Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 22 (2011) 46–52
Journal of Nutritional Biochemistry

Involvement of nucleophosmin/B23 in the cellular response to curcumin

Chien-Hui Weng[a], Benjamin Yat-Ming Yung[b], Jing-Jei Weng[c], Ming-Hsiu Wu[d,*]

[a]Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung 824, Taiwan
[b]Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong
[c]Institute of Biopharmaceutical Science, National Yang-Ming University, Taipei 112, Taiwan
[d]Department of Nutrition and Health Science, Fooyin University, 151 Chin-hsueh Road, Ta-liao Hsiang, Kao-hsiung Hsien 831, Taiwan, Republic of China

Received 3 May 2009; received in revised form 10 November 2009; accepted 16 November 2009

Abstract

Nucleophosmin (NPM/B23) is a nucleolar phosphoprotein involved in cellular response to many different stimuli. Herein, we studied the molecular mechanism of NPM/B23 induction by curcumin, a natural AP-1 inhibitor with antitumor properties. Exposure to 5–30 μM curcumin significantly and dose-dependently increased the level of NPM/B23 in non-transformed NIH 3T3 cells but not HeLa cells and F9 cells. Besides, the transformed F9 and HeLa cells are more sensitive to curcumin-induced cell death and growth inhibition than NIH 3T3 cells. Overexpression of c-Jun, but not c-Fos, decreased ~40% of NPM/B23 and enhanced the sensitivity of NIH 3T3 cells to 30 μM curcumin. Furthermore, down-regulation of NPM/B23 by transfection with NPM/B23 antisense plasmid enhanced the sensitivity to curcumin-induced cell death and growth inhibition. These results indicated that NPM/B23 expression regulates cellular sensitivity to curcumin. Besides, NPM/B23 knockdown may facilitate as a novel strategy to promote the sensitivity of cancer cells to curcumin.

Keywords: Curcumin; Nucleophosmin/B23; Activator protein-1; c-Jun; c-Fos

1. Introduction

Chemoprevention is defined as a pharmacological approach used to arrest or reverse the process of cancer development prior to invasion and metastasis occurring [1]. Many dietary compounds are believed to be chemopreventive agents, which suppress the transformative, hyperproliferative and inflammatory processes that initiate carcinogenesis [1,2]. Curcumin, a natural component deriving from the Curcuma species, is commonly used as a yellow coloring and flavoring agent in foods [2]. This agent has been shown to possess potent antitumor, anti-inflammatory and anti-oxidative properties [1–3]. Curcumin induces apoptosis in many tumor cell lines [1–8] and specifically displays protective activity against tumor in animal models, including colon cancers [3], histocytic tumor [7] and brain tumor [8]. Additionally, curcumin is an efficient inhibitor of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion on mouse skin [9] and carcinogen-induced tumorigenesis in rat intestine [10]. It has been previously suggested that the antitumor promotion activity of curcumin is attributed to its ability to suppress activator protein-1 (AP-1) signaling [11]. AP-1 is a sequence-specific transcriptional factor, including members of the Jun and Fos families [12]. Both c-Jun and c-Fos are implicated in many cellular processes [13]. The induction of c-Fos and c-Jun is suggested to protect cells from stress-induced apoptosis [12,13]. c-Jun regulates gene expression and cell function by participating in the formation of homodimers or heterodimers with c-Fos and subsequently binding to TPA-response elements [13]. The DNA-binding ability of AP-1 can be suppressed by curcumin, and thus, AP-1 transcriptional activity is blocked by the presence of curcumin [11].

Nucleophosmin/B23 (NPM/B23, also called protein B23), a nucleolar phosphoprotein, is more abundant in cancer cells than in normal resting cells [14]. Biosynthesis of NPM/B23 is increased markedly and promptly in association with mitogensis [15] or certain cell stresses [16–19]. NPM/B23 binds both DNA and RNA [20] and acts as a molecular chaperone during the assembly of new nucleosomes and DNA repair [21]. The expression of NPM/B23 is crucial for rendering cancer cells resistant to induction of differentiation and apoptosis [22,23]. NPM/B23 is rapidly up-regulated following UV irradiation as p53 or AP-1, and the UV-induced NPM/B23 expression is an immediate-early gene response induced by UV-damaged DNA [16–18]. Recent reports suggested that NPM/B23 interacts with MDM2 to stabilize p53 [24], and thus, NPM/B23 can regulate the stability and transcriptional activity of p53 [24,25]. Additionally, NPM/B23 binds p53 and prevents p53 phosphorylation at Ser 15 in response to low-dose UV irradiation and acts as a repressor of p53 by setting a threshold for p53 activation [26]. Similarly, NPM/B23 is also induced in response to hypoxia, thereby protecting against cell death through inhibition of p53 activation [19].

Although many biological functions of curcumin have been identified, the precise molecular mechanisms underlying its actions remain unknown. In the present study, we investigated the role of AP-1 in molecular mechanism underlying curcumin-mediated NPM/B23

* Corresponding author. Fax: +886 7 782 6740.
E-mail address: mt116@mail.fy.edu.tw (M.-H. Wu).

0955-2863/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2009.11.010

induction. Our results suggest that up-regulation of NPM/B23 in response to curcumin is important for cellular resistance to cytotoxicity of curcumin in certain cell species.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma (St. Louis, MO, USA), unless indicated otherwise. Curcumin was dissolved in dimethyl sulfoxide and diluted to 2 mM with ethanol. The range of curcumin concentrations used was in accordance with previous studies (Refs. [4,7,27–30]).

2.2. Antibodies

Anti-β-actin monoclonal antibody was purchased from Sigma. Anti-c-Jun polyclonal antibody and anti-c-Fos polyclonal antibody were from Santa Cruz (Santa Cruz, CA, USA). Polyclonal anti-NPM/B23-specific antibody was purified from anti-NPM/B23 rabbit serum by an affinity column packed with *Escherichia coli*-over-expressed NPM/B23-Sepharose 4B (GE Healthcare, Piscataway, NJ, USA). Secondary antibodies and horseradish peroxidase-conjugated goat anti-mouse IgG antibody were obtained from Jackson ImmunoResearch (Baltimore, PA, USA), and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was acquired from Chemicon (Temecula, CA, USA).

2.3. Cell cultures

Cells were cultured in Gibco Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated serum (Hyclone, Logan, UT) (bovine calf serum for NIH 3T3 cells; fetal bovine serum for HeLa cells and F9 cells), antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) and 3.7 g/L sodium bicarbonate (Merck, Darmstadt Germany) in a 5% CO_2 humidified incubator at 37°C.

2.4. Western blotting

Cell extracts were prepared as described previously [17]. After normalizing for protein content, extracts were separated by 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (GE Healthcare) and probed with antibodies against NPM/B23, c-Jun, c-Fos or β-actin. The resultant immunobands were detected by enhanced chemiluminescence reaction (Amersham Biosciences). For quantification of NPM/B23 induced by curcumin, NPM/B23 and β-actin immunoband intensities were determined by densitometric scanning. The values of NPM/B23 were normalized with respect to intensities of β-actin. Data were analyzed by LabWork analysis software (UVP, Upland, CA, USA).

2.5. RNA preparation and RT-PCR

Total RNA was isolated with UltraspecTM RNA isolation system (Biotecx, Houston, TX, USA) following the manufacturer's procedure. Reverse transcription was performed with random hexamers and Superscript II RT (Invitrogen Life Technologies, Carlsbad, CA, USA) and was carried out at 50°C for 60 min and then stopped by a transition to 75°C for 15 min. The products of reverse transcription were mixed with 1 μl of (10 μM) NPM/B23-specific primer (forward primer sequence: 5′ TCGGCTGTGAACTAAAGGCT 3′; reverse primer sequence: 5′ GAGCAGATCGCTTTCCA-GAC 3′) or β-actin (forward primer sequence: 5′ GGGAATGGGTCAGAAGGACT 3′; reverse primer sequence: 5′ ATACAGGGACAGCACAGCCT 3′), 1 μl of (10 μM) dNTP, 2 μl of 10×PCR buffer and 5 U Taq DNA polymerase (Violet, Taiwan) in 20 μl H_2O. These reactions were followed by PCR using the following procedure: 95°C for 5 min; 24 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min; and a final extension at 72°C for 7 min. PCR products were then run on a 1.0% agarose gel and analyzed by means of ethidium bromide staining.

2.6. Plasmids

The c-Jun expression plasmid (pECP4-c-Jun) and the pBluescript KS-c-Fos plasmid were generously given by Dr. Yu-Sun Chang (Graduate Institute of Basic Medical Science, Chang-Gung University). The characterization of pECP4-c-Jun and pBluescript KS-c-Fos plasmid was as described previously [31]. The c-Fos expression plasmid (pCR3.1-c-Fos) was constructed by inserting cDNA of c-Fos (cutting from pBluescript KS-c-Fos by restriction enzymes HindIII and BamHI) into pCR3.1 vector. The characterization of cDNA reverse orientation plasmid antisense NPM/B23 (pCR3-ASB23) plasmid was as described previously [16,17]. pCEP4-c-Jun, pCR3.1-c-Fos and pCR3-ASB23 utilize CMV promoter to express c-Jun, c-Fos or antisense B23.

2.7. Cell transfection and establishment of stable clones

Transient transfections were performed using the Lipofectamine Reagent (Invitrogen) method [17]. Before transfection, cells (8×10^5 per dish) were seeded in a 6-cm dish overnight. Plasmid DNA (4 μg) and Lipofectamine Reagent (25 μg) were each diluted in serum-free medium (300 μl). Plasmids and Lipofectamine Reagent were then mixed and incubated for 30 min at room temperature to allow for DNA–liposome complex formation. Cells were then rinsed twice with phosphate-buffered saline and then the medium was replaced by serum-free medium (1.4 ml). Finally, cells were overlaid with DNA–liposome complexes. After 6 h of incubation at 37°C in CO_2 incubator, the DNA-containing medium was replaced by fresh medium containing 10% serum. Two days post-transfection, the transfected cells were maintained in DMEM containing 200 μg/ml hygromycin B (Calbiochem, San Diego, CA) or 600 μg/ml G418 (Calbiochem) for selection of stably transfected c-Jun- or c-Fos-expressing plasmid clones, respectively. After selection with hygromycin B or G418 for 2 weeks, stably transfected clones were expanded to mass cultures and subsequently assayed for c-Jun, c-Fos and NPM/B23 expression. The transfectants were maintained in culture medium supplemented with 100 μg/ml hygromycin B or 250 μg/ml of G418.

2.8. Statistical analysis

Data were expressed as means±standard deviation (S.D.) throughout this article. All experiments were performed, independently, three times. Statistical analyses were performed with one-way ANOVA test. P values <.05 were considered to represent statistically significant difference between compared data sets.

3. Results

3.1. NIH3T3 cells were more resistant to curcumin than F9 cells and HeLa cells

The NIH 3T3 cell line is considered as a non-transformed cell line, and HeLa and F9 cells are derived from tumor cell lines. We compared the effect of curcumin on these three cell lines by detection of cell growth and death. Twenty-four hours of 5 μM curcumin treatment resulted in ~37% relative cell growth (~63% of cell growth inhibition) and ~38% cell death among F9 cells, but such conditions did not affect

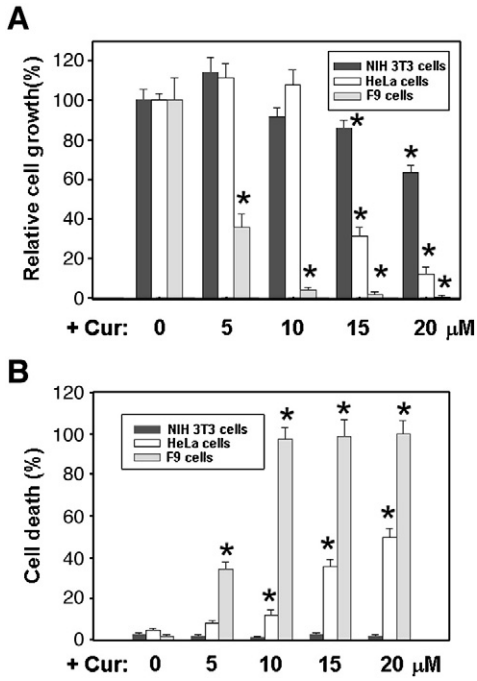

Fig. 1. HeLa cells and F9 cells were more sensitive to curcumin-induced cell death than NIH 3T3 cells. (A) The relative growth [proportion (%) of each untreated cell line at the same time point] for NIH 3T3 cells, HeLa cells and F9 cells at 24 h following curcumin treatment. Cell numbers were obtained by counting with a hemocytometer by trypan blue exclusion staining. Bars, means of triplicates±S.D. *P<.05, as compared with the relative growth of each untreated cell line. (B) Determination of the levels of viable cells and dead cells after NIH 3T3 cells, HeLa cells and F9 cells incubated with curcumin (5–20 μM) for 24 h. Viable and dead cells were determined by trypan blue exclusion staining. Bars, means of triplicates±S.D. *P<.05, as compared with the percentage of cell death in each of the corresponding untreated cell line.

the growth and death of HeLa cells and NIH 3T3 cells (Fig. 1). These doses of curcumin >10 μM caused nearly complete growth inhibition and cell death in F9 cells. The dose of curcumin-induced growth inhibition for HeLa cells and NIH 3T3 cells was greater than 15 μM (Fig. 1A). It was obvious that 15–20 μM curcumin induced some level of cell death for HeLa cells, but F9 cells were almost all dead under such conditions (Fig. 1B). In contrast to HeLa cells and F9 cells, most of NIH 3T3 cells showed no morphological changes or indication of cell death in response to 5–30 μM curcumin, and the dose of curcumin-induced NIH 3T3 cell death should be at least more than 40 μM (data not shown). These data indicated that HeLa cells and F9 cells were more sensitive to curcumin-induced cell death than NIH 3T3 cells. Therefore, we hypothesized that cancer cells and non-transformed cells may differ in their response to curcumin.

3.2. Curcumin dose-dependently induced NPM/B23 in NIH 3T3 cells but not in HeLa cells and F9 cells

Dose-dependent induction of NPM/B23 in NIH 3T3 cells was observed following 6 h of treatment with curcumin at a dose range of 10–30 μM (Fig. 2A). Further, NPM/B23 was also induced in NIH 3T3 cells by 20 μM curcumin, in a time-dependent manner (Fig. 2B). NPM/B23 was increased at the 3-h time point and reached a maximum at 12 h post-curcumin stimulation. Thereafter, NPM/B23 level remained high for at least 24 h (Fig. 2B). Similarly, NPM/B23 was induced by curcumin in HeLa cells, but the induction of NPM/B23 did not occur in a dose-dependent manner at curcumin concentration greater than 15 μM (Fig. 2C). F9 cells, an embryonal carcinoma cell line, are considered to lack endogenous AP-1 activity [31,32], and therefore, F9 cells were used as a model to examine the variation in NPM/B23 level following treatment of such cells with curcumin. As Fig. 2D reveals, stimulation of F9 cells with various doses of curcumin did not significantly increase the cellular level of NPM/B23 protein in F9 cells, even following treatment of cells with curcumin at a concentration of 30 μM. Moreover, the level of NPM/B23 protein did not change during exposure of such cells to curcumin at a concentration of 20 μM for 12 h (Fig. 2E). Practically, no F9 cell lysate was available following F9 cell exposure to 20 μM curcumin for 24 h, because most of the F9 cells were dead after 24 h treatment of curcumin. To compare precisely the level of NPM/B23 induced by various doses of curcumin, the immunoband intensities for NPM/B23 were quantified and normalized with respect to the corresponding intensities of β-actin (Fig. 2A, C and D). Fig. 2F depicts the quantification of NPM/B23 subsequent to the exposure of NIH 3T3 cells, HeLa cells and F9 cells to various doses of curcumin. In NIH 3T3 cells, curcumin dose-dependently stimulated expression of NPM/B23, and the level of NPM/B23 was increased by 1.8-, 2.3-, 3.2- and 4-fold in response to a 6-h exposure to 5, 10, 20 and 30 μM curcumin, respectively (Fig. 2F). In HeLa cells, the level of NPM/B23 was also increased after treatment of curcumin and peaked at a curcumin concentration of 10 μM (Fig. 2C and G). There was a 1.2-, 2-,

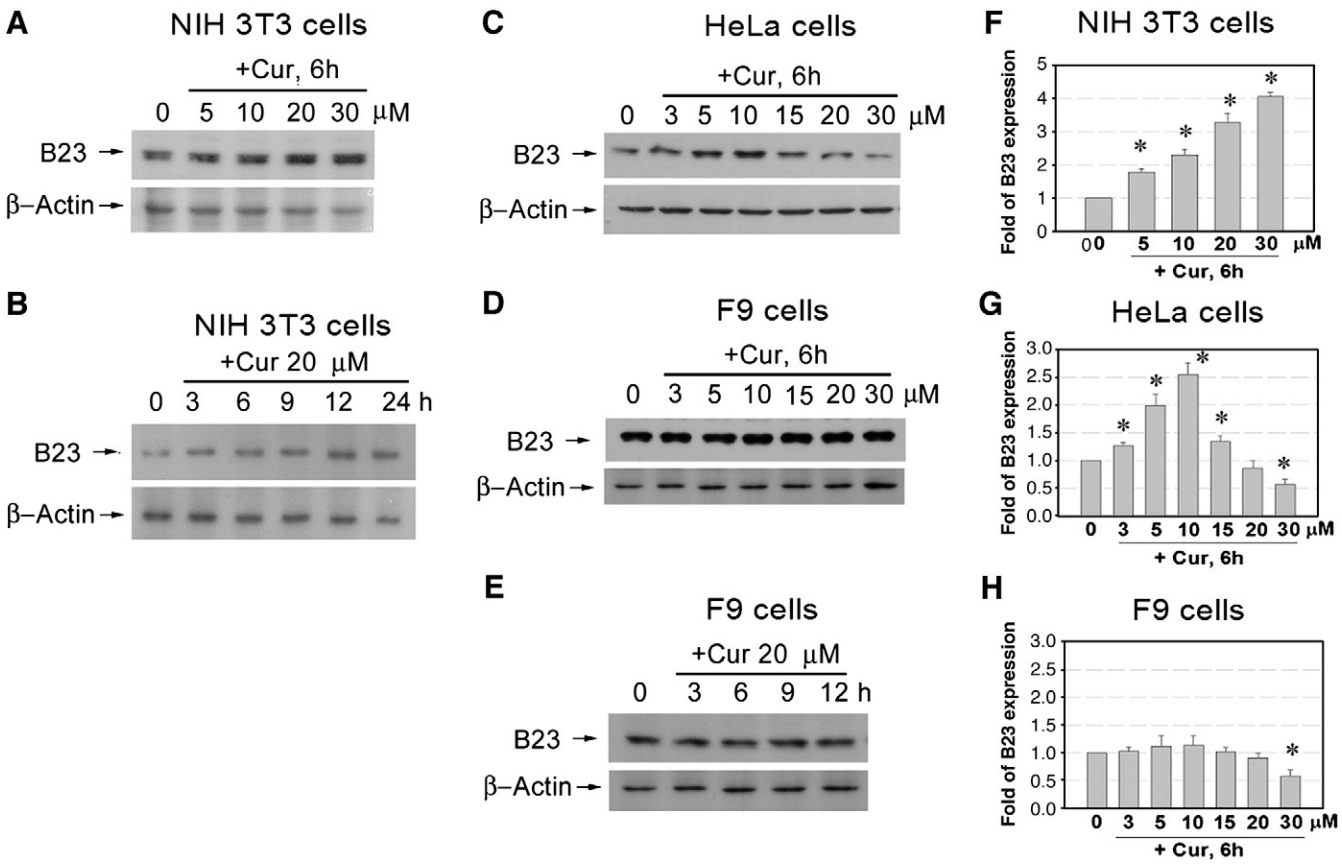

Fig. 2. NPM/B23 was significantly induced by 6 h of exposure curcumin in NIH 3T3, but not in HeLa cells and F9 cells. Cells were harvested and lysed at indicated conditions after curcumin treatment. Total cell lysates (20 μg/lane) were analyzed by Western blot using antibodies specific for NPM/B23 and β-actin. (A) The dose response of NPM/B23 induction after curcumin (Cur; 5–30 μM) treatment in NIH 3T3 cells. (B) The kinetics of NPM/B23 induction following 20 μM curcumin treatment in NIH 3T3 cells. (C) The induction of NPM/B23 after treatment of curcumin in HeLa cells. (D) The protein level of NPM/B23 subsequent to exposure of F9 cells to various concentration of curcumin (3–30 μM). (E) The NPM/B23 protein level during treatment of F9 cells with 20 μM curcumin. (F–H) Quantification of NPM/B23 protein level in cellular response to various concentration of curcumin in NIH 3T3, HeLa and F9 cells. These data are summarized from Fig. 1A, C and D. NPM/B23 and β-actin immunoband intensities were determined by densitometric scanning. Bars, means of triplicate±S.D. $^{*}P<.05$, as compared with the corresponding values for NPM/B23 normalized with respect to the immunoband intensities of β-actin in cells, which were not treated with curcumin (control group).

2.5- and 1.3-fold increase of NPM/B23 in response to a 6-h exposure of HeLa cells to 3, 5, 10 and 15 μM curcumin, respectively (Fig. 2G). In contrast to the 3- to 10-μM range of curcumin exposure for HeLa cells, where NPM/B23 increased, in the concentration range of 15–30 μM curcumin, NPM/B23 decreased, and at 30 μM, it was less than that for untreated cells (Fig. 2G). In F9 cells, NPM/B23 was not significantly induced by curcumin treatment, and NPM/B23 was decreased after treatment of 30 μM curcumin (Fig. 2D and H).

3.3. The up-regulation of NPM/B23 by curcumin does not occur through a transcriptional level

In order to study the mechanism by which curcumin elevates the intracellular level of NPM/B23, NPM/B23 mRNA was examined by RT-PCR method following curcumin treatment. Curcumin did not elicit a significant change in NPM/B23 mRNA level in NIH 3T3 cells (Fig. 3). Our previous investigations also revealed that curcumin did not affect NPM/B23 promoter activity and mRNA level in NIH 3T3 cells [18]. Therefore, curcumin-induced NPM/B23 may be regulated at a translational level, rather than at a transcriptional level.

3.4. Overexpression of c-Jun but not c-Fos down-regulated NPM/B23 and enhances cytotoxicity of curcumin in NIH 3T3 cells

Activity of AP-1 transcriptional factors might be an important signaling process for regulation of NPM/B23. AP-1 is a dimeric transcriptional factor that contains members of the Jun and Fos protein families [12]. It is hard to reconstitute with c-Jun expression in F9 cells, because F9 cells died rapidly after c-Jun-expressing plasmid transfection (data not shown). It has been reported that constitutive expression of the c-jun gene results in the differentiation of F9 cells [32], and the differentiated F9 cells were dead during hygromycin B selection in this study (data not shown). It is also reported that NPM/B23 is decreased in differentiated cells [23]. Overexpression of AP-1 did not cause cellular differentiation in NIH 3T3 cells (data not shown), and therefore, the transfection of AP-1-expressing plasmid studies were performed in NIH 3T3 cells. NIH 3T3 cells were transfected with c-Jun- or c-Fos-expressing plasmids, and the protein level of NPM/B23 was further examined after transfection. As shown in Fig. 4A, NIH 3T3 cells overexpressed c-Jun or c-Fos protein after transfection with c-Jun- or c-Fos-expressing plasmids, respectively. The level of NPM/B23 protein evidently decreased after transfection with c-Jun-expressing plasmids but did not significantly change after transfection with c-Fos-expressing plasmids (Fig. 4A). Fig. 4B illustrates the quantification of NPM/B23 after transfection with c-Jun- or c-Fos-expressing plasmids in NIH 3T3 cells, and NPM/B23 was decreased about 40% after transfection with c-Jun-expressing plasmids. Transfection with c-Fos-expressing vector did not cause significant change in NPM/B23 level.

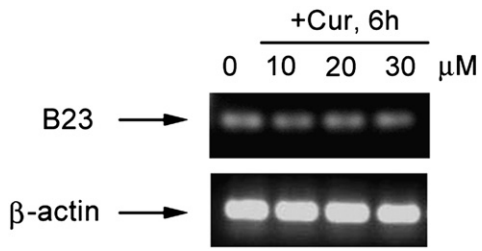

Fig. 3. The curcumin-induced expression of NPM/B23 was not mediated through a transcriptional level in NIH 3T3 cells. cDNA was synthesized from total RNAs extracted from NIH 3T3 cells incubated with 0 (as the control), 10, 20 or 30 μM curcumin and subjected to RT-PCR analysis. RT-PCR products were then run on a 1.0% agarose gel and analyzed by ethidium bromide staining.

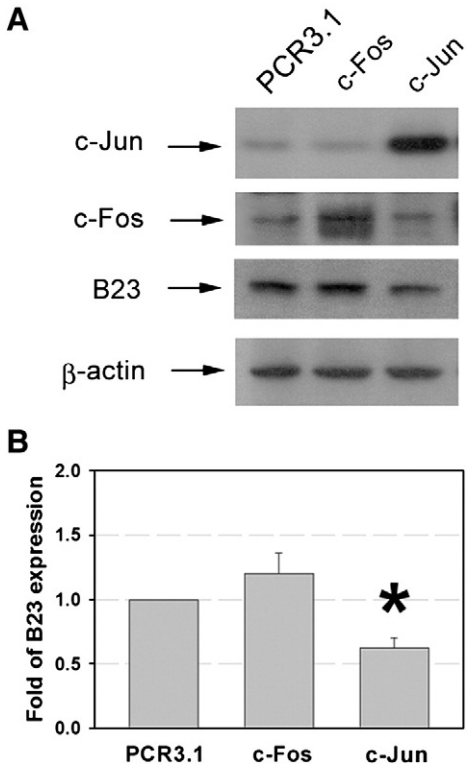

Fig. 4. The effect of AP-1 on the NPM/B23 protein level. (A) NPM/B23 was decreased in c-Jun-overexpressed cells. Transfections were performed by Lipofectamine Reagent method in NIH 3T3 cells. Transfected cells were lysed and cellular lysates of equal amount of proteins (30 μg/lane) from control-vector-transfected cells (lane 1: PCR3.1), c-Fos-expressing plasmid-transfected cells (lane 2: c-Fos) and c-Jun-expressing plasmid-transfected cells (lane 3: c-Jun) were analyzed by Western blot using antibodies specific for c-Fos, c-Jun, NPM/B23 and β-actin. (B) Folding expression of NPM/B23 subsequent to transfection of PCR3.1, c-Fos- or c-Jun-expressing plasmid. Bars, means of triplicate±S.D. *$P<.05$, as compared with the values of NPM/B23 normalized with respect to intensities of β-actin in cells transfected with control-vector plasmid (PCR3.1).

3.5. Overexpression of c-Jun enhances cytotoxicity of curcumin, but overexpression of c-Fos reduces cytotoxicity of curcumin

It has been reported that down-regulation of NPM/B23 makes cells more sensitive to stress-induced apoptosis [16,22,33]. We have noted herein that overexpression of c-Jun reduced cellular NPM/B23 and that c-Fos did not significantly affect the level of NPM/B23 (Fig. 4A). Therefore, cellular susceptibility to curcumin was tested in control vector, c-Jun-expressing plasmid or c-Fos-expressing plasmid stably transfected NIH 3T3 cells. After 24 h of curcumin (30 μM) treatment, curcumin inhibited most of cells' growth and caused ~25% relative cell growth (~75% of cell growth inhibition) (Fig. 5A) and low-level (~3%) cell death in control-vector-transfected cells (Fig. 5B). We observed that c-Jun-overexpressed cells were more sensitive to curcumin-induced cell growth inhibition, as compared with control-vector-transfected cells (~90% of cell growth inhibition) (Fig. 5A). By contrast, c-Fos-overexpressed cells were less sensitive to curcumin-induced cell growth inhibition (~65% of cell growth inhibition) and more resistant to curcumin-induced cell death compared with control-vector-transfected cells (Fig. 5). c-Jun-overexpressed cells increased cell death following curcumin treatment by nearly 7-fold as compared with control-vector-transfected cells, whereas c-Fos-overexpressed cells were significantly resistant to curcumin-induced cell death by a decrease of ~1.8-fold as compared with control-vector-transfected cells (Fig. 5B). Similar results were obtained from c-Jun- or c-Fos-expressing plasmid-transfected HeLa cells following curcumin treatment (data not shown).

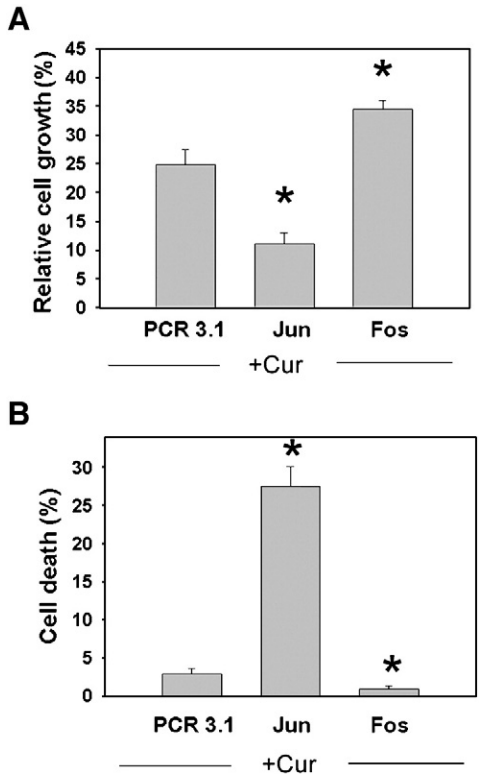

Fig. 5. The effect of AP-1 on cellular susceptibility to curcumin. NIH 3T3 cells were stably transfected with control vector (PCR 3.1 plasmid), c-Jun-expressing plasmid or c-Fos-expressing plasmid, and then the susceptibility of these cells to curcumin was tested. Transfections and establishment of stable clones were described under Section 2. (A) The relative growth (proportion of each untreated cell line at same time point) of control-vector-transfected cells (PCR 3.1), c-Jun-expressing plasmid-transfected cells (c-Jun) or c-Fos-expressing plasmid-transfected cells (c-Fos) at 24 h after curcumin (30 μM) treatment. Cell numbers were obtained by counting with a hemocytometer. Bars, means of triplicates±S.D. $^{*}P<.05$, as compared with the relative growth of each untreated cell line. (B) Determination of the levels of viable cells or dead cells after NIH 3T3 cells incubated with curcumin (30 μM) for 24 h. Viable and dead cells were determined by trypan blue exclusion method. Bars, means of triplicates±S.D. $^{*}P<.05$, as compared with the percentage of cell death in each untreated cell line.

3.6. NPM/B23 is involved in regulating the susceptibility of NIH 3T3 cells to curcumin-induced death

Overexpression of c-Jun or c-Fos altered cellular susceptibility to curcumin (Fig. 5), and overexpression of c-Jun also decreased the level of NPM/B23 (Fig. 3). Such findings initially tempted us to hypothesize that curcumin-induced cell death was associated with the intracellular level of NPM/B23. In order to test the hypothesis, NPM/B23 antisense cDNA plasmid was employed to reduce cellular NPM/B23 in NIH 3T3 cells. A stable clone of NPM/B23 cDNA antisense plasmid-transfected cells was established subsequent to G418 selection. As Fig. 6A shows, the cellular NPM/B23 protein was decreased after transfection of NPM/B23 cDNA antisense plasmid. The relative sensitivity of curcumin-induced cell death and growth inhibition was also partially enhanced after transfection of NPM/B23 cDNA antisense plasmid (Fig. 6B and C). NPM/B23 cDNA antisense plasmid-transfected cells increased cell death after curcumin (30 μM, 24 h) treatment by nearly threefold, as compared with control-vector-transfected cells (Fig. 6B). These data suggested that NPM/B23, at least in part, was associated with curcumin-induced cell death.

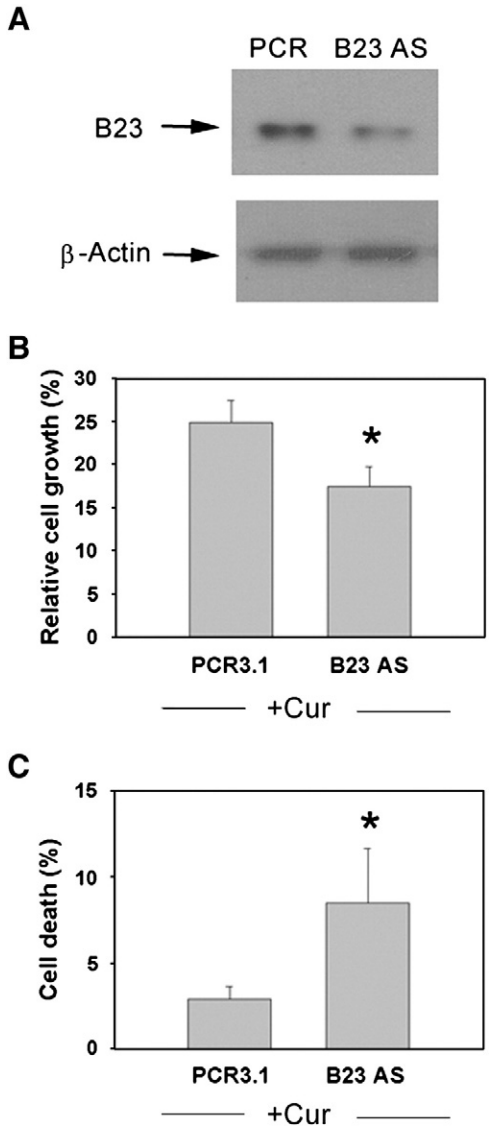

Fig. 6. Down-regulation of NPM/B23 increased cellular susceptibility to curcumin. (A) Cellular NPM/B23 was declined after transfection of NPM/B23 cDNA antisense expression plasmid. NIH 3T3 cells were stably transfected with control vector (PCR 3.1 plasmid) as control cells or NPM/B23 cDNA antisense expression plasmid (B23 AS) to reduce endogenous NPM/B23. Total cell lysates (30 μg/lane) were analyzed by Western blot using antibodies specific for NPM/B23 and β-actin. (B) The relative growth (proportion of each untreated cell line at the same time point) of control-vector-transfected cells (PCR 3.1) or NPM/B23 cDNA antisense-expressing plasmid-transfected cells at 24 h after curcumin (30 μM) treatment. Cell numbers were obtained by counting with a hemocytometer. Bars, means of triplicates±S.D. $^{*}P<.05$, as compared with the relative growth in vector (PCR3.1)-transfected cells, which were treated with the same dose of curcumin for 24 h. (C) Determination of the levels of viable cells or dead cells after control-vector-transfected cells (PCR 3.1) or NPM/B23 cDNA antisense-expressing plasmid-transfected cells incubated to curcumin (30 μM) for 24 h. Viable and dead cells were determined by trypan blue exclusion staining. Bars, means of triplicates±S.D. $^{*}P<.05$, as compared with the percentage of cell death in vector (PCR3.1)-transfected cells, which were treated with the same dose of curcumin for 24 h.

4. Discussion

Curcumin has been shown to possess potent antitumor activity in vivo [3,7,8]. The specific molecular mechanism for the selective cytotoxicity of curcumin may be important to elucidate the action of chemopreventive agents. In NIH 3T3 cells, NPM/B23 was induced by curcumin in a dose- and time-dependent manner (Fig. 2). In HeLa cells, NPM/B23 was also evidently increased at 6 h after exposure to curcumin at 5–10 μM (Fig. 2). However, induction of NPM/B23 was not dose-dependently responsive at 6 h subsequent to curcumin treatment at a concentration range of 15–20 μM curcumin (Fig. 2C and G),

with obvious cell death occurring at 24 h after exposure of 15–20 μM curcumin in HeLa cells (Fig. 1B). In F9 cells, NPM/B23 was not dose- and time-responsive to curcumin stimulation (Fig. 2D, E and H), and among these cell lines, F9 cells were the most sensitive to curcumin-induced cell death (Fig. 1). The specific expression pattern of NPM/B23 in response to curcumin stimulation might be important for cell survival. Previous studies have reported that NPM/B23 is an anti-apoptosis molecule [16–19] and that the anti-apoptosis effect of NPM/B23 is mediated through inhibition of p53, which is a pro-apoptosis molecule [16,24–26]. In addition, NPM/B23 acts as nuclear phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P_3] receptor, and the NPM/B23–PI(3,4,5)P_3 complex inhibits DNA fragmentation activity of caspase-activated DNase [34]. Transfection of antisense B23 plasmid enhanced the cytotoxicity of curcumin to NIH 3T3 cells (Fig. 6), revealing that the cellular level of NPM/B23 is associated with cytotoxicity of curcumin.

Overexpression of c-Jun decreased cellular NPM/B23 level (Fig. 4) and rendered cells more susceptible to cytotoxicity of curcumin (Fig. 5), indicating that the cellular level of c-Jun is an important factor that may determine curcumin-induced cell death and growth inhibition. By contrast, overexpression of c-Fos made cells more resistant to cytotoxicity of curcumin (Fig. 5), indicating that the cellular level of c-Fos is another important factor to prevent curcumin-induced cell death and growth inhibition. Because c-Fos tends to form heterodimers with c-Jun, it cannot form homodimers by itself [12]. Transcriptional factors, such as c-Jun/c-Fos heterodimers, may regulate genes involved in cell survival, while c-Jun/c-Jun homodimers may regulate genes involved in cell death and level of NPM/B23 protein. Therefore, the expression ratio of c-Jun and c-Fos may affect the level of NPM/B23 and other proteins, which may alter cellular susceptibility to curcumin's cytotoxicity.

Induction of NPM/B23 in NIH 3T3 cells following curcumin exposure is associated with inhibition of c-Jun, and curcumin-induced NPM/B23 did not arise via transcriptional level (Fig. 3). This is consistent with the fact that the promoter sequence of the NPM/B23 gene lacks an AP-1 binding site [14], and therefore, the AP-1 inhibitor, curcumin, did not directly affect the gene expression of NPM/B23. Thus, it may be that c-Jun regulates expression of other factors involving the translation or post-translation of NPM/B23.

The mechanisms responsible for curcumin-induced apoptosis appear to be quite varied, including effects such as the activation of caspase 3 [5,7] and caspase 9 [5], the release of cytochrome *c* and the generation of reactive oxygen species [4], the phosphorylation of p53 and the expression of certain apoptosis-related protein [4,27]. Curcumin has also been reported to selectively lead to apoptosis in deregulated cyclin D1-expressed cells and scleroderma lung fibroblasts without affecting normal mammary epithelial cells and normal lung fibroblasts, respectively [28,29]. Curcumin induces apoptosis in various cancer cells, whereas curcumin either is inactive or inhibits proliferation in many types of normal cells and primary cells [6]. However, curcumin is able to inhibit chemotherapeutic effects by reducing camptothecin-, mechlorethamine- or doxorubicin-induced apoptosis in breast cancer cells [35]. Furthermore, curcumin also decreases UV-induced apoptosis in A431 cells [30]. Therefore, curcumin on induction or prevention of cell death may be associated with factors such as cell species, kinds of stress and/or doses of curcumin administered.

In summary, curcumin induces NPM/B23 through inhibiting c-Jun signal transduction pathways, and the curcumin-increased NPM/B23 may be important for cell survival, whereas the selective cytotoxicity of curcumin may be associated with AP-1 and NPM/B23. These results appear to imply that the effect of curcumin on NPM/B23 might be useful for elucidation and application on cancer chemoprevention or chemotheraphy.

Acknowledgments

We thank Dr. Tsai Chi-Neu (Graduate Institute of Clinical Medical Science, Chang-Gung University) for her helpful suggestions as regards design of certain experiments. We also thank Dr. Tai Ming-Hong (Department of Medical Education and Research, Kaohsiung Veterans General Hospital) for carefully proofreading the manuscript. The work was supported by National Science Council (R.O.C.) Grants NSC93-2320-B242-006 and NSC95-2320-B242-009.

References

[1] Bode AM, Dong Z. Targeting signal transduction pathways by chemopreventive agents. Mutat Res 2004;555:33–51.
[2] Duvoix A, Blasius R, Delhalle S, Schnekenburger M, Morceau F, Henry E, et al. Chemopreventive and therapeutic effects of curcumin. Cancer Lett 2005;223:181–90.
[3] Huang MT, Lou YR, Ma W, Newmark HL, Reuhl KR, Conney AH. Inhibitory effects of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice. Cancer Res 1994;54:5841–7.
[4] Kuo ML, Huang TS, Lin JK. Curcumin, an antioxidant and anti-tumor promoter, induces apoptosis in human leukemia cells. Biochim Biophys Acta 1996;1317:95–100.
[5] Jana NR, Dikshit P, Goswami A, Nukina N. Inhibition of proteasomal function by curcumin induces apoptosis through mitochondrial pathway. J Biol Chem 2004;279:11680–5.
[6] Karunagaran D, Rashmi R, Kumar TR. Induction of apoptosis by curcumin and its implications for cancer therapy. Curr Cancer Drug Targets 2005;5:117–29.
[7] Khar A, Ali AM, Pardhasaradhi BV, Begum Z, Anjum R. Antitumor activity of curcumin is mediated through the induction of apoptosis in AK-5 tumor cells. FEBS Lett 1999;445:165–8.
[8] Purkayastha S, Berliner A, Fernando SS, Ranasinghe B, Ray I, Tariq H, et al. Curcumin blocks brain tumor formation. Brain Res 2009;1266:130–8.
[9] Huang MT, Smart RC, Wong CQ, Conney AH. Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-*O*-tetradecanoylphorbol-13-acetate. Cancer Res 1988;48:5941–6.
[10] Kawamori T, Lubet R, Steele VE, Kelloff GJ, Kaskey RB, Rao CV, et al. Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. Cancer Res 1999;59:597–601.
[11] Huang TS, Lee SC, Lin JK. Suppression of c-Jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells. Proc Natl Acad Sci U S A 1991;88:5292–6.
[12] Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1991;1072:129–57.
[13] Tyrrell RM. Activation of mammalian gene expression by the UV component of sunlight–from models to reality. Bioessays 1996;18:39–48.
[14] Chan WY, Liu QR, Borjigin J, Busch H, Rennert OM, Tease LA, et al. Characterization of the cDNA encoding human nucleophosmin and studies of its role in normal and abnormal growth. Biochemistry 1989;28:1033–9.
[15] Feuerstein N, Chan PK, Mond JJ. Identification of numatrin, the nuclear matrix protein associated with induction of mitogenesis, as the nucleolar protein B23. Implication for the role of the nucleolus in early transduction of mitogenic signals. J Biol Chem 1988;263:10608–12.
[16] Wu MH, Chang JH, Chou CC, Yung BY. Involvement of nucleophosmin/B23 in the response of HeLa cells to UV irradiation. Int J Cancer 2002;97:297–305.
[17] Wu MH, Chang JH, Yung BY. Resistance to UV-induced cell-killing in nucleophosmin/B23 over-expressed NIH 3T3 fibroblasts: enhancement of DNA repair and up-regulation of PCNA in association with nucleophosmin/B23 over-expression. Carcinogenesis 2002;23:93–100.
[18] Wu MH, Yung BY. UV stimulation of nucleophosmin/B23 expression is an immediate-early gene response induced by damaged DNA. J Biol Chem 2002;277:48234–40.
[19] Li J, Zhang X, Sejas DP, Bagby GC, Pang Q. Hypoxia-induced nucleophosmin protects cell death through inhibition of p53. J Biol Chem 2004;279:41275–9.
[20] Dumbar TS, Gentry GA, Olson MO. Interaction of nucleolar phosphoprotein B23 with nucleic acids. Biochemistry 1989;28:9495–501.
[21] Okuwaki M, Matsumoto K, Tsujimoto M, Nagata K. Function of nucleophosmin/B23, a nucleolar acidic protein, as a histone chaperone. FEBS Lett 2001;506:272–6.
[22] Chou CC, Yung BY. Increased stability of nucleophosmin/B23 in anti-apoptotic effect of ras during serum deprivation. Mol Pharmacol 2001;59:38–45.
[23] Hsu CY, Yung BY. Down-regulation of nucleophosmin/B23 during retinoic acid-induced differentiation of human promyelocytic leukemia HL-60 cells. Oncogene 1998;16:915–23.
[24] Kurki S, Peltonen K, Latonen L, Kiviharju TM, Ojala PM, Meek D, et al. Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. Cancer Cell 2004;5:465–75.
[25] Colombo E, Marine JC, Danovi D, Falini B, Pelicci PG. Nucleophosmin regulates the stability and transcriptional activity of p53. Nat Cell Biol 2002;4:529–33.

[26] Maiguel DA, Jones L, Chakravarty D, Yang C, Carrier F. Nucleophosmin sets a threshold for p53 response to UV radiation. Mol Cell Biol 2004;24:3703–11.
[27] Song G, Mao YB, Cai QF, Yao LM, Ouyang GL, Bao SD. Curcumin induces human HT-29 colon adenocarcinoma cell apoptosis by activating p53 and regulating apoptosis-related protein expression. Braz J Med Biol Res 2005;38: 1791–8.
[28] Choudhuri T, Pal S, Das T, Sa G. Curcumin selectively induces apoptosis in deregulated cyclin D1-expressed cells at G2 phase of cell cycle in a p53-dependent manner. J Biol Chem 2005;280:20059–68.
[29] Tourkina E, Gooz P, Oates JC, Ludwicka-Bradley A, Silver RM, Hoffman S. Curcumin-induced apoptosis in scleroderma lung fibroblasts: role of protein kinase cepsilon. Am J Respir Cell Mol Biol 2004;31:28–35.
[30] Chan WH, Wu CC, Yu JS. Curcumin inhibits UV irradiation-induced oxidative stress and apoptotic biochemical changes in human epidermoid carcinoma A431 cells. J Cell Biochem 2003;90:327–38.
[31] Liang CL, Chen JL, Hsu YP, Ou JT, Chang YS. Epstein–Barr virus BZLF1 gene is activated by transforming growth factor-beta through cooperativity of Smads and c-Jun/c-Fos proteins. J Biol Chem 2002;277:23345–57.
[32] de Groot RP, Kruyt FA, van der Saag PT, Kruijer W. Ectopic expression of c-jun leads to differentiation of P19 embryonal carcinoma cells. EMBO J 1990;9:1831–7.
[33] Liu WH, Yung BY. Mortalization of human promyelocytic leukemia HL-60 cells to be more susceptible to sodium butyrate-induced apoptosis and inhibition of telomerase activity by down-regulation of nucleophosmin/B23. Oncogene 1998; 17:3055–64.
[34] Ahn JY, Liu X, Cheng D, Peng J, Chan PK, Wade PA, et al. Nucleophosmin/B23, a nuclear PI(3,4,5)P(3) receptor, mediates the antiapoptotic actions of NGF by inhibiting CAD. Mol Cell 2005;18:435–45.
[35] Somasundaram S, Edmund NA, Moore DT, Small GW, Shi YY, Orlowski RZ. Dietary curcumin inhibits chemotherapy-induced apoptosis in models of human breast cancer. Cancer Res 2002;62:3868–75.