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Jun N-Terminal Kinase Pathway Enhances Signaling of Monocytic Differentiation of Human Leukemia Cells Induced by 1,25-Dihydroxyvitamin D_3

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Abstract Recent studies revealed that the MEK/ERK module of the mitogen-activated protein kinase (MAPK) signaling cascades is up-regulated in the early stages of 1α,25-dihydroxyvitamin D_3 (1,25D_3)-induced monocytic differentiation of human leukemia cells HL60. In the present study, we investigated whether another MAPK module, the JNK pathway, also participates in this form of differentiation. We found that the dependence on the concentration of the inducer, the vitamin-hormone 1,25D_3, in two types of human leukemia cells, HL60 and U937, and the kinetics of monocytic differentiation in HL60 cells, parallel the degree of the activation of the JNK pathway. A blockade of JNK signaling by a stable expression of dominant negative (dn) JNK1 mutant in U937 cells resulted in reduced *c-jun* phosphorylation, and the differentiation of these cells was markedly decreased. Similarly, inhibition of JNK1 and JNK2 activities by the selective inhibitor SP600125 led to both dose-dependent reduction of *c-jun* and ATF-2 phosphorylation, and of the differentiation of HL60 cells. In addition, we found that JNK activity is essential for the AP-1 DNA binding induced by 1,25D_3 in HL60 and U937 cells. The results indicate that in cultured human leukemia cells, the JNK pathway participates in the induction of monocytic differentiation by 1,25D_3, probably by activating the AP-1 transcription factor. J. Cell. Biochem. 89: 1087–1101, 2003. © 2003 Wiley-Liss, Inc.

Key words: vitamin D; jun N-terminal kinase; leukemia cells; differentiation; AP-1 transcription factor

HL60 and U937 cell lines are useful in vitro models for studying the differentiation of human leukemia. HL60 cells, derived from a patient with promyeloblastic leukemia with promyelocytic features [Gallagher et al., 1979], can be induced to differentiate along different hematopoietic lineages by a variety of different chemical compounds. For example, HL60 has been shown to undergo macrophage-like differentiation in response to phorbol esters [Rovera et al., 1979], granulocytic differentiation in response to retinoic acid, or dimethylsulfoxide [Collins et al., 1979; Breitman et al., 1980], and sequentially monocytic then macrophage-like differentiation in response to 1,25D_3 derivatives [McCarthy et al., 1983; Tanaka et al., 1983]. U937 cell line is a promonocytic leukemia cell line and can be induced to terminal monocytic differentiation by phorbol esters, 1,25D_3 or retinoic acid [Koren et al., 1979; Olsson et al., 1983]. In many studies, these cell lines have proven to be very valuable for investigating the differentiation mechanisms induced by chemical agents, including 1,25D_3. However, the precise sequence of signals is still not fully understood.

1,25D_3, a metabolite and an active form of vitamin D_3, has been reported to induce leukemia cell differentiation and control apoptosis [McCarthy et al., 1983; Wang and Studzinski, 1997]. It has been suggested that 1,25D_3 can exert its function through a genomic pathway and a non-genomic pathway. In the genomic pathway, 1,25D_3 enters target cells, binds to nuclear vitamin D receptor (VDR) which heterodimerizes with retinoid acid receptor (RXR),

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and then the heterodimer binds to vitamin D response element (VDRE) and initiates gene transcription for the required biological effects [Bouillon et al., 1995]. There may also be non-genomic pathways, like PKC activation [Wali et al., 1990], an increase of intracellular calcium concentration [Lieberherr, 1987], or an involvement of a poorly defined membrane receptor [Nemere et al., 1998; Fleet, 1999], and it is feasible that some or all of these signal by activation of the MAPK pathways. It was recently reported that ras might also activate the JNK as well as the ERK cascades [Davis, 2000; Kennedy et al., 2003]. Accumulating evidence now indicates that all MAPK pathways propagate signals important to the control of normal and neoplastic hematopoiesis [Platanias, 2003].

MAPKs are serine/threonine kinases that mediate numerous types of extracellular stimuli [Cobb and Goldsmith, 1995]. There are three major groups of MAP kinases: the extracellular signal-regulated protein kinases (ERKs); the *c-jun* N-terminus kinases (JNKs); and the p38MAPKs [Karin, 1995]. The ERKs are mainly activated by growth factors via a ras-dependent signal transduction pathway [Egan et al., 1993]. The JNKs and p38MAPKs, also designated stress activated protein kinases (SAPKs), are activated by stress-related stimuli [Mendelson et al., 1996], such as proinflammatory cytokines and environmental stress such as UV light, γ-irradiation, heat shock, osmotic shock, shear stress, growth factor withdrawal, ceramide, and protein synthesis inhibitors [Lee et al., 1998]. Like other MAPKs, JNKs contain the dual phosphorylation motifs and their activation requires phosphorylation at both residues, threonine (Thr-183) and tyrosine (Tyr-185), by upstream regulators such as the MAP kinase kinase SEK1/MKK4 kinase [Davis, 2000].

AP-1 has been demonstrated to regulate the activation of transcription of a variety of genes involved in cell growth, differentiation, and apoptosis [Wisdom, 1999]. Jun, fos family proteins, and ATF-2, which are transcription factors with a leucine zipper structure, form homo- or heterodimeric AP-1 and bind to the palindromic 12-*O*-tetradecanoylphorbol-13-acetate response element (TRE), which is found in the promoter region of many genes, and regulates their expression. The major down-stream effector of JNK is *c-jun*. Phosphorylation of *c-jun* on the sites that are phosphorylated by JNK (Ser-63 and Ser-73) results in increased AP-1 transcription activity. JNK also phosphorylates other AP-1 proteins, including Jun B, Jun D, and ATF-2 [Davis, 2000]. Thus, JNK appears to be a major regulator of AP-1 activation.

Our laboratory has previously found that the MEK/ERK pathway is activated in the early stage of the $1,25D_3$-induced monocytic differentiation of HL60 cells [Wang and Studzinski, 2001a], while an up-regulation of the JNK/*c-jun* pathway, which is enhanced by the inhibition of the p38MAPK pathway by inhibitors SB203580 and SB202190, was observed during monocytic differentiation induced by $1,25D_3$ [Wang and Studzinski, 2000]. However, a role for JNK activity in differentiation was not previously demonstrated. In the present study, we obtained evidence for the participation of the JNK pathway in monocytic differentiation induced by $1,25D_3$.

MATERIALS AND METHODS

Reagents

$1,25D_3$ (a kind gift from Dr. Milan Uskokovic, BioXell, Inc., Nutley, NJ) was prepared in absolute ethanol in a stock solution at 2.5×10^{-4} M. SP600125 was a generous gift from Signal Research Division, Celgene Corporation. Sigma supplied dimethyl sulfoxide (DMSO). Geneticin (G418 sulfate) was from Life Technologies (Rockville, MD). pcDNA3/dnJNK1 was a kind gift from Dr. R.J. Davis (University of Massachusetts, Worcester). The antibodies to Crk-L (c-20, rabbit polyclonal), and anti-rabbit IgG-HRP polyclonal antibody were purchased from Santa Cruz Co. (Santa Cruz, CA). Antibodies used to detect phospho-SAPK/JNK (Thr183/Tyr185, rabbit polyclonal), phospho-*c-jun* (Ser-63, rabbit polyclonal), phospho-ATF-2 (Thr-71, rabbit polyclonal), SAPK/JNK (rabbit polyclonal), *c-jun* (rabbit polyclonal), ATF-2 (rabbit polyclonal), and JNK assay kit were purchased from Cell Signaling Technology (Beverly, MA).

Cells and Cell Culture

HL60-G cells, a subclone of human promyeloblastic leukemia HL60 cells [Gallagher et al., 1979], and human promonocytic leukemia U937 cells [Sundstrom and Nilsson, 1976], were grown at 37°C in suspension culture in RPMI 1640 medium (Mediatech, Washington, DC) supplemented with 1% glutamine and 10% heat-inactivated, iron-enriched bovine calf serum (Hyclone, Logan, UT). The HL60 cells and U937

cells were passaged and fed 2–3 times weekly to maintain log phase growth. The cells were usually seeded at 2×10^5 cells/ml of fresh medium for experiments. The JNK inhibitor SP600125 was added to the cultures for the indicated times at the specified concentrations 1 h before the exposure to $1,25D_3$. Each experiment was repeated at least three times.

Assessment of Differentiation

The expression of differentiation markers CD14 and CD11b was determined by flow cytometry. Aliquots of 1×10^6 cells were harvested at indicated times, washed twice with PBS, then incubated for 45 min at room temperature with 0.5 μl MY4-RD-1 and 0.5 μl MO1-FITC (Coulter, Miami, FL) each containing 0.5 μg of the antibody, to detect the expression of surface cell markers CD14 and CD11b, respectively. The cells were then washed three times with ice cold 1× PBS, and resuspended in 0.5 ml 1× PBS. Two parameters analysis was performed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA), with CELL Quest software (Verity Software House, Inc., Topsham, ME). Isotypic mouse IgG1 was used to set threshold parameters for flow cytometry.

Monocytic differentiation was also monitored by cytochemical determination of the cytoplasmic enzyme monocytic serine esterase (MSE) activity, usually referred to as non-specific esterase (NSE). Smears were made by resuspending 2×10^6 cells in 100 μl 1× PBS and spreading on slides. The air-dried smears were fixed in formaldehyde and acetone mixture buffer for 30 s, then washed and stained for 45 min at room temperature with the following solution: 0.067 M sodium phosphate buffer, pH 7.6, 8.9 ml, hexazotized pararosaniline, 0.6 ml, 10 mg alpha-naphthyl acetate, and 0.5 ml ethylene glycol monomethyl ether. The MSE-positive cells were determined by counting 500 cells in each group.

Preparation of Cell Extracts

HL60 cells and U937 cells were collected by centrifugation and washed twice with ice-cold phosphate-buffered saline (PBS). Whole cell extracts were prepared by lysing the cells in protein lysis buffer consisting of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 1 mM phenylmethysulfonyl fluoride (PMSF). The cell lysates were centrifuged at 15,000*g* for 20 min. The supernatant was used for immunoblotting and for measurements of the activities of the protein kinases.

Nuclear extracts were prepared as previously described [Andrews and Faller, 1991]. All steps were performed at 4°C. Briefly, 2×10^7 cells were harvested and washed twice with ice-cold PBS, and resuspended in 0.2 ml ice-cold cell extract buffer (10 mM HEPES–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KC1, 0.5 mM DDT, and 0.2 mM PMSF). The cells were kept on ice for 10 min, vortexed for 10 s, and microfuged at 16,000*g* at 4°C for 30 s. The supernatant was saved as the cytoplasmic extract, and the pellet was resuspended in 20–40 μl of nuclear extraction buffer (20 mM HEPES–KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgC1_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF), placed on ice for 20 min, and centrifuged at 4°C at 16,000*g* for 2 min. The supernatant was saved as the nuclear extract and stored at −80°C.

Electrophoretic Mobility Shift Assay (EMSA)

The AP-1 oligonucleotide was 5′-end labeled by phosphorylation with T4 polynucleotide kinase in the presence of [γ-^{32}P] ATP (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The gel mobility shift assay was performed by using Stratagene Gelshift Assay Kit (La Jolla, CA). Nuclear extracts (10 μg) from HL60 or U937cells cells were incubated with 20 pg ^{32}P-labeled AP-1 probe 5′TAAAGCATGAGTCAGACACC-TC-3′ or 5-CGCTTGATGAGTCAGCCG GAA-3 (Santa Cruz Co.) carrying a consensus TRE sequence (underlined), respectively, in a binding buffer (1 μg poly (dI-dC), 20 mM HEPES, pH 7.9, 5 mM $MgC1_2$, 50 mM KC1, 1 mM DTT, 0.25 mg/ml BSA, 4% glycerol) at room temperature for 30 min. Competition was performed with tenfold or 50-fold excess of the unlabeled AP-1 consensus oligonucleotide, as indicated. The samples were then separated on a 4% polyacrylamide gel with a constant current of 22 mA for 3 h. The gel was dried and exposed to Kodak X-Omat LS film.

Western Blot Analysis

Whole cell lysates containing 40 μg of protein were subject to 10% SDS–PAGE gel electrophoresis and the proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Inc.). The membranes were blocked

with 5% non-fat milk in TBS with 0.1% (w/v) Tween 20 for 1 h at room temperature, probed for 1 h with the specified primary antibodies, and incubated with a horseradish-linked secondary antibody for 1 h. Bands of proteins on the membrane were visualized using a chemiluminescence assay system (Amersham Pharmacia Biotech, Inc.). The membranes were then stripped according to the manufacturer's protocol (Amersham Pharmacia Biotech, Inc.). The optical density (OD) of each band was quantitated using an image quantitator (Molecular Dynamics, Sunnyvale, CA).

JNK Kinase Assay

Two micrograms of recombinant N-terminal (amino acids 1–89) *c-jun* fusion protein bound to glutathione Sepharose beads were mixed with 250 μg of protein prepared from whole cell lysate of HL60 cells treated as indicated for individual experiments. This allowed the beads to selectively pull down SAPK/JNK from the cell lysates. The mixture was incubated with gentle rocking overnight at 4°C. The samples were then microcentrifuged for 30 s at 4°C, and the pellets were washed twice with 500 μl of

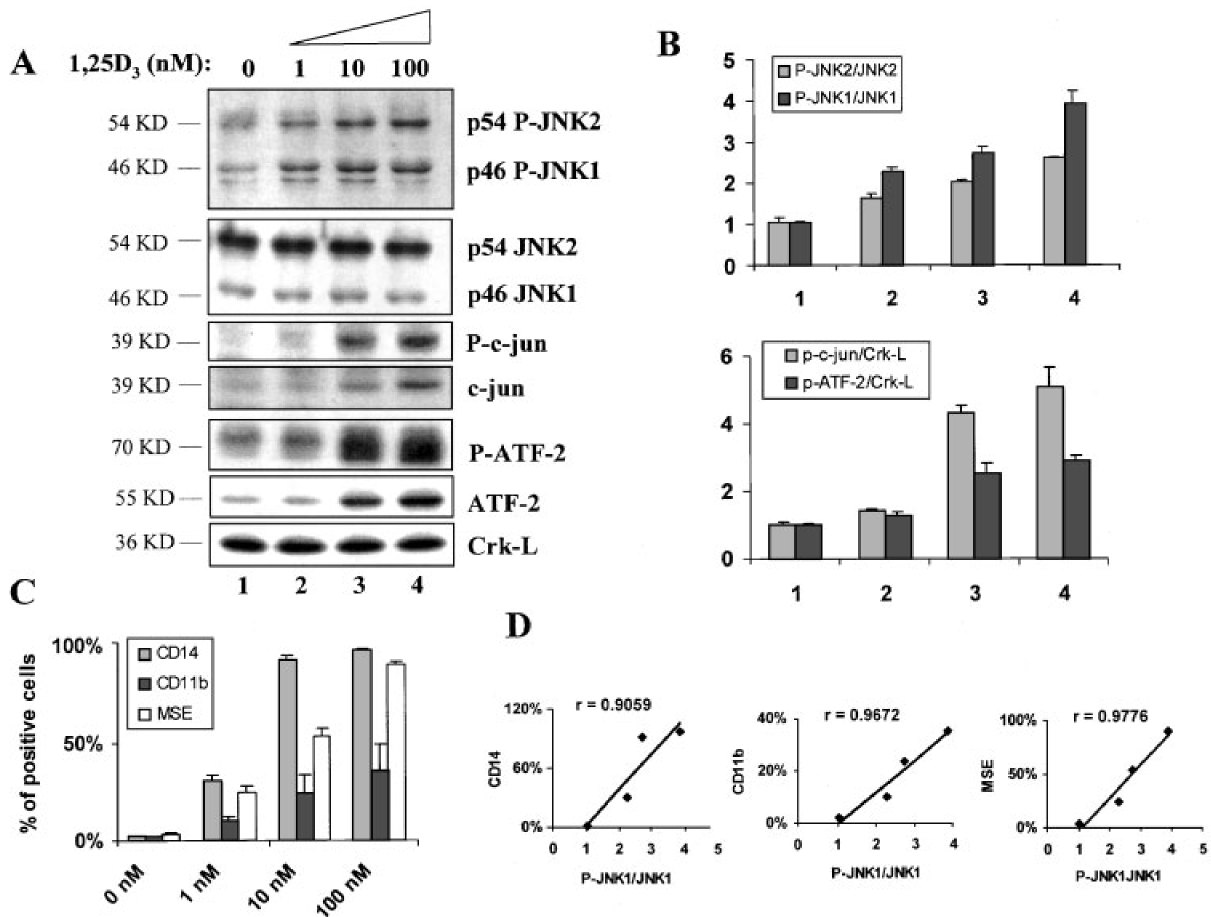


Fig. 1. Activation of JNK pathway correlates with monocytic differentiation induced by $1,25D_3$ in HL60 cells in a dose dependent manner. **A**: HL60 cells were treated for 48 h with $1,25D_3$ at the indicated concentrations. Proteins obtained from whole cell lysates were subjected to 10% SDS–PAGE gel electrophoresis, transferred onto nitrocellulose membranes, and incubated with the indicated antibodies, which were reprobed for Crk-L protein as a loading control. **B**: Optical densities (OD) of the protein levels were determined by Fluorimage with image Quant Software (Molecular Dynamics, Sunnyvale, CA) as described in Materials and Methods. The OD ratios of p46 P-JNK1 to total JNK1, p54 P-JNK2 to total JNK2, *P-c-jun* to Crk-L, and P-ATF-2 to Crk-L were plotted for the different concentration groups. Arithmetical means ± SD are shown, n = 3. **C**: The proportion of HL60 cells positive for monocytic markers CD14 and CD11b were determined by flow cytometry as described in Materials and Methods, and MSE was determined by counting 500 cells in each group. **D**: Correlation analysis: mean (n = 3) ratios for P-JNK1/JNK1 versus percentage of cells positive for differentiation markers CD14, CD11b, or MSE were plotted as XY scatter plot. Correlation coefficients (r) are shown above each graph. Similar analyses are summarized in Table I.

TABLE I. Activation of JNK Correlates With Monocytic Differentiation Induced by $1,25D_3$

Cells	Differentiation markers	Correlation coefficient (r)	
		JNK1	JNK2
HL60	CD14	0.9059	0.9430
	CD11b	0.9672	0.9887
	MSE	0.9776	0.9912
U937	CD14	0.9276	0.4981
	CD11b	0.9521	0.4539

Correlation analysis was performed using mean (n = 3) ratios of P-JNK1/JNK1 or P-JNK2/JNK2 versus percentage of cells positive for differentiation markers CD14, CD11b, or MSE.

1× lysis buffer and twice with 500 μl 1× kinase buffer and kept on ice.

The pellet that contained beads with the immunoprecipitated JNK and 2 μg of *c-jun* fusion proteins was suspended in 50 μl 1× kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM beta-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) supplemented with 100 μM ATP, and incubated for 30 min at 30°C. The reaction was terminated with 25 μl 3× SDS sampling buffer, boiled for 5 min, vortexed, and then microcentrifuged for 2 min. Samples (25 μl) were loaded on 10% SDS–PAGE gel and analyzed by Western blotting using phospho-GST-*c-jun* antibody.

Stable Transfections

Transfections were performed by electroporation with a Gene Pulser (Bio-Rad, Hercules, CA). U937 cells (2×10^6) were transfected with 20 μg of pcDNA3/dnJNK1 or pcDNA3 empty vector by electroporation (0.24 kV, 950 μF). After culture for 24 h, the cells were selected in the presence of 700 μg/ml geneticin, and drug

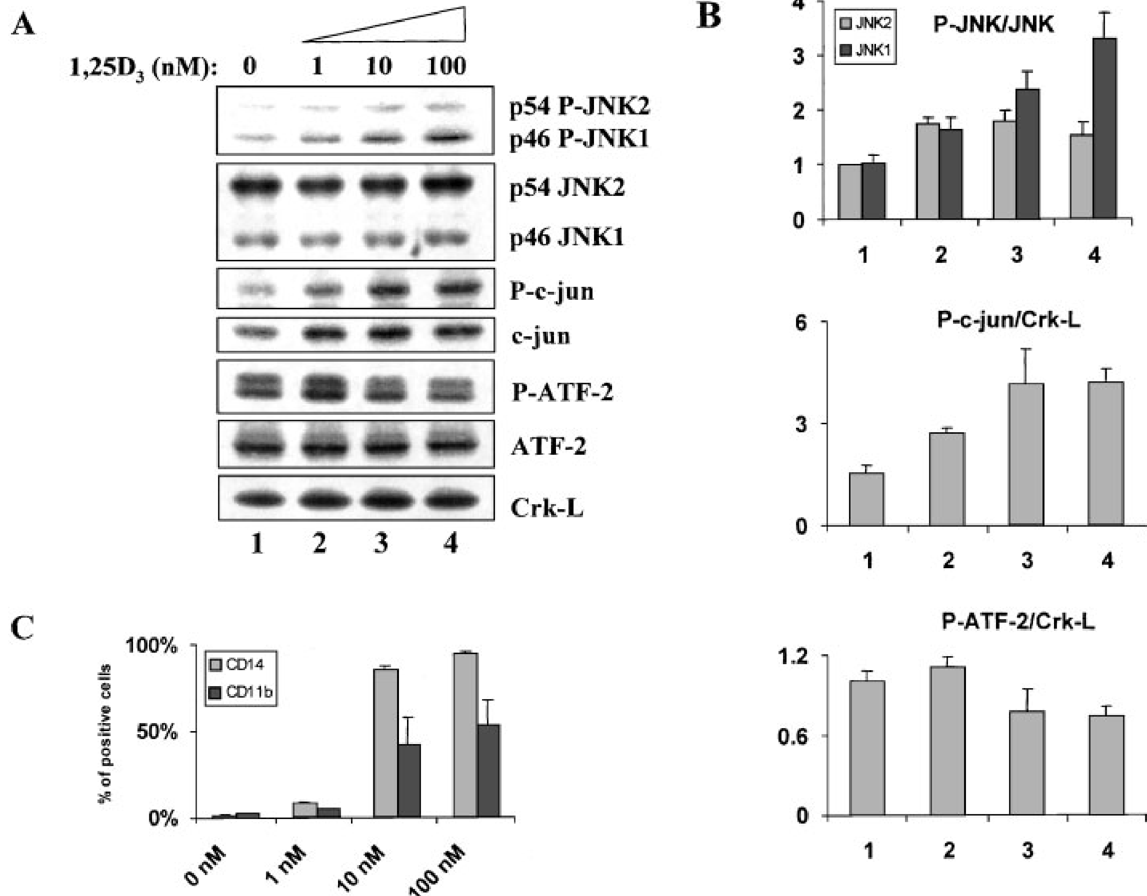


Fig. 2. Activation of JNK-1 and *c-jun* correlates in a dose dependent manner with differentiation induced by $1,25D_3$ in U937 cells. **A**: U937 cells were treated for 48 h with $1,25D_3$ at the indicated concentrations. Immunoblotting was performed as outlined in legend for Figure 1. **B**: The OD ratios for p46 P-JNK1 to JNK1, p54 P-JNK2 to JNK2, *P-c-jun* to Crk-L, and P-ATF-2 to Crk-L were plotted for the different $1,25D_3$ concentration groups. Arithmetical means ± SD are shown, n = 3. **C**: The proportion of HL60 cells positive for monocytic markers CD14 and CD11b determined by flow cytometry.

resistant cells were then subcloned by limiting dilution. The expression of FLAG-dnJNK was detected by RT-PCR.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total mRNA was extracted from 5×10^6 cells by using the RNeasy Kit (Quiagen, Valencia, CA). For RT-PCR, a 20-µl master mix of RT was prepared as follows: 5 mM $MgCl_2$, PCR buffer II (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 1 mM dNTPs, 1 U/µl RNase inhibitor, 2.5 µM random hexamers, a 1 µg sample of total cellular RNA, and diethyl pyrocarbonate-treated distilled water. The master mix was incubated in the Perkin-Elmer GeneAmp PCR system 9600 (Roche, Branchburg, NJ) at 42°C for 15 min and then at 99°C for 5 min and at 4°C for 5 min. After RT, 78 µl of PCR master mix containing 2 mM $MgCl_2$, PCR buffer II, 2.5 U/µl AmpliTaq DNA polymerase, and the following primers was added at a 0.15 µM concentration: *c-jun*, upstream primer (5′-GGATCAAGGCGGAG-AGGAAG-3′), downstream primer(5′-GCGTT-AGCATGAGTT-GGCAC-3′); β-actin, upstream primer (5′-TGACGGGGTCACCCACACTGTG-CCCAG-CTA-3′), downstream primer (5′-CTA-GAAGCATTTGCCGGTGGACGATGGAGGG-3′); FLAG-dnJNK1: upstream primer: (5′-TAT-AAGGACGATGATGACAAA-3′), downstream primer: (5′-TTTCTGTGGTGTGAAAACATT-3′). The complementary DNAs (cDNAs) in samples were amplified in the GeneAmpPCR System 9600 as follows: 10 min at 95°C as an initial step, followed by 35 cycles of 30 s each at 95°C, 30 s each at 55°C, and 45 s at 72°C; and then finally 7 min at 72°C. The RT-PCR products were separated on 2% agarose gels containing 0.5 µg/ml ethidium bromide. The intensities of the bands corresponding to *c-jun* and β-actin were measured by Image QuaNT Program (Molecular Dynamics).

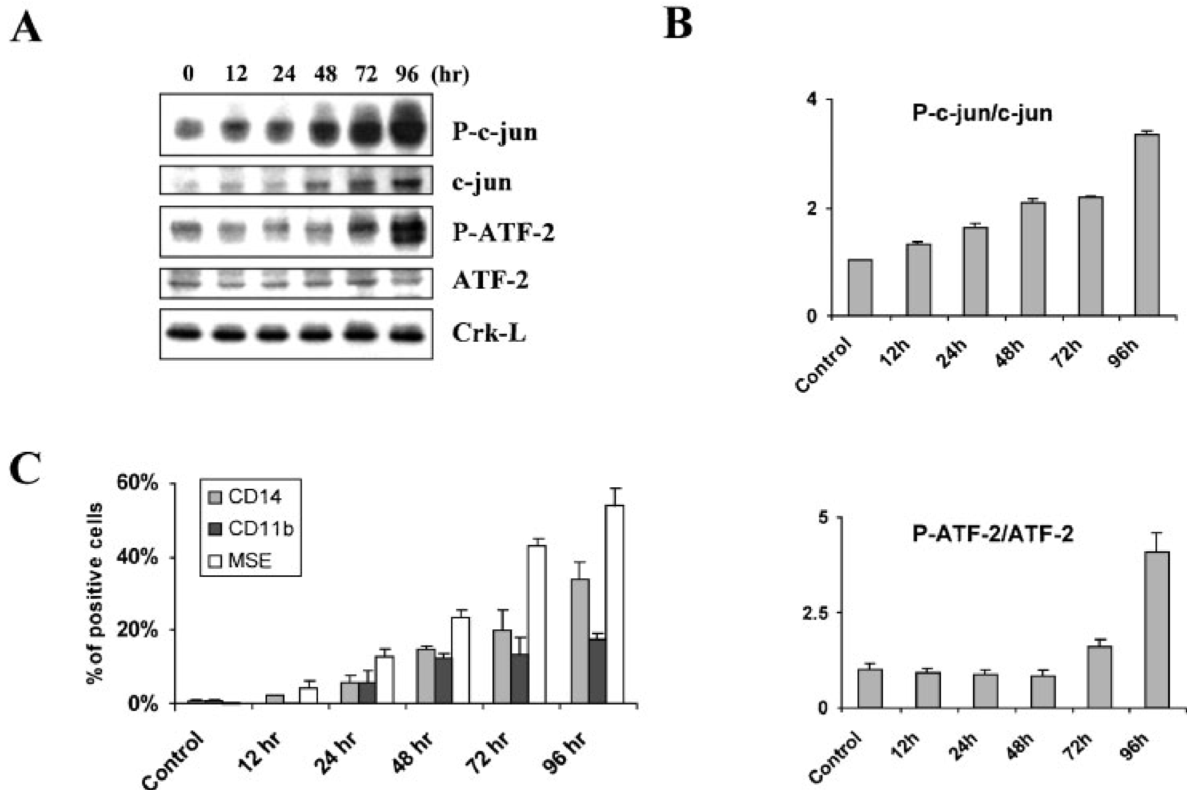


Fig. 3. The kinetics of activation of *c-jun* correlate with differentiation induced by $1,25D_3$ in HL60 cells. **A**: Immunoblots showing the time course of the activation of JNK pathway by $1,25D_3$. HL60 cells were treated with $1,25D_3$ (1 nM) for the indicated times, and Western blots were performed as outlined in legend for Figure 1. **B**: The ratios of OD for the signals of *P-c-jun* to *c-jun* and of P-ATF-2 to ATF-2 were plotted for the different time points. Means ± SD are shown, n = 3. **C**: The proportion of cells positive for monocytic markers CD14 and CD11b as determined by flow cytometry, and MSE were determined by counting 500 cells at each time point.

Statistical Analysis

All experiments were repeated at least three times. Differences between the means of various subgroups were assessed by two-tailed Student's *t*-test. Calculation of correlation coefficients (r) and other computations were performed with an IBM-compatible personal computer using Microsoft EXCEL program.

RESULTS

Monocytic Differentiation Induced by $1,25D_3$ Parallels the Up-Regulation of the JNK Pathway

Exposure of human myeloid leukemia cells HL60 or U937 to $1,25D_3$ results in the acquisition of a monocyte/macrophage-like phenotype, including surface markers CD14 [Wright et al., 1990; Zhang et al., 1994] and CD11b [Drayson et al., 2001]. These markers can be conveniently and accurately measured by flow cytometry, thus allowing a quantitative comparison of the differentiation response to $1,25D_3$ determined by flow cytometry with changes in gene expression. Figure 1 illustrates such a comparison. As shown in Figure 1C, HL60 cells differentiate in response to $1,25D_3$ at concentration as low as 1 nM, and increasing the concentration of $1,25D_3$ to 10 nM results in a near-maximal differentiation after 48 h of treatment, with almost all cells expressing the CD14 (the LPS receptor), and a lower proportion of cells expressing CD11b (β2-integrin) and the cytoplasmic marker "non-specific esterase" [Yam et al., 1971]. Also, the two principal isoforms of JNK that are expressed in these cells, p46 (JNK1) and p54 (JNK2), showed increased levels of phosphorylation, indicative of their activation [Davis, 2000], and increases in the abundance of phosphorylated forms of transcription factors *c-jun* and ATF-2 were also seen (Fig. 1A,B). Remarkably, there was a very tight correlation between the expression of the differentiation markers

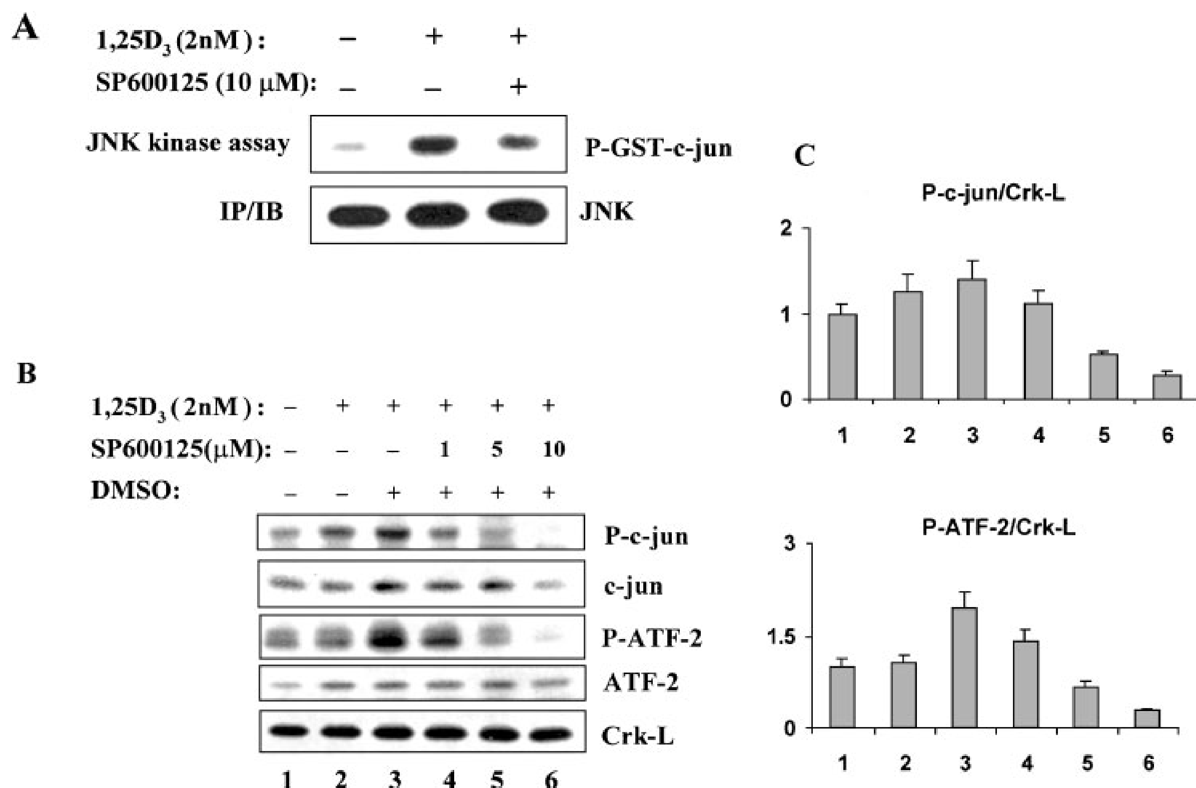


Fig. 4. Effect of SP600125 on JNK pathway. **A**: HL60 cells were treated for 72 h as follows: Control, $1,25D_3$ (2 nM) and $1,25D_3$ (2 nM) + SP600125 (10 μM). Total JNK in whole cell lysates was "pulled down" by incubation with 2 μg of N-terminal (amino acids 1–89) *c-jun* fusion protein bound to glutathione Sepharose beads. The kinase assay was then performed by adding 100 μM ATP, and SP600125 was added again to group 3 in vitro. The samples were loaded on SDS–PAGE 10% gel and analyzed by Western blotting using phospho-GST-*c-jun* antibody, and then reprobed by using JNK antibody. **B**: HL60 cells were pretreated in the absence or presence of 1, 5, or 10 μM SP600125, or pretreated with DMSO vehicle (0.1%) for 1 h, and then treated with $1,25D_3$ (2 nM) for 72 h. Cells were harvested and Western blots were performed as in Figure 1. **C**: The ratios of OD of *P-c-jun* to Crk-L and P-ATF-2 to Crk-L were plotted for the different groups. Means ± SD are shown, n = 3.

and ratios of P-JNK1 to JNK1, and P-JNK2 to JNK2, as illustrated in Figure 1D and summarized in Table I. Similarly, in U937 cells, $1,25D_3$ also increased phosphorylation of JNK isoforms and of *c-jun* in a dose-dependent manner (Fig. 2A,B), and phosphorylation of JNK1 also correlates with the effects of $1,25D_3$ on differentiation of these cells (Fig. 2C and

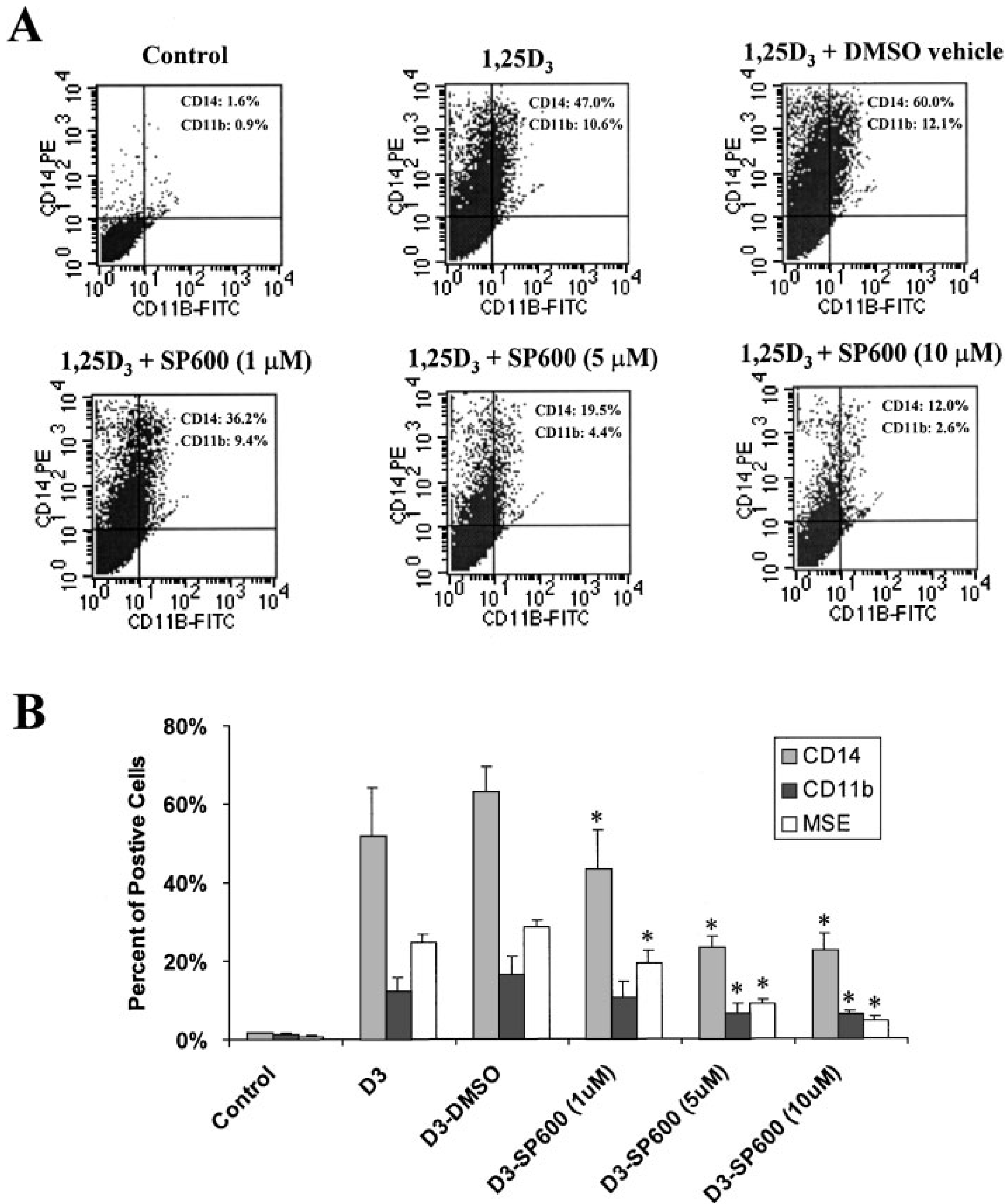


Fig. 5. Effect of SP600125 on differentiation induced by $1,25D_3$. **A**: HL60 cells were pretreated with 0.1% DMSO, the vehicle for SP600125, or with 1, 5, or 10 μM SP600125 for 1 h, and then $1,25D_3$ (2 nM) was added and the cells incubated for 72 h. The expression of monocytic differentiation markers CD14 and CD11b was then determined by flow cytometry, (**B**) summary of the percentages of cells exhibiting the indicated monocyte differentiation markers in the group of experiments illustrated in A. MSE was determined by counting 500 cells in each group. Means ± SD are shown, n = 3. The experimental groups marked with an asterisk were significantly reduced compared to groups treated with $1,25D_3$ and DMSO vehicle (*$P < 0.05$).

Table I). There was, however, a difference between these two cell lines, in that the activation of JNK2 did not correlate with differentiation in U937 cells (Table I), and the JNK target ATF-2 was not activated by 1,25D$_3$ exposure (Fig. 2A,B).

Examination of the time course of differentiation showed that evidence of differentiation can be detected in HL60 cells as early as 12 h at 1 nM concentration of 1,25D$_3$ (Fig. 3C), and this temporally corresponds to the activation of the JNK signaling pathway, as shown by the phosphorylation of *c-jun* (Fig. 3A,B). In contrast, increased phosphorylation of ATF-2 was only detected late, at 72 h of exposure to 1,25D$_3$. Thus, there is a strong correlation between 1,25D$_3$-induced monocytic differentiation and the activation of the JNK signaling pathway, which is particularly apparent at the level of *c-jun* phosphorylation, while phosphorylation of ATF-2 appears to be a secondary event in differentiation.

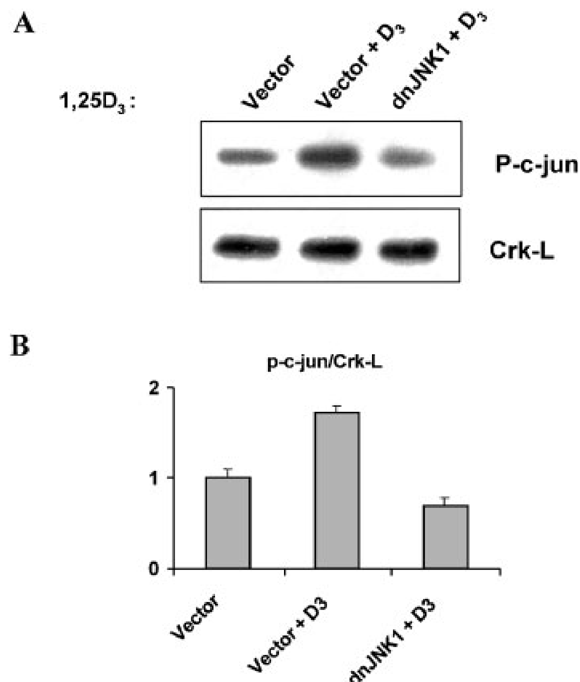


Fig. 6. Expression of a dominant negative (dn) JNK construct inhibits phosphorylation of *c-jun* induced by 1,25D$_3$ in U937 cells. **A**: U937 cells stably transfected with either an empty vector (pcDNA3) or pcDNA3–dnJNK1 were treated for 48 h with 1,25D$_3$ (2 nM). Protein from whole cell extracts were separated by 10% SDS–PAGE gel, transferred to a nitrocellulose membrane, detected by *P-c-jun* antibody, then the membrane was reprobed for the constitutively expressed protein Crk-L. **B**: The ratios of OD of *P-c-jun* to Crk-L were plotted for the different concentration groups. Means ± SD are shown, n = 3.

Down-Regulation of the JNK Pathway Activity by a Pharmacological Inhibitor Decreases Leukemia Cell Differentiation

A specific inhibitor of JNK activity, SP600125, has recently been synthesized [Bennett et al., 2001], and we confirmed its inhibitory activity in the HL60 cell system studied here. This anthrapyrazolone inhibited the in vitro JNK kinase activity immuno-precipitated from HL60 cells (Fig. 4A), or JNK kinase activity determined "in vivo" by the phosphorylation state of *c-jun* or ATF-2 in HL60 cells treated with graded concentrations of SP600125 (Fig. 4B,C). Reduced JNK kinase activity also resulted in lower steady-state levels of proteins that are constituents of the AP-1 transcription factor, i.e., *c-jun* and ATF-2 (Fig. 4B). Consistent with the results of the experiments described above, the SP600125 JNK inhibitor also significantly ($P < 0.05$) inhibited 1,25D$_3$-induced monocytic differentiation of HL60 cells in a concentration-dependent manner (Fig. 5). However, we were unable to detect inhibition by SP600125 of JNK pathway activation in intact U937 cells, or any effect on 1,25D$_3$-induced differentiation in these cells (data not shown).

Differentiation of U937 Cells Induced by 1,25D$_3$ Is Inhibited by the Expression of a Dominant-Negative JNK Construct

In order to extend the generality of the concept that JNK pathway is involved in monocytic differentiation, we stably transfected U937 cells, which are more easily transfected than HL60 cells, with a dominant negative (dn) construct of JNK. This construct expresses a mutated protein, which can bind to *c-jun* but does not phosphorylate it, thus preventing JNK kinase activity in a dn fashion [Derijard et al., 1994]. Multiple clones were generated, which expressed different levels of FLAG-dnJNK, as determined by RT-PCR (described in Materials and Methods). In contrast to an empty vector transfectant pcDNA3, the FLAG positive dn JNK1 clones did not show any evidence of activation of the JNK pathway following treatment with 1,25D$_3$ (Fig. 6), and 1,25D$_3$-induced differentiation of the FLAG positive clones was markedly inhibited (Fig. 7). Cumulatively, the data presented here strongly indicate that an active JNK pathway is either required for, or enhances, 1,25D$_3$-induced monocytic differentiation of human leukemia cells.

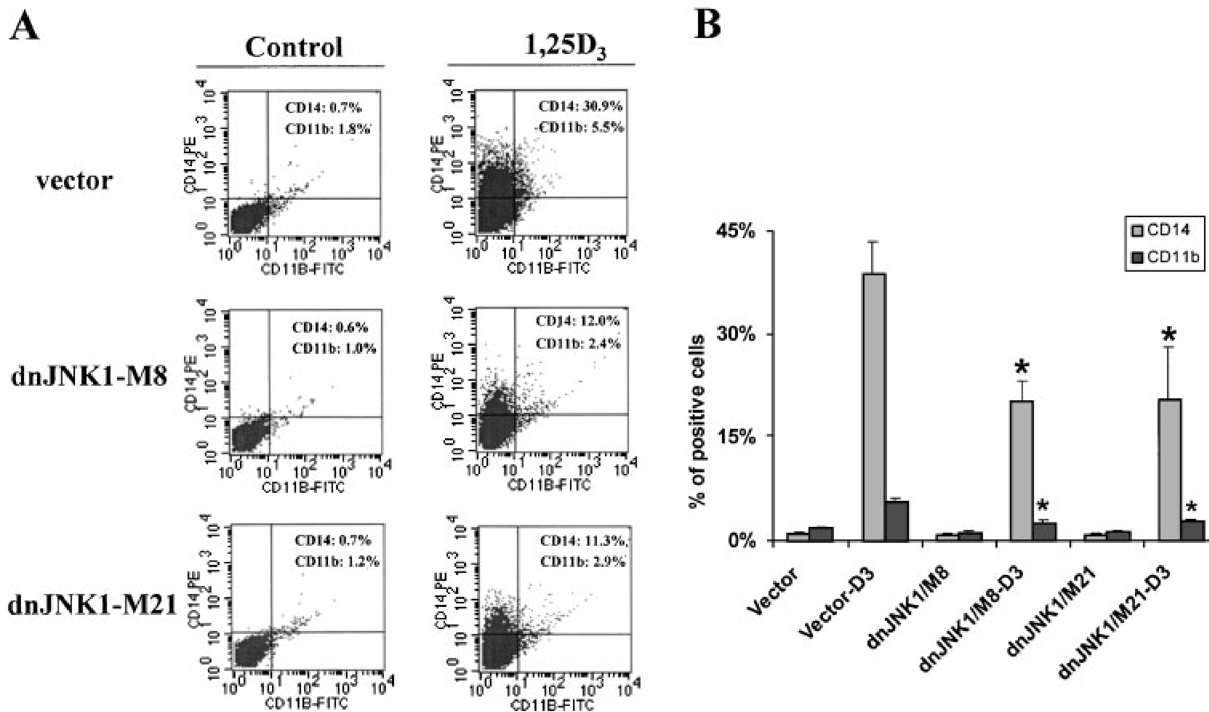


Fig. 7. Inhibition by dnJNK1 of differentiation induced by $1{,}25D_3$ in U937 cells. **A**: U937 cells stably transfected with the pcDNA3 empty vector or two clones (M8 and M21) stably transfected with pcDNA3–dnJNK1 were exposed to 2 nM $1{,}25D_3$ for 48 h, and the expression of CD14 and CD11b was determined by flow cytometry. **B**: Histogram summarizing three experiments, one of which is illustrated in A. Means ± SD are shown. The experimental groups marked with an asterisk were significantly reduced compared to cells transfected with empty vector and treated with $1{,}25D_3$ ($^*P < 0.05$).

JNK Pathway Regulates the Activity of AP-1 Transcription Factor in Differentiating Leukemia Cells

It has already been established that AP-1 activity is increased in HL60 cells induced to differentiate by $1{,}25D_3$ [Wang and Studzinski, 2001b]. To determine if this activity is under the direct control of JNK, as suggested by changes in *c-jun* and ATF-2 phosphorylation that accompany $1{,}25D_3$-induced differentiation described above, we inhibited JNK activity by SP600125 in HL60 cells and by the dn JNK construct in U937 cells (Fig. 8), and found inhibition of AP-1 binding to DNA, an indication of the activity of this transcription factor. Further evidence that JNK signals through AP-1 in this system was obtained by the demonstration of the down-regulation of *c-jun* mRNA expression in SP600125-treated HL60 cells (Fig. 9A,B), and in dnJNK-transfected U937 cells (Fig. 9C,D). Since *c-jun* gene is positively regulated by AP-1 [Karin, 1995], it can be considered a reporter gene as illustrated in for AP-1 activity in Figure 10. Thus, the data show that AP-1 activation by $1{,}25D_3$ leads to increased gene expression that appears to include differentiation-related gene products.

DISCUSSION

The studies presented here demonstrate an important role for the JNK pathway in $1{,}25D_3$-induced monocytic differentiation of human leukemia cells. Why would a pathway well known for mediating stress-related responses be activated in monocytic differentiation? One possible answer is that the acquisition of the monocytic phenotype may include preparations for stress that will result from events associated with phagocytosis by the mature monocyte, such as the release of reactive oxygen species. Thus, up-regulation of the JNK pathway may be a part of a complex network of changes in the cellular circuitry that occur when the gears are shifted from the rapid proliferation of the primitive precursor cell to the proliferative quiescence of the monocyte, and gene expression is re-directed to the functional repertoire of the mature cells. In this context, it should be

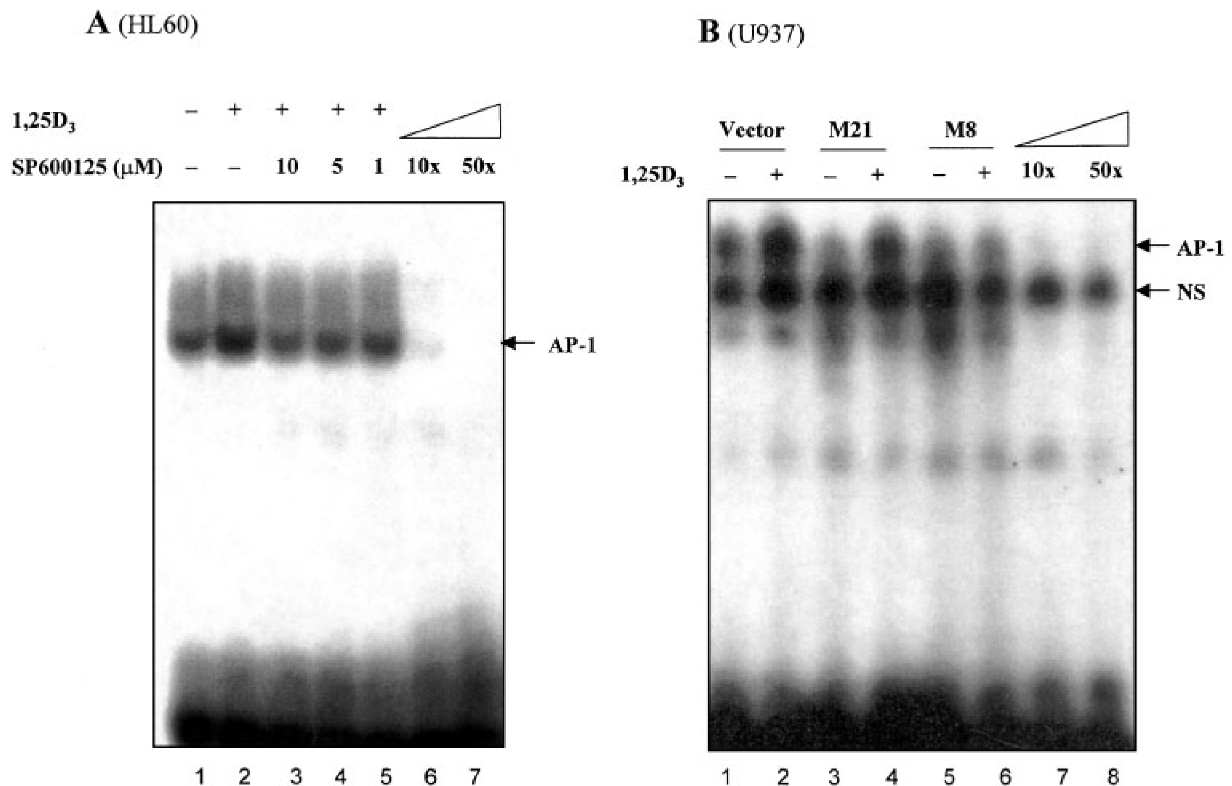
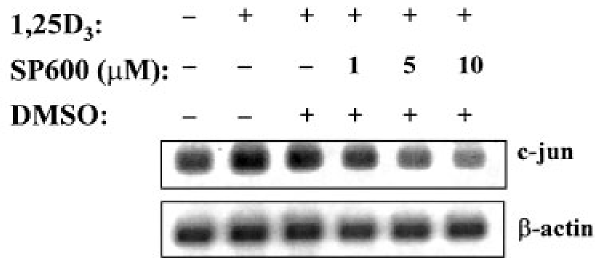
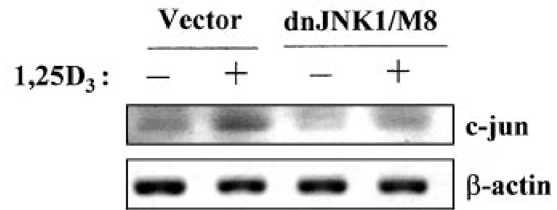


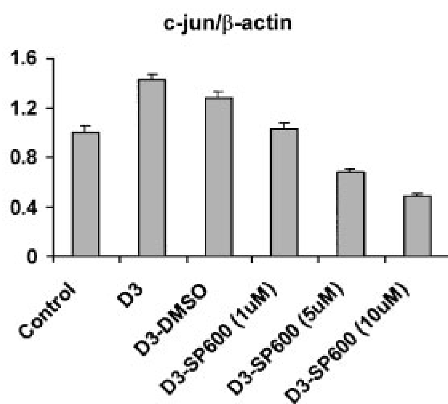
Fig. 8. Blockade of JNK activaty by SP600125, or stable transfection of dnJNK1, reduces the AP-1 DNA binding induced by $1,25D_3$ in HL60 and U937 cells. **A**: Extracts of nuclear proteins were prepared from HL60 cells treated for 72 h with $1,25D_3$ (2 nM) and SP600125 (10 μM), as indicated. Electrophoretic mobility shift assay were performed by incubation of the nuclear extracts with ^{32}P-labeled double-stranded oligonucleotides cognate to TRE, a consensus binding sequence for AP-1, as described in Materials and Methods. The arrow indicates the oligonucleotide–AP-1 complex. In competition assays, nuclear extracts from cells treated with $1,25D_3$ (2 nM) alone were used. Competition was performed with tenfold (**lane 6**) or 50-fold (**lane 7**) excess of unlabeled AP-1 consensus oligonucleotides. **B**: Extracts of nuclear proteins were prepared from pcDNA3 empty vector stably transfected U937 cells, or pcDNA3–dnJNK1 transfected clones M8 and M21, which were treated with 2 nM $1,25D_3$ for 48 h as indicated by (+). Electrophoretic mobility shift assays were performed as in Figure 8A. In competition assays, nuclear extract from empty vector pcDNA3 transfected cells treated with $1,25D_3$ (as **lane 2**) were used. Competition was performed with 10-fold (lane 7) or 50-fold (**lane 8**) excess of unlabeled AP-1 consensus oligonucleotides. NS, non-specific bands.

noted that the markers of differentiation utilized in this study have functional significance for the mature monocyte. CD14 serves as a receptor for a bacterial endotoxin, lipopolysaccharide (LPS), CD11b contributes to cell adhesion properties, and MSE, a monocyte-specific esterase, is one of the hydrolytic enzymes that aid digestion of phagocytosed material [Yam et al., 1971; Wright et al., 1990; Zhang et al., 1994; Drayson et al., 2001]. Interestingly, connections between LPS, CD14 signaling, and the JNK pathway have already been established in normal macrophages [Aderem and Ulevitch, 2000].

There are other examples of the involvement of JNK pathway in differentiation or in functions of differentiated cells. For instance, differentiation of rat neuronal cells PC12 was accompanied by persistent activation of JNKs, and over-expression of the JNK target *c-jun*-induced neurite outgrowths, a sign of differentiation, in these cells [Heasley et al., 1996]. Ectopic expression of *c-jun* also resulted in differentiation of P19 embryonal carcinoma cells [de Groot et al., 1990] and WEH1-3BD^{+} mouse myelocytic leukemia cells [Li et al., 1994], while expression of dn JNK blocked endodermal differentiation of P19 cells by retinoic acid [Jho et al., 1997]. Activation of JNK, together with p38 MAP kinase was observed in erythroid differentiation of mouse erythroleukemia cells induced to differentiate with erythropoietin [Nagata et al., 1998], or by environmental stress [Nagata and Todokoro,







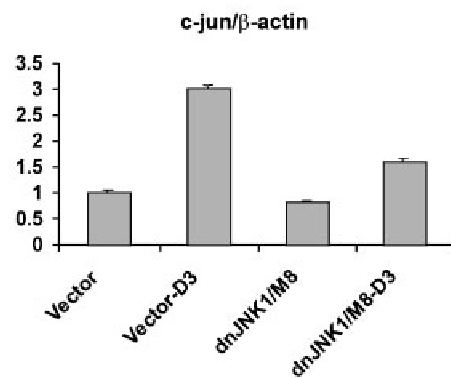


Fig. 9. Effect of SP600125 and of dnJNK1 transfection of on transcriptional expression of *c-jun* gene induced by $1,25D_3$ in human leukemia cells. **A**: HL60 cells were pretreated with 0.1% DMSO, the vehicle for SP600125, or with 1, 5, or 10 μM SP600125 for 1h, and then $1,25D_3$ (2 nM) was added and the cells incubated for 72 h. The cells were harvested and total RNA was extracted. The reverse transcription polymerase chain reaction for *c-jun* gene was performed, and the levels of β-actin transcripts were determined in the sample as internal controls. **B**: The ratios of (OD) of *c-jun* to β-actin were plotted for the different groups. Means ± SD are shown, n = 3. **C**: pcDNA3 empty vector stably transfected U937 cells or pcDNA3–dnJNK1 transfectant clones M8 were treated for 48 h in the absence or presence of $1,25D_3$ (2 nM). The reverse transcription polymerase chain reaction for *c-jun* gene was performed as in (A). **D**: The ratios of OD of *c-jun* to β-actin were plotted for the different groups. Means ± SD are shown, n = 3.

1999]. In mutant mice, T-cell differentiation was defective in the absence of JNK1 [Dong et al., 1998], and human $CD4^+$ lymphocytes were prevented from activation and differentiation by treatment with the JNK inhibitor SP600125 [Bennett et al., 2001], while expression of collagenase by cells derived from ascitic fluid of a patient with malignant adenocarcinoma of the ovary was inhibited by SP600125 [Han et al., 2001; Shin et al., 2002]. Thus, the rather unexpected link of the stress associated pathways to differentiation and to differentiated cellular functions is an emerging concept that deserves further, more detailed, exploration.

We were able to perform complementary experiments using two human myeloid leukemia cell lines, HL60 promyeloblastic cells [Gallagher et al., 1979] and U937 promonocytic cells [Sundstrom and Nilsson, 1976]. Each of these lines presents special experimental problems that are easier to overcome in the other line; for instance it is exceedingly difficult if not impossible to develop stably transfected clones of HL60 cells, but this can be accomplished using U937 cells. Conversely, HL60 cells take up the pharmacological agent SP600125, which inhibits JNK activity within the cells (Fig. 4), but we were unable to observe such inhibition in U937 cells (data not shown). However, we found that inhibition of JNK activity either by a dn construct or by a pharmacological inhibitor of its activity results in markedly inhibited differentiation (Figs. 5 and 7). In both cell lines, differentiation correlated with levels of phosphorylation of *c-jun* and AP-1 binding to TRE, but JNK2 and ATF-2 activation was found only in HL60 cells. These differences may reflect

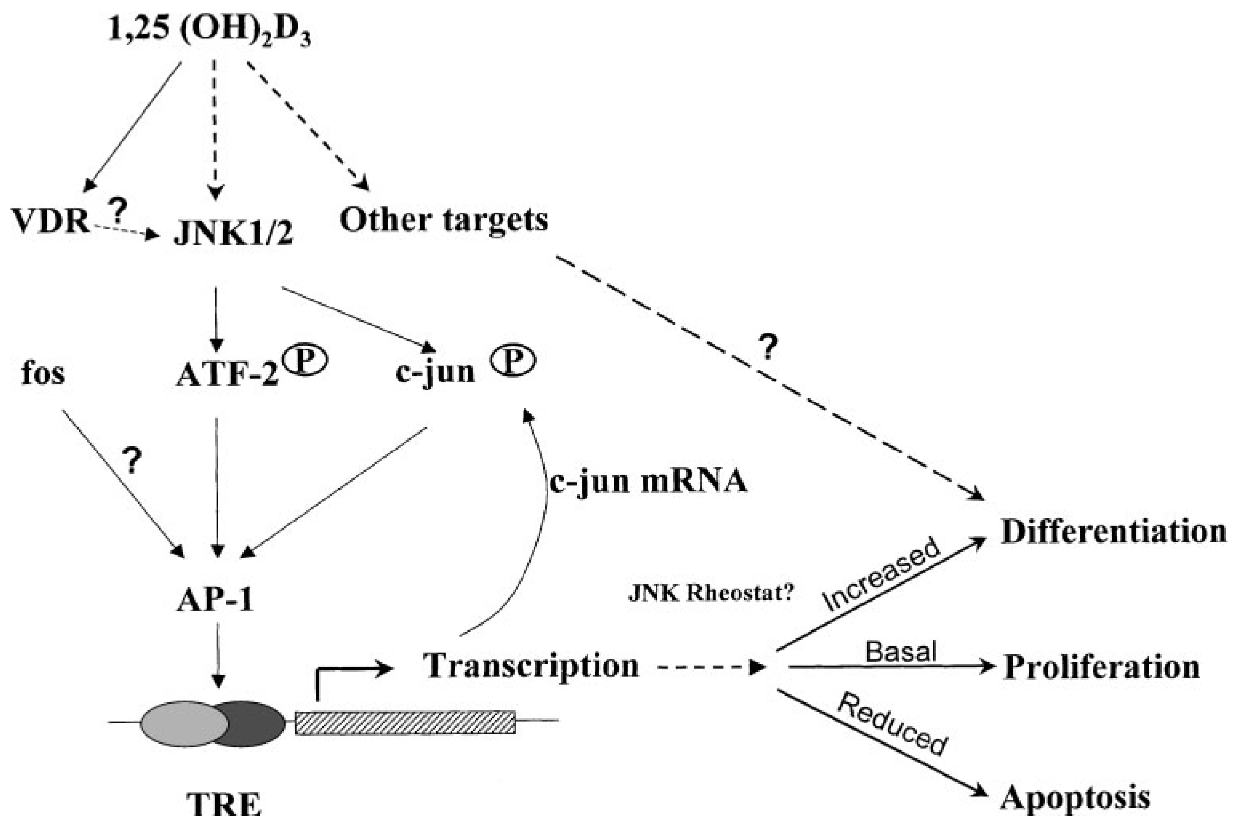


Fig. 10. Schematic representation of the findings presented here and their interpretation in the context of known mechanisms of $1,25D_3$ action. Vitamin D receptor (VDR) is activated by $1,25D_3$, and (combined with RXR) becomes a functional transcription factor, which activates genes involved in calcium homeostasis, as well as other, largely unknown, targets. One result of treatment with $1,25D_3$ is activation of the JNK MAPK pathway, which activates the AP-1 transcription factor, composed of phosphorylated proteins including *c-jun* and ATF-2 in the leukemia cells studied here. Since transcription of *c-jun* gene is under AP-1 control, *c-jun* mRNA expression can serve as a reporter of AP-1 activity. This activity at its basal level allows cells cycle progression and thus proliferation, and inhibition of this activity leads to death of leukemia cells as also reported for PC12 cells [Leppa et al., 2001], presumably due to a diminished capacity to deal with environmental stress. Conversely, increased JNK–AP-1 activity sensitizes leukemia cells to other signals provided by $1,25D_3$, resulting in enhanced monocytic differentiation. Dotted lines represent pathways not known at this time (Modified from Chen et al., 1999).

random individual variations between patients from whom the leukemia lines were derived, or different levels of progression along the monocytic differentiation lineage of the founder stem cells from which the lines originated. The finding of highly increased levels of *c-jun* phosphorylation in differentiating HL60 cells is consistent with a high level of signal amplification in the JNK cascade.

In this study, we were theoretically able to block the activity of all isoforms of JNK, of which there are at least 10 [Gupta et al., 1996; Davis, 2000], since both dn JNK and SP600125 interfere with all of these activities [Derijard et al., 1994; Bennett et al., 2001]. In reality, only three isoforms of JNK were detected on our immunoblots, the p54 JNK2, and two approximately p46 isoforms, generally referred to as JNK1. According to studies in human monocytic leukemia cells THP-1, the p54 isoform is JNK2α2, and the p46 isoforms are JNK1β1 and JNK2α1 [Dreskin et al., 2001]. No major differences were observed between these in HL60 cells, but in U937 cells the activation of one of the p46 isoforms clearly correlated with differentiation. The complexity of these isoforms precluded further analysis of these changes.

It remains unclear if an active JNK is absolutely essential for $1,25D_3$-induced differentiation, as we were unable to completely suppress its expression. Attempts to do so by increasing the concentration of SP600125 resulted in extensive cytotoxicity, suggesting that complete absence of JNK pathway activity is not compatible with cell survival. A scenario consistent with our results is illustrated in Figure 10; basal

levels of JNK pathway activity are posited to allow survival and cell proliferation, repression of JNK activity to increase the probability of apoptosis or another form of cell death [Leppa et al., 2001], while increased JNK activity appears to enhance other pathways, still unknown, that lead to monocytic differentiation. Further studies of the monocyte differentiation system may lead to better understanding of the distinctive roles of the signaling mediated by the JNK in the context of the simultaneous activation of other pathways.

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