ME). Surface expression of the TNF receptor was detected using a specific antibody against TNF-RI (MABTNFR1-B1; BD Biosciences Pharmingen, San Jose, CA) according to the standard protocol. We used purified mouse IgG as an isotype-matched control. Cells in the early phases of apoptosis were detected by annexin V staining (Annexin V-FITC apoptosis detection kit; MBL, Nagoya, Japan).

### Enzyme-linked immunosorbent assay (ELISA)

We measured the amounts of TNF-α protein in the conditioned medium of HL-60 and K562 cells using the TNF-α ELISA kit (R&D systems, Minneapolis, MN).

### Screening of gene expression profile by DNA chip analysis

We cultured HL-60 cells in the absence or presence of 20 nM depsipeptide (FK228) for 6 h, and isolated poly (A) RNA using a Poly (A) Quik mRNA isolation kit (Stratagene, La Jolla, CA). Poly (A) RNAs from depsipeptide-treated cells and the untreated control were labeled with Cy5 and Cy3, respectively, and hybridized to IntelliGene human cancer CHIP version 3.0 (Takara Bio Co. Ltd., Shiga, Japan), which contains cDNA fragments of 641 known cancer-related genes. Precise information of the array is available at the company's website (http://www.takara-bio.co.jp). The cDNA array was scanned at 560 nm using the Affimetrix 428 Array Scanner. The results were analyzed with BioDiscovery ImaGene version 4.2 software.

### Northern blotting

An equal amount (15 μg) of total cellular RNA was electrophoresed in 1% agarose gels containing formaldehyde, and blotted onto Hybond $N^+$ synthetic nylon membranes (Amersham Pharmacia Biotech., Buckinghamshire, England). The membranes were hybridized with $^{32}$P-labeled probes in Rapid-hyb buffer (Amersham Pharmacia Biotech.). We used a 1.1-kb full-length TNF-α cDNA (Wang et al., 1985), a 801 bp PCR fragment of type I TNF receptor cDNA (nt. 1213–2013) (Fuchs et al., 1992), a 1.4-kb full-length IL-1β cDNA (provided by Ajinomoto Pharmaceutical, Co., Tokyo, Japan), and a 598 bp PCR fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (nt. 146–743) as probes.

### Western blotting

Immunoblotting was carried out according to the standard method using the following antibodies: anti-type I TNF receptor (H-5; Santa Cruz Biotechnology), anti-procaspase-8 (B9-2; BD Pharmingen), anti-cleaved caspase-8 (11G10; Cell Signaling Technology, Beverley, MA), anti-procaspase-10 (#9752; Cell Signaling Technology), anti-procaspase-3 (clone 97; BD Transduction Laboratories, Lexington, KY), anti-cleaved caspase-3 (#9661; Cell Signaling Technology), anti-cleaved caspase-7 (#9492; Cell Signaling Technology), anti-poly(ADP-ribose) polymerase (PARP) (4C10-5; BD Pharmingen), anti-ASK1 (#3761; Cell Signaling Technology), anti-phosphorylated JNK (#9251; Cell Signaling Technology), and anti-β-actin (C4; ICN Biomedicals, Aurora, OH). The inhibition of HDACs by depsipeptide (FK228) was monitored with specific antibodies recognizing histones acetylated at the following sites: lysine 9 of histone H3 (H3-K9), lysine 18 of histone H3 (H3-K18), lysine 8 of histone H4 (H4-K8), and lysine 12 of histone H4 (H4-K12) (all purchased from Cell Signaling Technology).

### Nuclear run-on assay

HL-60 cells were cultured in the absence or presence of 20 nM depsipeptide (FK228) for 6 h. After being washed with phosphate-buffered saline, cells were disrupted in cell lysis buffer (10 mM Tris HCl, pH 8, 40 mM NaCl, 1.5 mM $MgCl_2$, and 0.02% nonidet P-40) containing protease inhibitor complex (Roche Diagnostics, Mannheim, Germany) on ice for 10 min, and nuclei were collected by microcentrifugation. Nascent nuclear RNA was transcribed in labeling buffer (20 mM Tris HCl, pH 8, 140 mM KCl, 10 mM $MgCl_2$, 1 mM $MoCl_2$, 20% glycerol, 14 mM β-mercaptoethanol, 10 mM phosphocreatine, 100 μg/mL phosphocreatine kinase, and 1 mM each of ATP, GTP and CTP) in the presence of 1 mCi/mL [$^{32}$P]UTP for 20 min at 30°C. The elongated RNA was purified after DNase and proteinase K treatment, and hybridized to immobilized plasmids containing cDNAs for TNF-α, β-globin, and GAPDH at $1 \times 10^6$ cpm/mL as previously described (Furukawa et al., 1990).

### Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as reported (Furukawa et al., 2002) with some modifications. Approximately $1 \times 10^6$ cells were resuspended in PBS, fixed with 1% formaldehyde at 37°C for 10 min, resuspended in 200 μL of SDS-lysis buffer (50 mM Tris HCl, pH 8, 10 mM EDTA, and 1% SDS), and sonicated on ice with 10-sec pulses 4× to disrupt chromatin at an average length of 500–1,000 bp. Sonicated cell suspensions were centrifuged at 13,000 rpm for 10 min, and 20 μL of each supernatant was heated at 65°C for 4 h after the addition of 0.8 μL of 5 M NaCl, which was used as an input. The rest of the supernatant was added to 1.8 mL of ChIP dilution buffer (167 mM NaCl, 16.7 mM Tris HCl, pH 8, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, 20 μg/mL salmon sperm DNA, and 50 μg/mL yeast tRNA) containing 10 μg of either anti-acetylated histone H3 antibody or anti-acetylated histone H4 antibody (Upstate Biotechnology, Lake Placid, NY). After incubation at 4°C for 16 h, the mixtures were further rocked with 60 μL of protein A agarose beads in the presence of BSA at 15 μg/mL and salmon sperm DNA at 12 μg/mL for 1 h. The immunoprecipitates were washed 3× each with four different buffers, then eluted with 0.1 M $NaHCO_3$ and 1% SDS. The eluents were heat-treated, digested with proteinase K, extracted with phenol/chloroform, ethanol-precipitated, and finally resuspended in 20 μL of TE (pH 8). We used 5 μL of the final suspension for PCR amplification of the promoter region of the TNF-α gene (−208 to +35) (Takashiba et al., 1993). The primer sequences are 5′-TATCCTTGATGCTTGTGTGTCC-3′ for the sense primer and 5′-CTCTGCTGTCCTTGCTGAGGGA-3′ for the antisense primer. In pilot experiments, we found 30 cycles to be the number most suitable for quantitative detection of the PCR product.

## RESULTS

### Screening of the changes in gene expression in depsipeptide-treated HL-60 cells

Because the principal action of HDIs is the modulation of transcription, it is reasonable to screen for changes in gene expression as an initial step in exploring the mechanisms of the cytotoxic effects of depsipeptide (FK228). To set optimal conditions for gene expression

analysis, we first determined the time-course of the effects of depsipeptide (FK228) using the human myeloid leukemia cell line HL-60. Cell cycle analysis was serially performed with HL-60 cells cultured in the absence or presence of depsipeptide (FK228) at a concentration of 20 nM, defined as the optimal concentration for myeloid leukemic cells in our pilot study (Kano, Y. et al., manuscript in preparation) and 50× lower than the $C_{max}$ of the drug (Sandor et al., 2002). As shown in Figure 1A, depsipeptide (FK228) induced cell cycle arrest at G2/M phase of the cell cycle after 24 h, followed by the appearance of sub-G1 fraction at 48 h of culture. The time course of the response to the drug was almost the same in K562 cells (data not shown). To confirm the induction of apoptosis by depsipeptide (FK228), we performed annexin V staining for depsipeptide-treated HL-60 cells. As shown in Figure 1B, annexin V-positive cells appeared after 24 h of culture, indicating that depsipeptide (FK228) causes apoptosis in leukemic cells.

According to this result, we decided to perform a DNA chip analysis using RNA samples isolated at 6 h of culture, at which time point no significant changes appeared on DNA histograms. The results of the analysis are summarized in Table 1—eight genes showed more than 2.5-fold increase in mRNA expression compared with the untreated control, and 12 genes showed more than twofold decrease among 641 cancer-related genes screened. It is of note that TNF-α mRNA expression was upregulated approximately threefold, and TNF-activated caspases (caspases-7 and -10) and TNF-related genes (TRAF-6, TNF2, TNF10, and TNF10b receptor) were included in the genes detected after depsipeptide treatment (data not shown). These results suggest the involvement of the TNF/TNF receptor system in the anti-leukemic effects of depsipeptide (FK228).

### Effects of depsipeptide (FK228) on the expression of TNF-α and its receptor in leukemic cells

To confirm the upregulation of TNF-α by depsipeptide (FK228), we carried out Northern blotting using HL-60 and K562 cell lines. Consistent with the result of the DNA chip analysis, the abundance of TNF-α transcript increased approximately three- and fivefold in depsipeptide-treated HL-60 and K562 cells, respectively, whereas no change was observed in the untreated control (Fig. 2A and data not shown). We then examined the production of TNF-α protein of these cells using ELISA. As shown in Table 2, TNF-α protein in the supernatants was below the detection limits in untreated HL-60 and K562 cells, but detectable after depsipeptide treatment, indicating that the increase in mRNA expression actually resulted in enhanced TNF-α protein production. To show the specificity of our observation, we reprobed the membrane filter with interleukin-1β probe. No changes were noted in the levels of IL-1β mRNA expression (Fig. 2A), suggesting that the upregulation of TNF-α is not part of the general increase in transcription of cytokine genes by depsipeptide (FK228).

Next, we investigated the presence of TNF-α receptors on these cell lines. As shown in Figure 2A, the type I TNF receptor (TNF-RI) mRNA was highly expressed in untreated HL-60 and K562 cells, and was downregulated after 12 h of treatment with depsipeptide (FK228). We then carried out flow cytometric and immunoblot analyses using specific antibodies against TNF-RI to confirm the expression of TNF receptors on HL-60 and K562 cells. TNF receptors were constitutively expressed on these cells, and were not affected by depsipeptide

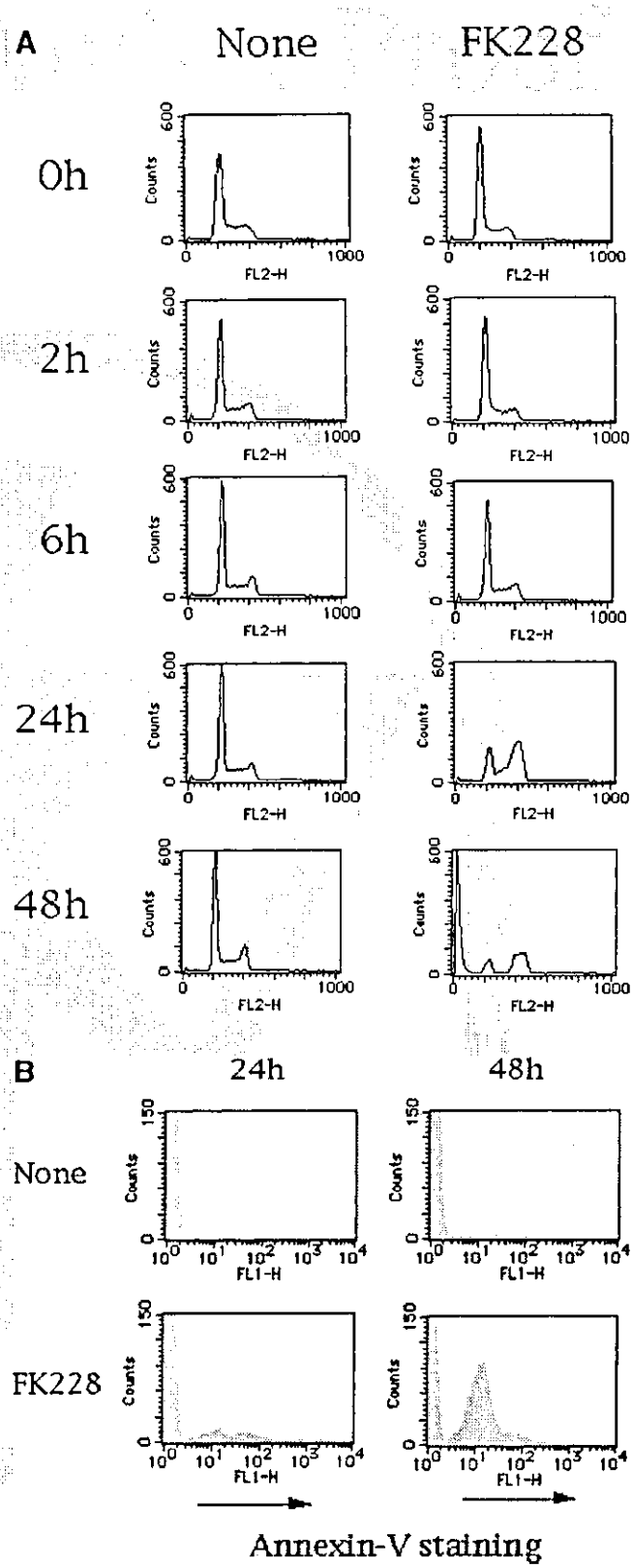


Fig. 1. Depsipeptide (FK228) induced cell cycle arrest and apoptosis in HL60 cells. HL-60 cells were cultured in the absence (none) or presence (delete) of depsipeptide (FK228) at a final concentration of 20 nM for up to 48 h. Cells were harvested at the indicated time points, and stained with propidium iodide for cell cycle analysis (A) and annexin V-FITC for the detection of apoptosis (B) by flow cytometry. The data shown are representative of multiple independent experiments.

Do it !h

TABLE 1. Results of DNA chip analysis of depsipeptide-treated HL60 cells

| Category | Gene name | Accession* | Fold changes |
|---|---|---|---|
| Genes whose expression was increased by FK228 | c-fyn | AJ310436 | 4.71 |
| | Proteasome subunit β9 | NM002800 | 4.28 |
| | Glutaredoxin (thioltransferase) | NM002064 | 3.49 |
| | Lysozyme | NM000239 | 3.40 |
| | TNF-α | NM004862 | 2.97 |
| | IFNγ-inducible protein 30 | NM006332 | 2.83 |
| | PMA-induced protein-1 | NM021127 | 2.78 |
| | NF-IL3 | NM005384 | 2.76 |
| Genes whose expression was decreased by FK228 | CHED | AJ297709 | −2.58 |
| | Wee1 | X62048 | −2.46 |
| | Ikaros | NM006060 | −2.45 |
| | A kinase (PRKA) anchor protein 1 | NM003488 | −2.36 |
| | RB-binding protein 6 | NM006910 | −2.32 |
| | BCR-related gene | NM021962 | −2.28 |
| | CD11a | NM002209 | −2.28 |
| | ATM | U82828 | −2.25 |
| | Lymphoid-restricted membrane protein | NM006152 | −2.23 |
| | ATP-dependent DNA ligase III | NM013975 | −2.22 |
| | Ki-67 | X65550 | −2.21 |
| | CDC7-like 1 | NM003503 | −2.20 |

*Poly(A) RNAs were isolated from HL-60 cells treated with 20 nM depsipeptide for 6 h and from the untreated control, labeled with Cy5 and Cy3, respectively, and hybridized to IntelliGene human cancer CHIP version 3.0 (Takara), which contains cDNA fragments of 641 cancer-related genes. Precise information of the array is available at the company's website (http://www.takara.com).

(FK228) at 12–24 h of culture, when TNF-α production was maximal at both mRNA and protein levels (Fig. 2B,C, and Table 3).

### Autocrine activation of the TNF-signaling pathway in depsipeptide-treated leukemic cells

The production of TNF-α in and the expression of its receptor on depsipeptide-treated HL-60 and K562 cells support the notion that TNF-α acts on these cells in an autocrine or paracrine manner to trigger apoptosis. To substantiate this hypothesis, we first investigated whether the TNF-signaling pathway is really activated in depsipeptide-treated leukemic cells. It is well known that, among initiator caspases, caspases-8 and -10 are cleaved and activated in the death-inducing signaling complex (DISC) formed upon the engagement of TNF-α to type I TNF receptors (Barnhart and Peter, 2003). The activated caspases-8 and -10, in turn, cleave executioner caspases such as caspases-3 and -7 (Budihardjo et al., 1999). Based on this knowledge, we examined the expression of these caspases using immunoblotting. As shown in Figure 3, the amounts of procaspases-8, -10, and -3 readily decreased and cleaved caspases-8, -3, and -7 appeared in HL-60 cells after 24 h of culture with depsipeptide (FK228), whereas no such changes were detected in the untreated control. Similar results were obtained with K562 cells (data not shown). Furthermore, the cleavage of PARP, a substrate of caspase-3, was observed after 48 h of the treatment, indicating that caspases were really activated in depsipeptide-treated cells (Fig. 3).

To obtain direct evidence that autocrine TNF-α mediates the activation of the caspase cascade and subsequent apoptosis, we examined the effect of anti-TNF-α neutralizing antibody on the cytotoxicity of depsipeptide (FK228) against HL-60 cells. Anti-TNF-α antibody alleviated the depsipeptide-induced apoptosis of HL-60 cells (a representative result is shown in Fig. 4A, and the results of three independent experiments are summarized in Table 4) as well as the activation of caspase-8 (Fig. 4B). In addition, we also examined whether siRNA-mediated targeting of TNF receptors affected the cytotoxic effects of depsipeptide (FK228). As shown in Figure 4C, siRNA against TNF-RI but not control siRNA suppressed FK228-induced apoptosis in accord with the reduction of TNF-RI expression. It is of note that the residual cells did not show an accumulation at G2/M phase, suggesting that autocrine TNF-α also plays a role in cell cycle arrest. Taken together, these results indicate that depsipeptide (FK228) induces production of TNF-α in certain subsets of myeloid leukemia cells, which in turn activates TNF receptor-mediated signal transduction pathways in an autocrine or paracrine manner, leading to apoptotic cell death and possibly cell cycle arrest.

In addition to the activation of the caspase cascade, TNF receptors appear to transduce death signals via a second pathway involving the Jun kinase cascade: ASK1-MKK4/7-JNK (c-Jun N-terminal kinase) (Baker and Reddy, 1998). We, therefore, investigated whether depsipeptide (FK228) simultaneously activated this pathway to induce apoptosis in leukemic cells. As shown in Figure 5, however, FK228 failed to activate JNK probably because of downregulation of its upstream activator ASK1.

### Depsipeptide (FK228) activates transcription of the TNF-α gene through hyperacetylation of its promoter

Finally, we investigated the mechanisms of upregulation of TNF-α mRNA by depsipeptide (FK228). First, we examined whether the enhanced expression of TNF-α mRNA is mediated through transcriptional or post-transcriptional mechanisms. Nuclear run-on assays revealed that transcription of the TNF-α gene was significantly augmented in depsipeptide-treated HL-60 cells (Fig. 6). Because the increase in TNF-α transcription is more than tenfold after adjusting to GAPDH transcription levels, upregulation of TNF-α mRNA can be explained solely by transcriptional activation, although the involvement of post-transcriptional mechanisms is not entirely excluded.

To clarify the mechanisms of transcriptional activation of the TNF-α gene by depsipeptide (FK228), we analyzed the status of histone acetylation in the TNF-α promoter using ChIP assays. Before going on to ChIP

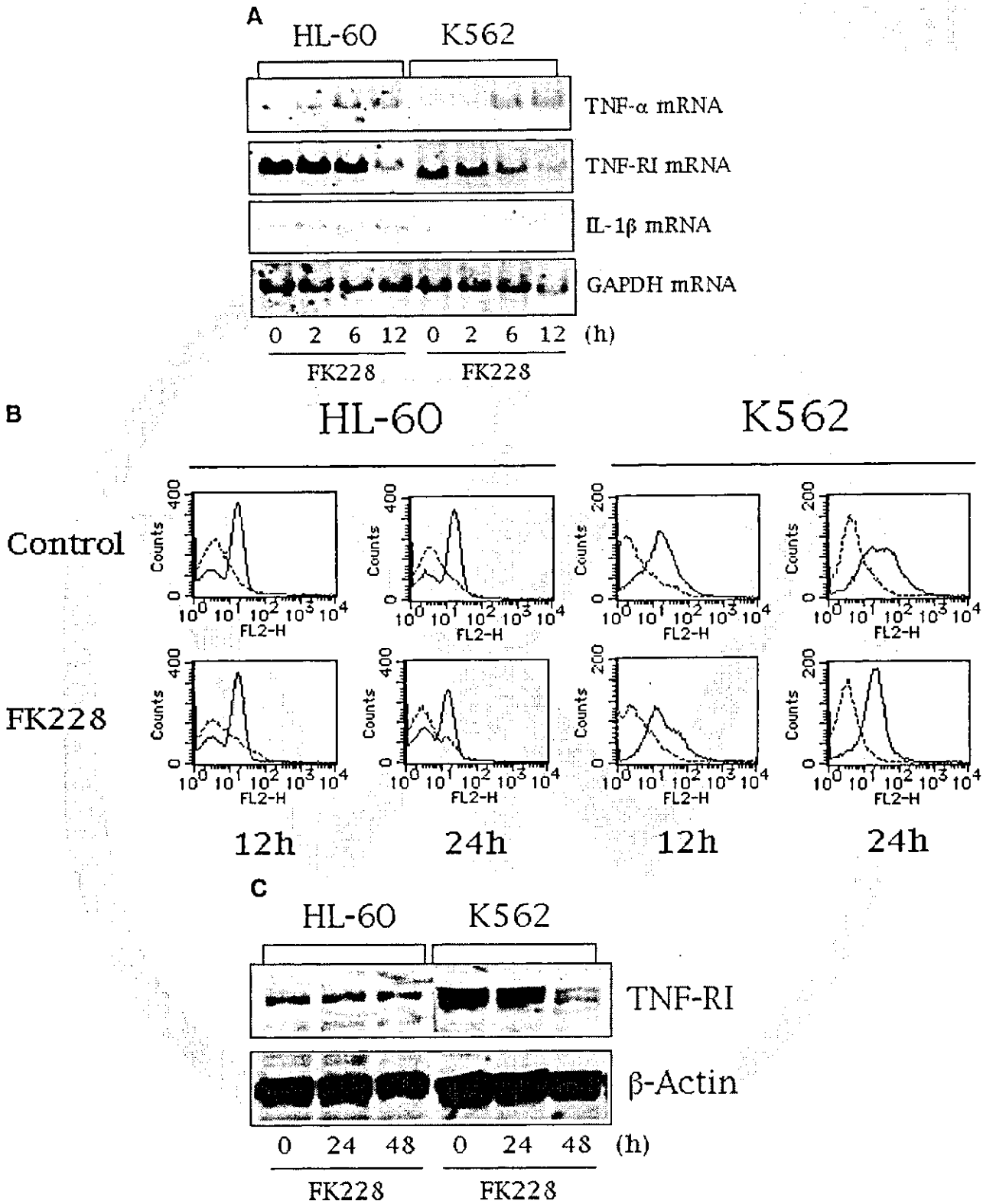


Fig. 2. Expression of TNF-α and its receptors in leukemic cells treated with depsipeptide (FK228). A: HL-60 and K562 cells were cultured with 20 nM depsipeptide (FK228) for up to 12 h. Total cellular RNA was isolated at the indicated time points, and subjected to Northern blot analysis for mRNA expression of TNF-α, TNF-RI, and IL-1β. The membrane filters were reprobed with GAPDH cDNA to serve as a loading control. B: TNF receptors on the surface of HL-60 and K562 cells were stained with a specific antibody against type I TNF receptor, and detected by Texas Red-conjugated secondary antibody using flow cytometry (straight lines). Purified mouse IgG was used as an isotype-matched control (dotted lines). C: Whole cell lysates were prepared from depsipeptide-treated HL-60 and K562 cells, and subjected to immunoblot analysis for TNF-RI expression. The membrane filters were reprobed with anti-β-actin antibody to verify the equal loading and integrity of samples. The data shown are representative of multiple independent experiments.

TABLE 2. TNF-α production in FK228-treated cells

| Cell line | HL-60 | | | | K562 | | | |
|---|---|---|---|---|---|---|---|---|
| Incubation time | 12 h | | 24 h | | 12 h | | 24 h | |
| FK228 | (−) | (+) | (−) | (+) | (−) | (+) | (−) | (+) |
| TNF-α[a] | <5.0 | 5.6 ± 2.4 | <5.0 | 18.0 ± 4.8 | <5.0 | 7.9 ± 1.7 | <5.0 | 12.8 ± 3.1 |

[a]The amounts of TNF-α in the supernatants determined by ELISA (pg/mL; mean ± SD, n = 3).

assays, we confirmed the effects of depsipeptide (FK228) as an HDI in vivo. As shown in Figure 7A, depsipeptide treatment caused the hyperacetylation of N-terminal lysine residues of histones H3 and H4 after 2 h of treatment in HL-60 cells. Previous studies have demonstrated that transcription of the TNF-α gene is governed by the formation of stimuli-specific enhancer complexes on its minimal promoter region between nucleotides -200 and -20 (Falvo et al., 2000). Notably, it has been shown that the enhancer complexes contain histone acetyltransferases CBP/p300, implying the importance of histone acetylation in the transcriptional regulation of TNF-α (Barthel et al., 2003). We, therefore, performed ChIP assays using specific antibodies against acetylated histones H3 and H4, and found that both histones were inducibly acetylated in the core promoter regions of the TNF-α gene after 2 h of culture with depsipeptide in HL-60 cells (Fig. 7B). These findings indicate that depsipeptide (FK228) enhances transcription of the TNF-α gene through hyperacetylation of its promoter.

## DISCUSSION

Given the anticipated role of HDIs in cancer treatment, it is essential to clarify their mechanisms of action in detail for better clinical applications in the future. Evidence is accumulating regarding the cellular consequences of HDI treatment for cancer, which include cell cycle arrest (Qiu et al., 2000), apoptosis (Bernhard et al., 1999), cellular differentiation (Warrell et al., 1998), suppression of tumor angiogenesis (Kim et al., 2001), and immunomodulation (Maeda et al., 2000). The molecular basis of these phenomena has also been studied extensively using conventional methods as well as global gene expression analysis. For example, HDIs accumulate target cells at either G1 or G2/M phase of the cell cycle, depending on the status of p53, through transcriptional activation of a CDK inhibitor, p21/Cip1 (Richon et al., 2000; Derjuga et al., 2001). HDI-induced cell cycle arrest may also be mediated by the altered expression of cyclin A, cyclin D, and p27/Kip1, resulting in a reduction in CDK2 and CDK4 activities (Sandor et al., 2000). As for apoptosis, the transcriptional activation of proapoptotic genes such as Fas and Bax is proposed to mediate HDI-induced apoptosis (Kwon et al., 2002). Other possible mechanisms of apoptosis include the perturbation of mitochondrial membranes, which results in the release of cytochrome *c* and subsequent activation of caspase-9 (Henderson et al., 2003), modulation of the expression of Bcl-2 family proteins (Amin et al., 2001), and the generation of reactive oxygen species (Ruefli et al., 2001). However, these findings were obtained using different HDIs in various cell systems, and it is unclear whether they are universally applicable to other cell types. This study is therefore aimed at understanding the specific mechanisms of action of HDIs against leukemias. We chose depsipeptide (FK228) as an HDI because it has proved to be one of the most effective HDIs against leukemias both in vitro and in vivo (Byrd et al., 1999; Murata et al., 2000; Piekarz et al., 2001; Sandor et al., 2002).

Because histone acetylation is directly linked to transcription and abnormal gene silencing is a hallmark of cancer, it is rational to carry out global gene expression profiling as an initial step to elucidate the mechanisms of action of HDIs. There are some studies dealing with this subject (Mariadason et al., 2000; Suzuki et al., 2002; Yamashita et al., 2002; Glaser et al., 2003). For example, Suzuki et al. (2002) reported that an HDI, trichostatin A, upregulated 23 genes in the colorectal cancer cell line RKO among 10,814 genes examined using a subtraction microarray. Most of them are classified as genes encoding enzymes and signal transducers, and are not growth-regulatory genes except TRADD (see below). In another study, Glaser et al. (2003) compared the gene expression profiles of three different bladder and breast cancer cell lines treated with three HDIs; SAHA, trichostatin A, and MS-27-275. They identified a common set of genes that are positively or negatively regulated by all of the HDIs in all of the cell lines tested. The common set includes 8 genes found to be upregulated and 5 genes found to be downregulated among 6,800 genes. Of the upregulated genes, p21/Cip1 seems to be most important for cell cycle arrest by HDIs. The genes encoding thymidylate synthase and CTP synthase were most prominently downregulated, which may be related to the growth arrest of these cancer cells. Because these studies were conducted with solid tumors, we adopted a similar approach in leukemic cells treated with depsipeptide (FK228), which is the most promising HDI for the treatment of leukemias. In the present study, depsipeptide (FK228) was shown to induce cell cycle arrest and apoptosis after 24 and 48 h of

TABLE 3. TNF receptor expression on FK228-treated cells

| Cell line | HL-60 | | | | K562 | | | |
|---|---|---|---|---|---|---|---|---|
| Incubation time | 12 h | | 24 h | | 12 h | | 24 h | |
| FK228 | (−) | (+) | (−) | (+) | (−) | (+) | (−) | (+) |
| Positivity[a] | 65.5% | 66.3% | 66.5% | 56.3% | 83.1% | 85.8% | 86.8% | 74.1% |
| MFI[a] | 12.7 | 12.9 | 12.8 | 10.6 | 57.5 | 26.5 | 60.3 | 32.9 |

[a]Positivity and mean fluorescence intensity were determined by flow cytometry.

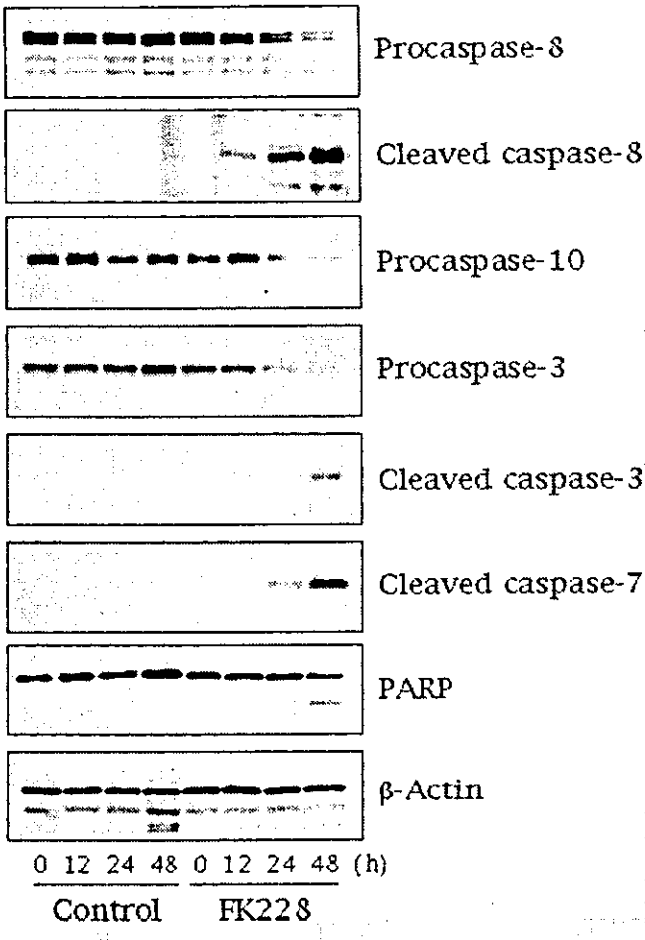


Fig. 3. Activation of the caspase cascade by depsipeptide (FK228) in HL-60 cells. HL-60 cells were cultured in the absence (control) or presence of 20 nM depsipeptide (FK228) for up to 48 h. Whole cell lysates were prepared at the indicated time points, and subjected to immunoblot analysis for procaspases-8, -10, and -3, cleaved caspases-8, -3, and -7, and PARP. The membrane filters were reprobed with anti-β-actin antibody to verify the equal loading and integrity of samples. The data shown are representative of multiple independent experiments.

culture, respectively, in HL-60 and K562 leukemic cell lines. Based on this data, we performed DNA chip analysis using RNA samples isolated at 6 h, when no apparent effect of the drug was observed. The global gene expression profiling revealed that depsipeptide (FK228) modulates a subset of genes related to growth regulation (Wee1, cdc25c, and Ki-67), checkpoint control (ATM), hematopoietic differentiation (CHED and Ikaros), cell adhesion (CD11a), signal transduction (c-fyn, NF-IL3, and A kinase anchor protein1), and apoptosis (caspases-7 and -10, DAP kinase, and FHIT) in HL-60 cells. Taking into account the time of preparing the samples, these changes are not a simple consequence of the effects of depsipeptide (FK228), but are considered to play causative roles. Our results disclose the changes in the expression of many genes that have been overlooked in similar attempts in the past, suggesting that HDIs exert cytotoxic effects via distinct mechanisms in leukemia and solid tumors.

TABLE 4. Effects of anti-TNF-α neutralizing antibody on the cytotoxicity of depsipeptide (FK228) against HL-60 cells

| FK228 | Additions | Proportion of cells in sub-G1 fraction (%)[a] | |
|---|---|---|---|
| − | Buffer | 3.6 ± 1.2 | |
| − | Anti-TNF-α | 3.3 ± 1.2 | |
| + | Buffer | 94.1 ± 3.1 | |
| | | | $P = 0.0248$* |
| + | Anti-TNF-α | 65.8 ± 11.9 | |
| | | | $P = 0.0145$** |
| + | Mouse IgG | 94.8 ± 4.2 | |

[a]Means ± SD of three independent experiments.
*$P$-value determined by a paired Student's $t$-test between buffer and anti-TNF-α.
**$P$-value determined by a paired Student's $t$-test between mouse IgG and anti-TNF-α.

In addition to TNF-α, a number of TNF-related cytokines and molecules involved in TNF signaling and function were detected in DNA chip analysis. Based on this finding, we examined the involvement of the TNF/TNF receptor system in the cytotoxicity of depsipeptide (FK228), and found that autocrine TNF-α was important for the induction of apoptosis and presumably of cell cycle arrest in myeloid leukemic cell lines. The similar role of TNF-α in interferon-mediated killing of hairy cell leukemia was reported by Baker et al. (2002). Importantly, depsipeptide (FK228) enhanced the expression of caspase-10, an initiator caspase directly activated by TNF-RI-associated DISC (Wang et al., 2001), and caspase-7, an executioner caspase activated in the TNF-mediated caspase cascade (Budihardjo et al., 1999). The induction of caspases-7 and -10 may strengthen the effects of autocrine TNF-α by supplying its effector molecules in depsipeptide-treated cells. According to a recent report by Aron et al. (2003), depsipeptide activates caspase-8 through downregulation of c-FLIP, a competitive inhibitor of caspase-8, thereby inducing cell death in chronic lymphocytic leukemia cells. It is possible that the suppression of c-FLIP is another factor strengthening the effects of depsipeptide (FK228) on myeloid leukemias. Furthermore, upregulation of TRADD may also contribute to depsipeptide-induced apoptosis as an enforcer of TNF action as suggested by Suzuki et al. (Suzuki et al., 2002). An investigation is currently underway in our laboratory to test these hypotheses.

It is surprising that depsipeptide (FK228) failed to activate TNF receptor-mediated Jun kinase cascade. Downregulation of ASK1 seemed to be responsible for the failure of JNK activation (Baker and Reddy, 1998). The downregulation of ASK1 may be part of the direct inhibitory effects of depsipeptide (FK228) on Ras-MAP kinase signaling pathways (Kobayashi, Y. et al., manuscript in preparation). Our observation is indicative of selective activation by FK228 of the caspase cascade downstream of TNF receptors. A similar dissociation of the caspase cascade and JNK pathways was demonstrated in a previous study using dominant-negative FADD (Wajant et al., 1998).

We obtained evidence suggesting that autocrine TNF-α also plays a role in an accumulation of HL-60 cells in G2/M phase. This is consistent with previous reports describing TNF-α-induced G2/M arrest (Darzynkiewicz et al., 1987; Kumakura et al., 2003). However, the extent of the accumulation by TNF-α is less prominent than that in HDI-treated cells. It is therefore unlikely that HDI-induced G2 arrest is entirely due to autocrine effects of TNF-α. Additional mechanisms such as the failure of cytokinesis via hyperacetylation of the centromere may be involved in this process (Taddei et al., 2001).

Finally, we investigated the mechanisms of depsipeptide-mediated upregulation of TNF-α. We demonstrated that depsipeptide (FK228) activated transcription of the

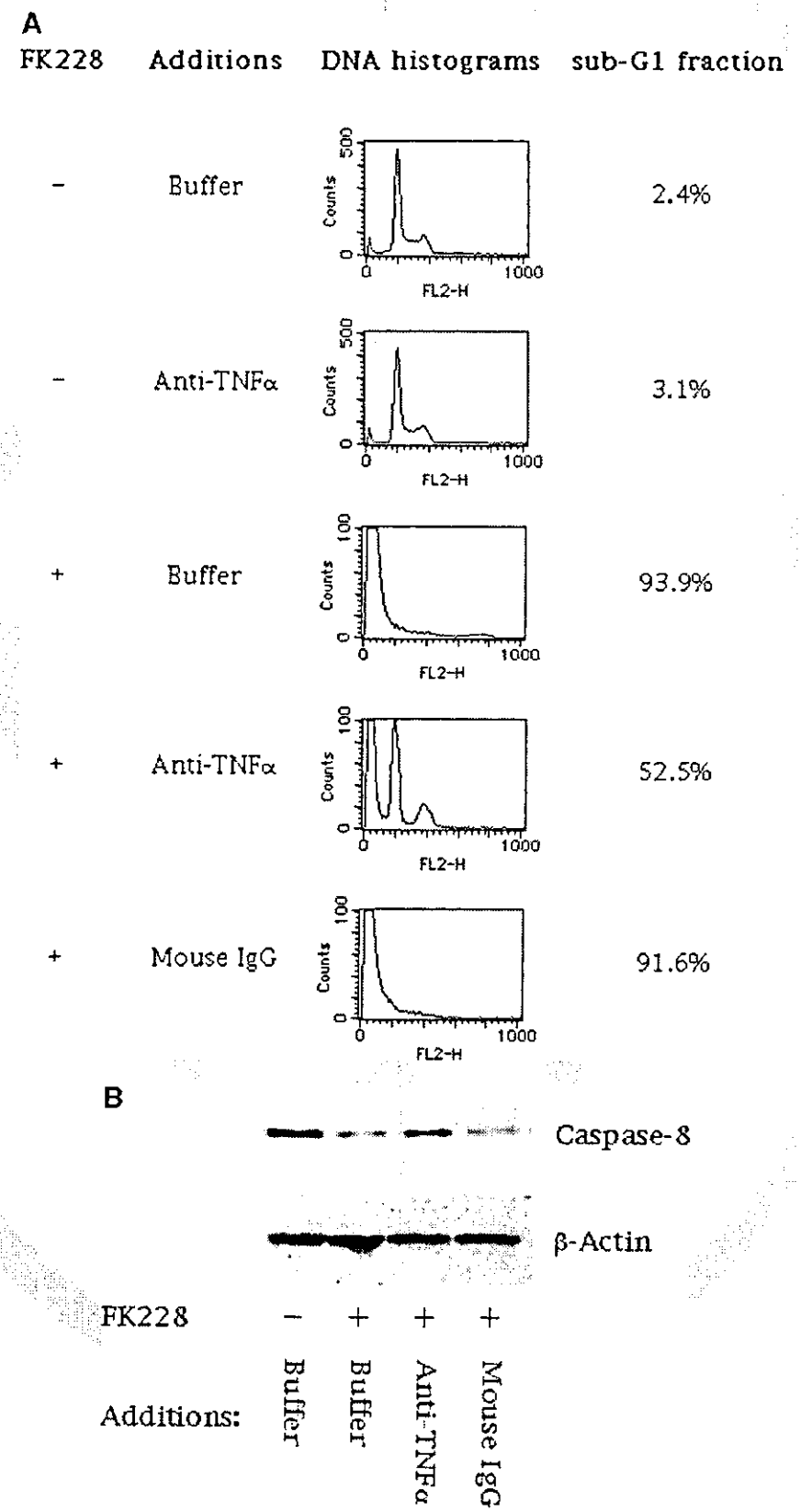


Fig. 4. Effects of a neutralizing anti-TNF-α antibody and siRNA against TNF-RI on the cytotoxicity of depsipeptide (FK228). **A**: HL-60 cells were cultured with either phosphate-buffered saline alone (buffer), purified mouse IgG (mouse IgG) or anti-TNF-α neutralizing antibody (Mab11; BD Pharmingen) (Anti-TNFα) at a final concentration of 20 μg/mL in the absence (−) or presence (+) of 20 nM depsipeptide (FK228). DNA histograms were obtained by staining cells with propidium iodide after 48 h of culture to determine the percentages of cells in sub-G1 fraction. **B**: Whole cell lysates were prepared at 48 h of culture, and subjected to immunoblot analysis for procaspases-8 and β-actin. **C**: HL-60 cells were pretreated with either siRNA against TNF-RI or its control at 50 nM for 30 h, and further cultured in the absence (−) or presence (+) of 20 nM depsipeptide (FK228). The effect of TNF-RI siRNA was confirmed by immunoblotting (left part) and flow cytometry (right upper part). DNA histograms were obtained after 48 h (right lower part). The data shown are representative of three independent experiments.

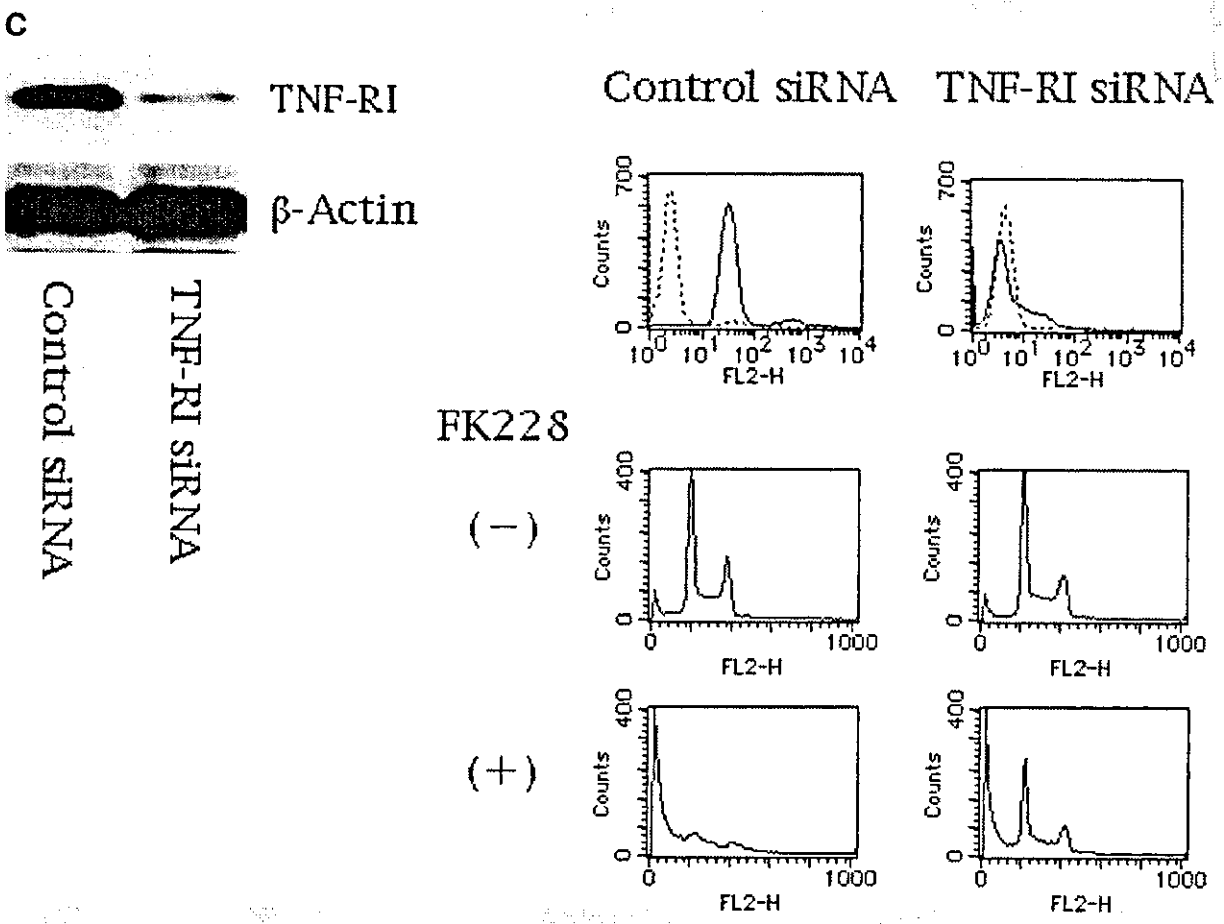


Fig. 4. *(Continued)*

TNF-α gene through hyperacetylation of histones H3 and H4 of its promoter regions. It has been shown that transcription of the TNF-α gene is governed by the formation of stimuli-specific enhancer complexes containing histone acetyltransferases CBP/p300 (Barthel et al., 2003). Depsipeptide (FK228) bypasses the requirement of the enhancer complexes, and aberrantly induces transcription of the TNF-α gene in myeloid leukemia cells. This information is not only useful for cancer treatment, but also applicable to pharmacological interventions for other inflammatory and immunological processes associated with activation of TNF-α.

In summary, the present study has defined autocrine production of TNF-α as an important mediator of the cytotoxic effects of depsipeptide (FK228) in a subset of myeloid leukemias. It is assumed, however, that many

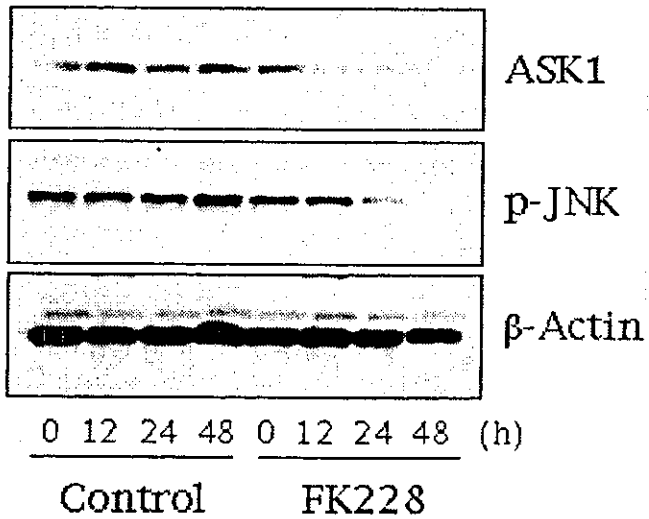


Fig. 5. Effects of depsipeptide (FK228) on the Jun kinase cascade. Whole cell lysates were prepared from HL-60 cells at the indicated time points, and subjected to immunoblot analysis for the expression of ASK1, phosphorylated JNK, and β-actin. The data shown are representative of two independent experiments.

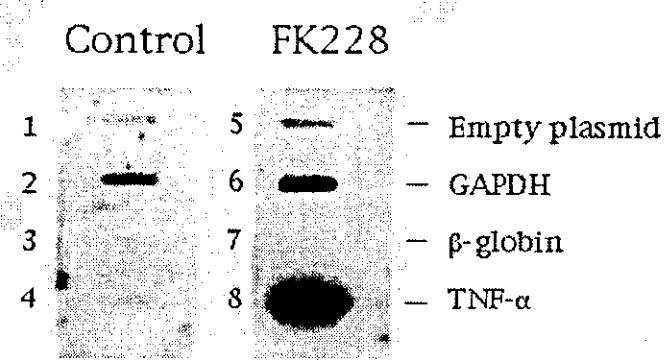


Fig. 6. Nuclear run-on assay for TNF-α transcription in depsipeptide-treated HL-60 cells. Nascent nuclear RNA was elongated in the presence of [$^{32}$P]UTP in HL-60 cells cultured with (control) or without 20 nM depsipeptide (FK228) for 6 h, and hybridized to immobilized plasmids containing cDNAs for GAPDH (lanes 2 and 6), β-globin (lanes 3 and 7), and TNF-α (lanes 4 and 8) on nylon membranes. Empty pCRII vector was used as a negative control (lanes 1 and 5). The data shown are representative of multiple independent experiments.

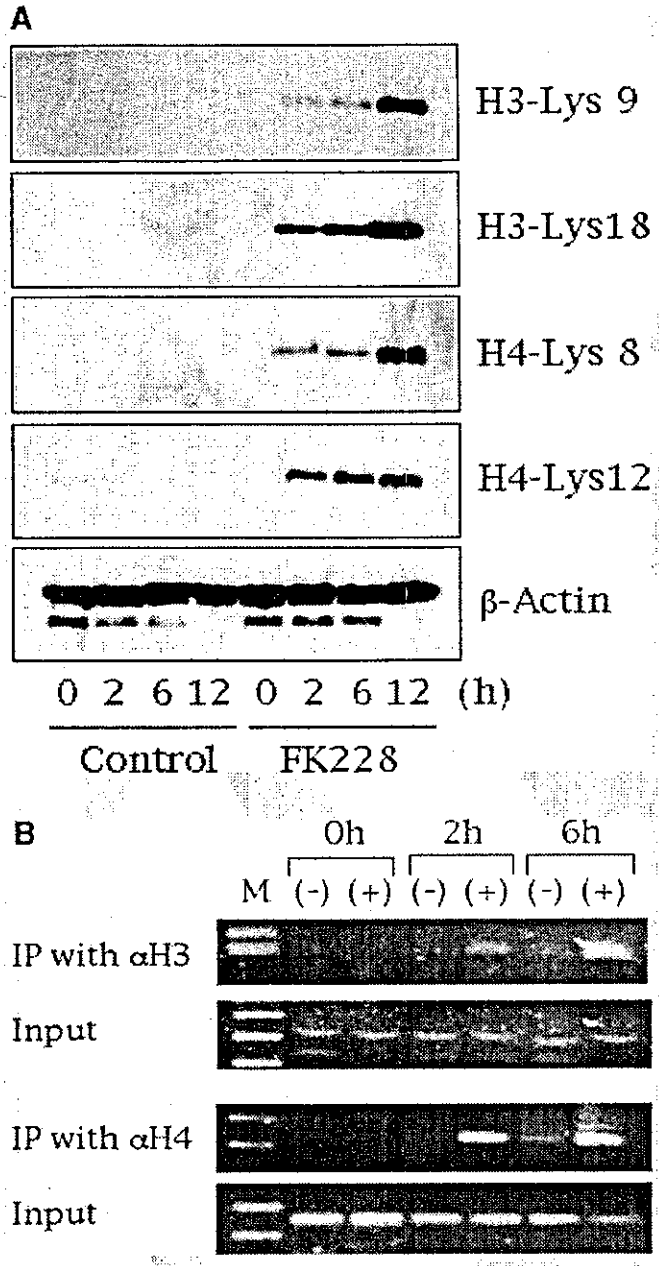


Fig. 7. Depsipeptide-induced hyperacetylation of TNF-α promoter in HL-60 cells. A: Acetylation of histone tails in depsipeptide-treated HL-60 cells. Whole cell lysates were prepared as described in Figure 3, and subjected to immunoblotting with the site-specific anti-acetylated histone antibodies indicated on the right. The membrane filters were reprobed with anti-β-actin antibody to verify the equal loading and integrity of samples. B: ChIP assay for acetylation of TNF-α promoter. After crosslinking with formaldehyde, chromatin suspensions were prepared from HL-60 cells treated with (+) or without (−) depsipeptide for 0, 2, and 6 h, and subjected to immunoprecipitation with antibodies against acetylated histones H3 and H4. The resulting precipitants were subjected to PCR using a specific primer pair corresponding to nucleotide positions −208 to +35 of the TNF-α promoter. PCR was carried out for 30 cycles, and the amplified products were visualized by ethidium bromide staining after 2% agarose gel electrophoresis. Input: Prior to the immunoprecipitation, 1/40 of the sonicated cell suspension was saved and used for PCR after reversal of the crosslinking. The data shown are representative of multiple independent experiments.

factors are involved in the pharmacological actions of depsipeptide (FK228). Our study will provide a clue as to further elucidate the molecular basis of the action of this potential drug for refractory leukemias.

## ACKNOWLEDGMENTS

The study is supported in part by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan, and by the Japan Leukemia Research Fund.

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