ARTICLE

Journal of Cellular Biochemistry 108:612–620 (2009)

Journal of Cellular Biochemistry

JNK1/c-Jun and p38α MAPK/ATF-2 Pathways Are Responsible for Upregulation of Fas/FasL in Human Chronic Myeloid Leukemia K562 Cells Upon Exposure to Taiwan Cobra Phospholipase A_2

Ku-Chung Chen, Yi-Ling Chiou, and Long-Sen Chang*

Institute of Biomedical Sciences, National Sun Yat-Sen University-Kaohsiung Medical University Joint Research Center, Kaohsiung 804, Taiwan

ABSTRACT

Fas and FasL expression upregulation was found in human leukemia K562 cells upon exposure to *Naja naja atra* phospholipase A_2 (PLA_2). PLA_2 treatment induced an increase in intracellular Ca^{2+} ($[Ca^{2+}]i$) and ROS generation levels, leading to activation of p38 MAPK and JNK. Suppression of both p38 MAPK and JNK abrogated Fas and FasL upregulation. Unlike PLA_2, catalytically inactive PLA_2 treatment did not markedly increase Fas and FasL protein expression, and p38 MAPK activation was exclusively responsible for catalytically inactive PLA_2-induced increase in Fas and FasL protein expression. Knockdown of p38α MAPK and JNK1 by siRNA proved that p38α MAPK and JNK1 were involved in ATF-2 and c-Jun phosphorylation, respectively. Compared with the p38α MAPK/ATF-2 pathway, the JNK1/c-Jun pathway played a crucial role in Fas/FasL upregulation. Unlike arachidonic acid, lysophosphatidylcholine mimicked the PLA_2 action in inducing Fas/FasL upregulation. Together with the previous finding that c-Jun and ATF-2 are involved in transcriptional regulation of Fas and FasL, our data suggest that PLA_2 induces Fas and FasL upregulation through p38α MAPK/ATF-2 and JNK1/c-Jun pathways in K562 cells, and PLA_2 catalytic activity is involved in this action. J. Cell. Biochem. 108: 612–620, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: PHOSPHOLIPASE A_2; p38α MAPK; JNK1; ATF-2; c-Jun; Fas/FasL UPREGULATION

Death receptors are members of the TNF receptor gene super family, which consists of more than 20 proteins with a broad range of biological functions including the regulation of cell death and survival, differentiation or immune regulation [Ashkenazi and Dixit, 1998; Krammer, 2000; Walczak and Krammer, 2000]. The best-characterized death receptors comprise Fas (CD95/APO-1), TNF receptor 1 (TNFRI), TRAIL-R1 and TRAIL-R2. Among them, the Fas/FasL system is a key signal pathway involved in apoptosis regulation in several different cell types [Walczak and Krammer, 2000]. Recent studies showed that *Naja naja atra* phospholipase A_2 (PLA_2)-induced apoptotic death of acute myeloid leukemia U937 cells was related to Fas and FasL upregulation [Liu et al., 2009a]. Given that the primary cause of treatment failure in acute myeloid leukemia is usually the emergence of multi-drug resistance [Schneider et al., 1995], therapeutic agents that restore the sensitivity of leukemic cells to apoptotic stimuli are potentially useful for treating leukemia [Wang, 2003; Testa and Riccioni, 2007]. Chronic myeloid leukemia is characterized by the Philadelphia chromosome, which results from a reciprocal translocation between chromosome 9 and chromosome 22 [Faderl et al., 1999]. This mutant gene encodes the constitutively active Bcr/Abl tyrosine kinase, which signals downstream to a variety of cytoprotective pathways including ERK, Akt, NF-κB and Jak/STAT [Hochhaus et al., 2002; Donata et al., 2004]. Although targeted therapy has been suggested to improve the efficacy of leukemia therapy, blockading cytoprotective pathways does not always succeed in treating chronic myeloid leukemia [Kuriakose, 2005; De Poeta et al., 2008]. Thus, understanding the molecular mechanism responsible for the transcriptional regulation of Fas and FasL may aid in developing novel therapeutic strategies for chronic myeloid leukemia. The present study was carried out in the hope of elucidating (1) whether *Naja naja atra* PLA_2 treatment led to Fas and FasL upregulation in chronic myeloid leukemia K562 cells; and (2) the underlying mechanism involved in transcriptional regulation of Fas and FasL genes after PLA_2 treatment.

Grant sponsor: National Science Council, ROC; Grant number: NSC98-2320-B110-002-MY3; Grant sponsor: National Sun Yat-Sen University-Kaohsiung Medical University Joint Research Center.

*Correspondence to: Prof. Dr. Long-Sen Chang, Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung 804, Taiwan. E-mail: lschang@mail.nsysu.edu.tw

Received 6 May 2009; Accepted 30 June 2009 • DOI 10.1002/jcb.22293 • © 2009 Wiley-Liss, Inc.

Published online 7 August 2009 in Wiley InterScience (www.interscience.wiley.com).

MATERIALS AND METHODS

Phospholipase A_2 (PLA_2) from the venom of *Naja naja atra* (Taiwan cobra) was isolated as previously described [Chang et al., 1998]. Recombinant PLA_2(D49K) were prepared according to our published procedure [Liu et al., 2009b]. PLA_2 activity was measured spectrophotometrically using the PLA_2 activity kit from Cayman Chemical (Ann Arbor, MI), and PLA_2(D49K) did not show significantly detectable enzymatic activity [Liu et al., 2009b]. *N*-Acetylcysteine (NAC), arachidonic acid, lysophosphatidylcholine (LysoPC), MTT, SB202190 and SP600125 were obtained from Sigma-Aldrich Inc., and BAPTA-AM, Fluo-4 AM and dichlorodihydrofluorescein diacetate (H_2DCFDA) were the products of Molecular Probes. Anti-Fas (N-18) and anti-phospho-c-Fos (Ser374) antibodies were obtained from Santa Cruz. Anti-FasL, anti-p38 MAPK, anti-p38α MAPK, anti-p38β MAPK, anti-phospho-p38 MAPK, anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-c-Jun, anti-phospho-c-Jun (Ser73), Anti-c-Fos, anti-ATF-2 and anti-ATF-2 (Thr71) antibodies were obtained from Cell Signaling Technology. Anti-β-actin antibodies were obtained from Chemicon. Anti-cytochrome c antibodies were purchased from BD Pharmingen, and horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce. Cell culture supplies were purchased from GIBCO/Life Technologies Inc. Unless otherwise specified, all other reagents were of analytical grade.

CELL CULTURE

Human chronic myeloid leukemic cell line K562 was obtained from ATCC (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1% sodium pyruvate, 2 mM glutamine and penicillin (100 units/ml)/streptomycin (100 μg/ml) in an incubator humidified with 95% air and 5% CO_2. For pharmacological experiments, K562 cells were pre-treated with 1 μM SB202190, 10 μM SP600125, 2 mM NAC, or 10 μM BAPTA-AM for 1 h before PLA_2 was added.

DETECTION OF Fas AND FasL mRNA EXPRESSION BY REVERSE TRANSCRIPTASE-PCR

Total RNA was isolated from untreated control cells or PLA_2-treated cells using the RNeasy minikit (QIAGEN Inc., Valencia, CA) according to the instructions of the manufacturer. Reverse transcriptase reaction was performed with 2 μg of total RNA using

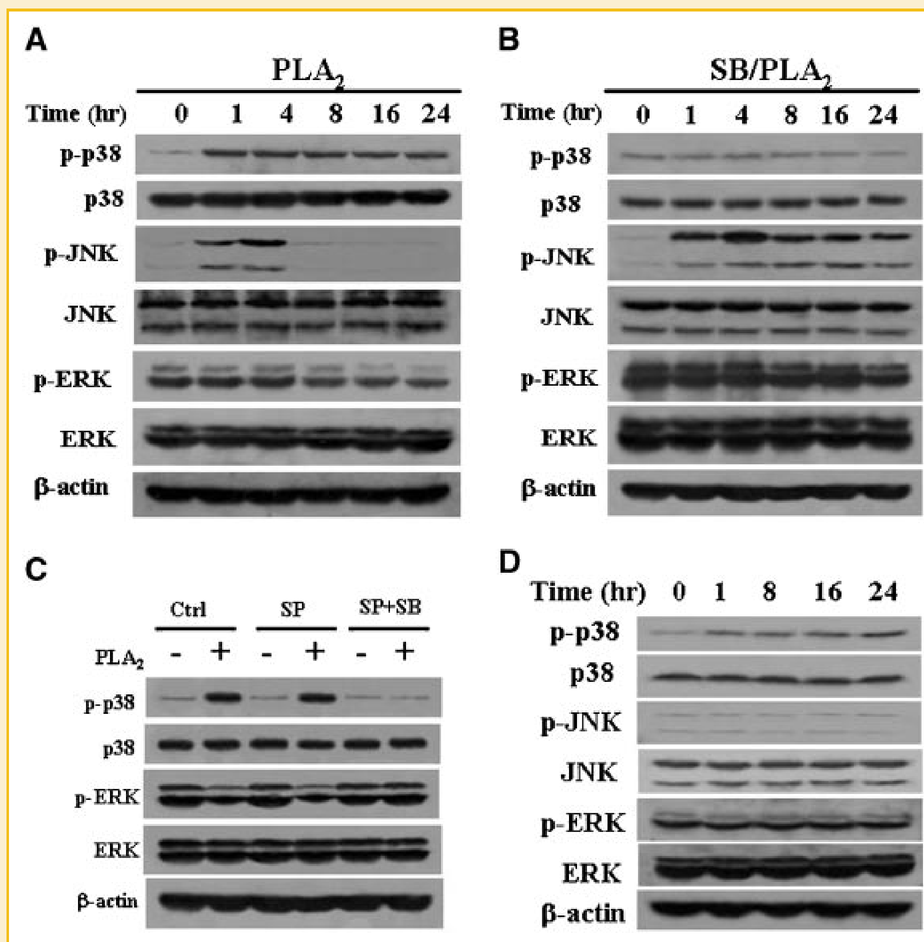

Fig. 1. Western blot analyses of phospho-ERK, phospho-p38 MAPK and phospho-JNK in K562 cells after treatment with PLA_2, PLA_2(D49K) or a combination of SB202190 and PLA_2. A: K562 cells were treated with 3 μM PLA_2 for indicated time periods. B: K562 cells were pretreated with 1 μM SB202190 (p38 MAPK inhibitor) for 1 h, and then incubated with 3 μM PLA_2 for indicated time periods. C: K562 cells were pretreated with 10 μM SP600125 (JNK inhibitor) or a combination of 1 μM SB202190 and 10 μM SP600125 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. D: K562 cells were treated with 10 μM PLA_2(D49K) for indicated time periods.

M-MLV reverse transcriptase (Promega) according to the manufacturer's recommendations. A reaction without reverse transcriptase was performed in parallel to ensure the absence of genomic DNA contamination. After initial denaturation at 95°C for 10 min, PCR amplification was performed using GoTaq Flexi DNA polymerase (Promega) followed by 35 cycles at 94°C for 50 s, 58°C for 50 s, and 72°C for 50 s. After a final extension at 72°C for 5 min, PCR products were resolved on 2% agarose gels and visualized by ethidium bromide transillumination under UV light. Primer sequences were as follows: Fas, 5′-CAAGGGATTGGAATTGAGGA-3′ (forward) and 5′-GACAAAGCCACCCCAAGTTA-3′ (reverse); and FasL, 5′-TCTCAGACGTTTTTCGGCTT-3′ (forward) and 5′-AAGACAGTCCCCCTTGAGGT-3′ (reverse). The PCR reaction yielded PCR products of 440 and 406 bp for Fas and FasL, respectively. Each reverse-transcribed mRNA product was internally controlled by glyceraldehyde-3-phosphate dehydrogenase (GADPH) PCR using primers 5′-GAGTCAACGGATTTGGTCGT-3′ (forward) and 5′-TGTGGTCATGAGTCCTTCCA-3′ (reverse), yielding a 512 bp PCR product. FasL and Fas reverse transcriptase-PCR products were subsequently confirmed by direct sequencing.

DNA TRANSFECTION AND LUCIFERASE ASSAY

The luciferase construct, pFLF1, containing the promoter region between −1435 and +236 of the Fas receptor gene was provided by Dr. Y. Nakanishi (Kanazawa University, Japan). This promoter region was inserted 18 bp upstream of the translation start codon of firefly luciferase gene in the pGV-B vector. The 1.2 kb FasL promoter in a eukaryotic expression vector HsLuc carrying a luciferase reporter gene downstream of the inserted FasL promoter was kindly provided by Dr. D.R. Green (University of Bern, Switzerland). pRSV-MKK3(Ala) encoding dominant-negative MKK3 was obtained from Dr. R.J. Davis (University of Massachusetts Medical School, USA), and pcDNA3-p38α and pcDNA3-p38β were kindly provided by Dr. J. Han (Xiamen University, China). The plasmids were transfected into K562 cells using Pipette-type Electroporator (MicroPorator-MP100, Digital Bio Tech. Co., Korea). Luciferase assay was performed with the Luciferase Reporter Assay System (Promega).

RNA INTERFERENCE

c-Fos siRNA (catalog number sc-29221) and negative control siRNA (catalog number sc-37007) were purchased from Santa Cruz

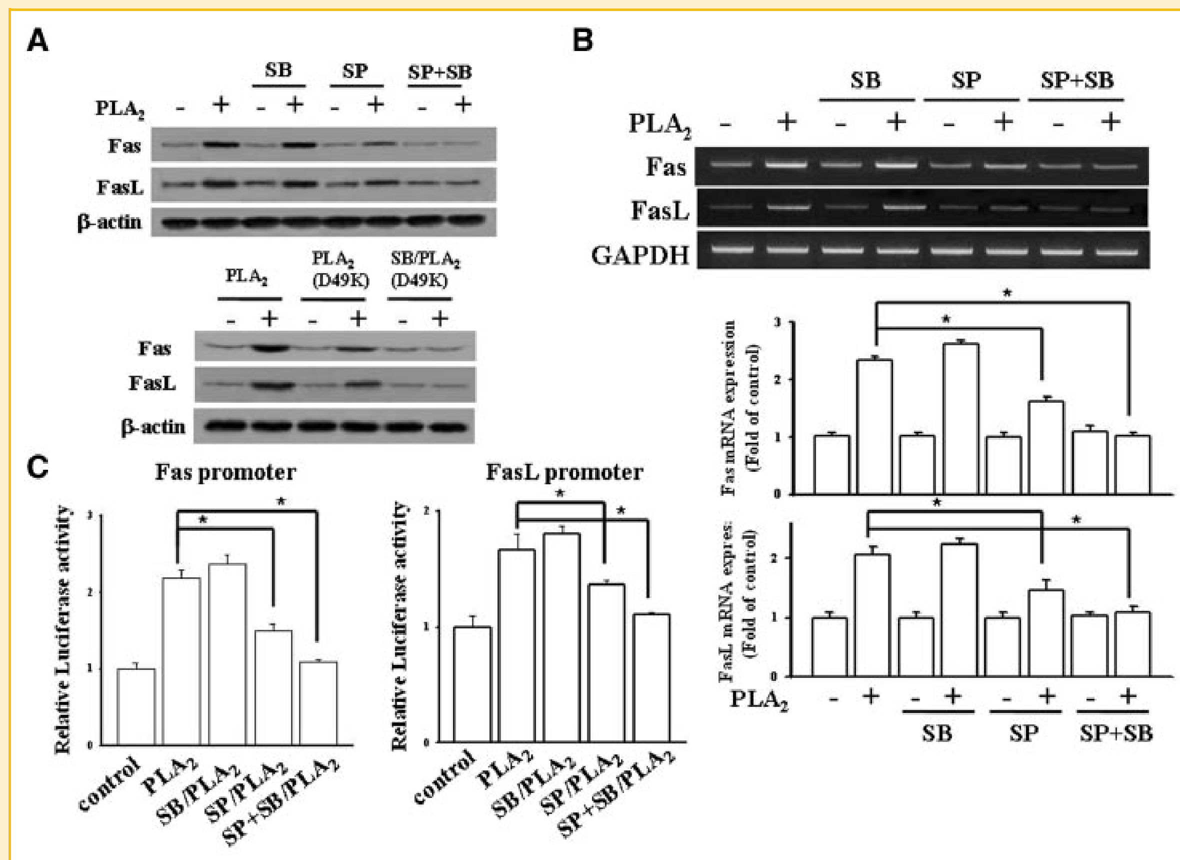

Fig. 2. Upregulation of Fas and FasL in PLA_2-treated K562 cells. K562 cells were treated with 3 μM PLA_2 or 10 μM PLA_2(D49K) for 24 h. Alternatively, K562 cells were pretreated with 1 μM SB202190, 10 μM SP600125 or a combination of 1 μM SB202190 and 10 μM SP600125 for 1 h, and then incubated with 3 μM PLA_2 or 10 μM PLA_2(D49K) for 24 h. A: Western blot analyses of Fas and FasL protein expression in PLA_2-treated cells (top panel) or PLA_2(D49K)-treated cells (bottom panel). B: Detecting the expression of Fas and FasL using RT-PCR (top panel). RT-PCR was conducted according to the procedure described in Materials and Methods Section. Bottom panel: Quantification of RT-PCR analyses of Fas and FasL mRNA ($^{*}P < 0.05$). C: PLA_2 treatment elicited increase in transcriptional activity of Fas promoter and FasL promoter ($^{*}P < 0.05$). After transfection with Fas or FasL promoter plasmids for 24 h, transfected cells were treated with 3 μM PLA_2 for 24 h with or without pretreatment with 1 μM SB202190, 10 μM SP600125 or a combination of 1 μM SB202190 and 10 μM SP600125 for 1 h.

Biotechnology, Inc. pKD-JNK1 shRNA plasmid was purchased from Upstate, and pSuper-p38α shRNA plasmid was obtained from Dr. A. Porras (Ciudad University, Spain). For the transfection procedure, cells were grown to 60% confluence and c-Fos siRNA, control siRNA, pKD-JNK1 shRNA or pSuper-p38α shRNA were transfected using Lipofetamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Lipofetamine 2000 reagent was incubated with serum free medium for 10 min, and respective siRNA was added subsequently. After incubation for 15 min at room temperature, the mixtures were diluted with culture medium and added to each well. At 24 h post-transfection, the cells were exposed to 3 μM PLA_2 for an additional 24 h, or pretreated with 1 μM SB202190, 10 μM SP600125 or a combination of 1 μM SB202190 and 10 μM SP600125 for 1 h and then treated with 3 μM PLA_2 for 24 h. Afterwards, cells were harvested for Western blot analyses.

MEASUREMENT OF INTRACELLULAR ROS

H_2DCFDA was employed to detect the intracellular generation of ROS. PLA_2-treated cells were collected and incubated with 10 μM H_2DCFDA (dissolved in DMSO) for 20 min prior to harvesting, then washed with PBS. The fluorescence intensity was measured by Beckman Coulter ParadigmTM Detection Platform with excitation at 485 nm and emission at 530 nm. Protein concentration was measured using the Bradford method (BIO-RAD) with bovine serum albumin as a standard. Results were shown as fold-increase in fluorescence intensity per microgram of proteins compared with the control group.

MEASUREMENT OF INTRACELLULAR CA^{2+} CONCENTRATION ($[CA^{2+}]I$)

The level of $[Ca^{2+}]i$ was quantified by fluorescence with Fluo-4 AM. The cells were treated with PLA_2 for the indicated time periods, and the treated cells were washed with ice-cold PBS. The cells resuspended in 1ml PBS were incubated with 5 μl of 1 mM Fluo-4 AM for 1 h. The fluorescence intensity of intracellular Ca^{2+} concentration was measured by Beckman Coulter ParadigmTM Detection Platform with excitation at 485 nm and emission at 530 nm. Results were shown as fold-increase in fluorescence intensity per microgram of proteins compared with the control group.

WESTERN BLOT ANALYSIS

After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1% Triton X-100, 1 mM EDTA,

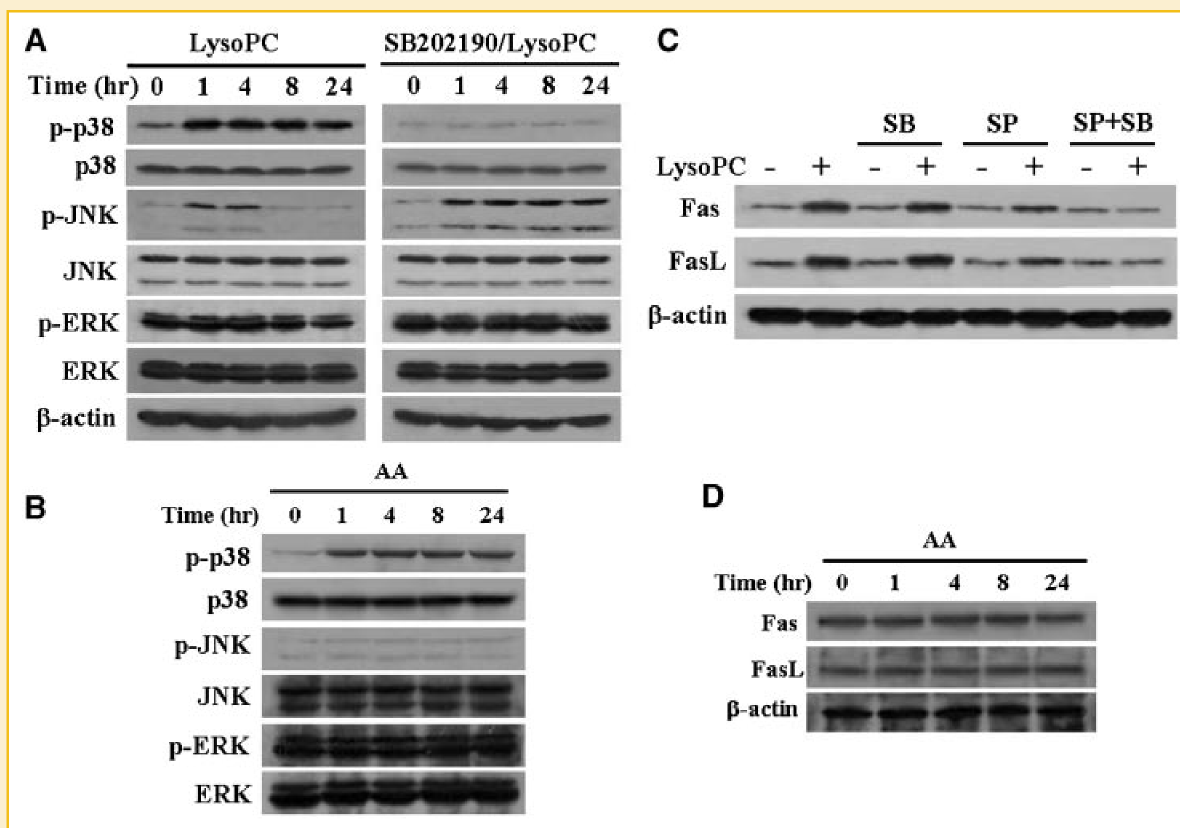

Fig. 3. Effect of LysoPC and arachidonic acid treatment on phosphorylation of MAPKs and Fas/FasL protein expression. Western blot analyses of phospho-MAPKs in (A) LysoPC-treated cells or (B) arachidonic acid-treated cells. K562 cells were treated with 25 μM LysoPC or 35 μM arachidonic acid (AA) for indicated time periods. Our unpublished data showed that viability of K562 cells was reduced by approximate 50% after treatment with 25 μM LysoPC or 35 μM AA. Alternatively, K562 cells were pretreated with 1 μM SB202190 for 1 h, and then incubated with 25 μM LyoPC for indicated time periods. C: Western blot analyses of Fas and FasL protein expression in LysoPC-treated cells. K562 cells were pretreated with 1 μM SB202190, 10 μM SP600125 or a combination of 1 μM SB202190 and 10 μM SP600125 for 1 h, and then incubated with 25 μM LysoPC for 24 h. D: Western blot analyses of Fas and FasL protein expression in AA-treated cells. K562 cells were treated with 35 μM AA for indicated time periods.

150 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF and protease inhibitor mixtures for 20 min on ice. After insoluble debris was precipitated by centrifugation at 13,000*g* for 15 min at 4°C, the supernatants were collected and assayed for protein concentration using the Bradford method. An equal amount of protein per sample (15 μg) was resolved on SDS–PAGE and transferred onto a PVDF membrane. The transferred membranes were blocked for 1 h in 5% nonfat milk in PBST (PBS containing 0.05% Tween 20) and incubated with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The immune complexes were detected by SuperSignal West Pico Chemiluminescent substrate kit (Pierce).

STATISTICAL ANALYSIS

All data are presented as mean ± SD. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $P < 0.05$ was taken as an indication of statistical significance. All the figures shown in this article were obtained from at least three independent experiments with similar results.

RESULTS AND DISCUSSION

Figure 1A shows that PLA_2 treatment led to phosphorylation of p38 MAPK and JNK and inactivate ERK in K562 cells. Unlike that in sustained p38 MAPK activation, JNK activation was diminished after PLA_2 treatment for 4 h. In terms of the findings that p38 MAPK negatively regulates JNK activation [Heinrichsdorff et al., 2008; Muniyappa and Das, 2008], the effect of SB202190 (p38 MAPK inhibitor) on phosphorylation of JNK was evaluated. Figure 1B shows that suppression of p38 MAPK resulted in a dynamically persistent JNK activation and attenuated PLA_2-elicited ERK inactivation. This reflected that PLA_2-induced p38 MAPK activation played a role in suppressing the levels of phospho-JNK and phospho-ERK in K562 cells. However, SP600125 (JNK inhibitor) pretreatment did not affect PLA_2-elicited p38 MAPK activation and ERK inactivation (Fig. 1C). Alternatively, treatment with recombinant PLA_2(D49K) (catalytically inactive PLA_2) elicited p38 MAPK activation, while the levels of phospho-ERK and phospho-JNK changed insignificantly after PLA_2(D49K) treatment (Fig. 1D).

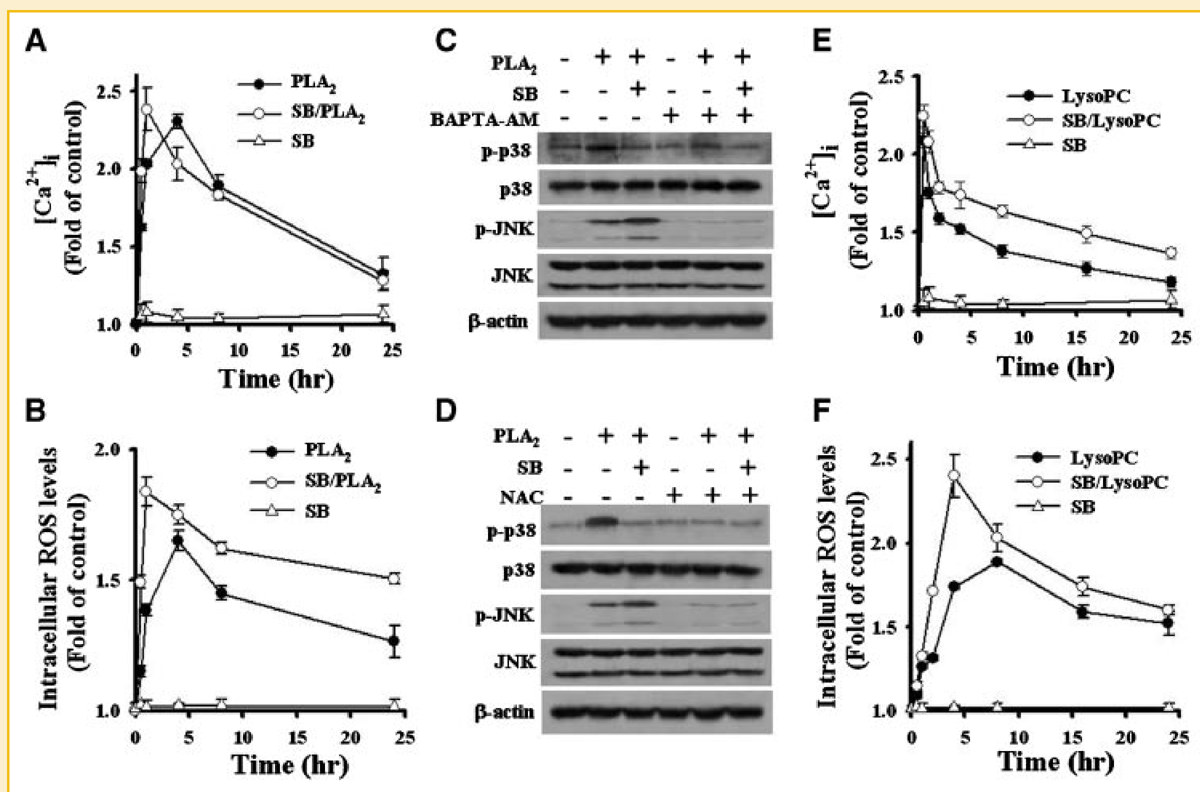

Fig. 4. Effect of PLA_2- and LysoPC-elicited $[Ca^{2+}]$i increase and ROS generation on p38 MAPK and JNK activation. A: Elevation of $[Ca^{2+}]$i was noted with K562 cells after treatment with 3 μM PLA_2. Alternatively, K562 cells were pretreated with 1 μM SB202190, and then incubated with 3 μM PLA_2 for indicated time periods. $[Ca^{2+}]$i was quantified by fluorescence plate reader after loading the cells with a calcium indicator (Fluo-4 AM). Results were shown as fold-increase in fluorescence intensity compared with the control group. The data represent the mean ± SD (n = 6). B: After treatment with 3 μM PLA_2 for indicated time periods, ROS generation was quantified by fluorescence plate reader. Results were shown as fold-increase in fluorescence intensity compared with the control group. The data represent the mean ± SD (n = 6). C: BAPTA-AM pretreatment abolished PLA_2-induced p38 MAPK and JNK activation. K562 cells were pretreated with 1 μM SB202190, 10 μM BAPTA-AM or a combination of 1 μM SB202190 and 10 μM BAPTA-AM for 1 h, and then incubated with 3 μM PLA_2 for 4 h. D: NAC pretreatment abolished PLA_2-induced p38 MAPK and JNK activation. K562 cells were pretreated with 1 μM SB202190, 2 mM NAC or a combination of 1 μM SB202190 and 2 mM NAC for 1 h, and then incubated with 3 μM PLA_2 for 4 h. LysoPC induced $[Ca^{2+}]$i increase (E) and ROS generation (F) in K562 cells. K562 cells were incubated with 25 μM LysoPC for indicated time periods. Alternatively, cells were pretreated with 1 μM SB202190 prior to treatment with 25 μM LysoPC.

This implied that PLA_2 catalytic activity contributed to evoke JNK activation and ERK inactivation in K562 cells.

Figure 2A shows that PLA_2 treatment upregulated Fas and FasL protein expression. Unlike SB202190, SP600125 (JNK inhibitor) pretreatment attenuated markedly Fas and FasL expression in PLA_2-treated K562 cells. Treatment with a combination of SP600125 and SB202190 abrogated PLA_2-induced Fas/FasL upregulation. The levels of both Fas mRNA and FasL mRNA were increased by PLA_2 treatment as evidenced by RT-PCR assay (Fig. 2B). Promoter assay revealed that, compared with control vectors, PLA_2 treatment enhanced the luciferase activity of the Fas and FasL promoters by approximately 2- and 1.8-folds, respectively (Fig. 2C). In contrast to SB202190, SP600125 attenuated transcription and translation of Fas and FasL. PLA_2-induced increased Fas and FasL promoter activity and mRNA levels were abrogated by treating with a combination of SB202190 and SP600125. Taken together, this suggests that PLA_2-induced Fas/FasL upregulation was mediated by p38 MAPK and JNK, and JNK played a crucial role in Fas/FasL upregulation after SB202190 pretreatment. Compared with PLA_2, PLA_2(D49K) treatment induced a slight increase in protein expression of Fas and FasL (Fig. 2A), but suppression of p38 MAPK abrogated PLA_2(D49K)-induced Fas and FasL upregulation. This reflected that PLA_2 catalytic activity heavily contributed to the signaling pathway responsible for Fas and FasL upregulation after SB202190 pretreatment.

Figure 3A shows that p38 MAPK and JNK activation was noted with LysoPC-treated cells. SB202190 pretreatment also led to persistent JNK activation in LysoPC-treated cells, and LysoPC-elicited ERK inactivation was restored by inactivation of p38 MAPK. As shown in Figure 3B, arachidonic acid (AA) treatment activated p38 MAPK but insignificantly altered the levels of phospho-ERK and phospho-JNK in K562 cells. In contrast to that of AA-treated cells (Fig. 3D), Fas and FasL upregulation was observed with LysoPC-treated cells (Fig. 3C). LysoPC-induced Fas and FasL upregulation was attenuated by SP600125 and abrogated by a combination of SB202190 and SP600125. Taken together, this indicated that LysoPC treatment mimicked the effect of PLA_2 treatment.

Given that PLA_2 treatment is reported to induce ROS or Ca^{2+}-elicited p38 MAPK activation in human neuroblastoma and leukemia cells [Chen et al., 2008; Liu et al., 2009a,b], the levels of Ca^{2+} and ROS were examined in PLA_2-treated K562 cells. PLA_2 treatment led to an increase in $[Ca^{2+}]i$ within 1 h, and PLA_2-induced $[Ca^{2+}]i$ increase was still noted after SB202190 pretreatment (Fig. 4A). Figure 4C shows that pretreatment with BAPTA-AM (Ca^{2+} chelator) abrogated JNK and p38 MAPK activation in PLA_2-treated cells. Figure 4B shows that SB202190 was unable to affect PLA_2-induced ROS generation. PLA_2-induced p38 MAPK and JNK activation was not observed after pretreatment with NAC (ROS scavenger) or a combination of NAC and SB202190 (Fig. 4D). Taken together, this reflected that PLA_2-induced $[Ca^{2+}]i$ increase and ROS generation were responsible for p38 MAPK and JNK activation. LysoPC treatment also led to an increase in $[Ca^{2+}]i$ and ROS generation in K562 cells regardless of SB202190 pretreatment (Fig. 4E,F), again suggesting that LysoPC mimicked the action of PLA_2.

p38 MAPK has four isomers including p38α, p38β, p38γ, and p38δ [Krishna and Narang, 2008]. Previous studies revealed that SB202190 mainly inhibits p38α MAPK and p38β MAPK, but it has no effect on p38γ and δ [Kumar et al., 1997]. Given that SB202190 pretreatment led to dynamically persistent JNK activation in PLA_2-treated cells, p38α MAPK and/or p38β MAPK were suggested to be involved in the JNK inactivation. Enslen et al. [1998, 2000]

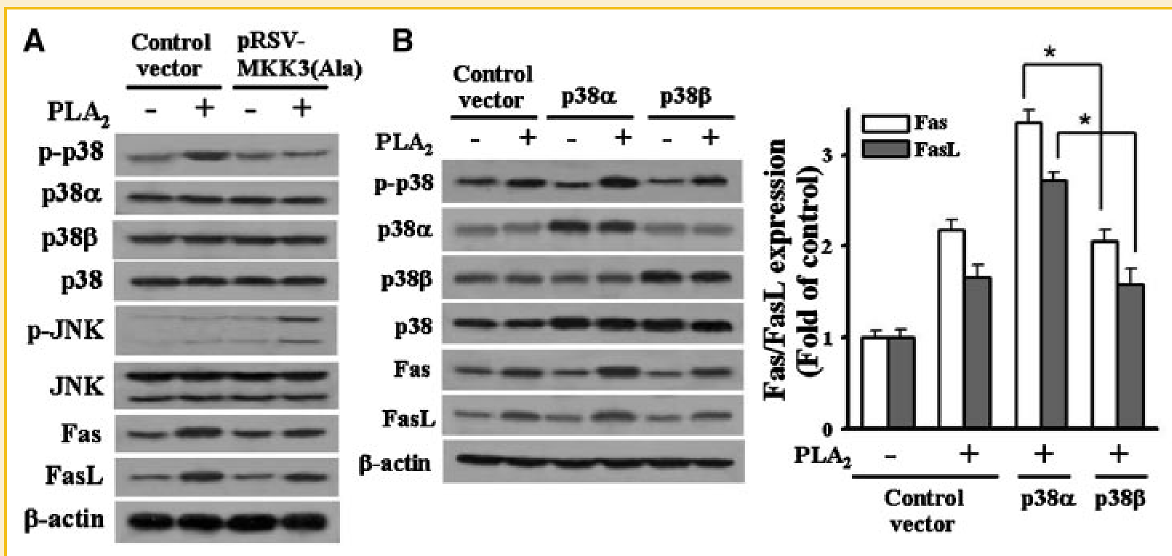

Fig. 5. Involvement of p38α MAPK in suppression of persistent JNK activation. A: Western blot analyses of JNK activation and Fas/FasL protein expression in PLA_2-treated cells that expressed dominant negative mutant of MKK3. K562 cells were transfected with an empty expression vector and pRSV-MKK3(Ala), respectively. After 24 h post-transfection, cells were treated with 3 μM PLA_2 for 24 h. B: PLA_2-elicited p38α MAPK activation was associated with Fas/FasL upregulation in K562 cells. K562 cells were transfected with an empty expression vector, pcDNA3-p38αr pcDNA3-p38β, respectively. After 24 h post-transfection, cells were treated with 3 μM PLA_2 for 24 h. Left panel: Western blot analyses of protein expression of Fas and FasL in pcDNA3-p38α- and pcDNA3-p38β-transfected cells. Right panel: Quantification of protein expression of Fas and FasL from Western blot analyses. Three independent experimental results were analyzed by densitometry ($^*P < 0.05$).

demonstrated that MKK3 activates only p38α, p38γ, and p38δ. Thus, expression of dominant negative mutant of MKK3 should inactivate p38α but did not affect p38β. Compared with that of control cells, p38α and p38β expression in pRSV-MKK3(Ala)-transfected cells was not significantly changed (Fig. 5A). Figure 5A shows that PLA_2 treatment was unable to evoke p38 MAPK activation in pRSV-MKK3(Ala)-transfected cells, while JNK activation was still noted after 24 h of PLA_2 treatment. This suggested that p38α MAPK was crucial for suppressing dynamically persistent JNK activation in PLA_2-treated cells. Fas and FasL upregulation was still noted when pRSV-MKK3(Ala)-transfected cells were treated with PLA_2. Unlike pcDNA3-p38β-transfected cells, pcDNA3-p38α-transfected cells showed marked p38 MAPK activation (Fig. 5B). Noticeably, upon exposure to PLA_2, Fas/FasL protein expression was higher in pcDNA3-p38α-transfected cells than in pcDNA3-p38β-transfected cells and control vector-transfected cells. Taken together, this suggested that p38α MAPK was involved in the suppression of persistent JNK activation and Fas/FasL upregulation in response to PLA_2 treatment.

Activated MAPKs-elicited downstream events including phosphorylation of c-Jun, c-Fos and ATF-2 have been demonstrated to be involved in Fas and FasL protein expression [Faris et al., 1998; Kasibhatla et al., 1998; Lasham et al., 2000; Crist et al., 2003; Christmann et al., 2007]. Figure 6A shows that phospho-c-Jun and phospho-ATF-2 were markedly increased after PLA_2 treatment, while the level of phospho-c-Fos changed insignificantly. SB202190 pretreatment increased significantly phosphorylation of c-Jun but abrogated phosphorylation of ATF-2. Pretreatment with a combination of SB202190 and SP600125 abolished PLA_2-induced phosphorylation of c-Jun and ATF-2, while SP600125 pretreatment eliminated c-Jun activation but did not affect the level of phospho-ATF-2 (Fig. 6A). Taken together, this suggested that p38 MAPK and JNK were responsible for phosphorylation of ATF-2 and c-Jun, respectively. Transfection with pSuper-p38α shRNA abrogated PLA_2-evoked phosphorylation of p38 MAPK and ATF-2, indicating p38α MAPK-mediated ATF-2 activation. Lauricella et al. [2006] found that JNK1-mediated c-Jun phosphorylation was associated with increased expression of FasL in bortezomib-treated HepG2

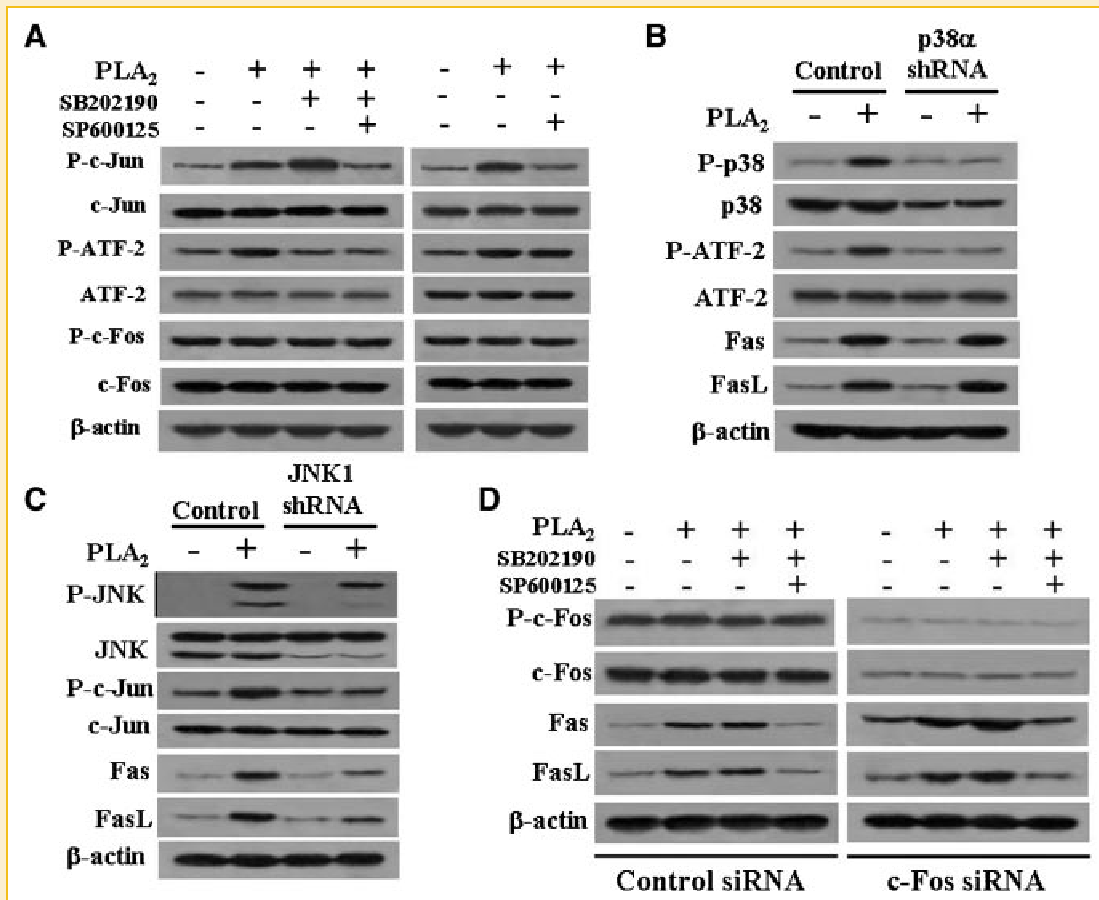

Fig. 6. Involvement of p38αMAPK/ATF-2 and JNK1/c-Jun in PLA_2-elicited Fas/FasL upregulation. A: Effect of SB202190 and SP600125 on phosphorylation of c-Jun, c-Fos and ATF-2 upon exposure to PLA_2. K562 cells were pretreated with 1 μM SB202190, 10 μM SP600125 or a combination of 1 μM SB202190 and 10 μM SP600125 for 1 h, and then incubated with 3 μM PLA_2 for 24 h. B: Down-regulation of p38α MAPK abolished ATF-2 phosphorylation. K562 cells were transfected with control vector or pSuper-p38α shRNA, respectively. After 24 h post-transfection, cells were treated with 3 μM PLA_2 for 24 h. C: Down-regulation of JNK1 abolished c-Jun phosphorylation. K562 cells were transfected with control vector or pKD-JNK1 shRNA, respectively. After 24 h post-transfection, cells were treated with 3 μM PLA_2 for 24 h. D: Effect of control siRNA and c-Fos siRNA on expression of Fas/FasL in PLA_2-treated cells. K562 cells were transfected with 100 nM control siRNA (Left panel) or c-Fos siRNA (Right panel), respectively. After 24 h post-transfection, cells were treated with 1 μM SB202190, 10 μM SP600125 or a combination of 1 μM SB202190 and 10 μM SP600125 for 1 h, and then incubated with 3 μM PLA_2 for 24 h.

cells. Consistent with this result, transfection with pKD-JNK1 shRNA markedly abolished c-Jun phosphorylation (Fig. 6C). Unlike knockdown of p38α MAPK, downregulation of JNK1 markedly attenuated but did not abrogate protein expression of Fas and FasL (Fig. 6B,C). Figure 6D shows that down-regulation of c-Fos did not abolish PLA_2-induced increase in Fas/FasL protein expression (Fig. 6D). Moreover, transfection of control siRNA did not affect PLA_2-induced upregulation of Fas/FasL. Taken together, our data suggested that JNK1/c-Jun and p38α MAPK/ATF-2 pathways regulated transcription of Fas and FasL genes, and JNK1/c-Jun pathway was exclusively responsible for Fas/FasL upregulation after abolition of p38α MAPK/ATF-2 pathway.

In this study, our data reveal that PLA_2 treatment induces Fas and FasL upregulation in K562 cells and LysoPC mimics the effect of PLA_2. Mounting evidences suggest that PLA_2-induced arachidonic acid release is closely related to the biological activity of PLA_2 [Triggiani et al., 2006; Lambeau and Gelb, 2008]. To our best knowledge, this is the first report showing the causal relationship between LysoPC and biological activity of PLA_2. As shown in Figure 7, PLA_2-induced $[Ca^{2+}]$i increase and ROS generation elicit p38α MAPK and JNK1 activation, and then p38α MAPK/ATF-2 and JNK1/c-Jun pathways upregulate the transcription of Fas and FasL genes. Abolishing the p38αMAPK/ATF-2 pathway makes JNK1/c-Jun to become an exclusive pathway responsible for Fas and FasL upregulation or vice versa. Noticeably, compared with the p38α MAPK/ATF-2 pathway, the JNK1/c-Jun pathway plays a crucial role in Fas and FasL upregulation. Targeted therapies that are designed to induce apoptosis are the most promising anti-leukemia strategies [Kuriakose, 2005; De Poeta et al., 2008]. A recent study suggests the use of TNF-family receptors (TNF-R1, Fas, TRAIL-R1 and TRAIL-R2) as targets for leukemia therapy in which FasL and TRAIL are found to selectively kill tumor cells but not normal cells [Testa and Riccioni, 2007]. In this regard, understanding the signal pathways responsible for PLA_2-induced Fas and FasL upregulation may afford the benefit of searching for effective strategies in improving leukemia treatment and overcoming therapy resistance.

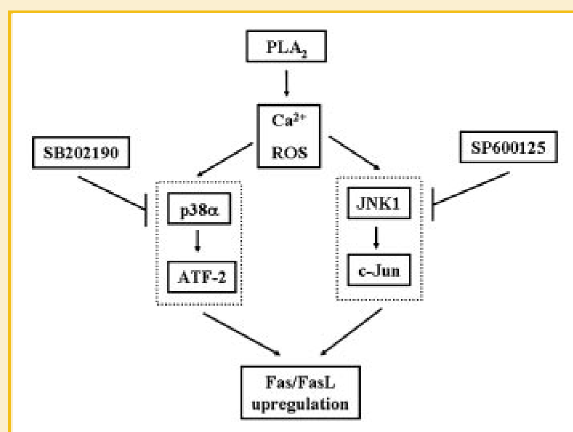

Fig. 7. Upregulation of Fas and FasL in PLA_2-treated K562 cells was mediated through p38α MAPK/ATF-2 and JNK1/c-Jun pathways. PLA_2 induced increase in $[Ca^{2+}]$i and ROS generation, leading to activation of p38α MAPK/ATF-2 and JNK1/c-Jun pathways. Both p38α MAPK/ATF-2 and JNK1/c-Jun pathways were involved in transcriptional regulation of Fas and FasL. Suppression p38α MAPK/ATF-2 pathway made JNK1/c-Jun pathway to become exclusive pathway responsible for upregulation of Fas and FasL in PLA_2-treated K562 cells and vice versa.

ACKNOWLEDGMENTS

This work was supported by grant NSC98-2320-B110-002-MY3 from the National Science Council, ROC (to L.S. Chang) and grant of National Sun Yat-Sen University-Kaohsiung Medical University Joint Research Center.

REFERENCES

Ashkenazi A, Dixit VM. 1998. Death receptors: Signaling and modulation. Science 281:1305–1308.

Chang LS, Lin SR, Chang CC. 1998. Identification of Arg-30 as an essential residue for the enzymatic activity of Taiwan cobra phospholipase A_2. J Biochem 124:764–768.

Chen KC, Kao PS, Lin SR, Chang LS. 2008. Upregulation of Fas and FasL in Taiwan cobra phospholipase A_2-treated human neuroblastoma SK-N-SH cells through ROS- and Ca^{2+}-mediated p38 MAPK activation. J Cell Biochem 106:93–102.

Christmann M, Tomicicm MT, Aasland D, Kaina B. 2007. A role for UV-light-induced c-Fos: Stimulation of nucleotide excision repair and protection against sustained JNK activation and apoptosis. Carcinogenesis 28:183–190.

Crist SA, Griffith TS, Ratliff FL. 2003. Structure/function analysis of the murine CD95L promoter reveals the identification of a novel transcriptional repressor and functional CD28 response element. J Biol Chem 278:35950–35958.

De Poeta G, Bruno A, Del Principe MI, Venditti A, Maurillo L, Buccisano F, Stasi R, Neri B, Luciano F, Siniscalchi A, de Fabritiis F, Amadori S. 2008. Deregulation of the mitochondrial apoptotic machinery and development of molecular targeted drugs in acute myeloid leukemia. Curr Cancer Drug Targets 8:207–222.

Donata NJ, Wu JY, Stapley J, Lin H, Arlinghaus R, Aggarwal BB, Shishodia S, Albitar M, Hayes K, Kantarjian H, Talpaz M. 2004. Imatinib mesylate resistance through BCR-ABL independence in chronic myelogenous leukemia. Cancer Res 64:672–677.

Enslen H, Raingeaud J, Davis RJ. 1998. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. J Biol Chem 273:1741–1748.

Enslen H, Brancho DM, Davis RJ. 2000. Molecular determinants that mediate selective activation of p38 MAP kinase isoforms. EMBO J 19:1301–1311.

Faderl S, Talpaz M, Estrov Z, O'Brien S, Kurzrock R, Kantarjian HM. 1999. The biology of chronic myeloid leukemia. N Engl J Med 341:167–172.

Faris M, Latinis KM, Kempiak SJ, Koretzky GA, Nel A. 1998. Stress-induced Fas ligand expression in T cells is mediated through a MEK kinase 1-regulated response element in the Fas ligand promoter. Mol Cell Biol 18:5414–5424.

Heinrichsdorff J, Luedde T, Perdiguero E, Nebreda AR, Pasparakis M. 2008. p38α MAPK inhibits JNK activation and collaborates with IκB kinase 2 to prevent endotoxin-induced liver failure. EMBO Rep 9:1048–1054.

Hochhaus A, Kreil S, Corbin AS, La Rosee P, Muller MC, Lahaye T, Hanfstein B, Schoch C, Cross NC, Berger U, Gschaidmeier H, Druker BJ, Hehlmann R. 2002. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. Leukemia 16:2190–2196.

Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A, Green DR. 1998. DNA damaging agents induce expression of Fas ligand and subsequent

apoptosis in T lymphocytes via the activation of NF-κB and AP-1. Mol Cell 1:543–551.

Krammer PH. 2000. CD95's deadly mission in the immune system. Nature 407:789–795.

Krishna M, Narang H. 2008. The complexity of mitogen activated protein kinases (MAPKs) made simple. Cell Mol Life Sci 65:3525–3544.

Kumar S, McDonnell PC, Gum RJ, Hand AT, Lee JC, Young PR. 1997. Novel homologues of CSBP/p38 MAP kinase activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. Biochem Biophys Res Commun 235:533–538.

Kuriakose P. 2005. Targeted therapy for hematologic malignancies. Cancer Control 12:82–90.

Lambeau G, Gelb MH. 2008. Biochemistry and physiology of mammalian secreted phospholipase A_2. Annu Rev Biochem 77:495–520.

Lasham A, Lindridge E, Rudert F, Onrust R, Watson J. 2000. Regulation of the human fas promoter by YB-1, Purα and AP-1 transcription factors. Gene 252:1–13.

Lauricella M, Emanuele S, D'Anneo A, Calvaruso G, Vassallo B, Carlisi D, Portanova P, Vento R, Tesoriere G. 2006. JNK and AP-1 mediate apoptosis induced by bortezomib in HepG2 cells via FasL/caspase-8 and mitochondria-dependent pathways. Apoptosis 11:607–625.

Liu WH, Cheng YC, Chang LS. 2009a. ROS-mediated p38α MAPK activation and ERK inactivation responsible for upregulation of Fas and FasL and autocrine Fas-mediated cell death in Taiwan cobra phospholipase A_2-treated U937 cells. J Cell Physiol 219:642–651.

Liu WH, Kao PS, Chou YL, Lin SR, Wu MJ, Chang LS. 2009b. Catalytic activity-independent pathway is involved in phospholipase A_2-induced apoptotic death of human leukemia U937 cells via Ca^{2+}-mediated p38 MAPK activation and mitochondrial depolarization. Toxicol Lett 185:102–109.

Muniyappa H, Das KC. 2008. Activation of c-Jun N-terminal kinase (JNK) by widely used specific p38 inhibitors SB202190 and SB203580: A MLK-3-MKK7-dependent mechanism. Cell Signal 20:675–683.

Schneider E, Cowan KH, Bader H, Toomey S, Schwartz GN, Karp JE, Burke PJ, Kaufmann SH. 1995. Increased expression of the multidrug resistance-associated protein gene in relapsed acute leukemia. Blood 85:186–193.

Testa U, Riccioni R. 2007. Deregulation of apoptosis in acute myeloid leukemia. Haematologica 92:81–94.

Triggiani M, Granata F, Frattini A, Marone G. 2006. Activation of human inflammatory cells by secreted PLA_2. Biochim Biophys Acta 1761:1289–1300.

Walczak H, Krammer PH. 2000. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res 256:58–66.

Wang ZY. 2003. Ham-Wasserman lecture: Treatment of acute leukemia by inducing differentiation and apoptosis. Hematology 1:1–13.