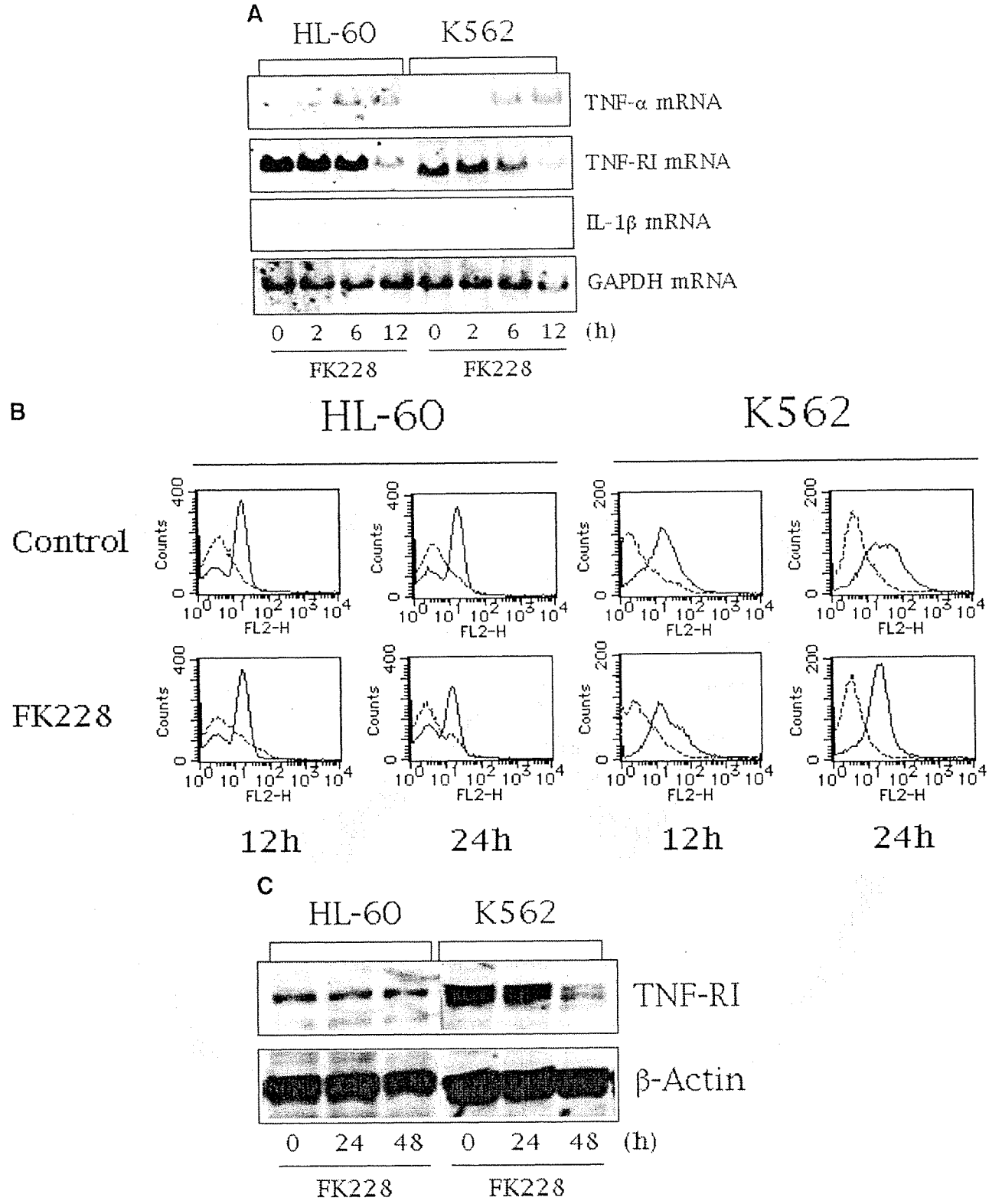

Fig. 2. Expression of TNF-α and its receptors in leukemic cells treated with depsipeptide (FK228). **A**: HL-60 and K562 cells were cultured with 20 nM depsipeptide (FK228) for up to 12 h. Total cellular RNA was isolated at the indicated time points, and subjected to Northern blot analysis for mRNA expression 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.

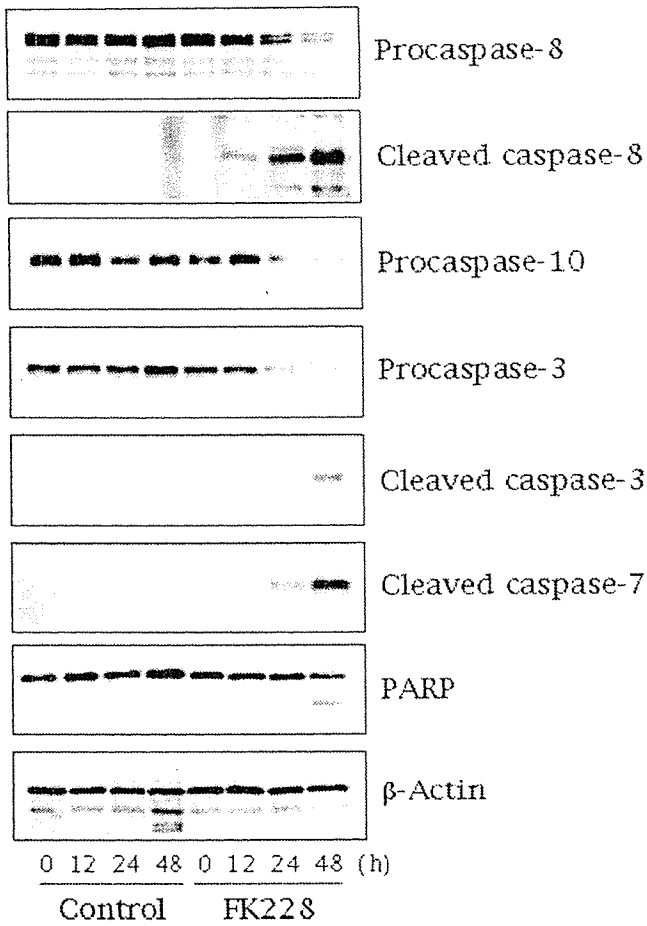

Fig. 3. Activation of the caspase cascade by depsipeptide (FK228) in HL-60 cells. HL-60 cells were cultured in the absence (control) or presence of 20 nM depsipeptide (FK228) for up to 48 h. Whole cell lysates were prepared at the indicated time points, and subjected to immunoblot analysis for procaspases-8, -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

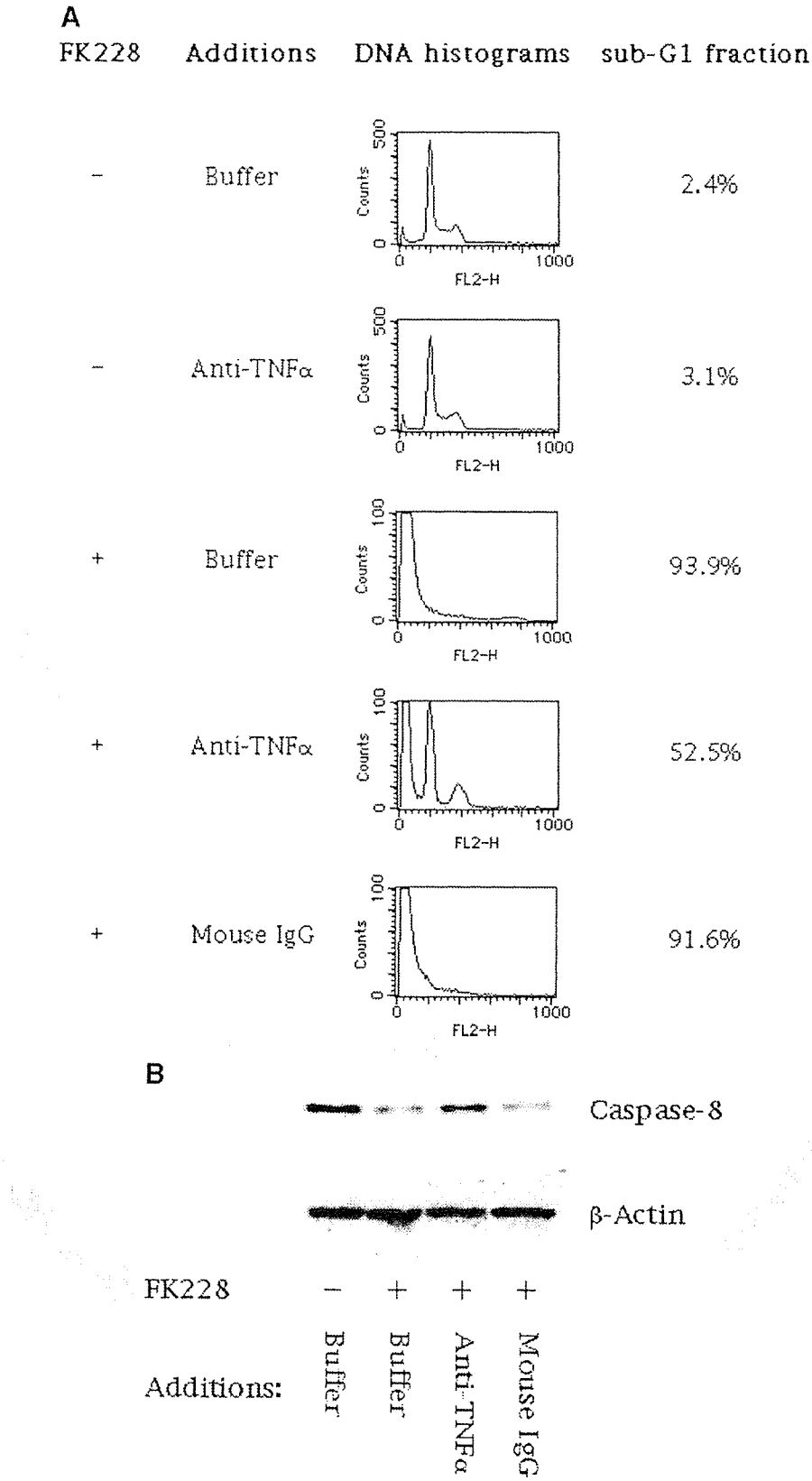

Fig. 4. Effects of a neutralizing anti-TNF-α antibody and siRNA against TNF-RI on the cytotoxicity of depsipeptide (FK228). **A**: HL-60 cells were cultured with either phosphate-buffered saline alone (buffer), purified mouse IgG (mouse IgG) or anti-TNF-α neutralizing antibody (Mab11; BD Pharmingen) (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.

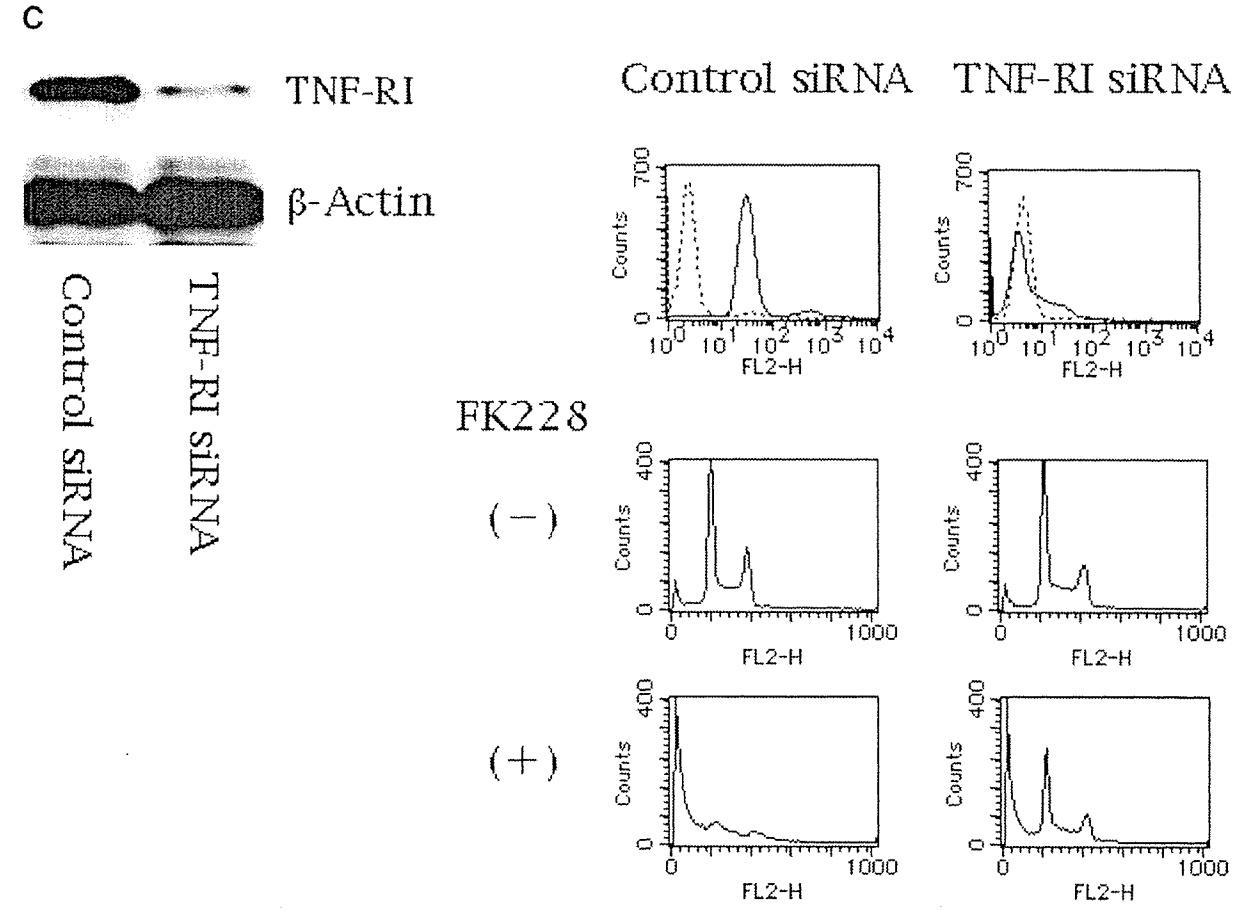

Fig. 4. (*Continued*)

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

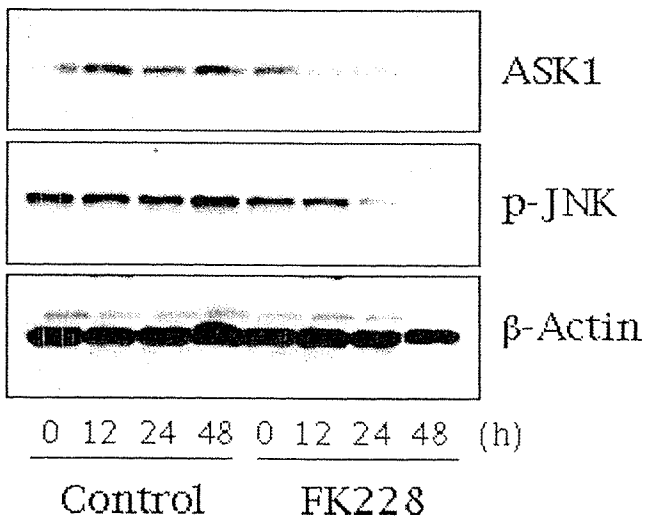

Fig. 5. Effects of depsipeptide (FK228) on the Jun kinase cascade. Whole cell lysates were prepared from HL-60 cells at the indicated time points, and subjected to immunoblot analysis for the expression of ASK1, phosphorylated JNK, and β-actin. The data shown are representative of two independent experiments.

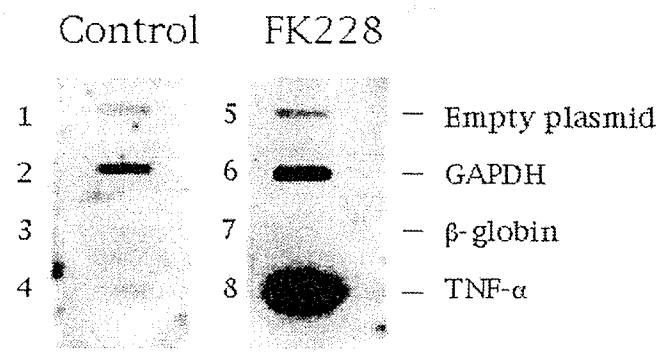

Fig. 6. Nuclear run-on assay for TNF-α transcription in depsipeptide-treated HL-60 cells. Nascent nuclear RNA was elongated in the presence of [^{32}P]UTP in HL-60 cells cultured with (control) or without 20 nM depsipeptide (FK228) for 6 h, and hybridized to immobilized plasmids containing cDNAs for 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.

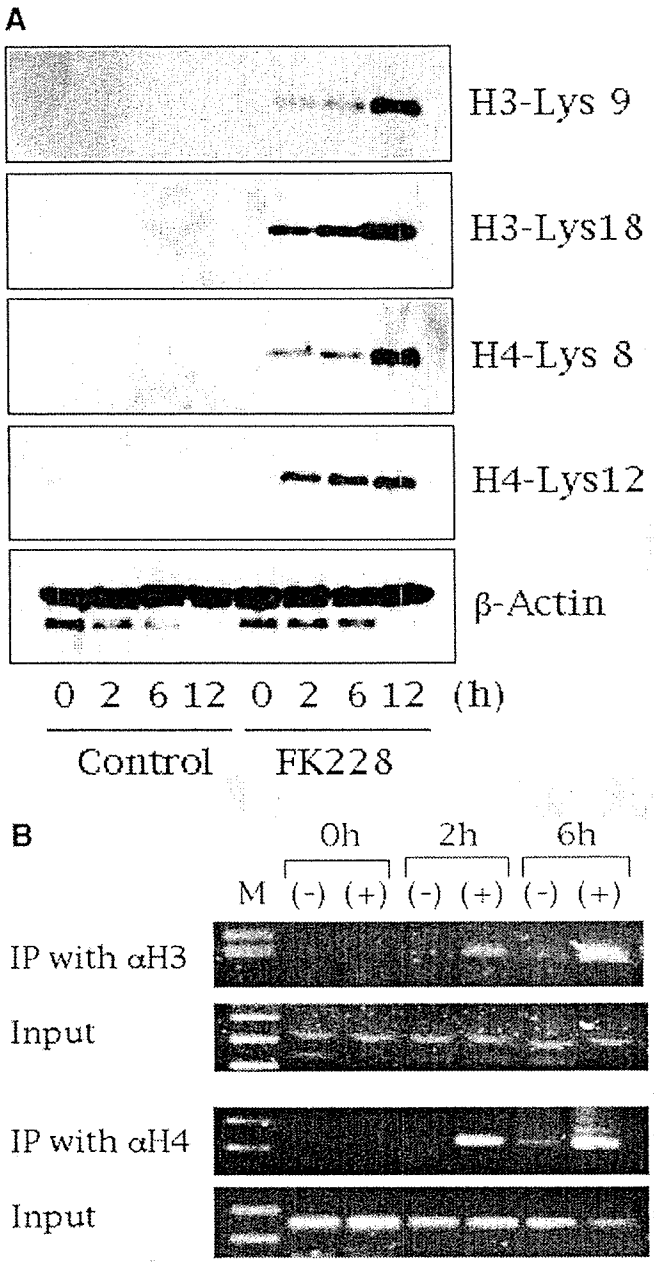

Fig. 7. Depsipeptide-induced hyperacetylation of TNF-α promoter in HL-60 cells. **A**: Acetylation of histone tails in depsipeptide-treated HL-60 cells. Whole cell lysates were prepared as described in Figure 3, and subjected to immunoblotting with the site-specific anti-acetylated histone antibodies 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.

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