Inhibition of Tumor Promotion by Parthenolide: Epigenetic Modulation of p21

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Abstract

The promotion stage in the multistep process of epidermal tumorigenesis is NF-κB–dependent, epigenetically regulated, and reversible, thus, a suitable target for chemoprevention. We investigated whether the NF-κB inhibitor, parthenolide, currently in cancer clinical trials, attenuates tumor promotion by modulating the epigenetically regulated NF-κB target genes, p21 and cyclin D1.

Parthenolide selectively inhibited the growth of neoplastic keratinocytes while sparing normal ones. Specifically, in JB6P+ cells, a model of tumor promotion, noncytotoxic parthenolide concentrations abrogated tumor promoter–induced cell proliferation and anchorage-independent growth. Furthermore, parthenolide decreased tumor promoter–induced NF-κB activity, increased p21, and decreased cyclin D1 expression. In parthenolide-treated cells, p21 transcription correlated with relaxed chromatin and p65/NF-κB binding at the p21 promoter. However, cyclin D1 transcription correlated more with p65/NF-κB binding than with chromatin structure at the cyclin D1 promoter. Epigenetic regulation by parthenolide seemed specific, as parthenolide did not alter global histone acetylation and methylation and histone deacetylase activity. Because p21 expression by parthenolide was sustained, we used p21-siRNA and p21−/− cancer cells and showed that the loss of p21 is cytoprotective against parthenolide.

Low parthenolide concentrations (0.25 mg/kg) inhibited tumor growth of promoted JB6P+ cells in xenograft immunocompromised mice using two different chemoprevention protocols. Tissue microarray of mouse tumors showed that parthenolide decreased scores of the cell proliferation marker Ki67 and p65/NF-κB, whereas it increased p21 expression.

These results show that low doses of parthenolide inhibit tumor promotion and epigenetically modulate p21 expression, highlighting the potential role of this drug as a chemopreventive agent and in epigenetic cancer therapy. Cancer Prev Res; 5(11); 1298–309. ©2012 AACR.

Introduction

The multistage in vitro murine (1) and human (2) squamous cell carcinoma (SCC) models are ideal to study the alterations that take place during the different stages of carcinogenesis. In particular, the promotion phase is a rate-limiting step in the tumorigenesis process (1), is mostly epigenetically regulated, hence, reversible, and is an attractive target for chemoprevention (3). The JB6-cell model is ideal to study tumor promotion and widely used to identify antitumor promotion agents (4).

Tumor promotion is epigenetically regulated and NF-κB–dependent, and high NF-κB activity has been associated with chemoresistance (4, 5). Therefore, targeting NF-κB and epigenetic mechanisms may inhibit tumor promotion. A candidate drug for this purpose is parthenolide because it is a pharmacologic NF-κB inhibitor and is currently being tested in cancer clinical trials. Therefore, new findings related to the anticancer potential of parthenolide, particularly pertaining to epigenetic therapy, may have chemopreventive and chemotherapeutic implications.

Parthenolide is a sesquiterpene lactone and the major bioactive molecule isolated from the medicinal plant feverfew (Tanacetum parthenium). Parthenolide inhibits NF-κB by suppressing the activity of inhibitor of IκB kinase (IKK) complex and the subsequent degradation of IκBα and IκBβ (6) or by directly modifying the p65/NF-κB protein (7). Recently, parthenolide was shown to specifically deplete histone deacetylase 1 (HDAC1; ref. 8) and DNA methyltransferase 1 (DNMT1; ref. 9) in tumor cells and is, so far, the only small molecule that kills cancer stem cells while sparing normal ones (10).
We hypothesized that parthenolide inhibits tumor promotion by suppressing promoter-induced NF-κB activity and by modulating the epigenetically regulated NF-κB target genes, p21 and cyclin D1. We used well-characterized in vitro models of epidermal SCC and focused on the JB6 model of tumor promotion. Low concentrations of parthenolide that do not affect cell growth of normal and premalignant cells were used to exclude the possibility that antitumor promotion effects are due to drug cytotoxicity. Using these noncytotoxic concentrations, the effect of parthenolide on tumor promoter–induced cell proliferation, anchorage-independent cell growth, cell-cycle regulation, and NF-κB activity. Chromatin structure and p65/NF-κB binding on p21 and cyclin D1 promoters were analyzed relative to p21 and cyclin D1 transcriptional regulation by parthenolide. The role of p21 in mediating sensitivity of tumor cells to parthenolide was investigated using p21-siRNA and p21-knockout cells. Finally, we studied the antitumor-promotion properties of low doses of parthenolide using in vivo chemoprevention models. Our results show that low concentrations of parthenolide have potent antitumor promotion properties in vitro and in vivo and epigenetically regulate the expression of the NF-κB target gene, p21, which is crucial for parthenolide’s antitumor potential.

Materials and Methods

Cell culture
Primary mouse keratinocytes (PMK) were prepared from newborn BALB/c mice as described in reference (11) and were approved by the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (Beirut, Lebanon). The murine (12, 13) and human (14) epidermal cell lines and JB6 cells (15) were generously provided in 1998 by Dr. Stuart H. Yusp (NIH, Bethesda, MD), in 2005 by Dr. Petra Boukamp (German Research Cancer Center, Heidelberg, Germany), and in 2007 by Dr. Nancy Colburn (National Cancer Institute (NCI), Frederick, Maryland), respectively. Cells were regularly tested for mycoplasma contamination. No cell authentication was done by the authors. Details about cell line description, maintenance, and testing are given in Supplementary Materials and Methods.

Parthenolide treatments
Parthenolide (BIOMOL-ENZO T) was prepared in dimethyl sulfoxide (DMSO). Control treatments contained up to 0.1% DMSO and did not affect the growth of cells (data not shown). Treatments were done at 40% to 50% cell confluency unless indicated otherwise.

Cell growth
Cell growth was determined by MTT (Roche Diagnostics) and/or Trypan blue dye exclusion assays. Details are given in Supplementary Materials and Methods.

Anchorage-independent transformation assay
Anchorage-independent growth was quantified using the CytoSelect 96-Well Cell Transformation Assay Kit (Cell Biolabs). JB6P+ cells were seeded in agar containing parthenolide ±5 nmol/L, 12-O-tetradecanoylphorbol-13-acetate (TPA) in complete media (10% FBS). Details are given in Supplementary Materials and Methods.

Cell-cycle analysis
Attached and detached cells were stained with 50 μg/ml propidium iodide (Sigma Chemical Co.) and analyzed by flow cytometry (16). Percentage of cells in the pre-G1, G1, S, and G2–M phases were determined using FlowJo software with the Watson pragmatic model (Tree Star).

Electrophoretic mobility shift assay
Nuclear protein extracts were prepared and NF-κB electrophoresis mobility shift assays (EMSA) were conducted as described (16). Details are in given in Supplementary Materials and Methods.

Dual luciferase assay for NF-κB transcriptional activity
Cells, at 60% to 80% confluency, were cotransfected with NF-κB (pGL2-IL-6–Luc, 0.2 μg) firefly luciferase reporter plasmid and renilla luciferase reporter plasmid (pRL-SV40, 0.04 μg) using Lipofectamine 2000 with PLUS reagent. The pGL2-IL-6–Luc uses the IL-6 promoter region containing 4 putative NF-κB binding sites and was kindly provided by Dr. Nancy Colburn. Transfected cells were pretreated with parthenolide for 1 hour then treated with or without 16 nmol/L TPA up to 48 hours. Luciferase activity was determined using the Dual Luciferase Reporter Assay Kit (Promega). NF-κB firefly luciferase was normalized to Renilla luciferase activity and plotted as percentage of control.

HDAC activity assay
Protein lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer, and HDAC activity was analyzed using the HDAC Assay Kit (Active Motif 56210).

Western blot analysis
Western blot analyses were conducted using total cellular protein extracts (30–50 μg) as described (16). Bands were quantified using ImageJ software (17). Details are given in Supplementary Materials and Methods.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation assay (ChIP) assay was carried out using the ChIP-IT Express Kit (Active Motif) using the antibodies: DiMeH3K4 (Active Motif 39141), DiMeH3K9 (Active Motif 39239), and p65 (sc-109X). R1 and R2 in the mouse p21 promoter (cdmn1a Mus musculus from Genbank, NCBI Reference Sequence: NC_000083.5) contained NF-κB-binding sites, as predicted by AliBaba2 software (18). The forward and reverse primers spanning R1 are 5′-GCAAGGCCCAGATGTTGTG-3′ and 5′-CTGCTGATCTGCTGACCTC-3′, respectively. The forward and reverse primers spanning R2 are 5′-GATCTGGAGGAGGAGAT-3′ and 5′-ACATCCGTGAGAGCA-3′, respectively. One region in the
mouse cyclin D1 promoter (cnd1 Mus musculus from Genbank, NCBI Reference Sequence: NC_000073.5) was studied and contained an evolutionary conserved NF-xB binding site (19, 20). The forward and reverse primers spanning this region are 5’-CCGGCTTTGATCTGCTGTA-3’ and 5’-CGCCGAGTCTGAGATTCTTC-3’, respectively. Primers were obtained from Metabion International AG and an isotype-matched IgG (Dianogen KCH-504-250) was used as a negative control. Primer amplification efficiencies ranged from 1.99 to 2.00. Real-time PCR was conducted using the QuantiTect SYBR Green PCR Kit (Qiagen) and iCycler (Bio-Rad). Details are given in Supplementary Materials and Methods.

**Real-time reverse transcription PCR**

Quantitative PCR (qPCR) was conducted using the QuantiTect SYBR Green PCR Kit (Qiagen) and iCycler (Bio-Rad). For mouse p21, the forward primer was designed as 5’-GATCCCTCAGCCCTGCTG-3’ and the reverse as 5’-CTGGTCCGTAGTGATTAGA-3’. For mouse cyclin D1, the forward primer was 5’-CTGCGGTCTGATGTTCTCT-3’ and the reverse as 5’-ATCCGCCTCCTGGAATTTG-3’ (21).

**siRNA transfection**

siRNAs transfactions were conducted using p21-siRNA (m2; sc-44271), p21-siRNA-m (sc-29428), and control-scrambled siRNAs, siRNA-B (sc-44230) and siRNA-C (sc-44231), according to the manufacturer’s instructions (Santa Cruz).

**Xenograft mouse model**

JB6P+ cells were promoted for 6 days with TPA (16 nmol/L) and 10% FBS and, then, 2 million cells in 100 µL PBS were subcutaneously injected into the shoulder of male NMRI-Nu immunocompromised mice as approved by the IACUC of the University of Erlangen-Nuremberg, Germany. Average mouse weight was 40 g, and the mice were 7 to 22 weeks old, evenly distributed across groups. Parthenolide of 400 µmol/L containing 1% DMSO in 100 µL PBS was injected intraperitoneally and was equivalent to 0.25 mg/kg. PBS or 1% DMSO was used as control treatment with no difference in mouse weights or tumor volumes between these 2 groups (data not shown). Tumors grew spherically, and their diameters were measured with a hole-mask. Tumor volumes were calculated as (4/3)πr3. Animals were sacrificed by cervical dislocation when the tumor diameter reached 25 mm.

**Tissue microarray**

Representative regions of mouse tumors were selected on hematoxylin and eosin-stained slices for inclusion in a tissue microarray (TMA) analysis. Samples were punched in triplicate and immunostained with Ki67 (Abcam 16667), p21 (sc-397), cyclin D1 (sc-718), or p65 (sc-109X). Immunostaining was expressed as a percentage of positively stained cells out of total cells per punch.

**Statistical analyses**

SPSS Version 16.0 and Microsoft Office Excel 2010 were used to calculate the best-fit regression model, the IC50 values, the independent sample t test, and ANOVA with associated posthoc tests: Dunnett t, Tukey, and Student–Newman–Keuls (SNK) tests. Syntax tool in SPSS was used to conduct multiple comparisons within interaction groups in 2-way ANOVA. Statistical significance was claimed when the P value was 0.05 or less. The best-fit regression functions were: “inverse” for PMKs, “logarithmic” for SP1, PAM212T, JB6P+, RT101, and HaCaT-I4, and “linear” for PAM212, I7, JB6P−, HaCaT, and HaCaT-A5. SEM for IC50 values (SEM IC50) were calculated using the following modified equations for logarithmic and inverse functions, respectively: SEM IC50 = IC50 × SEM ln(IC50) and SEM IC50 = (IC50)2 × SEM 1/IC50.

**Results**

Parthenolide differentially inhibits the growth of normal and tumor epithelial cells in human and mouse in vitro models

The anticancer activities of parthenolide have been reported at up to 100 µmol/L concentrations in *in vitro* cancer models (21). We tested for the growth inhibitory effects of parthenolide on murine and human *in vitro* epidermal cancer models (Fig. 1). Murine tumor cells comprised PMK as representatives of normal cells, SP-1 as benign tumor cells, PAM-212 as differentiated SCC cells, PAM-212T as nondifferentiated SCC cells, and I7 as metastatic spindle cells. At a concentration range of 5 to 10 µmol/L, parthenolide selectively inhibited the growth of the tumor cells with minor effects on PMKs (Fig. 1A).

Human cells comprised the clonal variants: HaCaT as representatives of nontumorigenic cells, HaCaT-A5 as benign tumor cells, and HaCaT-I4 as differentiated SCC cells. Parthenolide, at 10 µmol/L concentrations, did not significantly affect the growth of HaCaT cells, relative to control (P > 0.05), but decreased the growth of HaCaT-A5 and HaCaT-I4 cells (Fig. 1B).

Concentrations up to 10 µmol/L parthenolide showed differential growth inhibitory properties against epidermal tumor cells while sparing normal ones, so they were selected to study the effect of parthenolide on the JB6 model of tumor promotion (Fig. 1C). In this model, JB6P− and JB6P+ cells are immortalized nontumorigenic murine cells, with the former ones being promotion resistant, whereas the latter promotion responsive (15). Hence, JB6P+ cells become tumorigenic upon exposure to tumor promoters, such as TPA, and transform into metastatic RT-101 (15). Parthenolide, up to 10 µmol/L, differentially inhibited the growth of JB6P+ and RT-101 cells with nonsignificant effects on JB6P− cells (P > 0.05; Fig. 1C). Trypan blue counts showed similar growth...
Parthenolide inhibits tumor promotion

Parthenolide inhibits tumor promotion in 2D and 3D cultures

To investigate whether parthenolide inhibits tumor promotion, we tested if this drug reduces TPA-induced growth of JB6P+ cells at concentrations that do not affect their basal cell growth. Nontoxic concentrations of parthenolide of less than 10 μmol/L were selected.

In JB6P+ cells, TPA increased cell growth (Fig. 2A and B) as measured by MTT in 2-dimensional (2D) cultures. Parthenolide concentrations as low as 5 μmol/L did not decrease JB6P+ basal cell proliferation but significantly inhibited TPA-induced cell growth up to 72 hours (P < 0.01; Fig. 2A–B and data not shown).

We investigated whether parthenolide inhibits TPA-induced colony formation of JB6P+ cells in soft agar [3-dimensional (3D) cultures], a hallmark of malignant transformation. JB6P+ cells form colonies in soft agar mostly in the presence of TPA (Fig. 2C). Parthenolide decreased TPA-induced colony formation by at least 77% at concentrations as low as 3 μmol/L (Fig. 2C). Similar results were obtained by counting all colonies in the wells (Supplementary Fig. S1A). The ability of parthenolide to inhibit tumor transformation in 3D cultures was also observed in the human HaCaT-II4 cells (Fig. 2D). These SCC cells spontaneously form colonies in soft agar, independent of tumor promoters, and parthenolide inhibited their colony growth in a dose-dependent manner (Fig. 2D). These results show that parthenolide has antitumor promotion activities by inhibiting promoter-induced cell proliferation and anchorage-independent cell growth.

Parthenolide induces S-phase arrest in nonpromoted cells and blocks tumor-promoted cells at G2–M phases

We investigated the antitumor promotion properties of parthenolide on the cell-cycle distribution of TPA-promoted cells. JB6P+ cells were treated with parthenolide in the presence or absence of TPA, and their cell-cycle profiles and viabilities were analyzed.

The cell-cycle distribution of cells treated with 5 μmol/L parthenolide was similar to control (Fig. 3A). Treatment with 10 μmol/L parthenolide concentrations for 24 hours increased the S-phase by 58% relative to control and caused 9% accumulation in pre-G1. This S-phase increase was maintained higher than control values up to 72 hours, possibly indicating the S-phase arrest (Fig. 3A). This is supported by a decrease by at least 75% in the number of viable cells treated with 10 μmol/L parthenolide (P < 0.001; Supplementary Fig. S1B).

When TPA was added for 24 and 48 hours, the combined S to G2–M proportion of cells increased by 23% and 68%, respectively, relative to control (Fig. 3A). This S to G2–M increase was not sustained at 72 hours as G0–G1 phase increased to 79%, indicating that the promoted cells reentered the cell cycle rather than being arrested (Fig. 3A). This is supported by an increase by 80% in the number of TPA-treated cells at 72 hours, relative to control (P < 0.001; Supplementary Fig. S1B). The G0–G1 percentage in control cells increased from 59% at 24 hours to 84% at 72 hours, as cell cultures became more confluent (Fig. 3A).

Parthenolide at 5 μmol/L prevented TPA-induced increases in S to G2–M proportions at 24 hours, rendering the cell-cycle profile similar to control (Fig. 3A). When cells

Figure 1. Effect of parthenolide on the growth of normal and tumorigenic epidermal cells. At 40% to 50% confluence, cells were treated with parthenolide for 24 hours, and cell growth was determined by MTT assay. Cell growth values are expressed as percentage of control (Ct) cells for each group and represent the average ± SEM of the means of quadruplicate wells from at least 3 independent experiments. Murine (A) and human (B) cell lines represent different stages of epidermal carcinogenesis. C, murine cells representative of the JB6 model of tumor promotion. IC50 = SEM values are indicated for each cell type between parentheses. Other parthenolide concentrations ranging 4 to 14 μmol/L (not shown) were also included for some cell types for more accurate estimation of IC50. Statistical significance, if true, is reported at specific concentrations by 1-way ANOVA to indicate difference of PMK (A), HaCaT (B), or JB6P– (C) from any of the tumor cell lines in the corresponding figure. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
were cotreated with 5 μmol/L parthenolide and TPA for 48 hours, the combined S to G2-M proportions increased by 132% and were sustained as high as 185% at 72 hours relative to control (Fig. 3A). This indicated that promoted cells were blocked at S to G2-M phases by 5 μmol/L parthenolide. In fact, the G0-G1 proportion of cells, cotreated with TPA and 5 μmol/L parthenolide, did not increase back to control levels by 72 hours (Fig. 3A), and the viable counts of these cells remained similar to control values for up to 72 hours (Supplementary Fig. S1B). These results indicate that parthenolide induces S-phase arrest in nonpromoted cells and blocks TPA-promoted cells at S to G2-M phases.

**Parthenolide inhibits tumor promoter–induced NF-κB activity and modulates p21 and cyclin D1 NF-κB target genes**

NF-κB signaling is activated by various tumor promoters and is a key regulator in SCC development (4, 5, 22). Because parthenolide is a pharmacologic inhibitor of NF-κB (23), we tested whether its inhibition of tumor promotion associates with a reduction of TPA-induced NF-κB activity. The effect of parthenolide was assessed on the basal (Supplementary Fig. S1C and S2A) and TPA-induced (Supplementary Fig. S1C and S2B) NF-κB activities in JB6P+ cells. TPA increased NF-κB luciferase activity (Supplementary Fig. S1C; ref. 24) and DNA binding affinity (Supplementary Fig. S2A and S2B; ref. 25). Parthenolide, at 5 μmol/L, did not affect basal but significantly inhibited TPA-induced NF-κB luciferase and DNA binding activities (Supplementary Fig. S1C and S2A and S2B). Parthenolide of 10 μmol/L concentrations significantly decreased basal and TPA-induced NF-κB luciferase and DNA binding activities (Supplementary Fig. S1C and S2A and S2B; P < 0.05). Similar results were obtained using the HaCaT-II4 cells, in which 10 μmol/L parthenolide decreased NF-κB DNA binding affinity (Supplementary Fig. S2C) and it reduced NF-κB luciferase activity by 65% ± 21% at 48 hours (data not shown).

The NF-κB target genes in keratinocytes, p21 (26, 27), and cyclin D1 (19, 20), play crucial roles in tumor promotion (20, 28–30), epigenetically regulated (31, 32), and are important targets of epigenetic therapy (33). p21 inhibits cyclin-dependent kinases (CDK) that play a direct role in G1–S transition (34), and p21 overexpression can cause S-phase arrest (35). Parthenolide, at concentrations as low as 5 μmol/L, induced p21 protein expression in JB6P+ cells peaking at 6 hours relative to control (Fig. 3B). The increased p21 expression by parthenolide was sustained up
to 48 hours (Fig. 3B), p21 at lower protein levels can act as a cyclin D1-CDK assembly factor, hence, inducing cell proliferation; whereas, at higher levels, p21 functions as a CDK inhibitor causing cell-cycle arrest (36). In parthenolide-treated cells, it is more plausible that p21 causes cell-cycle arrest, instead of cell proliferation, concomitant with the observed S-phase arrest (Fig. 3A). On the other hand, TPA transiently induced at 3-hour p21 protein levels, which then decreased to basal levels by 24 hours (Fig. 3C). TPA and parthenolide synergistically increased p21 protein levels as early as 3 hours, reaching 35 folds of control values at 6 hours of treatment (Fig. 3B and C, Supplementary Fig. S2D). In cells cotreated with TPA and parthenolide, p21 proteins remained above control levels up to 48 hours (Fig. 3C), correlating with blocked S to G2–M phases observed in parthenolide-treated TPA-promoted cells (Fig. 3A).

Conversely to p21, cyclin D1 enhances tumor promotion and is overexpressed in many cancers (19). Cyclin D1 induction is a rate-limiting event during cell-cycle entry from quiescence into the G1 phase (19). For DNA synthesis to occur during S-phase, cyclin D1 proteins should be downregulated (37). Parthenolide treatment at 10 μmol/L concentrations for 3 and 6 hours transiently decreased cyclin D1 protein levels (Fig. 3B), which was associated with the observed S-phase arrest (Fig. 3A). Cyclin D1 protein levels decreased in control cells in a time-dependent manner up to 48 hours (Fig. 3B), possibly due to increased confluency, hence, decreased number of cycling cells (Fig. 3A). In TPA-treated cells, cyclin D1 protein levels increased 2 folds of control by 24 hours, and 10 μmol/L parthenolide prevented this increase (Fig. 3C). However, 5 μmol/L parthenolide did not decrease cyclin D1 protein levels relative to control and to TPA-treated cells (Fig. 3B and C).

These results indicate that parthenolide at low 5 μmol/L concentrations decrease basal and promoter-induced NF-κB activities concomitant with increases in p21 protein levels. These properties of parthenolide could be observed in JB6P+ cells also at 10 μmol/L concentrations, up to 6 hours, at which this drug is not cytotoxic, as determined by lactate dehydrogenase release (data not shown).

Figure 3. Effect of parthenolide (PTL) on the cell-cycle regulation of JB6P + cells in the presence or absence of tumor promoters. At 40% to 50% confluency, cells were cotreated with PTL ± TPA (5 μmol/L) for the indicated time points in hours (h). A, pre-G1, G0–G1, S, and G2–M phases of the cell cycle were plotted as percentage of the total cell population and obtained by flow cytometric analyses of propidium iodide–stained DNA content and analyzed by FlowJo. Results represent the averages of 3 independent experiments. B and C, whole-cell protein lysates were immunoblotted with antibodies for p21 and cyclin D1, and reprobed with anti-GAPDH to normalize for equal loading. Densitometry values were expressed relative to starting control (0 μmol/L PTL) in each of the 3 gels shown and standardized relative to GAPDH for each condition. Results are representative of 3 independent experiments.
Parthenolide differentially modulates p65 binding and chromatin structure on p21 and cyclin D1 promoters regulating their gene expression

We further investigated whether parthenolide modulation of p21 and cyclin D1 expression correlates with this drug’s ability to regulate NF-κB DNA binding and chromatin structure in the promoter regions of these genes. The activity of the p65 NF-κB subunit is a limiting factor for NF-κB activation and transformation responses (38). Overexpression of p65 in JB6P- and HaCaT cells is sufficient for their transformation response to tumor promoters (38, 39). Moreover, p65/NF-κB induces the transcription of cyclin D1 (27) in epidermal cells.

The binding of p65 to p21 promoter and to its effect on p21 transcripts in JB6P+ cells (Fig. 4A–C). Two putative NF-κB binding regions in the p21 promoter were studied: a promoter distal R1 and a promoter proximal R2. Chromatin structure was analyzed using DiMeH3K9 and DiMeH3K4, which are markers of the transcriptionally inactive and active chromatin, respectively (40). Therefore, the ratio, DiMeH3K9/DiMeH3K4, abbreviated as DiMeH3(K9/K4), positively correlates with the transcriptionally inactive chromatin. By 6 hours, parthenolide at 10 μmol/L significantly inhibited p65 binding to R1 and R2 (Fig. 4A, P < 0.01) and decreased DiMeH3(K9/K4) indicating relaxed chromatin structure at the p21 promoter relative to control (Fig. 4B). Interestingly, p21 mRNA expression peaked at 6 hours (Fig. 4C), suggesting that induction of p21 transcription by parthenolide at 6 hours occurs when the chromatin region of the p21 promoter is relaxed relative to control, even if the binding of p65 is

![Figure 4. Parthenolide differentially modulates p65 binding and chromatin structure on p21 and cyclin D1 promoters. At 40% to 50% confluence, JB6P+ cells were treated PTL for the indicated time points in hours (h). A and B, p65 and DiMeH3[K9/K4] ChIPs on p21 promoter. Two regions in the p21 promoter region were studied: a promoter distal R1 and a promoter proximal R2. C, p21 mRNA quantitated by qRT-PCR was normalized over GAPDH mRNA. p65 (D) and DiMeH3[K9/K4] E ChIPs on cyclin D1 promoter. F, cyclin D1 mRNA quantitated by qRT-PCR was normalized over GAPDH mRNA. Values are expressed as folds of control (Ct) for each group and represent the average ± SD of triplicate wells. Threshold cycle (Ct) values of Ct qRT-PCR samples were similar, and Ct measurements were set as 1.0 at different time points. ChIP data were normalized over input DNA and presented as fold-enrichment over IgG control. Statistical significance is compared with solvent-treated controls for each group using 1- and 2-Way ANOVA. *, P < 0.05; **, P < 0.01; ***, P < 0.001.](https://cancerpreventionresearch.aacrjournals.org/content/5/11/1304)
reduced at this promoter. At 24 hours, the inhibition of p65 binding to R1 and R2 by parthenolide (Fig. 4A) was concomitant with increases in DiMeH3(K9/K4) ratios (Fig. 4B). This chromatin closure and inhibition of p65 binding correlated with decreased p21 transcription at 24 hours relative to 6 hours (Fig. 4C). These results suggest that parthenolide regulation of p21 transcription correlates with chromatin structure and with p65 binding at its promoter.

Next, the binding of p65 to cyclin D1 promoter was investigated by ChIP assays in relation to chromatin structure on this promoter and to its effects on cyclin D1 mRNA expression in JB6P+ cells (Fig. 4D–F). A conserved and defined NF-kB binding region in the cyclin D1 promoter (19) was used in ChIP assays. By 6 hours, parthenolide at 10 \( \mu \)mol/L significantly decreased p65 binding to cyclin D1 promoter (\( P < 0.05 \); Fig. 4D) and significantly decreased DiMeH3(K9/K4) ratio (\( P < 0.05 \)) indicating relaxed chromatin structure at the cyclin D1 promoter relative to control (Fig. 4E). Cyclin D1 mRNA expression decreased maximally at 6 hours by parthenolide (Fig. 4F) correlating more with reduced p65 binding than with chromatin relaxation at the cyclin D1 promoter. At 24 hours, p65 binding by parthenolide was restored to control levels (Fig. 4D), and the DiMeH3(K9/K4) increased at the cyclin D1 promoter indicating chromatin closure relative to control (Fig. 4E). Cyclin D1 transcripts significantly increased at 24 hours (\( P < 0.001 \); Fig. 4F) correlating more with p65 binding at the cyclin D1 promoter rather than with chromatin structure. These results suggest that cyclin D1 transcriptional regulation by parthenolide correlates more with p65 binding, at the cyclin D1 promoter, than with chromatin structure.

Epigenetic regulation by parthenolide seems to be specific to certain genes rather than a global chromatin effect. In fact, parthenolide treatment of JB6P+ cells did not alter global DiMeH3(K9/K4) protein level (Supplementary Fig. S3A). Furthermore, 5 \( \mu \)mol/L parthenolide did not cause major decreases in acetyl histone 3 (AcH3) and acetyl histone 4 (AcH4) protein levels normalized to their corresponding histone proteins (Supplementary Fig. S3A). This correlated with the observation that parthenolide did not cause changes in general HDAC activities (Supplementary Fig. S3B and S3C), although it caused HDAC1 cleavage (Supplementary Fig. S3D) and presumably inhibition as recently reported (41).

p21 mediates sensitivity of tumor cells to parthenolide

p21-increased expression by parthenolide was sustained and was epigenetically regulated. To investigate the role of p21 in parthenolide-mediated growth inhibition, we transiently transfected JB6P+ cells with control scrambled (Ct-siRNA) or with p21-siRNA and then treated them with parthenolide (Fig. 5A). As expected, parthenolide increased p21 protein levels at 6 hours in Ct-siRNA but not in p21-siRNA–transfected cells (Fig. 5B). Interestingly, p21-siRNA–transfected cells were more viable than Ct-siRNA counterparts upon parthenolide treatment (\( P < 0.05 \); Fig. 5A). In these transfection assays, JB6P+ cells were treated at higher confluency (60%–80% after transfection), so the 10 and 15 \( \mu \)mol/L parthenolide concentrations were less cytotoxic than in the observed MTT assays of Fig. 1, where cells were treated at 40% to 50% confluency.

To further support the role of p21 in mediating sensitivity to parthenolide, another epithelial cell line model of solid tumors was used, consisting of p21−/− knockout colon cancer HCT-116 cells, HCT-116 p21−/−, and their wild-type variants, HCT-116 Wt. MTT-based cell growth assay showed that HCT-116 p21−/− cells were significantly more resistant to parthenolide than HCT-116 Wt cells, at all tested concentrations (\( P < 0.001 \); Fig. 5C). Trypan blue viability assay showed similar trends (Fig. 5D). As expected, parthenolide was able to induce p21 protein expression in HCT-116 Wt cells as early as 6 hours (Fig. 5E). These results indicate that loss of p21 is cytoprotective against parthenolide and support our earlier finding that increased p21 protein levels by parthenolide in JB6P+ cells (Fig. 3B) correlate with cell-cycle arrest (Fig. 3A) rather than cell proliferation.

Parthenolide inhibits TPA-induced tumor growth in vivo

Parthenolide-mediated inhibition of tumor promotion prompted us to investigate whether these effects can be observed in vivo. We used the tumor promoted JB6P+ mouse xenograft model, in which the JB6P+ cells were promoted for 6 days in the presence of TPA and 10% serum and then, subcutaneously injected into NMR1-Nu immunocompromised mice. JB6P+ cells do not form tumors in vivo unless they are initially exposed to tumor promoters (42; data not shown). To our knowledge, the parthenolide dose of 0.25 mg/kg/d is the lowest concentration reported in animal models (43). Therefore, we tested whether parthenolide at this low concentration inhibits promoted JB6P+ cells from forming tumors in vivo using 2 chemoprevention protocols.

In the first method, parthenolide was administered to the mice intraperitoneally every other day over a 10-day period and stopped upon injection of tumor cells (Fig. 6A). Solvent controls, PBS, and 1% DMSO, showed similar results (\( P > 0.05 \)) and were, hence, grouped into 1 control. Parthenolide significantly inhibited tumor volume by a range of 34% to 50% relative to control (\( P < 0.001 \)) from days 31 to 34 of drug administration (Fig. 6A).

In the second protocol, parthenolide was administered to the mice intraperitoneally every other day over a 10-day period but was not stopped until the first appearance of the tumors, which was approximately on day 20 from the start of drug administration (Fig. 6B). Parthenolide significantly inhibited the tumor volumes by 63% relative to control as early as day 25 (\( P < 0.05 \)) and by a range of 51% to 58% from days 28 to 34 (\( P < 0.001 \); Fig. 6B). Therefore, parthenolide showed an earlier and stronger effect when administered for 10 additional days after tumor cell injection. There was no overall difference in mouse survival and weight, up to day 34, between control and parthenolide-treated mice in both experimental protocols (data not shown).

To investigate whether low parthenolide concentrations affect tumor cell proliferation and modulate p65, p21, and
cyclin D1 protein levels in vivo, TMA was conducted on the tumors removed from the first chemoprevention protocol (Fig. 6A and C). Parthenolide significantly reduced the scores of the cell proliferation marker Ki67 and p65, whereas increased p21 scores ($P<0.05$; Fig. 6C). Parthenolide caused nonstatistically significant decreases in cyclin D1 scores (data not shown).

**Discussion**

Tumor promotion is a rate-limiting step in tumorigenesis, NF-κB–dependent, epigenetically regulated, and reversible (1, 4). In this study, we have investigated whether the NF-κB inhibitor parthenolide attenuates tumor promotion at noncytotoxic concentrations and modulates the epigenetically regulated NF-κB target genes, p21 and cyclin D1.

Low parthenolide concentrations did not affect the basal growth of JB6P+ cells but inhibited their promotion in 2D, 3D, and animal models, highlighting that the antitumor promotion properties of parthenolide are not due to drug cytotoxicity. At low doses, parthenolide decreased in vitro and in vivo promoter-induced NF-κB activities and p65 immunostaining, respectively. Furthermore, parthenolide regulated in opposite directions p21 and cyclin D1 gene expression.
expression and suppressed the growth of tumor cells through partial p21-dependent mechanisms.

The critical role of NF-κB in skin physiology and pathology has been well studied and shows that NF-κB has antagonistic functions in the skin (39). Whereas NF-κB in normal keratinocytes reduces the propensity of malignant transformation, its activation serves as an important survival mechanism for neoplastic keratinocytes. This dual role may be because of the fact that p21, an NF-κB target gene expressed in normal but not neoplastic epidermal cells (39).

Induction of p21 transcription by parthenolide occurred when the chromatin in the vicinity of its promoter region was similar or more relaxed relative to control, even if p65 binding at this promoter was reduced. The relaxed chromatin state may allow factors, other than p65, to bind to the p21 promoter triggering its expression. Transcriptional regulation of p21 by parthenolide could be a balance between chromatin structure and other factors, including p65 that target the p21 promoter. Histone marks, such as trimethyl H3K27, which indicates repressed p21 expression (44), could also affect chromatin structure altered by parthenolide. Another epigenetic player, HDAC1, was recently shown to regulate cellular proliferation and differentiation by directly targeting the p21 gene, as shown by ChIP assays (45). Interestingly, parthenolide is the first example of small molecules that specifically inhibits HDAC1 without affecting other HDACs, possibly resulting in less deleterious effects to patients with cancer than pan-HDAC inhibitors.
Parthenolide’s effect on cyclin D1 gene was opposite to p21 causing an early decrease in cyclin D1 transcription, which correlated mostly with decreased p65 binding, at the cyclin D1 promoter, rather than with further chromatin condensation.

NF-κB activity may be induced by a variety of tumor promoters, including UVB, leading to SCC development. Parthenolide was shown to protect against UVB-induced skin cancer and photocaging by inhibiting AP-1/MAPK (46) and matrix metalloproteinase-1 (43) signaling, respectively. Parthenolide reduced papilloma multiplicity by 30% when administered as 1 mg/mouse/d by food pellets in UVB-irradiated SKH-1 hairless mice (46). In the TPA-induced xenograft tumor model that we used in NMRI-Nu mice, we have observed up to 63% inhibition of tumor volumes, relative to control, by parthenolide at lower concentrations than in the previous study (46). This increased sensitivity to parthenolide may be due to differences in the experimental protocols or may be due to parthenolide administration orally (46) versus intraperitoneally in our studies. In fact, parthenolide is not orally bioavailable (47), resulting in the recent synthesis of an orally bioavailable derivative, dimethylamino-parthenolide (48), which is currently being tested in cancer clinical trials (10). Furthermore, in the xenograft models used in this study, parthenolide may be reducing tumor volumes by modifying host cells and subsequent interactions with tumor cells.

Ideal epigenetic drugs function at low nontoxic concentrations, allowing cell division to occur in preneoplastic or tumorigenic cells so that the epigenetic effects are incorporated in the chromatin and manifested into the cell machinery (33, 49). Consistently, clinical trials have confirmed that low-dose exposure to epigenetic drugs leads to more effective responses whether administered alone or in combination treatment (50). Feverfew tea, rich in parthenolide, is commonly used against migraine and inflammation, and parthenolide derivatives are in cancer clinical trials only in hematologic malignancies and not in solid tumors, such as the skin and colon tumor models we used (10). Our study shows that low doses of parthenolide inhibit tumor promotion and epigenetically modulate p21 expression, highlighting the potential role of this drug as a chemopreventive agent and in epigenetic cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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