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

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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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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