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Biochemical Pharmacology 69 (2005) 1603–1611
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Morin inhibits 12-*O*-tetradecanoylphorbol-13-acetate-induced hepatocellular transformation via activator protein 1 signaling pathway and cell cycle progression

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Received 12 January 2005; accepted 14 March 2005

Abstract

Flavonoids are constituents of fruits, vegetables, and plant-derived beverages, as well as components in herbal containing dietary supplements. They exhibit a remarkable spectrum of biochemical and pharmacological activities. In this study, we examined morin (3,5,7,2′,4′-pentahydroxyflavone) for its effect on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated human hepatocytes. Morin inhibited TPA-induced cellular transformation in Chang liver cells in a dose-dependent manner. Luciferase assay and electrophoretic mobility shift assay revealed that morin suppressed TPA-induced AP-1 activity, and the inhibition of AP-1 activity by morin was mediated through the inhibition of p38 kinase. Moreover, morin induced the S-phase arrest and inhibited the DNA synthesis in TPA-treated hepatocytes, suggesting that a cell cycle checkpoint was activated by morin to block DNA synthesis in S phase. In conclusion, our results suggested that morin was a potent anti-hepatocellular transformation agent that inhibited cellular transformation by suppressing the AP-1 activity and inducing the S-phase arrest in human hepatocytes.

Keywords: Morin; Hepatocyte; Transformation; Activator protein 1; Mitogen-activated protein kinase; Cell cycle

1. Introduction

Flavonoids are a large and important group of natural products that include flavonols, flavones, flavanones, and isoflavones [1]. Over 4000 flavonoids have been identified in fruits, vegetables, nuts, seeds, as well as in tea and wine. Morin (3,5,7,2′,4′-pentahydroxyflavone), a member of flavonols, is a yellowish pigment found in the old fustic (*Chlorophora tinctoria*) and osage orange (*Maclura pomifera*) as well as in many Chinese herbs [2]. Morin displays a variety of biological actions. For examples, morin exhibits an anti-inflammatory activity [3,4]. It is an antioxidant that protects various human cells, like myocytes, endothelial cells, hepatocytes, and erythrocytes against oxyradicals damage [5,6]. Furthermore, morin exhibits an anti-tumor promotion effect by significantly inhibiting the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus early antigen activation and TPA-induced skin tumor promotion [7,8]. Moreover, morin acts as a chemopreventive agent against oral carcinogenesis in vitro and in vivo [9,10]. Although morin was suggested as the anti-cancer agent, the molecular mechanism of morin remained to be elucidated.

Activator protein 1 (AP-1), a nuclear transcription factor, consists of homo- and heterodimers of the proto-oncogene families Fos (c-Fos, FosB, Fra-1, and Fra-2), Jun (c-Jun, JunB, and JunD), and activating transcription factor (ATF2, ATF3/LRF1, and B-ATF) [11]. AP-1 activity is induced by ultraviolet radiation, DNA damage, growth factors, TPA, and cytokines [11,12]. Its activity is controlled by signaling through the mitogen-activated protein (MAP) kinases [12]. When stimulated, AP-1 binds to the TPA-responsive element and induces transcription of several genes involved in cell proliferation, differentiation,

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; AP-1, activator protein 1; MAP, mitogen-activated protein; ATCC, American Type Culture Collection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco modified Eagle medium; FBS, fetal bovine serum; INT, *p*-iodonitrotetrazolium violet; RLU, relative luciferase unit; EMSA, electrophoretic mobility shift assay; JNKs, c-Jun N-terminal kinases; ERKs, extracellular signal-regulated kinases; MEK 1/2, MAP kinase kinase 1/2

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doi:10.1016/j.bcp.2005.03.008

apoptosis, and neoplastic transformation [11,13]. Blocking of TPA-induced AP-1 activity inhibits the induced transformation in vitro and in vivo, suggesting that inhibition of AP-1 activity leads to the suppression of cellular transformation [14–16]. Furthermore, some chemopreventive agents, including aspirin, polyphenols and retinoid acid, have been shown to inhibit cellular transformation and tumor promotion by suppressing the AP-1 activity [17,18]. All these studies strongly indicated that the inhibition of AP-1 activity results in the suppression of tumor promotion.

Therefore, the aim of this study was to elucidate the anticancer mechanism of morin. We chose human hepatocytes as the model system because hepatocellular carcinoma is the fifth most common cause of cancer in the world [19]. We investigated the effects of morin on TPA-induced hepatocellular transformation, TPA-induced AP-1 activity, MAP kinase pathway, and cell cycle progression. Our results indicated that morin inhibited cellular transformation by suppressing the AP-1 activity and inducing the S-phase arrest in human hepatocytes.

2. Materials and methods

2.1. Materials

Flavonoids and TPA were purchased from Sigma (St. Louis, MO, USA) and dissolved in ethanol. The chemical structures of flavonoids used in this study are shown in Fig. 1. Plasmid DNAs pAP1-Luc and pSV3-neo were purchased from Stratagene (La Jolla, CA, USA) and American Type Culture Collection (ATCC) (Rockville, MD, USA), respectively. Neomycin G-418 was obtained from Promega (Madison, WI, USA) and dissolved in water. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma and dissolved in PBS. MAP kinase inhibitors PD98059, SB203580, and curcumin were purchased from Calbiochem® (San Diego, CA, USA) and dissolved in methanol, water, and ethanol, respectively.

2.2. Cell culture and stable transfection

Human Chang liver cell line was purchased from ATCC and maintained in Dulbecco modified Eagle medium (DMEM) (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, Utah, USA) at 37 °C. Plasmid pAP1-Luc, containing the AP-1 response element driving a luciferase reporter gene, was linearlized by *Alw*NI. The cells were co-transfected with 2.5 μg linear pAP1-Luc DNA and 2.5 μg *Eco*RI-linearlized pSV3-neo DNAs by SuperFect® transfection reagent as manufacturers protocol (Qiagen, Valencia, CA, USA). Forty-eight hours later, the cells were subcultured and selected with 400 μg/ml G-418. The cell clone which showed the highest luciferase activity was selected and designated as Chang/AP-1 cells. The recombinant cell line was maintained in DMEM supplemented with 10% FBS and 400 μg/ml G-418.

2.3. Anchorage-independent transformation assay

Chang/AP-1 cells (1×10^4) were exposed to TPA and various amounts of morin in 2 ml of 0.4% DMEM agar over 4 ml of 0.6% DMEM agar containing TPA and morin. The cultures were maintained at 37 °C for 21 days. The cell colonies were then stained with 250 ng/ml *p*-iodonitrotetrazolium violet (INT) (Sigma) overnight and visualized under a phase-contrast microscope. Colonies larger than 25 cells were scored as described [20].

2.4. Luciferase assay

Chang/AP-1 cells were cultured in 96-well plates at 37 °C for 24 h, washed with DMEM, and starved by DMEM supplemented with 0.1% FBS for an additional 24 h [14]. The cells were then treated with various amounts of morin and incubated at 37 °C. After a 16-h incubation, the cells were washed with ice-cold PBS, lysed with 350 μl Triton lysis buffer (50 mM Tris–HCl, 1% Triton X-100, 1 mM dithiothreitol, pH 7.8), and centrifuged at $12{,}000 \times g$ for 2 min at 4 °C. The luciferase activity was measured by mixing 20 μl of cell lysate with 100 μl of luciferase reagent (470 μM luciferin, 33.3 mM dithiothreitol, 270 μM coenzyme A, 530 μM ATP, 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, pH 7.8), and determined with a luminometer (FB15, Zylux Corp., Maryville, TN, USA). Relative luciferase activity was calculated by dividing the relative luciferase unit (RLU) of treated cells by the RLU of untreated cells.

2.5. MTT assay

Cell viability was monitored by MTT colorimetric assay. Briefly, cells were cultivated in 96-well culture plates. After a 24-h incubation at 37 °C, various amounts of compounds were added to confluent cell monolayers and incubated for another 24 h. One-tenth volume of 5 mg/ml MTT was then added to the culture medium. After a 4-h incubation at 37 °C, equal cell culture volume of 0.04N HCl in isopropanol was added to dissolve the MTT formazan, and the absorbance value was measured at 570 nm using a microplate reader.

2.6. Electrophoretic mobility shift assay (EMSA)

Chang/AP-1 cells were cultured in 25 cm^2 flasks at 37 °C for 24 h, washed with DMEM, and then treated with 20 ng/ml TPA and 100 μM morin for various periods. The crude nuclear extracts were prepared as previously

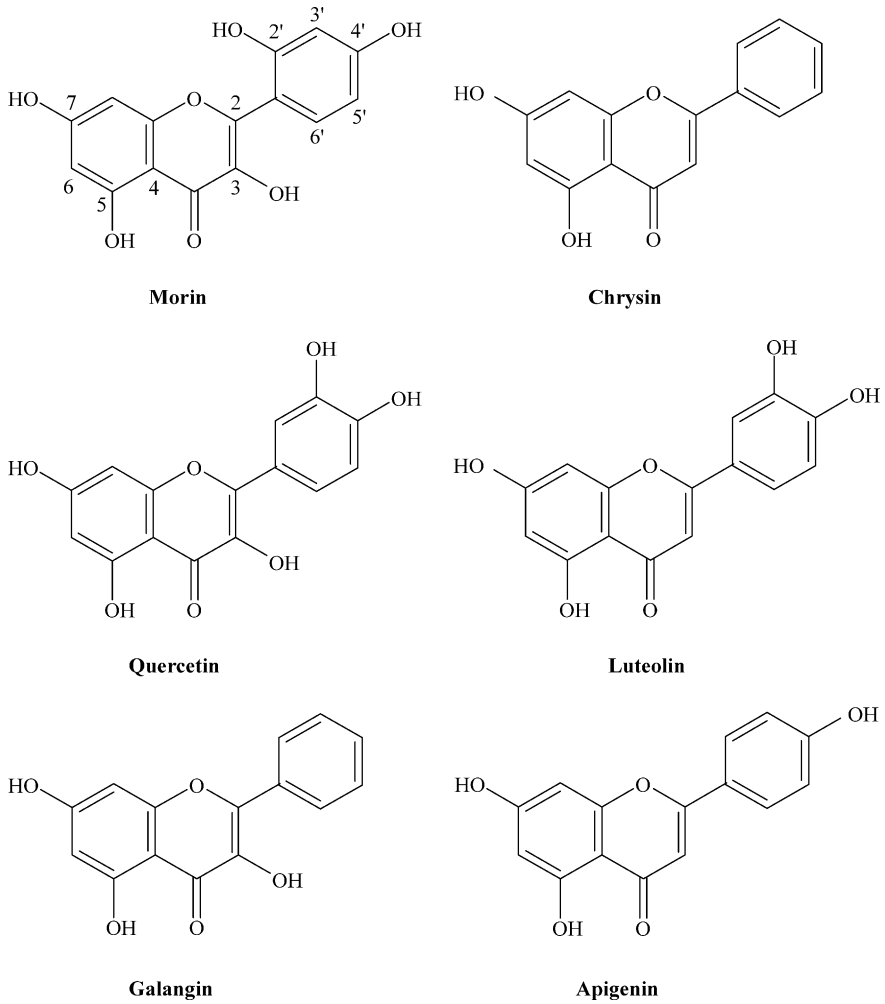

Fig. 1. Chemical structures of flavonoids used in this study.

described [21], and the protein was quantitated with a Bradford assay (Bio-Rad, Hercules, CA, USA). The double-stranded oligonucleotides corresponding to the AP-1 binding sites were purchased from Promega. The oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and EMSAs were performed as previously described [21].

2.7. Western blot analysis

Chang/AP-1 cells were cultured in 25 cm^2 flasks at 37 °C for 24 h, washed with DMEM, and treated with 20 ng/ml TPA and 100 μM morin for various periods. The cells were then collected using a cell scraper after being washed with ice-cold PBS, and lysed with 250 μl sample buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue, pH 6.8). The protein concentration of the cell lysate was determined with a Bradford method. The proteins (15–20 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and the protein bands were then transferred electrophoretically to nitrocellulose membranes. Membranes were blocked in blocking buffer (20 mM Tris–HCl, 140 mM NaCl, 0.1% Tween 20, 5% skim milk powder, pH 7.6) and probed with primary anti-c-Jun N-terminal kinases (JNKs), anti-phosphorylated JNKs, anti-p38, anti-phosphorylated p38, anti-extracellular signal-regulated kinases (ERKs), or anti-phosphorylated ERKs antibodies (Cell Signalling, Beverly, MA, USA). The bound antibody was detected with horseradish peroxidase-conjugated anti-mouse antibody followed by chemiluminescence (ECL system, Amersham, Buckinghamshire, UK) and exposed by autoradiography.

2.8. Cell cycle analysis

The percentage of cells in G1, S, and G2/M phases of the cell cycle was determined by flow cytometry analysis [22]. The cells were treated with 20 ng/ml TPA and various amounts of morin for 24 h, harvested by trypsin digestion, fixed with 70% cold ethanol, and then stained with staining buffer (0.1% Triton X-100, 200 μg/ml RNase A, 20 μg/ml propidium iodide) for 30 min at room temperature. For flow cytometric analysis, at least 10,000 cells were used in each sample. The DNA content histogram was abstracted, and the percentage of cells in each phase was calculated using ModFit LT software.

2.9. DNA synthesis analysis

The relative rates of DNA synthesis were measured by incorporation of methyl-[^{3}H] thymidine (NEN, Boston, MA, USA) as described previously [23]. Briefly, the cells were treated with 20 ng/ml TPA and various amounts of morin for 24 h, and then pulsed with 1 μCi of methyl-[^{3}H] thymidine for 1 h. The cells were then harvested and the radioactivity was measured by scintillation counting.

2.10. Statistical analysis

Data were presented as mean ± standard error. Student's *t*-test was used for comparisons between two experiments. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Morin inhibited TPA-induced hepatocellular transformation

The anchorage-independent transformation assay was carried out to measure the ability of cells to grow in an anchorage-independent manner. Chang/AP-1 cells were treated with 100 ng/ml TPA and/or various amounts of morin for 21 days in soft agars. As shown in Fig. 2, cells treated with TPA developed the transformed phenotype of colony formation in soft agars, while the TPA-induced cell transformations on soft agars were significantly suppressed by morin. TPA induced 750–800 transformed colonies in soft agars, whereas there were 50–100 soft agar colonies in the morin treatment group (100 μM). These results indicated that morin inhibited TPA-induced transformation in human hepatocytes.

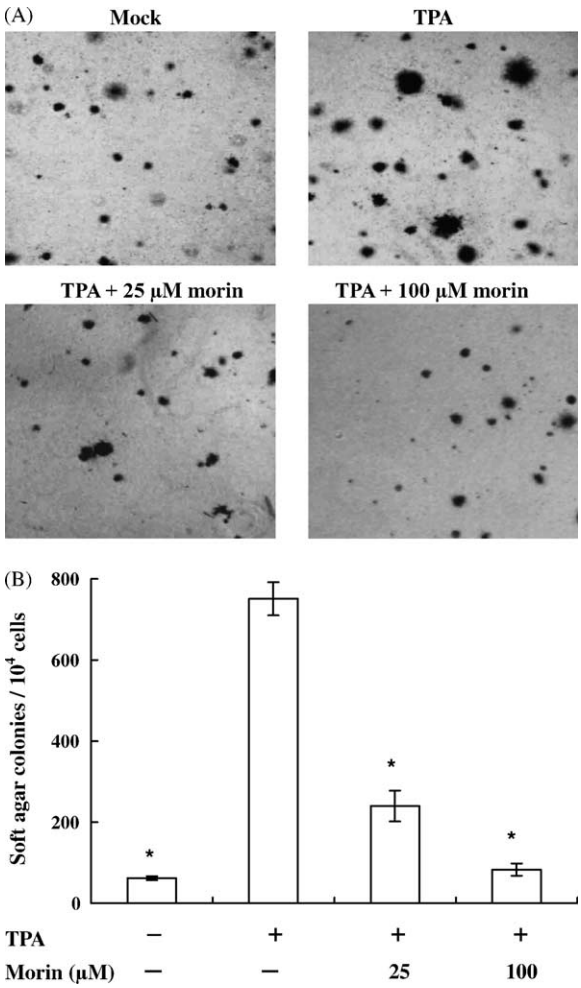

Fig. 2. Anchorage-independent transformation assay. (A) Colony formation in soft agars. Chang/AP-1 were incubated with 100 ng/ml TPA and/or various amounts of morin in 0.4% agars for 21 days. The viable colonies were then stained with INT overnight and visualized by phase-contrast microscopy (40×). (B) Quantitative analysis. Transformation response is expressed as the number of soft agar colonies per 10^4 cells. Data are presented as mean ± standard error of triplicate assays. $^*p < 0.05$, compared with TPA treatment.

3.2. Morin suppressed TPA-induced AP-1 activity in human hepatocytes

The AP-1 not only mediates cell proliferation, survival and apoptosis, but also appears to be involved in cell transformation and tumorigenesis [14]. To test whether the inhibition of colony formation by morin resulted from the suppression of AP-1 activity, Chang/AP-1 cells were serum-starved for 24 h and then treated with morin and TPA for 16 h. Morin inhibited the TPA-induced AP-1 activity in a dose-dependent manner (Fig. 3). Moreover, no significant loss of mitochondrial function was detected by MTT assay. Ethanol used to dissolve morin and TPA had no effect on AP-1 activity (data not shown). The inhibitory effect of morin on AP-1 activity agreed with its inhibitory effect on cell transformation. Furthermore, AP-1 inhibitor, such as retinoic acid, inhibited both the TPA-induced AP-1 activity and the TPA-induced colony formation [15]. Therefore, these results suggested that the inhibition of cell transformation by morin was through its suppression of AP-1 activity.

3.3. Morin suppressed TPA-induced AP-1 DNA-binding ability in human hepatocytes

To further study the molecular basis of morin on AP-1 suppression, the DNA-binding activity of AP-1 was analysed by EMSA. The double-stranded oligonucleotides corresponding to the AP-1 responsive element were incubated with Chang/AP-1 nuclear extracts, which were treated with 20 ng/ml TPA and 100 μM morin at indicated time points. As expected, TPA treatment induced the DNA-binding ability of AP-1 (Fig. 4). Morin induced a biphasic suppression of the binding with an early inhibition at 6 h followed by a second decrease at 18 h. These results

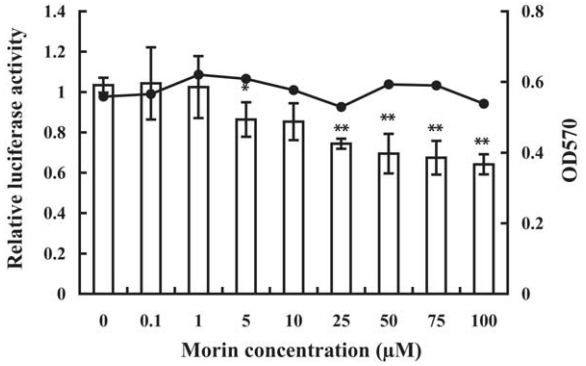

Fig. 3. Effect of morin on TPA-induced AP-1 activity in hepatocytes. Chang/AP-1 cells were seeded into 96-well plates and treated with 20 ng/ml TPA and indicated doses of morin. The luciferase activity and cell viability were determined at 16 and 24 h, respectively. The bars represent the relative luciferase activity, which is presented as comparison with the RLU relative to untreated cells. The lines represent the cell viability during treatment. Values are mean ± standard error of triplicate assays. $^{*}p < 0.05$, and $^{**}p < 0.01$, compare with TPA treatment.

indicated that morin suppressed TPA-induced AP-1 DNA-binding ability in Chang liver cells.

3.4. Structure/function relationships

Comparison of the structures of morin (3,5,7,2′,4′-pentahydroxyflavone) with quercetin (3,5,7,3′,4′-pentahydroxyflavone) or galangin (3,5,7-hydroxyflavone) suggested that AP-1 suppression of morin was independent

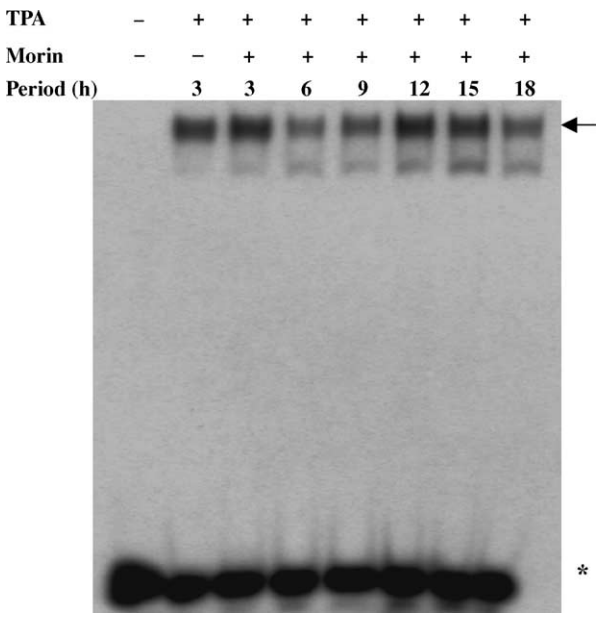

Fig. 4. Effect of morin on TPA-induced AP-1 DNA-binding ability in hepatocytes. Chang/AP-1 cells were treated with 20 ng/ml TPA and 100 μM morin for 3, 6, 9, 12, 15, or 18 h. The nuclear extracts were then prepared and incubated with 5′-^{32}P-labeled double-stranded oligonucleotides corresponding to the AP-1 sequence as described in Section 2. The arrowhead points to the location of AP-1/DNA complex and the asterisk indicates the unbound oligonucleotides. Similar results were obtained in three different experiments.

Table 1
Inhibition of TPA-induced AP-1 activity in Chang liver cells by different flavonoids

Flavonoid[a]	OH substitutions	Relative AP-1 activity[b]
Morin	3, 5, 7, 2′, 4′	$0.75 \pm 0.02^{*}$
Quercetin	3, 5, 7, 3′, 4′	0.78 ± 0.24
Galangin	3, 5, 7	0.85 ± 0.03
Chrysin	5, 7	$2.70 \pm 0.33^{**}$
Luteolin	5, 7, 3′, 4′	$1.51 \pm 0.09^{**}$
Apigenin	5, 7, 4′	$3.67 \pm 0.14^{**}$

[a] The concentration of flavonoid used in this assay was 25 μM.
[b] The data are expressed as relative AP-1 activity, which is presented as comparison with the RLU relative to TPA-treated cells. Values are mean ± standard error of triplicate assays.
$^{*}p < 0.05$, compared with TPA treatment.
$^{**}p < 0.01$, compared with TPA treatment.

of 2′ or 4′ hydroxyl groups (Table 1). Therefore, we further tested the other related flavonoids that lack the hydroxyl group at carbon 3, such as chrysin (5,7-dihydroxyflavone), luteolin (5,7,3′,4′-tetrahydroxyflavone), or apigenin (5,7,4′-trihydroxyflavone). Neither of these flavonoids showed any AP-1 inhibition, indicating that hydroxyl group located at position 3 of morin was required for its AP-1 suppression.

3.5. Morin regulated TPA-induced AP-1 activity via p38 kinase pathway

All three MAP kinase cascades, the ERKs, JNKs and p38 pathways, have been shown to mediate AP-1 induction in response to extracellular signals; we therefore asked whether one of these pathways was inhibited following exposure to morin. The Chang/AP-1 cells were treated with 20 ng/ml TPA and 100 μM morin, and the MAP kinase proteins and their phosphorylated (activated) forms in hepatocytes were analysed by Western blotting. Fig. 5A shows that the levels of MAP kinase proteins were similar in cells treated with TPA and morin for various times. The phosphorylations of ERKs, p38, and JNKs were induced by TPA treatment. However, exposure to morin significantly diminished TPA-induced phosphorylation of p38 kinase, slightly inhibited the phosphorylation of ERKs, and exhibited no effect on the phosphorylation of JNKs. These data indicated that p38 kinase was the target of morin that leads to the suppression of TPA-induced AP-1 activity in hepatocytes.

Several specific inhibitors of the MAP kinase cascades have been described. PD98059 specifically inhibits MAP kinase kinase 1/2 (MEK1/2), the kinases that phosphorylate ERK, but has no effect on other kinases [24]. Curcumin, an inhibitor of c-Jun/AP-1 complex, is also known to have inhibitory effect on JNKs [25]. SB203580, in contrast, selectively inhibits p38 and has no effect on MEK1/2 [26]. To further elucidate the pathway involved in morin activity, Chang/AP-1 cells were treated with inhibitors for 1 h and exposed to 20 ng/ml TPA and 100 μM morin in the presence of inhibitors for 16 h. Fig. 5B shows that SB203580

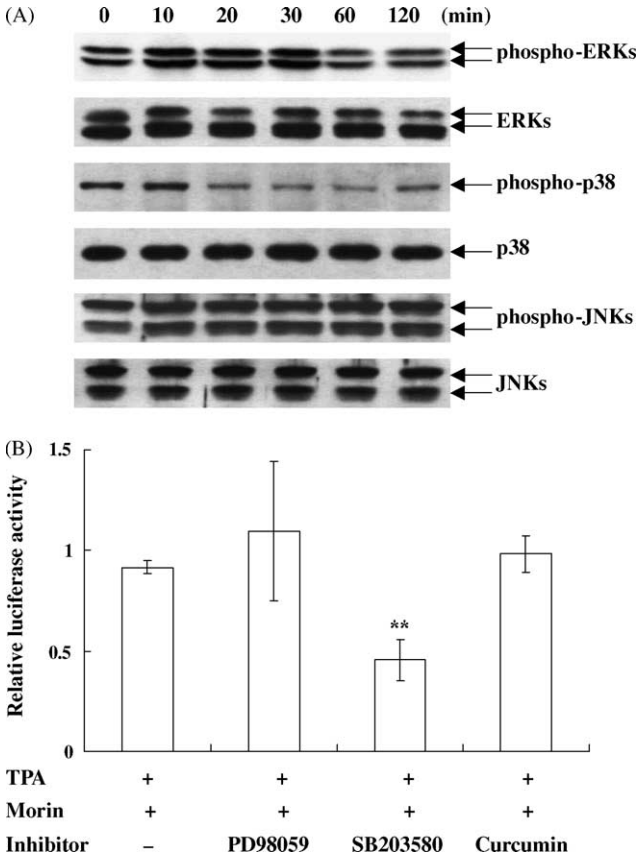

Fig. 5. Signal transduction pathway contributing to the suppression of TPA-induced AP-1 activity by morin in hepatocytes. (A) Western blotting analysis. Chang/AP-1 cells were exposed to 20 ng/ml TPA and 100 μM morin for 0, 10, 20, 30, 60, or 120 min. The phosphorylated (phospho-ERKs, phospho-p38, phospho-JNKs) and non-phosphorylated MAP kinase proteins (ERKs, p38, JNKs) were detected by PhosphoPlus® MAP kinase antibody kit and visualized by chemiluminescence. Similar results were obtained in three different experiments. (B) MAP kinase inhibitors analysis. Chang/AP-1 cells were pretreated with the PD98059 (50 μM), SB203580 (20 μM), or curcumin (15 μM) for 1 h, and exposed to 20 ng/ml TPA and 100 μM morin in the presence of inhibitors for 16 h. The results are expressed as relative luciferase activity, which is presented as comparison with the RLU relative to untreated cells. Values are mean ± standard error of triplicate assays. $^{**}p < 0.01$, compared with no inhibitor present.

significantly inhibited the TPA-induced AP-1 activity, while PD98059 and curcumin had no effect on the TPA-induced AP-1 activity. Taken together, the inhibition of p38 kinase phosphorylation was one of the mechanisms contributing to the suppression of TPA-induced AP-1 activity and subsequent cell transformation by morin.

3.6. Morin induced S-phase arrest in human hepatocytes

Because p38 is involved in inflammation, cell differentiation, and cell cycle regulation [27,28], we further analysed the effect of morin on cell growth arrest. The cell cycle progression was investigated in asynchronous cultures of TPA-treated Chang/AP-1 cells after a 24-h treatment. Fig. 6 shows that morin caused an increase at S phase in a dose-dependent response. The cells accumulated in S phase to a level of 55.25% after exposure to 100 μM morin, with a decrease in cells in the G1 phase and strong reduction of cells in the G2/M phase. These results demonstrated that morin caused a cell cycle arrest predominately in S phase.

We further looked whether DNA synthesis, a hallmark of S-phase progression thought to be negatively regulated in response to S-phase checkpoint activation, was inhibited by morin. Fig. 7 shows that the rate of [^{3}H]-thymidine incorporation into DNA was inhibited by morin in a dose-dependent manner. Moreover, there was no significant increase in cell number between morin treatment groups. Thus, these data reinforced the suggestion that a cell cycle checkpoint has been activated by morin to block DNA synthesis in S phase.

4. Discussion

Our finding revealed that morin inhibited TPA-induced hepatocellular transformation. The strong decline of TPA-induced AP-1 activity by morin was linked to a dramatic inhibition of cell cycle progression that has a predominant feature of an S-phase arrest. The S-phase arrest was also associated with severe inhibition of DNA synthesis. It has been described that morin inhibited ATPase and double-stranded DNA unwinding activity of RepA, resulting in the inhibition of DNA synthesis [29]. Therefore, we speculated that a cell cycle checkpoint was activated by morin to block DNA synthesis in S phase.

Comparison of the structure of morin with other flavonoids suggested that suppression of AP-1 activity by morin depended on the hydroxyl group at carbon 3. The structure–function relationships have been revealed for the antioxidant and apoptotic properties of flavonoids. It has been described that the double bond between carbon 2 and 3, and the additional presence of hydroxyl groups at carbon 3 and 5, are important structural determinants for the antioxidant effects of flavonoids [30]. Structure–activity relationship also showed that at least two hydroxylations at carbons 3, 5, and 7 are needed to induce apoptosis [31]. Results of this study demonstrated that flavonoids, which possess the hydroxyl group located at carbon 3, suppressed TPA-induced AP-1 activity. Because blocking of TPA-induced AP-1 activity inhibits the induced transformation [14], we proposed that flavonoids with a hydroxy group at carbon 3 were potential anti-cancer agents and deserved to be further analysed.

Morin suppressed the TPA-induced AP-1 DNA-binding ability in a biphasic manner with an early inhibition at 6 h followed by a second later decrease at 18 h. We speculated that early inhibition is related to the suppression of the phosphorylation of a pool of constitutive AP-1 complex, and late inhibition is related to the suppression of synthesis of a new pool of AP-1 proteins. MAP kinases, including ERKs, JNKs and p38 kinase, are the most common pathways to mediate AP-1 activity [13]. Many reports indicated

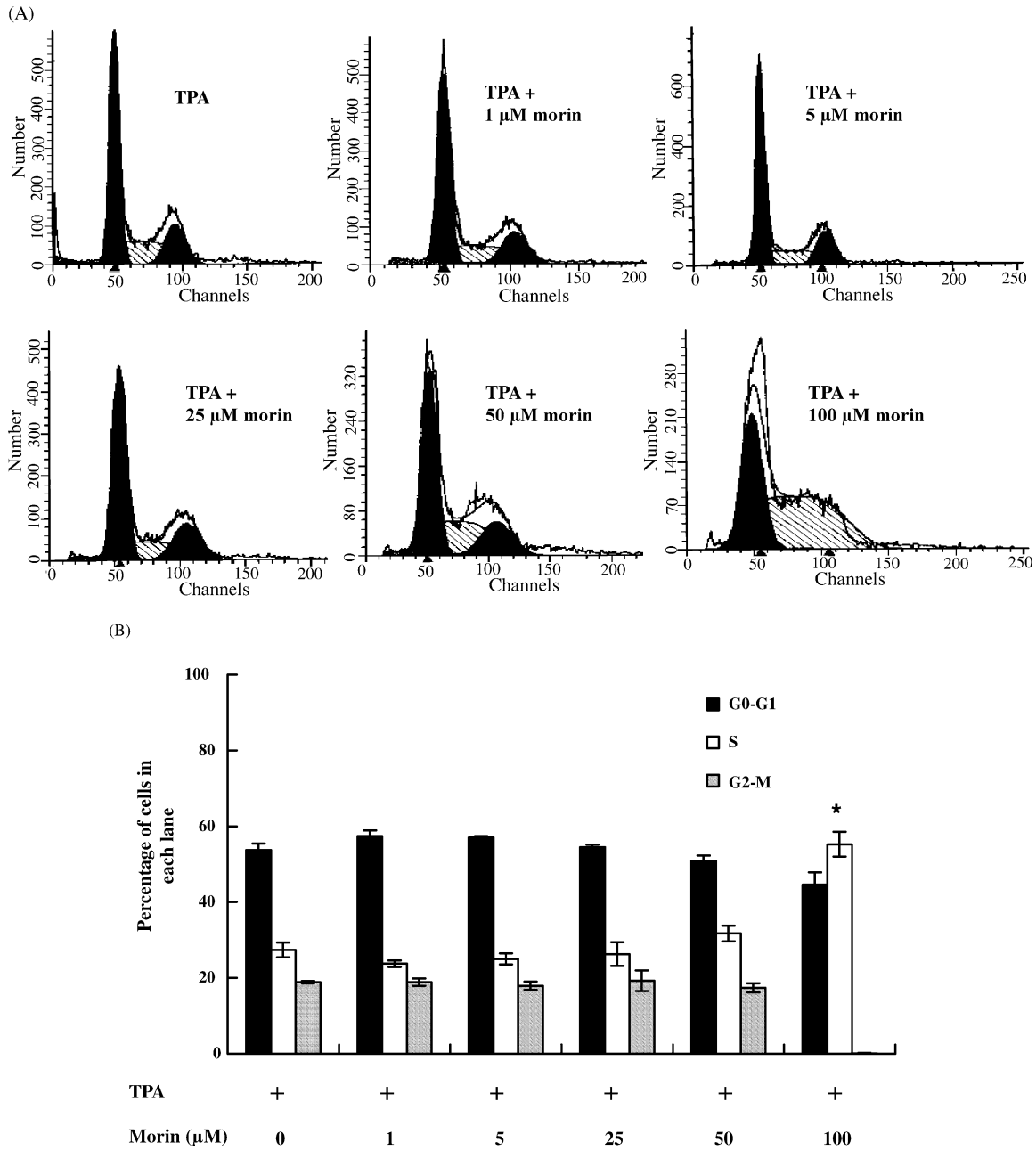

Fig. 6. Flow cytometric analysis of morin-treated Chang/AP-1 cells. (A) Representative cell cycle distribution. Cells were treated with 20 ng/ml TPA and 0, 1, 5, 25, 50, or 100 μM for 24 h, and then stained by propidium iodide. The DNA content was measured by flow cytometry. Data are from a single preparation representative of three independent experiments. (B) Fraction of cells in G1, S, and G2/M, exposed with 20 ng/ml TPA and various amounts of morin. Values are mean ± standard error of triplicate assays. $^{*}p < 0.05$, compared with TPA treatment.

that p38 kinase mediates cell growth arrest and apoptosis [27,28]. For instance, tumor necrosis factor-induced human melanoma growth-inhibition is mediated by p38 kinase-dependent cell cycle arrest [32]. Aplidin^{TM}, a novel antitumor agent of marine origin, induces growth arrest and apoptosis in human MDA-MB-231 breast cancer cells via JNKs and p38 kinase pathways [33]. Arsenic trioxide was able to induce the apoptotic activity in chronic myelogenous leukemia K562 cells, and its apoptotic mechanism may be associated with the p38 kinase [34]. Our data showed that there was no inhibitory effect of morin on ERKs and JNKs. However, the phosphorylation of p38 kinase was inhibited by morin. The SB203580, a specific p38 kinase inhibitor, also suppressed the AP-1 activity. Therefore, these results indicated that morin inhibited the hepatocellular transformation, suppressed AP-1 activity, and induced S-phase arrest in hepatocytes via p38 kinase pathway.

The bio-availability of morin has been studied [35]. Serum profiles after oral administration of morin at dose of 50 mg/kg in rats showed that the peak serum concentration of morin is 84.9 ± 18.1 μM. In our study, morin at

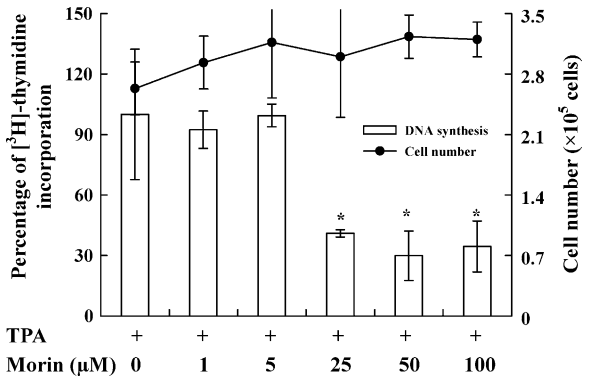

Fig. 7. Effect of morin on TPA-induced DNA synthesis and cell number in hepatocytes. Chang/AP-1 cells were treated with 20 ng/ml TPA and various amounts of morin for 24 h. The cells were then pulsed with [^{3}H]-thymidine for 1 h and harvested. The radioactivity and cell number were counted by scintillation counter and microscopic counting, respectively. Values are mean ± standard error of triplicate assays. $^*p < 0.05$, compared with TPA treatment.

concentration of 25 μM was sufficient to inhibit both the TPA-induced AP-1 activity and cellular transformation. Therefore, we speculated that the anti-cancer effect of morin measured in vitro may occur in vivo.

In conclusion, we demonstrated that morin blocked the TPA-induced hepatocellular transformation by inhibiting AP-1 activity via p38 kinase pathway. Furthermore, cell cycle analysis and [^{3}H]-thymidine incorporation assay showed that a cell cycle checkpoint was activated by morin to block DNA synthesis in S phase. Taken together, our results suggested that morin was a potent anti-transformation agent that inhibited cellular transformation by suppressing the AP-1 activity and inducing the S-phase arrest in human hepatocytes.

Acknowledgements

We thank Dr. Chin-Chen Lien for a critical review of the manuscript. We thank Miss I-Lu Lai for her technical assistant. This work was supported by grants from National Science Council and China Medical University, Taiwan.

References

[1] Das DK. Naturally occurring flavonoids: structure, chemistry, and high-performance liquid chromatography methods for separation and characterization. Methods Enzymol 1994;234:410–20.
[2] Windholz M. The Merck Index, eleventh ed. Merck & Co., Inc; 1989 p. 986–7.
[3] Fang SH, Hou YC, Chang WC, Hsiu SL, Chao PD, Chiang BL, et al. Morin sulfates/glucuronides exert anti-inflammatory activity on activated macrophages and decreased the incidence of septic shock. Life Sci 2003;74:743–56.
[4] Galvez J, Coelho G, Crespo ME, Cruz T, Rodriguez-Cabezas ME, Concha A, et al. Intestinal anti-inflammatory activity of morin on chronic experimental colitis in the rat. Aliment Pharmacol Ther 2001;15:2027–39.
[5] Kitagawa S, Sakamoto H, Tano H. Inhibitory effects of flavonoids on free radical-induced hemolysis and their oxidative effects on hemoglobin. Chem Pharm Bull 2004;52:999–1001.
[6] Wu TW, Zeng LH, Wu J, Fung KP. Morin hydrate is a plant-derived and antioxidant-based hepatoprotector. Life Sci 1993;53:PL213–8.
[7] Iwase Y, Takemura Y, Juichi M, Mukainaka T, Ichiishi E, Ito C, et al. Inhibitory effect of flavonoid derivatives on Epstein-Barr virus activation and two-stage carcinogenesis of skin tumors. Cancer Lett 2001; 173:105–9.
[8] Lee SF, Lin JK. Inhibitory effects of phytopolyphenols on TPA-induced transformation, PKC activation, and c-Jun expression in mouse fibroblast cells. Nutr Cancer 1997;28:177–83.
[9] Brown J, O'Prey J, Harrison PR. Enhanced sensitivity of human oral tumours to the flavonol, morin, during cancer progression: involvement of the Akt and stress kinase pathways. Carcinogenesis 2003; 24:171–7.
[10] Kawabata K, Tanaka T, Honjo S, Kakumoto M, Hara A, Makita H, et al. Chemopreventive effect of dietary flavonoid morin on chemically-induced rat tongue carcinogenesis. Int J Cancer 1999;83:381–6.
[11] 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.
[12] Karin M, Liu Z, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol 1997;9:240–6.
[13] Shaulian E, Karin M. AP-1 in cell proliferation and survival. Oncogene 2001;20:2390–400.
[14] Dong Z, Birrer MJ, Watts RG, Matrisian LM, Colburn NH. Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. Proc Natl Acad Sci USA 1994; 91:609–13.
[15] Hsiang CY, Wu SL, Ho TY. Activation of activator protein 1 and extracellular signal-regulated kinases in human hepatocellular transformation. Tumor Biol 2004;25:313–20.
[16] Jochum W, Passegue E, Wagner EF. AP-1 in mouse development and tumorigenesis. Oncogene 2001;20:2401–12.
[17] Agadir A, Chen G, Bost F, Li Y, Mercola D, Zhang X, et al. Differential effect of retinoic acid on growth regulation by phorbol ester in human cancer cell lines. J Biol Chem 1999;274:29779–85.
[18] Dong Z, Huang C, Brown RE, Ma WY. Inhibition of activator protein 1 activity and neoplastic transformation by aspirin. J Biol Chem 1997; 272:9962–70.
[19] Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907–17.
[20] Huang RP, Peng A, Hossain MZ, Fan Y, Jagdale A, Boynton AL, et al. Tumor promotion by hydrogen peroxide in rat liver epithelial cells. Carcinogenesis 1999;20:485–92.
[21] Hsiang CY, Lai IL, Chao DC, Ho TY. Differential regulation of activator protein 1 activity by glycyrrhizin. Life Sci 2002;70:1643–56.
[22] Ormerod MG, Kubbies M. Cell cycle analysis of asynchronous cell populations by flow cytometry using bromodeoxyuridine label and Hoechst-propidium iodide stain. Cytometry 1992;13:678–85.
[23] Mirault ME, Tremblay A, Beaudoin N, Trembley M. Overexpression of seleno-glutathione peroxidase by gene transfer enhances the resistance of T47D human breast cells to clastogenic oxidants. J Biol Chem 1991;266:20752–60.
[24] Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 1995;92:7686–9.
[25] 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 USA 1991;88:5292–6.
[26] New L, Jiang Y, Zhao M, Liu K, Zhu W, Flood LJ, et al. A novel protein kinase regulated by the p38 MAP kinase. EMBO J 1998; 17:3372–84.
[27] Ambrosino C, Nebrada AR. Cell cycle regulation by p38 MAP kinases. Biol Cell 2001;93:47–51.

[28] Perce AK, Humphrey TC. Integrating stress-response and cell cycle checkpoint pathways. Trends Cell Biol 2001;11:426–33.
[29] Xu H, Ziegelin G, Schroder W, Frank J, Ayora S, Alonso JC, et al. Flavones inhibit the hexameric replicative helicase RepA. Nucleic Acids Res 2001;29:5058–66.
[30] Rice-Evans CA, Miller NJ, Paganga G. Structure–antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 1996;20:933–56.
[31] Monasterio A, Urdaci MC, Pinchuk IV, Lopez-Moratalla N, Martinez-Irujo JJ. Flavonoids induce apoptosis in human leukemia U937 cells through caspase- and caspase–calpain-dependent pathways. Nutr Cancer 2004;50:90–100.
[32] Hattori T, Hayashi H, Chiba T, Onozaki K. Activation of two distinct anti-proliferative pathways, apoptosis and p38 MAP kinase-dependent cell cycle arrest, by tumor necrosis factor in human melanoma cell line A375. Eur Cytokine Netw 2001;12:244–52.
[33] Cuadrado A, Garcia-Fernandez LF, Gonzalez L, Suarez Y, Losada A, Alcaide V, et al. Aplidin™ induces apoptosis in human cancer cells via glutathione depletion and sustained activation of epidermal growth factor receptor, Src Jun N-terminal kinase and p38 kinase. J Biol Chem 2003;278:241–50.
[34] Shim MJ, Kim HJ, Yang SJ, Lee LS, Choi HI, Kim T, et al. Arsenic trioxide induces apoptosis in chronic myelogenous leukemia K562 cells: possible involvement of p38 MAP kinase. J Biochem Mol Biol 2002;35:377–83.
[35] Hou YC, Chao PD, Ho HJ, Wen CC, Hsiu SL. Profound difference in pharmacokinetics between morin and its isomer quercetin in rats. J Pharm Pharmacol 2003;55:199–203.