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Involvement of Erks activation in cadmium-induced AP-1 transactivation *in vitro* and *in vivo*

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Abstract

Cadmium is a potent and effective carcinogen in rodents and has recently been accepted by IARC (International Agency for Research on Cancer) as a category 1 carcinogen. Cadmium-induced up-regulation of intracellular signaling pathways leading to increased mitogenesis is thought to be a major mechanism for the carcinogenic activity following chronic cadmium exposure. In the present study, we found that exposure of cells to cadmium induced significant activation of AP-1 and all three members of the MAP kinase family in mouse epidermal JB6 cells. The induction of AP-1 activity by cadmium appears to involve activation of Erks, since the induction of AP-1 activity by cadmium was blocked by pretreatment of cells with PD98058. Interestingly, the induction of AP-1 by cadmium was greatly enhanced by the chemical tumor promoter, TPA and the growth factor EGF, but not by ultraviolet C radiation. *In vivo* studies demonstrated that cadmium could also induce transactivation of AP-1 in AP-1-luciferase report transgenic mice. Considering the role of AP-1 activation in tumor promotion, the results presented in this study provide a possible molecular mechanism for cadmium-induced carcinogenesis. (Mol Cell Biochem **222**: 141–147, 2001)

Key words: cadmium, AP-1, transgenic and MAP kinase

Abbreviations: AP-1 – activator protein-1; Erks – extracellular signal-regulated protein kinases; MAPKs – mitogen-activated protein kinases; JNKs – c-Jun N-terminal kinases; TPA – 12-O-tetradecanoylphorbol-13-acetate; EGF – epidermal growth factor; FBS – fetal bovine serum; MEM – minimal essential medium

Introduction

Cadmium is a toxic transition metal that is widely used in industry. Cadmium is a potent and effective carcinogen in rodents, and has recently been accepted by the International Agency for Research on Cancer as a Category 1 (human) carcinogen (IARC, 1994) [1]. The main sources of human exposure to cadmium are tobacco smoke, food and industrial pollution. Cadmium is absorbed by inhalation and ingestion and has a biological half-life > 25 years [2]. For cigarette smoke, up to 60% of the cadmium deposited in the lung is absorbed. Epidemiological studies have identified lung, prostate, and, to a lesser extent, kidney and stomach as primary targets for cadmium-induced tumorigenesis [1].

Carcinogenesis is a lengthy, multistep process that may be arbitrarily divided into initiation, promotion, and progression of the tumor. DNA mutations and alterations in cell signaling are presumed to play a major role in the entire process [3, 4]. Cadmium is not a strong initiator because it is only a weak mutagen [5]. The effect of enhancement of endogenous mutations through inhibition of DNA repair may be a major mechanism for tumor initiation by cadmium [4]. With regard to the promotion and progression stages, the induction of immediate early genes by cadmium points to a probable

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mechanism of stimulation of proliferation of mutated cells and progression to malignancy [4, 5]. It has been reported that several protooncogenes, such as c-jun and c-myc, have been shown to be activated by cadmium in cell culture models [6–13]. The induction of these protooncogens is suggested to be mechanistically important for cadmium-induced carcinogenicity [4, 14, 15]. It was also reported that cadmium could induce the activation of protein kinase C (PKC) and mitogen-activated protein kinases (MAP kinases) [3, 16]. In the present study, we investigated the involvement of transcription factor, AP-1, in cadmium-induced signaling both *in vitro* and *in vivo*.

Materials and methods

Cell culture and agents

Mouse epidermal JB6 Cl41 and its AP-1-luciferase reporter stable transfectant, P^+1-1, were cultured in monolayers in Eagle's minimal essential medium containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 μg of gentamicin/ml [17, 18]. Fetal bovine serum was from Life Technologies, Inc. (Rockville, MD, USA); Eagle's minimal essential medium (MEM) was from Calbiochem (San Diego, CA, USA); luciferase assay substrate was from Promega (Madison, WI, USA); cadmium chloride was purchased from Sigma (St. Louis, MO, USA); the phospho-specific antibodies against phosphorylated forms or nonphosphorylated forms of Erks, p38 kinase and JNKs were purchased from New England Biolabs (Beverly, MA, USA).

Animals

2X TRE-luciferase reporter transgenic mice were originally established by R.A. Flavell *et al*. [19]. A C57BL/6 male mouse carrying the 2X TRE-luciferase transgene was crossed with DBA/2 (SASCO, Omaha, NE, USA) females [19, 20]. The offspring were screened by testing both the basal level and TPA-induced level of luciferase activity for the presence of the AP-1-luciferase reporter gene. Males and females were housed separately in solid bottom polycarbonate cages on ventilated animals racks (~ 4–5 mice/cage, individualized by incisions in the ears) under temperature-, humidity-, and light-controlled conditions. Food and water were available *ad libitum* and the dorsal skin of the mice was shaved every week during the experiment period.

Assay for AP-1 activity in vitro

Confluent monolayers of JB6 P^+1-1 cells were trypsinized, and 8×10^3 viable cells suspended in 100 l of 5% FBS MEM were added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. Twelve to 24 h later, cells were starved by culturing them in 0.1% FBS MEM for 24 h. Then, the cells were exposed to cadmium for AP-1 induction and maintained in culture. After different periods of time in culture, the cells were extracted with lysis buffer, and luciferase activity was measured using a luminometer (Monolight 2010). The results are expressed as relative AP-1 activity [21].

Phosphorylation analysis for Erks, P38 kinase, and JNKs

The cells were seeded in 6-well plates and cultured until 80–90% confluent. Then the cells were starved for 48 h in 0.1% FBS MEM and treated with arsenic at the concentration indicated. The cells were then extracted with SDS sample buffer. Immunoblots for detection of phosphorylated proteins were carried out using phospho-specific MAPK antibodies against phosphorylated sites of Erks, P38 Kinase, or JNKs as described previously [20, 21]. Antibodies were purchased from New England Biolabs and used according to manufacturer's recommendations. Antibody proteins were detected by chemiluminescence (ECL, New England Biolabs, Beverly, MA, USA).

Assay of AP-1 activity in vivo

All the mice were characterized by testing both the basal level and UVB-induced level of luciferase activity. The AP-1-luciferase reporter-bearing male and female mice (6–9 weeks old) were randomly divided into two groups (19–21 mice/group), including a negative control group (control) and cadmium-treated group. Two weeks after grouping, the mice were exposed to cadmium by topical application. The dorsal skin of the mice was punch biopsied at different time points to determine the induction of AP-1 activity in the epidermis. The luciferase activity of punch biopsied epidermis was measured as described previously [19, 20].

Statistical analysis

The significance of the difference in the AP-1 activity was determined with the Student's *t*-test.

Results

Induction of AP-1 in AP-1 luciferase transgenic mice by cadmium. To determine activation of AP-1 *in vivo* by cadmium, each AP-1 luciferase reporter transgenic mouse was treated

topically with 500 μg of cadmium chloride, and the dorsal skin of the mice was punch biopsied to determine the luciferase activity at 0, 16, 30 and 48 h after treatment with cadmium. The results show that exposure of mice to cadmium induced significant increases of AP-1 activity in the skin of mice (Fig. 1). Maximal induction of AP-1 activity in skin of mice was observed from 16–48 h after cadmium exposure (Fig. 1). These results provide the first evidence that cadmium could induce AP-1 transactivation *in vivo*.

Cadmium induces AP-1 activation in mouse epidermal JB6 cells

In order to investigate the signal transduction pathways involved in cadmium-induced AP-1 transactivation, the mouse epidermal JB6 cell was employed as cell culture model. As shown in Fig. 2, cadmium treatment of P^+1-1 cells, a stable JB6 Cl41 transfectant with the AP-1-luciferase reporter, markedly increased of AP-1 activity in dose-dependent manner (Fig. 2). The dose for maximal induction of AP-1 activity ranged between 0.8 and 3.2 μM (Fig. 2). A time course study suggested that treatment of cells with cadmium resulted in increased AP-1 activity within 12 h after treatment (Fig. 3). Maximal induction of AP-1 activity was observed between 24 and 48 h post-cadmium treatment. AP-1 activity gradually returned toward basal levels after 3 days (Fig. 3). These results are consistent with those from *in vivo* studies, suggesting that cadmium is a strong stimulator for AP-1 transactivation both in a cell culture model and in the mouse skin *in vivo* model.

Activation of Erks, JNKs, and P38 kinase by cadmium

AP-1 is a known target of the MAP kinase family, including Erks, JNKs, and p38 kinases. A previous study reported that

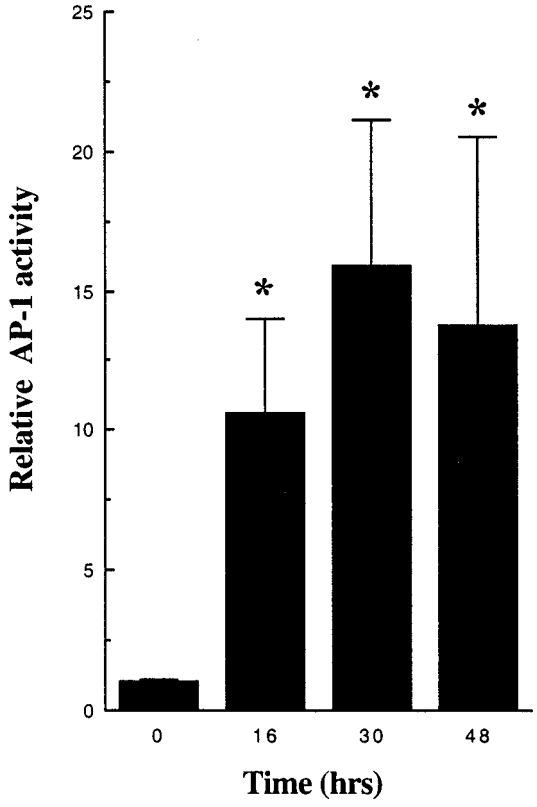

Fig. 1. Induction of AP-1 by cadmium in AP-1-luciferase transgenic mice. The AP-1-luciferase transgenic mice were described under 'Materials and methods.' The mice were randomly divided into two groups, including negative controls and cadmium-treated groups. Two weeks after grouping, the mice were exposed topically to cadmium chloride (500 μg/mouse). The dorsal skin of the mice was punch biopsied to determine the induction of AP-1 activity in the epidermis at different time points. The luciferase activity of punch biopsied epidermis was measured as described previously [25, 26]. The results were presented as relative AP-1 activity (means ± S.E. of 4 independent experiments. *indicates a significant increase from control ($p < 0.05$).

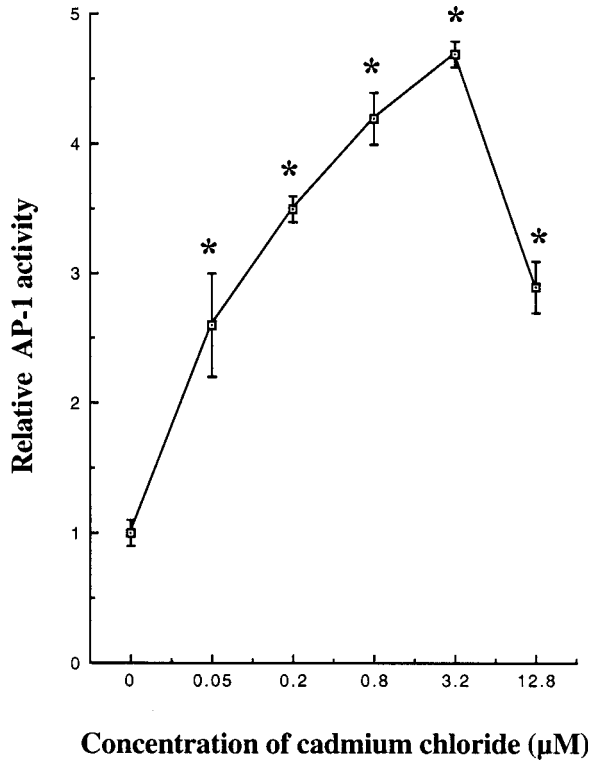

Fig. 2. Transactivation of AP-1 by cadmium in mouse epidermal JB6 cells. 8×10^3 P^+1-1 cells were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were starved for 12 h by replacing medium with 0.1% FBS MEM. Then the cells were treated with different concentrations of cadmium chloride. After being cultured for 24 h, the luciferase activity was measured, and the results presented as relative AP-1 activity. Each value indicates the mean and S.E. of 4 identically treated assay wells. *indicates a significant increase from control ($p < 0.05$).

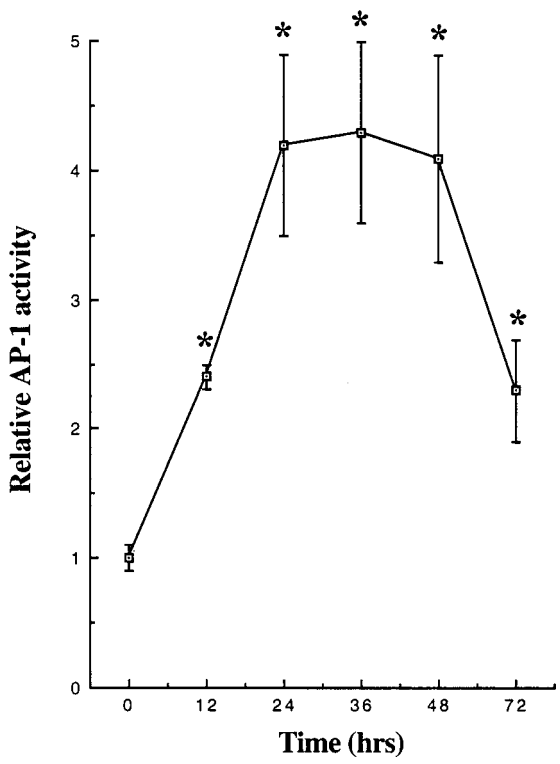

Fig. 3. Time-course studies on cadmium-induced AP-1 activity. 8×10^3 P⁺1-1 were seeded, cultured and starved as described for Fig. 2. The cells were then treated with cadmium chloride (1 μM) and harvested at the time points indicated for luciferase activity assay. The results are presented as relative AP-1 activity. Each value indicates the mean and S.E. of 4 identically treated assay wells. *indicates a significant increase from control ($p < 0.05$).

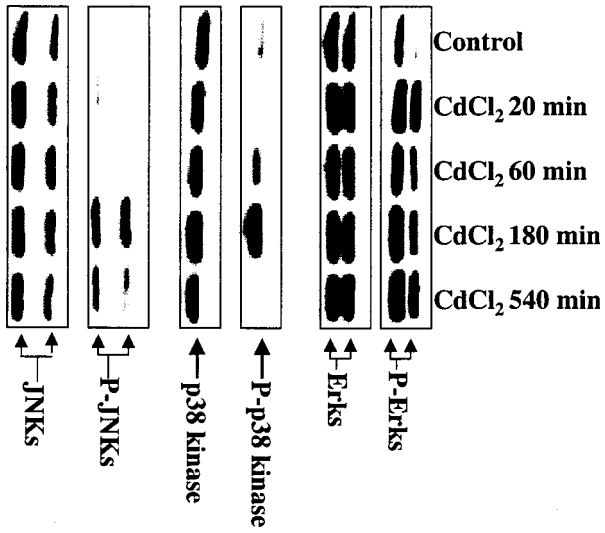

Fig. 4. Activation of Erks, JNKs, and p38 kinase by cadmium. JB6 P⁺1-1 cells were seeded into each well of 6-well plates. After being cultured until 80–90% confluent, the cells were starved for 48 h in 0.1% FBS MEM. Then the cells were treated with of cadmium chloride (1 μM) and incubated for different times as indicated. The cells were extracted with SDS sample buffer. Immunoblots for detection of nonphosphorylated and phosphorylated proteins were carried out using antibodies against nonphosphorylated Erks, JNKs or p38 kinases and phosphorylated sites of Erks, JNKs, and p38 kinase, respectively.

treatment of non-small cell lung carcinoma cell line CL3 with high doses (> 80 μM) of cadmium induced activation of Erks, JNKs and p38 kinases, while a low dose of cadmium caused inhibition of Erk and had no effect on p38 activity [14]. To determine the effects of cadmium on MAP kinase activation in mouse epidermal cells, we incubated the JB6 cells with 1 μM of cadmium chloride. The activation of Erks, JNKs and p38 kinases was determined using specific antibodies recognizing the dual phosphorylation sites of the specific kinase. The results showed that exposure of cells to 1 μM cadmium chloride caused significant activation of Erks, JNKs, as well as p38 kinases (Fig. 4). The Erks activation appeared to be sustained in all time points observed, while phosphorylation of JNKs and p38 kinases was delayed and transient (Fig. 4). The peak activation of JNKs and p38 kinase was observed at 180 min post-cadmium treatment (Fig. 4).

Blockade of cadmium-induced AP-1 activation by PD98059

PD98059 is a specific inhibitor of MEK1, a kinase immediately upstream of Erks. To investigate the role of Erks in cadmium-induced AP-1 activation, we determined the effects of PD98059 on cadmium-induced AP-1 activation. The results show that pretreatment of cells with PD98059 resulted in significant inhibition of cadmium-induced AP-1 activity (Fig. 5). These results suggest that activation of Erks plays a role in cadmium-induced AP-1 activation.

Effect of cadmium on TPA-, EGF- or UV-induced AP-1 activation

TPA, EGF, and UVC are well known tumor promoters and induce high levels of AP-1 activity [17, 19–21]. To assess the combined effects of cadmium with these tumor promoters on AP-1 activation, P⁺1-1 cells were treated with combination of cadmium and TPA, EGF or UVC, respectively. The results show that cadmium had an additive effect on either TPA- or EGF-induced AP-1 activity, while it did not enhance UVC-induced the AP-1 activation (Fig. 6). These data suggest that cadmium may enhance the carcinogenic effect of TPA and EGF.

Discussion

Cadmium has been classified as a human carcinogen by IARC in 1993 [1]. Several mechanisms to explain cadmium

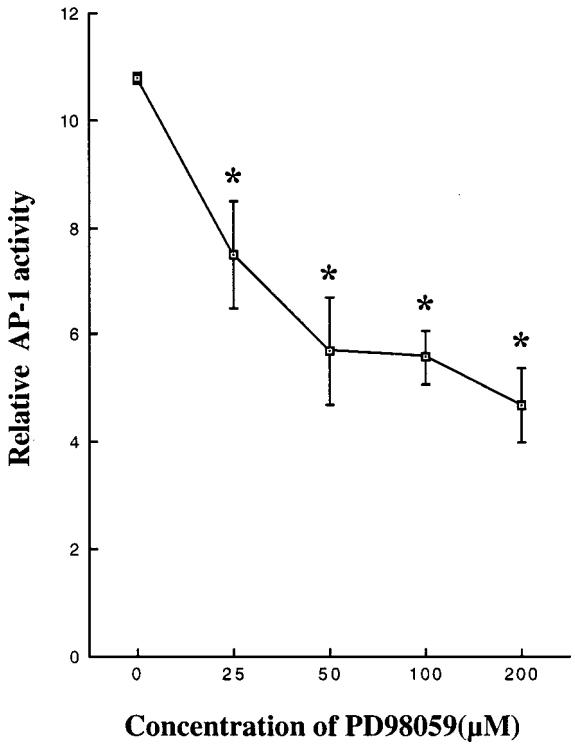

Fig. 5. Inhibition of cadmium-induced AP-1 activity by PD98059. JB6 P⁺1-1 cells were seeded, cultured and starved as described for Fig. 2. The cells were first treated with different concentrations of PD98059 as indicated for 45 min. Then the cells were exposed to 2 μM of cadmium chloride for 24 h, and the luciferase activity was measured. The results are presented as relative AP-1 activity (means ± S.E. of 4 independent experiments). *indicates a significant increase from control ($p < 0.05$).

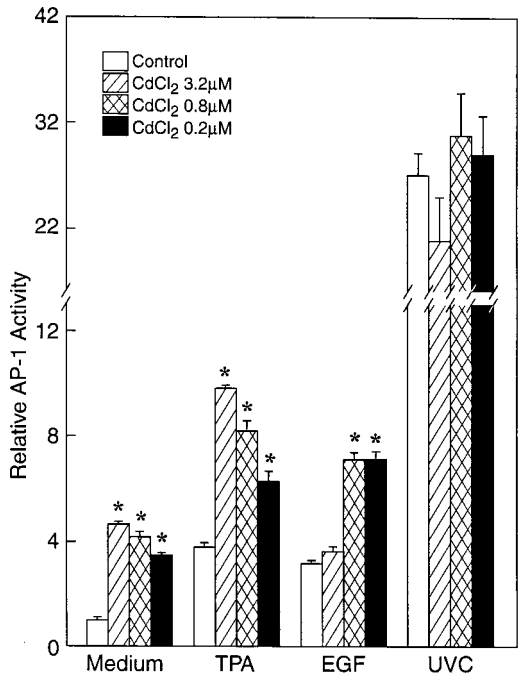

Fig. 6. Effects of cadmium on TPA-, EGF- or UVC-induced AP-1 activation. JB6 P⁺1-1 cells were seeded and cultured as described for Fig. 2. After being cultured overnight, the cells were starved for 12 h. Then the cells were exposed to cadmium chloride (concentrations as indicated) plus TPA (10 ng/ml), EGF (10 ng/ml) or UVC (60 J/m^2). The cells were then incubated for 24 h. The luciferase activity was measured and results are presented as relative AP-1 activity (means ± S.E. of 4 independent experiments). *indicates a significant increase from the respective control ($p < 0.05$).

carcinogenesis have been proposed. It is likely that multiple mechanisms are involved in cadmium-induced tumor development [3, 6–18]. The present study focused on the effects of cadmium on activation of AP-1, a transcription factor that is required for tumor promotion in many cases. The results show that cadmium exposure caused transactivation of AP-1 in both a cell culture model and a transgenic mouse model. Exposure of cells to cadmium also induced activation of Erks, p38 and JNKs. Pretreatment of cells with PD98059 significantly inhibited cadmium-induced AP-1 activity. Furthermore, cadmium had an additive effect on TPA- and EGF-induced AP-1 activation. The data from this study demonstrate that cadmium is a strong inducer of AP-1 activation, which may be one of the mechanisms involved in cadmium-induced carcinogenesis.

Cadmium is widely used in industry in plating, batteries, plastics and semiconductors [22]. It is not only highly toxic to human, but also is a human carcinogen [1, 22]. Exposure of cadmium is associated with cancer of lung and prostate in human [23, 24]. In rats, cadmium exposure by subcutaneous injection gives rise to tumors at the site of injection, also in the kidney and in the testes [22, 24]. Inhalation and other methods of cadmium exposure give rise to tumors of the lung and prostate [22, 24]. Cadmium exposure can promote altered gene expression and modify intra- or inter-cellular communication [22]. These and other alterations in signal transduction are generally considered to be related to tumor promotion [22]. It was also found that the enhancement of endogenous mutations through inhibition of DNA repair is a major mechanism reinforcing gene mutation [4]. Thus, cadmium is not considered a strong tumor initiator [5], and it is believed that the carcinogenic activity of cadmium is mainly through tumor promotion rather than initiation [22]. Previous investigations showed that cadmium could induce the activation of protein kinase C (PKC) and MAP kinase [3, 16]. Recently, Chuang *et al.* [14] reported that cadmium induced persistent activation of JNKs or p38 kinases and moderate activation of Erks at high cytotoxic doses, while activities of Erks were decreased by treatment of cells with low doses of cadmium. The results from the same report suggested that JNKs and p38 kinase cooperatively participate in apoptosis induction by cadmium, while decreased Erk activity induced by low doses of Cd contributed to growth arrest and apoptosis [14]. MAP kinases, including Erks, JNKs and p38 kinases, are

direct upstream kinases that regulate the transcription factor, AP-1, in cell response to extracellular stimulations, such as EGF, TPA, and UV radiation . In this study, we investigated the involvement of AP-1 in cadmium-induced signal transduction. We found that cadmium induced activation of AP-1 both *in vitro* and *in vivo*. The activation of AP-1 by cadmium appears to mediated by MAP kinases, at least Erks, because treatment of cells with cadmium not only induced activation of all three members of the MAP kinase family, but also induction of AP-1 by cadmium could be dramatically inhibited by pretreatment of cells with PD98059. These data suggest that cadmium-induced the activation of MAP kinases, especially Erks, is involved in its AP-1 induction.

The important role of AP-1 for tumor promotion has been demonstrated in different cell models by different groups [25–35]. AP-1 was first considered to be a mediator of tumor promotion because of its ability to alter gene expression in response to tumor promoters, including epidermal growth factor, TPA, and UV radiation [25]. AP-1 activity was found to be progressively elevated in mouse epidermal JB6 cells representing various stages of tumor promotion [27]. The activation of AP-1 appears to be required for the preneoplastic-to-neoplastic progression of JB6 cells. Overexpression of c-Jun in P^+ cells caused neoplastic transformation and the introduction of a dominant negative mutant of c-Jun blocked tumor promoter-induced cell transformation [27]. Also, blocking AP-1 activity by either pharmacological inhibitors or molecular biological inhibitors could impair tumor development. These inhibitors include PD98059, SB202190, Wortmannin, Ly294002, Insp6, fluocinolane acetonide, retinoic acid, ascorbic acid, dominant negative Erks, dominant negative JNKs and dominant negative P1-3 kinase [19, 21, 28–31]. Moreover, acquisition of a tumor promotion-resistant phenotype is associated with the loss of responsiveness to tumor promoter-induced AP-1 activation [32]. Introducing wild-type Erk_2 converts tumor promotion-resistant phenotype to tumor promotion-sensitive phenotype [21]. In contrast, overexpression of dominant negative mutant Erk_2 blocks TPA- or EGF-induced AP-1 activation and cell transformation [33]. The requirement of AP-1 in tumor promotion was further supported by findings that the retinoid SR11302, an AP-1 inhibition-specific retinoid, markedly inhibited TPA-induced papilloma formation and AP-1 activation *in vivo*, whereas repeated application of another retinoid, SR11235, a transcriptional activator of the retinoic acid response element, did not inhibit papilloma formation and AP-1 activation [16]. The promoter region of most TPA-response genes includes several protooncogenes, such as c-fos, c-Jun, and matrix degrading metalloproteinases contain the AP-1 binding sequence or TRE [25, 34, 35]. Downstream genes with a regulatory element AP-1 may be involved in the process of tumor promotion. Wilson *et al.* [36] reported that an absence of metalloproteinase matrilysin resulted in a reduction in mean tumor multiplicity of ~ 60% and metalloproteinase matrilysin contributed to the very early stage of tumor development [36]. In light of the important role of AP-1 activation in tumor development, we investigated the signal transduction pathways leading to AP-1 activation by cadmium *in vitro* and *in vivo*. The data obtained from this study may provide a molecular mechanism to explain the induction of some proto-oncogenes, such as c-jun, in previous reports. In addition cadmium-induction of AP-1 activation may play a role in cadmium-induced carcinogenesis.

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