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Award Number DAMD17-97-1-7078

TITLE: c-Jun N-terminal Kinase and Apoptosis in Breast Cancer

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REPORT DATE: June 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20000303 105

DTIC QUALITY INSPECTED 3

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1999	Annual (15 May 98 - 14 May 99)

4. TITLE AND SUBTITLE
c-Jun N-terminal Kinase and Apoptosis in Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7078

6. AUTHOR(S)
Yi-Rong Chen

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030-3498

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Our previous studies show that c-Jun N-terminal kinase (JNK) participates in apoptosis signaling. JNK is differentially regulated by mitogenic and apoptotic stimuli in Jurkat T cells and in MCF-7 breast cancer cells, suggesting that duration of JNK activation may determine cell fate. A tetracycline-inducible system was used to control the expression of JNK1 and dominant-negative JNK1. This inducible system will be used to examine the duration hypothesis. In addition, we identified curcumin as an effective inhibitor for JNK activation by various stimuli. Curcumin may inhibit JNK activation by suppressing activation of JNK activators at the MAP3K level. The possibility to use curcumin to control the duration of JNK activation is currently under investigation. Tumor suppressor p53 is not required for radiation induced JNK activation. It is also not required for apoptosis induced by JNK activation. However, our data does not exclude that p53 may mediate JNK-induced apoptosis, which needs to be further studied. Both JNK activity and Fas expression can be induced by γ radiation; however, Fas expression is closely associated with a wild-type p53 status but not with the JNK activation. Our results show that induction of Fas expression is not the downstream effect for the JNK pathway.

14. SUBJECT TERMS
Breast Cancer ; JNK; MAP kinase; apoptosis

15. NUMBER OF PAGES
21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Yi-Rong Chen 6-11-99

PI - Signature Date

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INTRODUCTION

Apoptosis is a unique biochemical and morphological pattern of cell death characterized by internucleosomal DNA cleavage, chromatin condensation, membrane blebbing, and cell fragmentation. Apoptosis is important in regulating the development and maintenance of homeostasis in multicellular organisms. Apoptosis is positively and negatively regulated by many physiological and pathological factors, and this regulation is initiated by genetic and biochemical programs. Apoptosis occurs in normal physiological conditions, and can be induced by ionizing radiation, UV radiation, DNA-damaging drugs, oxidants, viral infection, and deprivation of growth factors. Disorders in apoptosis play critical roles in carcinogenesis and in the development of resistance to radiation or chemotherapy in cancer cells. Therefore, understanding the molecular mechanisms of the apoptotic process is important for the prevention and treatment of cancers.

JNK (c-Jun N-terminal kinase; also named as stress-activated protein kinase, SAPK) family members belong to the mitogen-activated protein kinase (MAPK) superfamily which also includes extracellular signal-regulated kinases (ERKs) and the p38-MAPK family. MAPKs are serine/threonine kinases that target other kinases, transcription factors, and membrane receptor tails, causing diverse effects such as cell proliferation, transformation, differentiation, and apoptosis. To date, the JNK family consists of three genes, JNK1, JNK2, and JNK3, which are subdivided into ten isoforms. Substrates for JNK family members include the transcription factors c-Jun, JunD, ATF-2, ATFa, Elk-1, Sap-1a, and p53; phosphorylation of c-Jun, ATF-2, Elk-1, and Sap-1a increases their transcriptional activity. JNK kinase activity is activated by proinflammatory cytokines (TNF-α and IL-1), G protein-coupled receptors, lymphocyte activation stimuli, osmotic shock, heat shock, protein synthesis inhibitors, ceramides, DNA-damaging chemicals, UV radiation, and γ radiation. Activation of JNK involves MKK4 (MAPK kinase 4, also called SEK1 [SAPK/ERK kinase] and JNKK1), MKK7 (JNKK2), and MEKK1 (MAPK kinase kinase1). MEKK1 phosphorylates and activates the dual-specificity kinase MKK4/SEK or MKK7. MKK4/SEK (or MKK7) then activates JNK via threonine and tyrosine phosphorylation of the T-P-Y motif on JNK. Recently, several other kinases, e.g., TAK1 (TGF-β activating kinase 1) and ASK1 (apoptosis signal-regulating kinase 1), were also identified to activate the JNK pathway through MKK4 or MKK7.

JNK is activated in apoptosis induced by growth factor withdrawal, UV-C, γ radiation, ceramide, heat shock, and DNA-damaging drugs. Activation of the JNK pathway can lead to cell death. Interference with the JNK pathway by the dominant-negative mutant of MEKK1, MKK4/SEK, or JNK1 suppresses apoptosis. JNK's substrate, c-Jun, is required for ceramide-induced apoptosis and apoptosis of neuronal cells caused by NGF withdrawal. All these results indicate the importance of the JNK pathway in apoptosis. However, reports on the necessity of JNK in Fas-mediated apoptosis are controversial, and JNK activation induced by TNF-α is not required for apoptosis induction.

The JNK pathway participates in cellular responses to mitogens, stresses, and apoptotic agents. The mechanisms by which JNK integrates with other cellular signaling to achieve these diverse functions are intriguing. We have found that the induction of JNK in response to mitogenic and apoptotic signals have different activation patterns, transient versus persistent, respectively. Co-treatment of a tyrosine phosphatase inhibitor (sodium orthovanadate) and T-cell activation signals (phorbol 12-myristate 13-acetate [PMA] plus ionomycin) prolongs the JNK induction by T-cell activation agents and results in T-cell apoptosis. A similar phenomenon was also observed in breast cancer cells. These results suggest that the duration of JNK activation may be the determining factor for the outcome of signaling. **The aims of this study are (i) to examine the role of the JNK pathway in proliferation apoptosis in breast cancer cells, (ii) to study the mechanism of JNK-mediated apoptosis, (iii) to identified genes involved in JNK-mediated apoptosis.**

BODY

A. Induction of JNK by Mitogenic and Apoptotic Stimuli in Breast Cancer Cells *(details were included in the 1998 annual report; these experiments are related to specific aim #1 in the Statement of Work)*

We found that the induction of JNK in response to mitogenic and apoptotic signals in breast cancer MCF7 cells have different activation patterns, transient versus persistent, respectively. We treated MCF-7 cells with either PMA or epidermal growth factor (EGF). Both PMA and EGF induce an immediate and transient JNK activation in MCF-7 cells. The kinase activity increased in 15 min and decreased to basal levels in 90 min. MCF-7 were also treated with various apoptotic agents, and examined for endogenous JNK activation. Among the agents tested, UV-C and 5-FU showed strong JNK inducing ability. Both agents induced persistent JNK activation, which followed by apoptosis as determined by morphological change and nuclear straining of the cells. To our surprise, γ radiation induced only weak JNK activation in MCF-7 cells. We also did not detect apparent apoptosis induction in MCF-7 cells by propidium iodide staining of the nuclei (less than 5% condensed nuclei) 48 h after irradition.

These data reveal that mitogenic agents induced transient JNK activation in MCF-7 cells, while apoptotic agents induced persistent JNK activation. These results are consistent with our previous observation in T cells , and further support our hypothesis that duration of JNK activation may determine cell fate. We were unable to induced significant JNK activation and apoptosis induction in MCF-7 with γ radiation. We will test our hypothesis by modulating the duration of JNK activation using inducible system for JNK and dominant-negative JNK (as described below) in combination with either mitogenic or apoptotic stimuli.

B. Tumor Suppressor p53, Death Receptor Fas, and JNK-mediated Apoptosis *(these results were published in attached manuscript # 2; these experiments are related to specific aim#2 in the Statement of Work)*

Tumor suppressor p53 plays an important role in apoptosis induced by γ radiation and by the adenovirus E1A gene. It has been shown that p53 may facilitate the expression of Bax, an apoptosis accelerator, and the expression of the death receptor Fas. The p53 protein is a direct substrate for JNK. JNK-mediated p53 phosphorylation has been shown to disrupt MDM2-p53 interaction and prevent p53 degradation and, subsequently, enhance p53-mediated transcription. A recent report also showed that p53 can up-regulate Fas expression through a translation-independent pathway. We studied whether p53-mediated Fas expression is one of downstream events of JNK activation by examining the correlation between JNK activation and Fas expression in cells with different p53 status.

Induction of Fas expression is associated with wild type p53 but not with JNK activation in leukemia cells.

To study the correlation between JNK induction, Fas expression, and p53 status, we examined the effect of γ radiation on three leukemia cell lines with different p53 status. γ radiation induced JNK activation in three myeloid cells lines HL-60, KBM-7, and BV173. Nevertheless, γ radiation only induced Fas expression in BV-173 which has wild type p53 genes, but not in $p53^{-/-}$ HL-60 cells or in KBM-7 cells, which have mutated p53. The induction of Fas expression was also irrelevant to the basal expression levels of Fas in each cell line. These results, which are consistent with previous reports, indicate that the induction of Fas expression is associated with the presence of functional p53 proteins; however, JNK activation is not correlated with expression of Fas receptors.

Induction of Fas expression is associated with a wild type p53 status but not with JNK activation in prostate cancer cells.

To study the relationship between enhancement of Fas expression and JNK activation further, two prostate carcinoma cell lines, $p53^{+/+}$ LNCaP cells and $p53^{-/-}$ PC-3 cells, were treated with apoptotic stimuli— γ radiation or

a retinoic acid analog 4-hydroxyphenyl retinamide (4-HPR). γ radiation induced JNK activation in both LNCaP and PC-3 cells, while 4-HPR induced JNK activation only in LNCaP cells. γ radiation significantly enhanced Fas expression in $p53^{+/+}$ LNCaP but not in p53-null PC-3 cells. However, no induction in Fas expression was observed in either LNCaP or PC-3 cells treated with 4-HPR. These results indicate that Fas expression is associated with the wild type p53 status; however, JNK activation is not necessarily associated with Fas expression in $p53^{+/+}$ or $p53^{-/-}$ cells.

Expression of Fas in the absence of JNK induction is not sufficient for apoptosis induction.

JNK activation is involved in and required for radiation-induced apoptosis. Other reports suggested that Fas expression may be involved in apoptosis induced by DNA damaging agents. However, we observed no correlation in JNK activation and Fas expression. We further examined whether Fas expression can be induced in the absence of JNK activation, and whether Fas expression alone is sufficient for apoptosis induction. MCF-7 breast cancer cells, which contain wild type p53 alleles, were treated with various apoptotic agents, and examined for Fas expression, endogenous JNK activation, and apoptosis induction. Among the agents tested, γ radiation (20 Gy) induced significant levels of Fas expression in MCF-7 cells, but the same treatment induced only transient and weak JNK activation and no apoptosis. Addition of a Fas cross-linking antibody after γ radiation significantly augmented apoptosis induction in MCF-7 cells. This apoptosis may be triggered by death signaling from the newly synthesized and cross-linked Fas proteins. In contrast, UV-C caused strong JNK activation and apoptosis in MCF-7 cells in the absence of significant Fas induction. The addition of anti-Fas antibody did not enhance UV-induced apoptosis in MCF-7 cells.

In conclusion, Induction of Fas expression by DNA-damaging agents is dependent on the expression of functional p53, and has been suggested to play an important role in apoptosis induction. JNK (c-Jun N-terminal kinase), which is capable of phosphorylating p53, is also involved in apoptotic signaling induced by various apoptotic stimuli. Here, we report that although Fas induction is closely linked to the expression of wild type p53, it is not correlated with JNK activation induced by apoptotic stimuli. JNK activation does not necessarily lead to Fas expression, even in cells containing wild type p53. In addition, Fas expression can be induced without significant JNK activation. Furthermore, induction of Fas expression is not sufficient for apoptosis induction; however, it may sensitize cells to Fas-ligation induced apoptosis.

C. Curcumin is an effective inhibitor for JNK activation by various stimuli *(These results were published in **attached manuscript #1**; this research was not included in the original proposal but is related to specific aim#1 in the Statement of Work)*

Curcumin (diferuloylmethane), a dietary pigment from *Curcuma longa*, gives the golden-yellow color and unique flavor to curry. The anticarcinogenic effects of this compound are demonstrated by its ability to inhibit tumor initiation by azoxymethane, benzpyrene and 7,12-dimethyl-benz(a)anthracene, and to suppress tumor promotion by phorbol esters. It has been shown that curcumin strongly inhibits both c-Jun and NF-κB activation by phorbol 12-myristate 13-acetate (PMA) or tumor necrosis factor-α (TNF-α) treatments. This inhibitory effect may account for the anti-inflammatory and anti-oxidant properties of curcumin; however, the mechanism of these inhibitory effects remains unclear.

We found that curcumin inhibits JNK activation by various agonists including PMA plus ionomycin, anisomycin, UV-C, γ radiation, TNF-α, and sodium orthovanadate. Although both JNK and ERK activation by PMA plus ionomycin were suppressed by curcumin, the JNK pathway was more sensitive. The IC_{50} (50% inhibition concentration) of curcumin was between 5-10 μM for JNK activation, and was 20 μM for ERK activation. In

transfection assays, curcumin moderately suppressed MEKK1-induced JNK activation; however, it effectively blocked JNK activation caused by co-transfection of TAK1, GCK (germinal center kinase), or HPK1 (hematopoietic progenitor kinase). Curcumin did not directly inhibit JNK, SEK1, MEKK1, or HPK1 activity. Although curcumin suppressed TAK1 and GCK activities at high concentrations, this inhibition cannot fully account for the JNK inhibition by curcumin *in vivo*. Our data suggest that curcumin may affect the JNK pathway by interfering with the signaling molecule(s) at the same level or proximally upstream of the MAPKKK level. Taken together, the inhibition of the MEKK1-JNK pathway reveals a possible mechanism of suppression of AP-1 and NF-κB signaling by curcumin, and may explain the potent anti-inflammatory and anti-carcinogenic effects of this chemical.

Since curcumin is an effective inhibitor for JNK activation, we are currently testing whether curcumin can be used to terminate JNK activation induced by apoptotic signals. If so, the association of duration of JNK induction and apoptosis will be examined.

D. Establishment of the tetracycline-inducible system for JNK1 and JNK1(APF) expression *(This work is related to specific aim#1 in the Statement of Work)*

The Tc-regulated inducible system was adapted from the *E. coli* tetracycline (tet) resistance operon with modifications as described by Gossen et al. The inducible system consists of the **Tet-operator** sequence and a **reverse Tc-controlled transactivator (rtTA)**, which is a fusion molecule of a reverse Tet repressor and the herpes simplex virus VP-16 transactivating domain. The fusion rtTA has the ability to bind to Tet-operators and to activate the downstream gene in the presence of tetracycline or its analogs. We have construct two plasmids **pUHD-Hyg+-HA-JNK1** and **pUHD-Hyg+-HA-JNK1(APF)** which contain the JNK genes under the control of the Tet-operator sequence fused with minimal CMV promoter. We have co-transfected pUHD-Hyg+-HA-JNK1 or pUHD-Hyg+-HA-JNK1(APF) with the pUHD172-1neo plasmid, which encoding rtTA, into 293 cells. The transfected cells were then cultured in the presence or absence of doxycycline (a tetracycline analog). The induction of transfected genes by doxycycline was confirmed by Western blot using anti-HA and anti-JNK1 antibodies.

Currently, we are establishing the permanently transfected MCF-7 cells containing the tet-inducible JNK1 or JNK1(APF). The Tc-inducible system can be used in different ways to test our hypothesis. We can modulate the duration of JNK activation by inducing JNK1(APF) expression in the presence of apoptotic signals. High levels of JNK1(APF) will compete with the endogenous JNK for the upstream activator, therefore, suppress the activation of endogenous JNK by apoptotic signals. By adjusting the timing of deoxycycline addition and of the treatment of apoptotic agents, we may be able to create different durations of JNK induction and examine whether a persistent JNK activation is required for apoptosis. In addition, Jurkat (tet-JNK1) cells will be incubated in deoxycycline containing medium for different periods, and then be washed and cultured in fresh medium. The different length of induction of JNK may cause different duration of JNK activation, then we can examine the correlation of the kinetics of JNK activation and apoptosis induction. In addition, this system will also be used to identify downstream genes that are regulated by the JNK pathway.

APPENDICES

A. List of Key Research Accomplishments

- Identification of curcumin as an effective inhibitor for JNK activation by various stimuli
- Revealing the lack of correlation in JNK activation and p53-mediated Fas expression
- Construction of tetracycline-inducible system for JNK1 and dominant-negative JNK1 (JNK1[APF])

B. Reportable Outcomes

Poster Abstract

Fourteenth Annual Meeting on Oncogene, June 24-27, 1998, La Jolla, California, poster abstract (#95) entitled "Molecular Mechanisms of c-Jun N-terminal Kinase (JNK)-mediated Apoptosis"

Manuscripts(*#1 was attached with the 1998 annual report; #2 and 3 are attached with this report*)

1. Chen, Y.-R., W. Wang, A.-N.T. Kong, and T.-H. Tan. 1998. Molecular mechanisms of c-Jun N-terminal kinase (JNK)-mediates apoptosis induced by anticarcinogenic isothiocyanates. *J. Biol. Chem.* 273: 1769-1775.

2. Y.-R. Chen and T.-H. Tan (1998) Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin. *Oncogene* **17**, 173-178

3. Y.-R. Chen and T.-H. Tan (1999) Lack of correlation in JNK activation and p53-dependent Fas expression induced by apoptotic stimuli. *Biochem. Biophys. Res. Com.* **256**, 595-599

Award

Dr. Alexander Y. M. Wang Memorial Scholarship in Biomedical Research Studies (provided by Dr. Alexander Y. M. Wang Memorial Foundation, Houston)

Degree obtained

The PI (Yi-Rong Chen) has fulfilled the requirements and received the degree of **Doctor of Philosophy** from the Graduate School of Biomedical Sciences, Baylor College of Medicine, Houston on December 31, 1998. The title of the dissertation is "Molecular Mechanisms of c-Jun N-terminal Kinase-mediated apoptosis signaling". The diploma was awarded on May 25, 1999.

Employment received

The PI is currently staying in Dr. Tse-Hua Tan's laboratory as a postdoctoral associate to continue the work on JNK-mediated apoptotic signaling.

C. Attached Abstract and Manuscripts

Abstract

Fourteenth Annual Meeting on Oncogene, June 24-27, 1998, La Jolla, California, poster abstract (#95) entitled "Molecular Mechanisms of c-Jun N-terminal Kinase (JNK)-mediated Apoptosis"

Manuscripts

1. Y.-R. Chen and T.-H. Tan (1998) Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin. *Oncogene* **17**, 173-178

2. Y.-R. Chen and T.-H. Tan (1999) Lack of correlation in JNK activation and p53-dependent Fas expression induced by apoptotic stimuli. *Biochem. Biophys. Res. Com.* **256**, 595-599

MOLECULAR MECHANISMS OF C-JUN N-TERMINAL KINASE (JNK)-MEDIATED APOPTOSIS

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Previously, our laboratory found that the JNK pathway is required for apoptotic signaling induced by γ radiation and UV-C (J. Biol. Chem. 272: 631 & 31929, 1996). We further examined the molecular mechanisms of JNK-mediated apoptosis with various agents. We found that JNK also participates in apoptosis induced by anticarcinogenic isothiocyanates (J. Biol. Chem. 273: 1796, 1998). Isothiocyanate- and γ radiation-induced JNK activation were blocked by antioxidants, suggesting that the death signaling was triggered by oxidative stress. Over-expression of Bcl-2 suppressed isothiocyanate-induced JNK activation and apoptosis, but failed to protect cells from death induced by over-expression of activated JNK1. These results suggest that Bcl-2 is upstream of the JNK pathway. Tumor suppressor p53 is not required for both JNK activation and JNK-mediated apoptosis. Despite the occasional co-induction of JNK and Fas expression in apoptosis, Fas expression is not the downstream event of JNK activation. A caspase inhibitor blocked UV-C-, γ radiation-, or isothiocyanate-induced apoptosis without inhibiting the JNK activation, which suggests that JNK activation is an independent or upstream event of the activation of caspases. However, JNK activation induced by Fas-ligation is suppressed by a caspase inhibitor, suggesting that the JNK pathway can be regulated by a caspase-dependent mechanism.

Oncogene (1998) 17, 173–178
© 1998 Stockton Press All rights reserved 0950–9232/98 $12.00
http://www.stockton-press.co.uk/onc

Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin

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Curcumin, a dietary pigment in curry, suppresses tumor initiation and tumor promotion. Curcumin is also a potent inhibitor for AP-1 and NF-κB activation. In this report, we show that curcumin inhibits JNK activation by various agonists including PMA plus ionomycin, anisomycin, UV-C, γ radiation, TNF-α, and sodium orthovanadate. Although both JNK and ERK activation by phorbol 12-myristate 13-acetate (PMA) plus ionomycin were suppressed by curcumin, the JNK pathway was more sensitive. The IC_{50} (50% inhibition concentration) of curcumin was between 5–10 μM for JNK activation and was 20 μM for ERK activation. In transfection assays, curcumin moderately suppressed MEKK1-induced JNK activation; however, it effectively blocked JNK activation caused by co-transfection of TAK1, GCK, or HPK1. Curcumin did not directly inhibit JNK, SEK1, MEKK1 or HPK1 activity. Although curcumin suppressed TAK1 and GCK activities at high concentrations, this inhibition cannot fully account for the JNK inhibition by curcumin *in vivo*. Our data suggest that curcumin may affect the JNK pathway by interfering with the signaling molecule(s) at the same level or proximally upstream of the MAPKKK level. Taken together, the inhibition of the MEKK1-JNK pathway reveals a possible mechanism of suppression of AP-1 and NF-κB signaling by curcumin, and may explain the potent anti-inflammatory and anti-carcinogenic effects of this chemical.

Keywords: curcumin; JNK; c-Jun; AP-1; NF-κB; cell signaling

Introduction

c-Jun and c-Fos are members of the bZip transcription factor family, which contain a basic DNA-binding domain and a leucine-zipper dimerization motif. AP-1 complexes consist of a homodimer of c-Jun or a heterodimer of c-Jun/c-Fos family members (Angel and Karin, 1991; Johnson and McKnight, 1989). These AP-1 factors can bind to the TRE (TPA-response element) sequence (Angel *et al.*, 1987) and enhance gene expression. AP-1 factors also interact with other transcription factors including NF-κB (Stein *et al.*, 1993), NF-AT (Jain *et al.*, 1993) and glucocorticoid receptor (Jonat *et al.*, 1990; Schule *et al.*, 1990) to regulate gene expression.

AP-1 transcriptional activity (c-Jun and c-Fos) is regulated at both transcriptional and post-translational levels (Whitmarsh and Davis, 1996). c-Jun N-terminal kinases (JNKs; also named stress-activated protein kinases, SAPKs) were recently shown to be important for the expression and post-translational modification of c-Jun proteins (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). JNK phosphorylates the Ser63/Ser73 residues in the N-terminal transactivating domain of c-Jun, and strongly augments its transcriptional activity (Whitmarsh and Davis, 1996). JNK is involved in cellular responses to environmental stress, proinflammatory cytokines, mitogen stimulation, and apoptotic stimuli (reviewed in Kyriakis and Avruch, 1996). The JNK subfamily, including the various isoforms of JNK1, JNK2, and JNK3, are members of the MAP kinase family (Gupta *et al.*, 1996). Like other MAP kinases, JNK is regulated by a kinase cascade-MEKK1 (MAPKKK)→SEK1/MKK7 (MAPKK)→JNK (MAPK) (Derijard *et al.*, 1995; Kyriakis and Avruch, 1996; Lin *et al.*, 1995; Sanchez *et al.*, 1994; Tournier *et al.*, 1997; Yao *et al.*, 1997). Recent work revealed that multiple MEKK1-like kinases activate the JNK pathway via SEK1/MKK4. These kinases include MEKK2-4, TAK1 (TGF-β activated kinase-1), MAPKKK5/ASK, Tpl-2/Cot, MLK3/SDPRK and MUK/DLK (reviewed in Fanger *et al.*, 1997). Furthermore, several yeast STE20-like kinases including p21 (Rac1/Cdc42)-activated kinases (PAKs) (Bagrodia *et al.*, 1995; Frost *et al.*, 1966), germinal center kinase (GCK) (Pombo *et al.*, 1995), and hematopoietic progenitor kinase 1 (HPK1) (Hu *et al.*, 1996; Wang *et al.*, 1997) were found to be potential upstream kinases for MEKK1 or TAK1 (reviewed in Sells and Chernoff, 1997). However, others proposed that MAPKKKs (e.g. MEKKs and MLK) are parallel to, but not downstream of, PAK and GCK (Fanger *et al.*, 1997; Gerwins *et al.*, 1997; Teramoto *et al.*, 1996).

Besides c-Jun, the JNK pathway also activates the transcription factors ATF-2 (Gupta *et al.*, 1995), Elk-1 (Whitmarsh *et al.*, 1995) and Sap-1a (Janknecht and Hunter, 1997), and interacts with the NF-κB pathway (Meyer *et al.*, 1996). MEKK1, an upstream kinase of JNK, can induce NF-κB dependent transcription activity (Hirano *et al.*, 1996; Lee *et al.*, 1997; Meyer *et al.*, 1996). NF-κB activation may be due to the activation of the IκB-α kinase complex by MEKK1 and the subsequent degradation of the phosphorylated IκB-α (Lee *et al.*, 1997). Additionally, the JNK protein physically interacts with the c-Rel protein, a member of the NF-κB family, and thus, may play a role in the NF-κB signaling pathway (Meyer *et al.*, 1996), although the exact function of this interaction is not clear.

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Received 27 August 1997; revised 18 February 1998; accepted 19 February 1998

Curcumin (diferuloylmethane), a dietary pigment from *Curcuma longa*, gives the golden-yellow color and unique flavor to curry. The anticarcinogenic effects of this compound are demonstrated by its ability to inhibit tumor initiation by azoxymethane, benzpyrene and 7,12-dimethyl-benz(α)anthracene, and to suppress tumor promotion by phorbol esters (Huang *et al.*, 1994, 1988; Rao *et al.*, 1995). It has been shown that curcumin strongly inhibits both c-Jun and NF-κB activation by PMA or tumor necrosis factor-α (TNF-α) treatments (Hanazawa *et al.*, 1993; Huang *et al.*, 1991; Singh and Aggarwal, 1995). This inhibitory effect may account for the anti-inflammatory and anti-oxidant properties of curcumin (Ruby *et al.*, 1995; Singh and Aggarwal, 1995); however, the mechanism of these inhibitory effects remains unclear. In the present report, we show that curcumin completely blocked JNK activation by various agents including PMA plus ionomycin, γ radiation, UV-C, anisomycin, TNF-α, and sodium orthovanadate. Curcumin inhibited both JNK and ERK activation induced by PMA plus ionomycin; however, JNK induction is more sensitive than ERK induction. By the data of *in vitro* and *in vivo* inhibition assays, we propose that curcumin may inhibit JNK induction by suppressing the regulator(s) at the same level or proximally upstream of the MAPKKK level. Curcumin has both cytostatic and cytotoxic effects, which may be associated to its inhibition of ERK and JNK activation.

Results

Curcumin blocks c-*jun* gene expression induced by phorbol esters (Huang *et al.*, 1991). JNK is capable of phosphorylating c-Jun and enhancing its transcriptional activity, which is important for c-*jun* gene expression. It is possible that curcumin blocks c-*jun* expression by suppressing JNK activation. We therefore tested whether curcumin can inhibit JNK activation induced by PMA plus ionomycin, the T-cell activation signals, in Jurkat cells. JNK activation induced by PMA plus ionomycin was completely blocked by pretreatment of Jurkat T cells with curcumin (50 μM) for 1 h (Figure 1). To examine whether curcumin could inhibit the effects of other JNK stimuli, several other agonists were tested. We found the pretreatment of curcumin suppressed JNK activation induced by all of the tested agents including UV-C, γ radiation, anisomycin and TNF-α (Figure 1). This inhibition of JNK activation could be due to either the suppression of the upstream activator(s) of JNK or the enhancement of the activity of dual-specificity JNK phosphatases, which are known to suppress JNK activity (Chu *et al.*, 1996; Muda *et al.*, 1996). However, curcumin also inhibited JNK activation induced by the phosphatase inhibitor, sodium orthovanadate (Figure 1), which inhibits the dual-specificity JNK phosphatases. Therefore, the results suggest that curcumin most likely blocked JNK activation by inhibiting the upstream activator(s) of the JNK pathway.

To examine whether curcumin inhibits the JNK pathway specifically, we studied the dose response of curcumin's inhibitory effects on both JNK and ERK activation induced by PMA plus ionomycin in Jurkat cells. Curcumin blocked JNK activation in a dose-dependent manner and a complete inhibition of JNK activation was observed in treatments with around 40 μM of curcumin (Figure 2a and b). The IC_{50} (50% inhibitory concentration) for JNK inhibition was between 5–10 μM (Figure 2b). Similar inhibition curves were observed in anisomycin and UV-C-

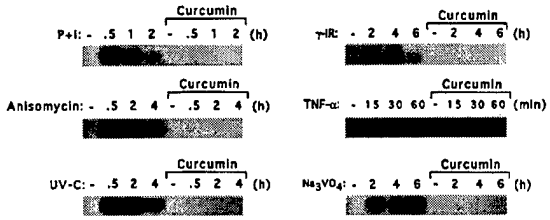

Figure 1 Curcumin inhibits JNK activation by various agonists. Jurkat T cells were incubated in medium with or without curcumin (50 μM) for 1 h, and then treated with various JNK agonists including PMA (50 ng/ml) plus ionomycin (1 μM), γ radiation (80 Gy), UV-C (200 J/m^2), anisomycin (2 μg/ml), and sodium orthovanadate (1.5 mM). MCF-7 cells were cultured with or without curcumin (50 μM) for 1 h, and then treated with TNF-α (100 ng/ml). Cells were collected at different time points as indicated, and the endogenous JNK1 activity was determined by immunocomplex kinase assays

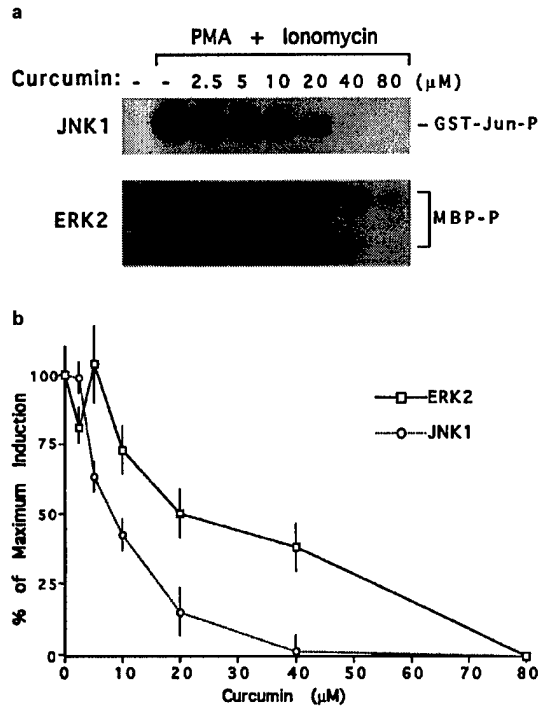

Figure 2 Curcumin inhibits both JNK and ERK activation. (**a**) Jurkat T cells were incubated in medium containing different concentrations of curcumin for 1 h, and then were stimulated with PMA (50 ng/ml) plus ionomycin (1 μM) for 30 min. Endogenous JNK1 and ERK2 activities were determined by immunocomplex kinase assays using GST-Jun(1–79) and MBP, respectively, as substrates. (**b**) The kinase activation shown in panel a was quantitated with a densitometer. Residual kinase activity after curcumin treatment was presented as a percentage of maximum induction, which is kinase activation by PMA plus ionomycin in the absence of curcumin. For quantitation of MBP phosphorylation, both phosphorylated protein bands were measured and the average value were presented. Data presented are the means and standard deviations of three measurements

induced JNK activation (data not shown). ERK2 induction by PMA plus ionomycin was also sensitive to curcumin treatment (Figure 2a and b); however, the IC_{50} was 20 μM, which was higher than that for JNK inhibition (Figure 2b). Furthermore, although curcumin suppressed ERK2 induction by PMA plus ionomycin, incubation with curcumin did not abolish the ERK2 basal activity even at a concentration of 80 μM (Figure 2a).

JNK and ERK activities are required for mitogenic signaling and cell proliferation (reviewed in Robinson and Cobb, 1997; Whitmarsh and Davis, 1996). Therefore, inhibition of these pathways by curcumin should affect cell growth and survival. Jurkat T cells were incubated in media containing 0, 10, 20 or 40 μM of curcumin, and were examined for the next 3 days. The cells cultured with normal medium had a doubling time of around 36 h. Incubation of the cells in 10 μM curcumin slowed the growth rate in comparison with the control (Figure 3a); however, it did not change the survival rate significantly (Figure 3b). Higher concentrations (20 and 40 μM) of curcumin completely suppressed the cell growth and induced significant cell death after day 2 (Figure 3a and b). These results indicate that the ability of curcumin to suppress cell proliferation was correlated with its ability to suppress JNK and ERK activities (Figure 2b) and that curcumin has both cytostatic and cytotoxic effects depending on drug concentrations and the duration of treatment.

Because of the effective inhibition of JNK activation by curcumin, we further examined the molecular target of curcumin. *In vitro* incubation of curcumin with purified JNK recombinant proteins did not inhibit their ability to phosphorylate GST-Jun proteins (Figure 4a). Incubation of curcumin with GST-SEK1 proteins also failed to suppress SEK1 activity, as assayed by using GST-SAPK(KR), a kinase-dead mutant, as a substrate (Figure 4a). Similarly, curcumin did not abolish *in vitro* phosphorylation of GST-SEK1 by purified GST-MEKK1 (Figure 4a). These data indicate that curcumin does not directly inhibit the kinase function of JNK, SEK1, and MEKK1. We then transfected the HA-tagged JNK plasmid with plasmids encoding various upstream kinases including SEK1 (Sanchez *et al.*, 1994), MEKK1 (Yan *et al.*, 1994), TAK1 (Yamaguchi *et al.*, 1995), GCK (Pombo *et al.*, 1995), and HPK1 (Hu *et al.*, 1996) into 293 cells. The transfected cells were cultured in complete medium for 6 h for recovery, and then were treated with or without 20 μM of curcumin for 24 h. The JNK activity was determined by immunoprecipitating the kinase with an anti-HA antibody, followed by kinase assays. Curcumin treatment did not inhibit JNK activity isolated from cells transfected with HA-JNK alone or in combination with SEK1, in contrast, it enhanced the JNK activation by SEK1 *in vivo* in repeated experiments (Figure 4b and data not shown). JNK activity induced by MEKK1 was only slightly inhibited by curcumin (Figure 4b). These results are consistent with the *in vitro* inhibition experiments that JNK, SEK1 and MEKK1 may not be direct targets for curcumin's inhibitory effect. In contrast, curcumin significantly suppressed JNK induction by co-expression of TAK1, GCK, and HPK1 (Figure 4b). Curcumin did not significantly affect the expression of HA-JNK (Figure 4b), indicating that this *in vivo* inhibition was not due to the suppression of HA-JNK expression.

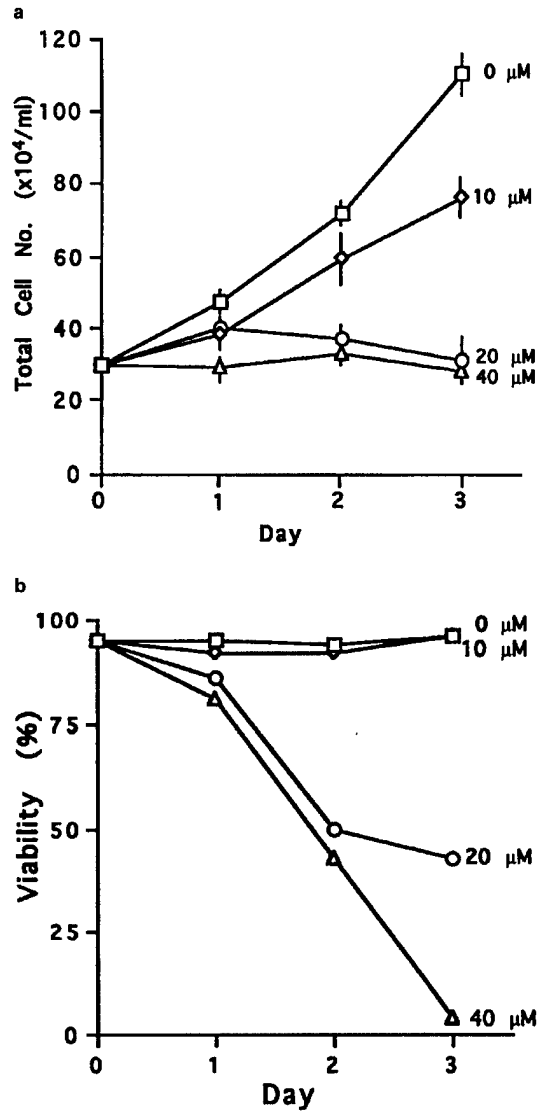

Figure 3 Curcumin has both cytostatic and cytotoxic effects. Jurkat cells were incubated in culture media containing 0, 10, 20, or 40 μM of curcumin with an initial cell density of 3×10^5 cells/ml. (**a**) The total cell numbers were counted by a hemacytometer for the next 3 days. (**b**) The percentage of viable cells in each treatment was determined by a trypan blue exclusion method

Because curcumin suppressed the JNK activation by TAK1, HPK1 and GCK *in vivo*, we further examined whether these JNK upstream kinases are the direct targets of curcumin. We immunoprecipitated the individual kinases from 293 cells transfected with HPK1, TAK1, or GCK and performed *in vitro* kinase assays using myelin basic protein (MBP) as a substrate in the presence of various concentrations of curcumin. The kinase activity of HPK1 was not affected by coincubation with curcumin (Figure 5). Curcumin inhibited MBP phosphorylation by TAK1 and GCK at high concentrations; however, as observed in repeated experiments, it did not abolish the kinase activities even at the highest concentration tested

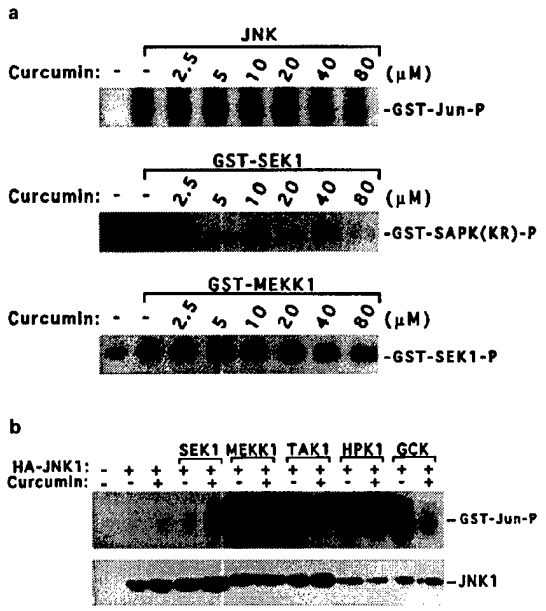

Figure 4 Effect of curcumin on different JNK regulators. (**a**) Purified recombinant JNK, GST-SEK1, and GST-MEKK1 protein were incubated with different concentrations of curcumin in kinase buffer for 10 min at room temperature, then GST-Jun(1–79), GST-SAPK(KR), and GST-SEK1 were added as substrates, respectively, in the presence of [γ-^{32}P]ATP for kinase reactions (30 min at 30°C). Phosphorylation of the substrate was analysed by SDS–PAGE and autoradiography. (**b**) 293 cells were transfected with the HA-JNK plasmid with or without plasmids encoding various upstream kinases including SEK, MEKK1, TAK1, GCK, and HPK1. Cells were cultured in complete medium for 6 h after transfection and then treated with 20 μM of curcumin for 24 h. The HA-JNK activity in the transfected cells was immunoprecipitated by an anti-HA antibody and determined by immunocomplex kinase assays. Expression levels of JNK proteins were examined by Western blotting

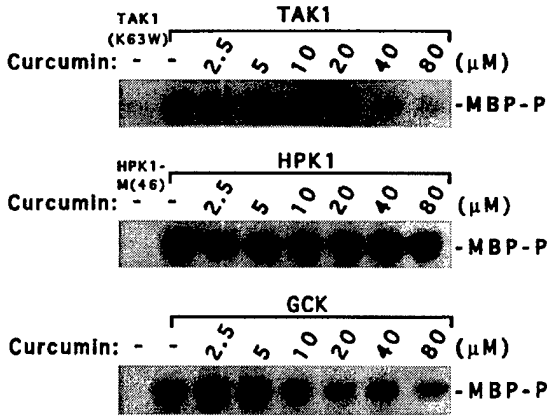

Figure 5 Effect of curcumin on TAK1, HPK1 and GCK kinase activities *in vitro*. TAK1, HPK1, or GCK was immunoprecipitated from cell lysates prepared from 293 cells transfected with the individual wild-type or dominant-negative kinases. The precipitated kinases were incubated with different concentration of curcumin in kinase buffer for 10 min at room temperature, then myelin basic proteins (MBP) were added as substrates in the presence of [γ-^{32}P]ATP for kinase reactions (30 min at 30°C). Phosphorylation of the substrate was analysed by SDS–PAGE and autoradiography

(80 μM) (Figure 5 and data not shown). Because curcumin inhibited JNK activation effectively at lower concentrations in experiments *in vivo* (Figures 1 and 2), this moderate inhibition of TAK1 and GCK does not fully account for the curcumin's inhibitory effect on the JNK pathway *in vivo*.

Discussion

Curcumin is a dietary compound with the known properties of antioxidation, anti-inflammation, and anticarcinogenesis (Huang *et al.*, 1994; 1988; Rao *et al.*, 1995; Ruby *et al.*, 1995; Singh and Aggarwal, 1995). Curcumin blocks c-*jun* expression and NF-κB activation induced by various agents (Hanazawa *et al.*, 1993; Huang *et al.*, 1991; Singh and Aggarwal, 1995). This inhibitory effect is believed to be the major mechanism for the biological effect of this chemical. However, the mechanism by which curcumin inhibits transcriptional activation remains unknown. In this report, we showed that curcumin completely blocked JNK activation by various agonists. Curcumin was more effective in blocking JNK activation than ERK activation induced by PMA plus ionomycin, and it failed to eliminate basal ERK activity (Figure 2). This may explain the observation in a previous report that curcumin preferentially inhibits c-*jun* gene expression, but not c-*fos* expression, induced by PMA stimulation (Huang *et al.*, 1991). c-Jun transcriptional activity, which is dependent on JNK activation, is essential for its own gene expression (Karin, 1995). Thus inhibition of JNK by curcumin would result in the inhibition of c-Jun activation and transcription of the c-*jun* gene. In contrast, c-*fos* gene expression is more dependent on ternary complex factors, which can be activated by the ERK pathway and bind to the serum-response element (Karin, 1995). Therefore, c-*fos* expression induced by PMA is less affected by curcumin.

By the *in vitro* inhibition assays, we showed that kinase activities of JNK, SEK1, MEKK1, and HPK1 may not be the direct targets of curcumin's inhibitory effect. However, curcumin moderately inhibit MEKK1-induced JNK activation and effectively inhibited JNK activation by coexpression of TAK1, HPK1, and GCK. Although curcumin suppressed TAK1 and GCK activities at high concentrations *in vitro*, these inhibitory effects probably cannot fully account for the inhibition of JNK activation *in vivo*. The fact that curcumin inhibited JNK induction by various agonists (Figure 1) implies that curcumin interferes with the proximal upstream rather than the distal upstream of the JNK pathway. Our results suggest that curcumin may act on regulators at the same level or proximally upstream of the MAPKKK level. The inhibition of upstream regulators can lead to the unresponsiveness of the JNK cascade (MEKK1-SEK-JNK). MEKK1 has been shown to enhance the NF-κB transcription activity (Hirano *et al.*, 1996; Meyer *et al.*, 1996), and is a candidate for the kinase that activates the IκB kinase complex (Lee *et al.*, 1997). The IκB kinase complex phosphorylates IκB and this results in its degradation and the activation of NF-κB (Chen *et al.*, 1996c). Our data suggest that inhibition of the MEKK1-JNK pathway by curcumin may also contribute to the suppression of NF-κB activation. Therefore, through

inhibition of the JNK signaling module, curcumin can effectively block both AP-1 (c-Jun) and NF-κB signaling pathways.

AP-1 and NF-κB activities are important for cell proliferation and both activities are required for expression of immediate-early or early genes, such as IL-2 and IL-2 receptor in lymphocytes (Kelly and Siebenlist, 1995). In this report, we show that curcumin inhibited both the JNK and ERK pathways. The inhibition of the ERK and JNK pathways and the suppression of AP-1 and NF-κB can block cell growth. Thus, the anticarcinogenic effect of curcumin may be due to its inhibition of these cellular pathways and suppression of cell growth. Besides cell proliferation, AP-1 and NF-κB are also involved in the cellular reaction to inflammatory cytokines such as interleukin-1 and TNF-α (Brenner *et al.*, 1989; Finco and Baldwin, 1995; Muegge *et al.*, 1989), which play important roles in many immune responses. Curcumin's ability to block AP-1 and NF-κB could be a useful tool in controlling inflammatory and autoimmune diseases.

Materials and methods

Cells, antibodies and reagents

Human Jurkat T cells (clone J.LEI) were cultured in RPMI-1640 supplemented with 10% fetal calf serum and streptomycin/penicillin. MCF-7 cells and human embryonic kidney 293 cells were cultured in DMEM supplemented with 10% fetal calf serum and streptomycin/penicillin. Rabbit anti-JNK1 (Ab101) and anti-HPK1 (Ab2025) antibodies were described previously (Chen *et al.*, 1996a; Hu *et al.*, 1996). Anti-ERK2 (#C-14), anti-TAK1 (#M-17), and anti-GCK (#N-19) antibodies were purchased from Santa Cruz. The anti-HA antibody (#12CA5) was purchased from Boehringer Mannheim. Curcumin, PMA, ionomycin, and anisomycin were purchased from Sigma. Recombinant JNK protein was purchased from Stratagene. Recombinant human TNF-α was purchased from R&D System.

Plasmids

GST-Jun(1-79), pCIneo-HPK1, pCIneo-HPK1-M(46), and HA-JNK plasmids were described previously (Hu *et al.*, 1996; Wang *et al.*, 1996). The GST-SAPK(KR) and pUna3-MEKK1 were kindly provided by D Templeton (Case Western Reserved U, Cleveland, OH), the GCK plasmid was a gift from J Kehrl (NIH, Bethesda, MD), pEBG-SEK1 was a gift from L Zon (Harvard Medical School, Boston, MA), and the TAK1 and TAK1(K63W) plasmids were gifts from K Matsumoto (Nagoya University, Nagoya, Japan).

Cell transfection and treatment

Briefly, 293 cells were plated 24 h before transfection at a density of 1.5×10^5 per 35 mm well. Cells were co-transfected with the HA-JNK plasmid in combination with different expression plasmids for the upstream kinases as indicated. The transfections were performed by a calcium phosphate precipitation protocol (Specialty Media) with duplicates in each transfection. After removing the transfection mixture, the cells were incubated in complete medium for 6 h for recovery, then treated with or without curcumin for 24 h.

Cell extracts preparation and immunocomplex kinase assays

Whole cell lysate was prepared by suspending 2×10^6 cells in 200 μl lysis buffer (20 mM HEPES [pH 7.4], 2 mM EGTA, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothrietol [DTT], 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM sodium orthovanadate). The cell lysates were kept on ice and vigorously vortexed every 5 min for 20 min. The lysates were cleared by centrifugation at 15 000 *g* for 3 min, and the supernatants were stored at −80°C. Kinase assays were carried out as described (Chen *et al.*, 1996b). Kinases were precipitated by incubation with indicated specific antibodies and protein A-agarose beads (Bio-Rad) in the lysis buffer at 4°C for 3 h. The precipitates were washed twice with the lysis buffer, twice with the LiCl buffer (500 mM LiCl, 100 mM Tris-Cl [pH 7.6] and 0.1% Triton X-100), and twice with the kinase buffer (20 mM 4-morpholinepropane-sulfonic acid [MOPS; pH 7.6], 2 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100 and 1 mM sodium orthovanadate), then mixed with 5 μg of substrate (GST-Jun(1−79) or MBP), 15 μM of ATP, and 10 μCi of [γ-^{32}P]ATP in 30 μl of kinase buffer. The kinase reaction was performed at 30°C for 30 min, then terminated by adding SDS sample buffer. The reaction mixtures were boiled and analysed by SDS–PAGE and autoradiography.

Abbreviations

JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; SEK, SAPK and ERK kinase; MEKK1, MAPK kinase kinase-1; TGF-β, transforming growth factor-β; TAK1, TGF-β activated kinase-1; GCK, germinal center kinase; HPK1, hematopoietic progenitor kinase-1; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor-α; MBP, myelin basic protein.

Acknowledgements

We thank Drs R Davis, MC-T Hu, M Karin, J Kehrl, K Matsumoto, D Templeton and L Zon for providing valuable reagents, members of Tan laboratory for their helpful discussions and critical reading of this manuscript, A Brown and S Lee for technical assistance, and M Lowe for secretarial assistance. This work was supported by the National Institutes of Health grant R01-AI38649 to T-H Tan, who is a Scholar of the Leukemia Society of America. YR Chen is a recipient of Department of Defense Predoctoral Fellowship DAMD17-97-1-7078 in the Breast Cancer Research Program.

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Biochemical and Biophysical Research Communications **256**, 595–599 (1999)
Article ID bbrc.1999.0383, available online at http://www.idealibrary.com on IDEAL®

Lack of Correlation in JNK Activation and p53-Dependent Fas Expression Induced by Apoptotic Stimuli

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Received February 4, 1999

Induction of Fas expression by DNA-damaging agents is dependent on the expression of functional p53, and has been suggested to play an important role in apoptosis induction. JNK (c-Jun N-terminal kinase), which is capable of phosphorylating p53, is also involved in apoptotic signaling induced by various apoptotic stimuli. Here, we report that although Fas induction is closely linked to the expression of wild type p53, it is not correlated with JNK activation induced by apoptotic stimuli. JNK activation does not necessarily lead to Fas expression, even in cells containing wild type p53. In addition, Fas expression can be induced without significant JNK activation. Furthermore, induction of Fas expression is not sufficient for apoptosis induction; however, it may sensitize cells to Fas-ligation induced apoptosis. © 1999 Academic Press

c-Jun N-terminal kinase (JNK), also called stress-activated protein kinase (SAPK), belongs to the mitogen-activated protein kinase (MAPK) superfamily which also includes extracellular signal-regulated kinases (ERKs) and the p38-MAPK. Substrates for JNK include the transcription factors c-Jun, JunD, ATF-2, ATFa, Elk-1, and Sap-1a (reviewed in 1-3). The JNK pathway is involved in apoptotic signaling (reviewed in 3). JNK is induced by growth factor withdrawal, UV-C, γ radiation, ceramide, heat shock, and DNA-damaging drugs (4–9). Activation of the JNK pathway can lead to cell death (4, 8). Interference with the JNK pathway by the dominant-negative mutant of MEKK1, MKK4/SEK, or JNK1 suppresses apoptosis (4, 6–8). JNK's substrate, c-Jun, is required for ceramide-induced apoptosis (10) and apoptosis of neuronal cells caused by NGF withdrawal (11, 12).

Tumor suppressor p53 plays an important role in apoptosis induced by γ radiation and by adenovirus E1A gene (13, 14). It has been shown that p53 may facilitate the expression of Bax (15), an apoptosis accelerator, and the expression of the death receptor Fas (16, 17). The p53 protein is a direct substrate for JNK (18, 19). JNK-mediated p53 phosphorylation has been shown to disrupt MDM2-p53 interaction and prevent p53 degradation and, subsequently, enhance p53-mediated transcription (20). A recent report also showed that p53 can up-regulate Fas expression through a translation-independent pathway (21). We studied whether p53-mediated Fas expression is one of downstream events of JNK activation by examining the correlation between JNK activation and Fas expression in cells with different p53 status. We found that enhancement of Fas expression was associated with wild-type p53 expression; however, it did not associate with JNK activation. Furthermore, induction of Fas expression alone is not sufficient to induce apoptosis in γ irradiated cells.

MATERIALS AND METHODS

Cell culture. MCF-7 breast cancer cells, prostate carcinoma cell lines LNCaP and PC-3 (from ATCC), and myeloid leukemia cell lines HL-60, BV173, and KBM7 (provided by Dr. W. Zhang, M. D. Anderson Cancer Center, Houston) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, gentamycin, and streptomycin/penicillin.

Reagents and radiation treatments. 4-Hydroxyphenyl retinamide was obtained from Sigma. Rabbit anti-JNK1 antibody (Ab101) was described previously (5). The monoclonal anti-Fas IgM (CH-11) and fluorescein isothiocyanate (FITC)-conjugated anti-Fas antibody were purchased from Kamiya Biomedical Company and Pharmingen, respectively. Ultraviolet C (UV-C) irradiation was performed by using a UV Stratalinker 1800 (Stratagene). Gamma irradiation was performed using a Gammacell 1000 ^{137}Cs source (Atomic Energy of Canada Limited).

Cell extract preparation and immunocomplex kinase assays. Whole cell lysate was prepared by lysing 2×10^6 cells in 200 μl lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM EGTA, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothrietol [DTT], 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM Na_3VO_4). The cell lysates were kept on ice and vigorously vortexed every 5 min for 20 min. The lysate was cleared by centrifugation at 15,000 g for 3 min, and the supernatant was stored at −80 °C. Endogenous JNK1 was precipi-

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TABLE 1

Human Cell Lines Used in This Study

Name	Cell type	p53 status	References
HL-60	Monocytic leukemia	−/−	22
KBM-7	Myeloid leukemia	Mutated	23
BV178	Myeloid leukemia	+/+	
LNCaP	Prostate cancer	+/+	24, 25
PC-3	Prostate cancer	−/−	24, 25
MCF-7	Breast cancer	+/+	27, 28

tated by incubation with an anti-JNK1 antibody (Ab101) and protein A-agarose beads (Bio-Rad) in the lysis buffer at 4 ℃ for 3 h. The precipitates were washed twice with the lysis buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris-Cl [pH 7.6], and 0.1% Triton X-100), twice with kinase buffer (20 mM 4-morpholinepropanesulfonic acid [MOPS; pH 7.6], 2 mM EGTA, 10 mM $MgCl_2$, 1 mM [DTT], 0.1% Triton X-100, and 1 mM Na_3VO_4), and then mixed with 5 μg of GST-Jun(1–79), 15 μM of ATP, and 10 mCi of [γ-^{32}P]ATP in 30 μl of kinase buffer. The kinase reaction was performed at 30 ℃ for 30 min, then terminated by adding SDS sample buffer. The reaction mixtures were boiled and analyzed by SDS-PAGE and autoradiography.

Nuclear staining. For nuclear morphology staining, the harvested cells were fixed with 1% paraformaldehyde (in 1× PBS) for 10 min, washed once with 1× PBS, then incubated in Hoechst 33258 (2.5 ng/ml in PBS) solution. The nuclear morphology was examined by a fluorescence microscope. Cells with condensed or fragmented nuclei were considered as apoptotic cells.

Flow cytometry analyses. One million cells with or without treatments were harvested and stained with FITC-conjugated anti-Fas antibody (20 μl) in 100 μl of 1× phosphate buffered saline (PBS) containing 2% of fetal calf serum (FCS) on ice for 30 min. The stained cells were then washed twice with PBS containing 2% FCS, and fixed with 1% paraformaldehyde in PBS. The stained cells were analyzed by flow cytometry assays (Profile and XL, Coulter Co.).

RESULTS AND DISCUSSION

Induction of Fas expression is associated with wild type p53 but not with JNK activation in leukemia cells. To study the correlation between JNK induction, Fas expression, and p53 status, we examined the effect of γ radiation on three leukemia cell lines with different p53 status (Table 1) (22, 23; W. Zhang, personal communication). γ radiation induced JNK activation in three myeloid cells lines HL-60, KBM-7, and BV173, although KBM-7 cells were more resistant to γ radiation in JNK activation than the other two (Fig. 1A). Nevertheless, γ radiation only induced Fas expression in BV-173 which has wild type p53 genes, but not in $p53^{-/-}$ HL-60 cells or in KBM-7 cells, which have mutated p53 (Fig. 1B). The induction of Fas expression was also irrelevant to the basal expression levels of Fas in each cell line. These results, which are consistent with previous reports (16), indicate that the induction of Fas expression is associated with the presence of functional p53 proteins; however, JNK activation is not correlated with expression of Fas receptors.

Induction of Fas expression is associated with a wild type p53 status but not with JNK activation in prostate cancer cells. To study the relationship between enhancement of Fas expression and JNK activation further, two prostate carcinoma cell lines, $p53^{+/+}$ LNCaP cells and $p53^{-/-}$ PC-3 cells (24, 25), were treated with apoptotic stimuli—γ radiation or a retinoic acid analog 4-hydroxyphenyl retinamide (4-HPR). γ radiation induced JNK activation in both LNCaP and PC-3 cells (Fig. 2A), while 4-HPR induced JNK activation only in LNCaP cells (Fig. 2B). γ radiation significantly enhanced Fas expression in $p53^{+/+}$ LNCaP but not in p53-null PC-3 cells (Fig. 2C). However, no induction in Fas expression was observed in either LNCaP or PC-3 cells treated with 4-HPR (Fig. 2C), although 4-HPR induced a persistent JNK activation in LNCaP cells. These results indicate that Fas expression is associated with the wild type p53 status; however, JNK activation is not necessarily associated with Fas expression in $p53^{+/+}$ or $p53^{-/-}$ cells.

Expression of Fas in the absence of JNK induction is not sufficient for apoptosis induction. JNK activation is involved in and required for radiation-induced apoptosis (5–8). Other reports suggested that Fas expression may be involved in apoptosis induced by DNA damaging agents (16, 26). However, we observed no

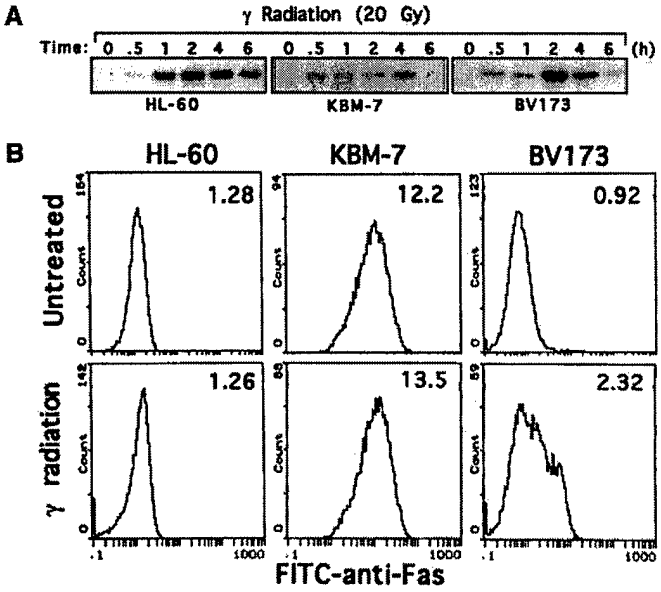

FIG. 1. Induction of Fas expression was associated with the status of p53 but not with the JNK activation in leukemia cells. (A) HL-60 ($p53^{-/-}$), KBM-7 (p53 mutant), and BV173 ($p53^{+/+}$) cells were irradiated with γ radiation (20 Gy). The cells were collected at different time points, and the endogenous JNK activities were analyzed by immunocomplex assays. (B) HL-60, KBM-7, and BV173 cells were irradiated with or without γ radiation (20 Gy), and were harvested 8 h after irradiation. Surface expression of Fas on irradiated cells were examined as described in Materials and Methods. The numbers presented were means of fluorescence intensity.

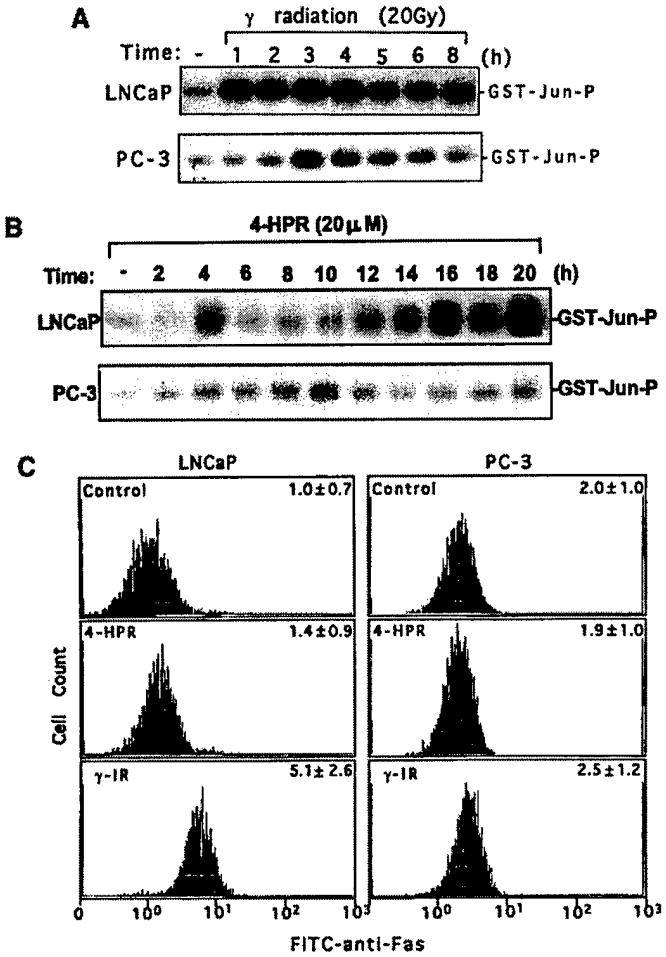

FIG. 2. JNK activation and Fas expression in LNCaP and PC-3 cells by γ radiation and 4-HPR. LNCaP and PC-3 cells were treated with (A) γ radiation (20 Gy) or (B) 4-HPR (20 μM). The cells were harvested at indicated time points and the endogenous JNK activity was determined by immunocomplex kinase assays as described in Materials and Methods. (C) LNCaP and PC-3 cells were treated with or without γ radiation (20 Gy) or 4-HPR (20 μM). Cells were harvested 20 h after treatments, and stained with FITC-conjugated anti-Fas antibody. The expression levels of Fas were examined by flow cytometry assays. The numbers presented were mean fluorescence intensity and standard deviation.

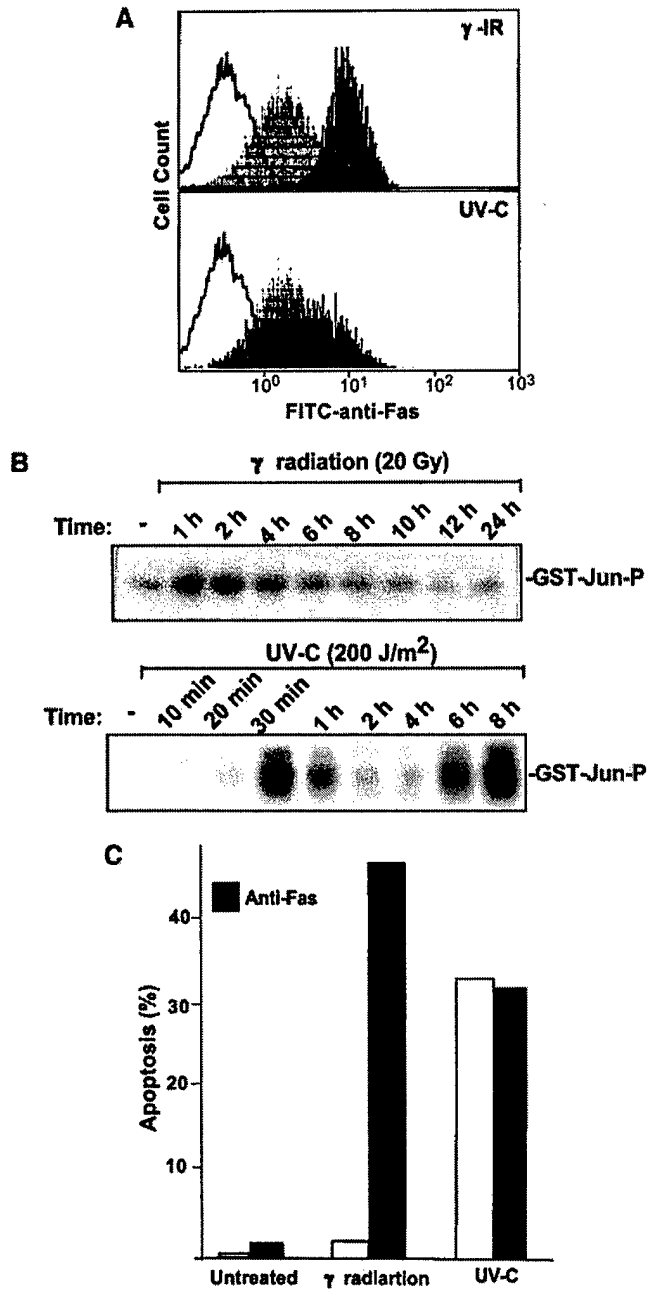

FIG. 3. Lack of correlation between Fas expression and apoptosis in MCF-7 cells treated with radiation. MCF-7 cells were treated with γ radiation (20 Gy) or UV-C (200 J/m²). (A) Cells were harvest 12 h after irradiation, and surface Fas expression was examined as described in Material and Methods. Solid curve: background, gray shade: basal Fas expression, black shade: γ or UV radiation-induced Fas expression. (B) Cells were collected at indicated time points and endogenous JNK was examined by immunocomplex kinase assays. (C) MCF-7 cells were untreated or treated with γ radiation (20 Gy) or UV-C (200 J/m²), then incubated in culture medium with or without an anti-Fas (CH-11, 100 ng/ml) for 36 h. Apoptosis induction were examined by nuclear staining. Cells with condensed or fragmented nuclei were identified as apoptotic cells.

correlation in JNK activation and Fas expression. We further examined whether Fas expression can be induced in the absence of JNK activation, and whether Fas expression alone is sufficient for apoptosis induction. MCF-7 breast cancer cells, which contain wild type p53 alleles (27, 28), were treated with various apoptotic agents, and examined for Fas expression, endogenous JNK activation, and apoptosis induction. Among the agents tested, γ radiation (20 Gy) induced significant levels of Fas expression in MCF-7 cells (Fig. 3A), but the same treatment induced only transient and weak JNK activation (Fig. 3B). We did not observe apparent apoptosis induction in MCF-7 cells by nuclei staining with Hoechst dye 36 h after γ irradiation (Fig.

3C). Addition of a Fas cross-linking antibody after γ radiation significantly augmented apoptosis induction in MCF-7 cells (Fig. 3C). This apoptosis may be triggered by death signaling from the newly synthesized and cross-linked Fas proteins. In contrast, UV-C caused strong JNK activation and apoptosis in MCF-7 cells (Fig. 3, B and C) in the absence of significant Fas induction (Fig. 3A). The addition of anti-Fas antibody did not enhance UV-induced apoptosis in MCF-7 cells (Fig. 3C).

It has been implicated that the enhancement of Fas expression, a p53-dependent event, may be involved in apoptosis induced by γ radiation and DNA damaging chemicals (16, 26). We and others also showed that JNK, which phosphorylate p53 proteins (18, 19), plays an important role in apoptotic signaling (4–9). Here, we show that JNK activation is not associated with the enhancement of Fas expression on the cell surface. JNK activation does not necessarily lead to Fas expression, even in the presence of wild type p53. In addition, Fas expression can be induced in the absence of significant JNK activation. These data suggest that p53-dependent Fas expression is not a downstream event of JNK activation. Induction of Fas expression was not sufficient for apoptosis induction; however, it may cause cells become susceptible to Fas ligation-induced apoptosis. It has been suggested that JNK may induce apoptosis by up-regulating expression of Fas ligand through c-Jun activation (29, 30). We did not detect significant surface expression of the Fas ligand in the conditions that induced JNK activation (data not shown). However, Fas ligand may be cleaved and released from the cell surface (31) and, therefore, be undetectable by surface staining. It is possible that upon receiving apoptotic stimuli, the combination of JNK-induced Fas ligand expression and p53-dependent Fas expression leads to the activation of death signaling. Several evidences suggest that Fas-Fas ligand interaction is not the only mechanism for JNK-mediated apoptosis. First, many cells with mutated p53 or null mutation in p53 genes undergo apoptosis after receiving radiation treatments, and activation of the JNK pathway induces apoptosis in p53-null cells, p53 mutant cells, or cells containing viral oncogenes that suppress p53 function (8, 32). These results suggest that a functional p53, which is required for Fas induction, may not be necessary for JNK-mediated apoptosis. Secondly, cells without FADD or containing dominant-negative FADD, which are defective in Fas signaling, are still sensitive to γ radiation and DNA-damaging chemicals (33, 34). This suggests that Fas/FasL, although induced, may not be required for apoptosis induced by DNA-damaging agents.

ACKNOWLEDGMENTS

We thank the members of Tan laboratory for their helpful discussions and critical reading of this manuscript, R. Afshar and S. Lee for technical assistance, and M. Lowe for secretarial assistance. This work was supported by the National Institutes of Health grants R01-AI38649 and R01-AI42532 (T.-H. Tan). T.-H. Tan is a Scholar of the Leukemia Society of America. Y.-R. Chen is supported by a predoctoral fellowship (DAMD17-97-1-7078) from the Breast Cancer Research Program, Department of Defense.

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