Inhibition of Tetrandrine on Epidermal Growth Factor-induced Cell Transformation and its Signal Transduction

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Abstract. Background: The mouse epidermal JB6 cell system is a model for studying tumor promotion. We used the JB6 Cl 41 cell line to examine the mechanism of the anti-tumor-promoting effect of tetrandrine, an alkaloid isolated from Stephania tetrandra S Moore. Materials and Methods: The anti-tumor-promoting effect of tetrandrine was evaluated by assay of inhibition of epidermal growth factor (EGF)-induced transformation of JB6 Cl 41 cells in soft agar. The activity of activator protein-1 (AP-1), a transcription factor, was analyzed using the AP-1-dependent reporter assay. Phosphorylation of extracellular-signal-regulated kinases (ERKs) and Akt, a pivotal effector of phosphatidylinositol 3-kinase (PI3K), was detected by Western blotting. Results: Tetrandrine significantly blocked EGF-induced cell transformation, attenuated EGF-induced AP-1 activation, and inhibited phosphorylation of ERKs, which regulates AP-1 activation. It also tended to suppress EGF-induced Akt phosphorylation. Conclusion: Our results indicate that tetrandrine inhibits EGF-induced transformation of JB6 cells by blocking the activation of ERKs, AP-1 and Akt.

Tetrandrine, a bisbenzylisoquinoline alkaloid found in Stephania tetrandra S Moore, has been used in China to treat patients with silicosis, asthma and hypertension for decades (1-4). It possesses a remarkable pharmacological profile (4-8), including anti-tumor and anti-inflammatory activities. However, the mechanisms of its anti-tumor and anti-inflammatory effects remain unclear. We previously showed that tetrandrine inhibits angiogenesis of cultured choroids in streptozotocin-diabetic rats (9). We also found that tetrandrine inhibits in vitro angiogenesis in normal choroids and tube formation of vascular endothelial cells induced by mitogenic stimuli, including platelet-derived growth factor (PDGF)-BB and FBS (9, 10). Tetrandrine inhibits FBS- and PDGF-BB-induced proliferation of rat aortic smooth muscle cells by modulating extracellular signal-regulated kinases (ERKs) and altering the cell cycle progression (11). Wu et al. (12) reported that tetrandrine inhibits the expression of proinflammatory cytokines, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in human monocyte cells. iNOS and COX-2 are inducible enzymes mediating inflammatory responses and abnormally increased expression of these enzymes is thought to be involved in the pathogenesis of some tumors (13). Many of the molecular alterations that occur in carcinogenic processes, as well as in inflammatory responses, are associated with intracellular signal transduction pathways converging on the activation of transcription factors, such as nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1), which regulate cell proliferation and differentiation (14-16). Zhang et al. (17) have shown that tetrandrine protects acinar cells from
lipopolysaccharide-induced injury by inhibiting NF-κB activation. Tetrandrine was also found to inhibit mitogen-activated protein (MAP) kinases such as c-jun N-terminal kinases (JNKs), p38 kinases and ERKs, and AP-1, as well as inducing down-regulation of the NF-κB signaling pathway (11, 18, 19). Expression of iNOS and COX-2 is also tightly controlled by the transcription factors NF-κB and AP-1 (13, 20, 21). Therefore, the anti-tumor and anti-inflammatory effects of tetrandrine may be due to inhibition of signal transduction pathways.

The JB6 cell system of clonal genetic variants, which are promotion-sensitive (P+) or promotion-resistant (P–), is an excellent model for studying signal transduction at the molecular level in various stages of carcinogenesis (22-27). The JB6 P+–P– and transformed variants are a series of cell lines representing ‘earlier-to-later’ stages of preneoplastic-to-neoplastic progression (23, 24, 27). JB6 P+ cells are transformed when stimulated with epidermal growth factor (EGF) or 12-O-tetradecanoylphorbol-13-acetate (TPA), forming colonies in soft agar (22-27). The transformation is thought to involve activation of activator protein 1 (AP-1) (22, 25-27), which regulates the transcription of various genes related to cellular inflammation, proliferation and apoptosis (28). AP-1 plays a key role not only in tumor promotion (26, 27), but also in tumor progression and metastasis (29). In addition, we have demonstrated that phosphatidylinositol 3-kinase (PI3K) and its downstream effector, Akt, are closely involved in EGF-induced cell transformation and cell development, such as cell proliferation, apoptosis and migration (32, 33), and Akt plays a pivotal role in the PI3K-signaling pathway (34). Therefore, tetrandrine might suppress neoplastic transformation in JB6 P+ cells through inhibition of the AP-1 and PI3K/Akt pathways. In this study, we investigated the effect of tetrandrine on EGF-induced cell transformation and associated signaling pathways in JB6 P+ cells.

**Materials and Methods**

**Materials.** Eagle’s minimal essential medium (MEM), L-glutamine and basal medium Eagle (BME) were from Life Technologies (Rockville, MD, USA); fetal bovine serum (FBS), EGF, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), gentamycin and tetrandrine were from Sigma (St. Louis, MO, USA). Akt antibody, phospho-specific Akt (serine 473), and PhosphoPlus p44/42 mitogen activated protein kinase antibody kits were from Cell Signaling Technology Inc. (Beverly, MA, USA).

**Cell culture.** The mouse epidermal JB6 Cl 41 cell line and its stable transfectant P+1-1 (AP-1 reporter transfectant) were gifts from Dr. Zigang Dong of the Hormel Institute, University of Minnesota (Austin, MN, USA), and were cultured at 37°C in MEM supplemented with 5% heat-inactivated FBS, 2 mM L-glutamine and 25 μg/ml gentamycin.

**Cell growth inhibition assay.** JB6 Cl 41 cells (1x10³) were seeded into each well of 96-well plates and allowed to attach overnight. The cells were then treated with tetrandrine (0.01, 0.1, 0.3 or 1 μM) or the vehicle alone (0.1% DMSO; control) in 1 ml of 0.33% BME agar containing 10% FBS over 3.5 ml of 0.5% BME agar medium containing 10% FBS. The cultures were maintained in an incubator at 37°C under 5% CO₂ in air, and the number of colonies formed was scored under a microscope 14 days after exposure to EGF.

**Assay of AP-1 activity.** JB6 Cl 41 cells (5x10⁴) were seeded into a 96-well plate. After 24 h incubation, the cells were starved by replacing the medium with 0.1% FBS/MEM for 24 h. The cells were then pretreated with tetrandrine (0.01, 0.1, 0.3 or 1 μM) or the vehicle (0.1% DMSO) for 24 h in an incubator at 37°C under 5% CO₂ in air. Subsequently, MTT (25 μL of 2 mg/mL in phosphate-buffered saline (PBS) was added to each well, then incubation was continued for 4 h at 37°C. Formazan crystals were dissolved in DMSO and absorbance was determined at 540 nm with a microplate reader (Multiskan Bichromatic; Labsystems Japan, Tokyo, Japan).

**Western blotting.** Western blotting was carried out as described elsewhere (30, 31, 35). In brief, JB6 Cl 41 cells were cultured to 80% confluence. The cells were starved in 0.5% FBS/MEM for 24 h at 37°C. The medium was then changed to fresh 0.1% FBS/MEM and the cells were incubated for another 2-4 h at 37°C. Before exposure of the cells to EGF, they were treated or not treated with tetrandrine (0.1, 0.3 or 1 μM) or vehicle (0.1% DMSO) for 1 h and cultured with 10 ng/ml EGF in the presence or absence of tetrandrine for 24 h. The cells were then treated with lysis buffer, and luciferase activity was measured with a Luminescencer-PSN AB-2200 (ATTO, Tokyo, Japan). Relative AP-1 activity was calculated as described elsewhere (30, 31, 35).
phosphorylated proteins. Antibody-bound proteins were detected by fluorescence assay (ECF Western Blotting Kit, Amersham Biosciences Corp, Little Chalfont, Buckinghamshire, England) and bands were analyzed using a Typhoon 9410 imaging analyzer (Amersham Biosciences Corp).

Statistical analysis. Experiments were performed at least three times. Statistical analysis was carried out using Student's t-test and Welch's t-test.

Results

Effect of tetrandrine on EGF-induced cell transformation. EGF induced 2000-3000 transformed colonies of the mouse epidermal JB6 promotion-sensitive (P+) cell line Cl 41 in soft agar (cell transformation), whereas almost no colony formation was observed in the control group (0.1% DMSO). Tetrandrine (Figure 1) inhibited EGF-induced cell transformation in a concentration-dependent manner (Figure 2A). In the concentration range that blocked cell transformation, tetrandrine had no effect on cell proliferation (Figure 2B).

Effect of tetrandrine on EGF-induced AP-1 activation. Previous studies have demonstrated that blocking of tumor promoter-induced AP-1 activation inhibited neoplastic transformation in JB6 cells (26, 36). We therefore examined the effect of tetrandrine on EGF-induced AP-1 activation.

As shown in Figure 3, tetrandrine appeared to attenuate EGF-induced AP-1 activation dose-dependently, though the effect only became statistically significant at 1 μM.

Effect of tetrandrine on EGF-induced ERK phosphorylation. Watts et al. (37) have shown that ERKs play a central role in AP-1 transactivation and neoplastic transformation induced by TPA and EGF in JB6 cells. We therefore studied the effect of tetrandrine on EGF-induced phosphorylation of ERKs. Tetrandrine inhibited ERK phosphorylation in a concentration-dependent manner (Figure 4), and the effect was statistically significant at 0.3 and 1 μM.

Effect of tetrandrine on EGF-induced Akt phosphorylation. We have demonstrated that Akt, a pivotal effector of PI3K, is also associated with EGF-induced cell transformation in JB6 cells (30, 31). Therefore, we also investigated the effect of tetrandrine on EGF-induced Akt phosphorylation. As shown in Figure 5, tetrandrine tended to inhibit EGF-induced Akt activation at 0.3 and 1 μM, but without statistical significance.

Discussion

The results of this study indicate that tetrandrine significantly inhibited EGF-induced cell transformation of mouse epidermal JB6 Cl 41 cells, which is a well-developed model used to screen chemopreventive agents (25, 35).
Figure 3. Effect of tetrandrine on EGF-induced AP-1 activation. JB6 AP-1 reporter stable P+1-1 cells were exposed to 10 ng/ml EGF with or without the indicated concentration of tetrandrine for 24 h. Relative AP-1 activity is expressed as mean±S.E. (n=3). *Significantly different from EGF alone at p<0.05.

Figure 4. Effect of tetrandrine on EGF-induced ERK phosphorylation. JB6 CI 41 cells were pretreated with the indicated concentration of tetrandrine for 1 h. The cells were then treated or not treated with EGF (10 ng/ml) and further cultured for 15 min. The phosphorylation levels were estimated by immunoblotted as described in Materials and Methods. Relative ERK phosphorylation is expressed as mean±S.E. (n=4). *Significantly different from EGF alone at p<0.05.
Tetrandrine has been reported to inhibit cell proliferation in a variety of tumor cells (5, 8). However, in the present study, inhibition of EGF-induced cell transformation by tetrandrine did not appear to be due to inhibition of cell proliferation because tetrandrine had no effect on cell proliferation in the concentration range that inhibited transformation.

The transcription factor AP-1 plays a critical role in neoplastic transformation, as well as tumor promotion (26-28). Blocking of tumor promoter-induced AP-1 activation inhibited neoplastic transformation in JB6 cells (26, 36). AP-1 is a well-characterized transcription factor composed of either homo- or heterodimers of JUN and FOS family members (28), and is regulated, at least in part, by the MAP kinase-signaling cascade (37-39). Although three classes of MAP kinases are known, including ERKs, JNKs and p38 kinases (40-43), activation of ERKs appears to be essential for activation of AP-1 and for transformation by TPA and EGF in JB6 cells (37, 38). We found that tetrandrine inhibited EGF-induced AP-1 activation and phosphorylation of ERKs. However, the inhibition of AP-1 activation by tetrandrine was significant only at highest concentration examined (1 μM). Therefore, other mechanisms may also be involved in the inhibition of cell transformation by tetrandrine. Interestingly, the inhibition pattern of phosphorylation of ERKs by tetrandrine was similar to that of cell transformation. Huang et al. (38) have showed that overexpression of ERK2 in JB6 P– (promotion-resistant) cells resulted in conversion to the promotion-sensitive phenotype. Furthermore, inactivation of ERKs in JB6 P+ (promotion-sensitive) cells was shown to inhibit neoplastic transformation, as well as AP-1 activation (39). These findings suggest that ERKs are critical for neoplastic transformation in the JB6 model, although activation of AP-1 is also important. Therefore, the inhibitory action of tetrandrine on EGF-induced cell transformation is considered to be closely related to the blocking of ERK phosphorylation.

PI3K is central to the coordinated control of multiple cell-signaling pathways leading to cell growth, proliferation, survival and migration (32), and the PI3K-signaling pathway is suggested to play a role in carcinogenesis (32, 33). Akt, a downstream effector of PI3K, plays a key role in PI3K signaling (34). We have shown that overexpression of a dominant negative mutant of Akt1, which antagonizes Akt function, blocked cell transformation induced by EGF, but
not TPA, in JB6 P+ cells (30, 31). We also showed that caffeine inhibited both EGF-induced cell transformation and Akt activation, without greatly inhibiting AP-1 activation or ERK phosphorylation (31). Therefore, Akt may also play an important role in cell transformation induced by EGF, though not TPA (30, 31). Tetrandrine appeared to inhibit EGF-induced Akt activation, and even though the inhibition did not reach statistical significance, tetrandrine-induced inhibition of Akt may play some part in blocking cell transformation.

Although tetrandrine inhibited EGF-induced activation of ERKs, AP-1 and Akt, as well as neoplastic transformation, it did not inhibit tyrosine phosphorylation of the EGF receptor (EGFR) induced by EGF in JB6 Cl 41 cells (data not shown), indicating that the inhibitory effects on ERKs, Akt and AP-1 are independent of the blocking of EGFR phosphorylation. We and others have suggested the involvement of PI3K in AP-1 activation and cell transformation induced by EGF (22, 30). We have also shown that treatment with LY294002, a PI3K inhibitor, and the expression of a dominant negative mutant of PI3K p85 subunit inhibited ultraviolet B (UVB)-induced phosphorylation of ERKs, as well as Akt activation, in JB6 Cl 41 cells (44). In that study, treatment with U0126, a MAP kinase/ERK (MEK) inhibitor, and the expression of a dominant negative mutant of ERK2 both reduced UVB-induced Akt activation. Thus, the activation of Akt was suggested to be regulated, at least in part, through the PI3K/ERK pathway following UVB stimulation. Therefore, the inhibition of activation of ERKs, Akt and AP-1 might be related to the blocking of PI3K by tetrandrine. However, it is unclear if the activation of Akt is regulated via the PI3K/ERK pathway in the case of EGF stimulation, because LY294002 had no effect on the EGF-induced phosphorylation of ERKs in JB6 Cl 41 cells (30). Further investigations are required to clarify in detail the mechanisms of tetrandrine inhibition of cell transformation.

The carcinogenic process, including inflammatory responses, results from numerous molecular alterations and is associated with a number of intracellular signal transduction pathways that converge upon activation of transcription factors, including AP-1 (14-16). Therefore, the MAP kinase and PI3K pathways are considered as prime targets for cancer-chemopreventive and anti-inflammatory phytochemicals (14-16, 25, 45). Our findings in this study indicate that tetrandrine inhibited EGF-induced activation of ERKs, Akt and AP-1, as well as neoplastic transformation. The inhibition of activation of ERKs is suggested to be particularly important for tetrandrine-inhibition of cell transformation. Our results provide further insight into the pleiotropic actions, including anti-tumor and anti-inflammatory activities, of tetrandrine.

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References


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