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Role of Rad51 Down-Regulation and Extracellular Signal-Regulated Kinases 1 and 2 Inactivation in Emodin and Mitomycin C-Induced Synergistic Cytotoxicity in Human Non–Small-Cell Lung Cancer Cells

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ABSTRACT

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) is a natural anthraquinone derivative found in the roots and rhizomes of numerous plants. It is a tyrosine kinase inhibitor and has anticancer effects on lung cancer. Rad51 plays a central role in homologous recombination, and high levels of Rad51 expression are observed in chemo- or radioresistant carcinomas. Our previous studies have shown that the mitogen-activated protein kinase kinase (MKK) 1/2-extracellular signal-regulated kinase (ERK) 1/2 signal pathway maintains the expression of Rad51. Therefore, in this study, we hypothesized that emodin could enhance the effects of the antitumor antibiotic mitomycin C (MMC)-mediated cytotoxicity by decreasing the expression of Rad51 and the phosphorylation of ERK1/2. Exposure of the human non–small-cell lung cancer H1703 or A549 cell lines to emodin decreased the MMC-elicited phosphorylated ERK1/2 and Rad51 levels. Moreover, emodin significantly decreased the MMC-elicited Rad51 mRNA and protein levels by increasing the instability of Rad51 mRNA and protein. In emodin- and MMC-cotreated cells, ERK1/2 phosphorylation was enhanced by constitutively active MKK1/2 (MKK1/2-CA), thus increasing Rad51 protein levels and protein stability. The synergistic cytotoxic effects induced by emodin combined with MMC were remarkably decreased by MKK1-CA-mediated enhancement of ERK1/2 activation. Depletion of endogenous Rad51 expression by small interfering Rad51 RNA transfection significantly enhanced MMC-induced cell death and cell growth inhibition. In contrast, overexpression of Rad51 protects lung cancer cells from the synergistic cytotoxic effects induced by emodin and MMC. We conclude that suppression of Rad51 expression or a combination of emodin with chemotherapeutic agents may be considered as potential therapeutic modalities for lung cancer.

Lung cancer is the leading cause of cancer-related death in the world and can be broadly classified into small cell lung cancer (SCLC) and non–small-cell lung cancer (NSCLC) (Bhattacharjee et al., 2001). NSCLC accounts for approximately 85% of all lung cancers (Landis et al., 1999), and unlike SCLC, NSCLC is less sensitive to chemotherapeutic agents; the average 5-year survival rates are 10 to 15% (Chang et al., 2005). Mitomycin C (MMC) is an anticancer drug that forms monoadducts and intrastrand cross-links between the N-2 guanines of the d(CpG) sequence in the minor groove of DNA (Warren and Hamilton, 1996; Dronkert and Kanaar, 2001). MMC is typically used as a first- or second-line regimen to treat NSCLC and is often combined with other chemotherapeutic agents during advanced NSCLC treatment (Babiak et al., 2007). The therapeutic value of MMC depends on the ability of cells to remove DNA damage (McHugh et al., 2001).

Mammalian Rad51, a key protein in the homologous recombination repair (HRR) pathway, is implicated in the re-

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ABBREVIATIONS: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; MKK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; MMC, mitomycin C; MMK1/2-CA, constitutively active mitogen-activated protein kinase kinase 1/2; HRR, homologous recombination repair; CK2, casein kinase 2; ALLN, *N*-acetyl-Leu-Leu-norleucinal; DMSO, dimethyl sulfoxide; si, small interfering; siRNA, small interfering RNA; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; CI, combination index; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; SN-38, 7-ethyl-10-hydroxycamptothecin; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal.

pair of DNA double-strand breaks that may arise at stalled DNA replication forks or DNA cross-links that are caused by agents such as MMC (Haaf et al., 1995; Daboussi et al., 2002). Rad51 has ATPase capable of forming a right-handed helical nucleoprotein filament on single-stranded DNA (Sung and Robberson, 1995) and can then undergo strand transfer and heteroduplex DNA formation (Sung, 1994). Rad51 knockout in mice results in early embryonic lethality (Lim and Hasty, 1996; Tsuzuki et al., 1996).

High levels of Rad51 expression are observed in a variety of tumor cell lines (Richardson, 2005) and are associated with a poor prognostic outcome in lung cancer (Qiao et al., 2005). Elevated Rad51 levels promote instability of the genome (Richardson et al., 2004) and can enhance the frequency of spontaneous recombination and increase the resistance to chemotherapeutic agents and ionizing radiation in mammalian cells (Vispé et al., 1998). Disruption of Rad51 function in the Chinese hamster ovary cell line also promotes genome instability and tumor formation in nude mice (Bertrand et al., 2003), whereas loss of Rad51 function in the chicken DT40 cell line induces chromosome breaks before cell death (Richardson, 2005).

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an anthraquinone derivative from the rhizome of *Rheum palmatum*, a herb widely used in traditional Chinese medicine (Shi et al., 2001). Emodin induces apoptosis and growth arrest in various cancer cells in humans, such as those of the lung (Su et al., 2005), breast (Zhang et al., 1998, 1999), cervix, bone marrow, liver, and prostate (Chen et al., 2002; Shieh et al., 2004; Wang et al., 2007a). Because of its antiproliferative activity, emodin is a potent tyrosine kinase inhibitor of HER-2/neu tyrosine kinase (Zhang et al., 1998, 1999), casein kinase 2 (CK2) (Yim et al., 1999), p56lck (Zhang et al., 1995), epidermal growth factor receptor tyrosine kinase (Zhang et al., 1999), and p38 mitogen-activated protein kinase (Kwak et al., 2006; Wang et al., 2007b). In addition, emodin antagonizes AKT and ERK1/2 signaling and triggers apoptosis in human lung carcinoma cells (Zhang et al., 1999; Su et al., 2005). However, there is no information to address the function of emodin in the MMC-induced cytotoxicity of human NSCLC cells.

In this study, we investigated the role of emodin in suppressing MMC-induced cell viability in two NSCLC cell lines and examined the possible underlying molecular mechanisms. Moreover, we examined the role of Rad51 in the resistance to chemotherapeutic agents and emodin in lung cancer therapy. Our study shows that emodin has a synergistic effect on MMC-induced cytotoxicity via decreasing the MMC-induced expression of Rad51 and the phosphorylation of ERK1/2. Understanding the combined effects of MMC and emodin on NSCLC may further help to improve therapeutic modalities for advanced lung cancer, especially for patients in whom lung cancer cells are resistant to chemotherapeutic agents.

Materials and Methods

Drugs and Reagents. Emodin was purchased from Sigma-Aldrich (St. Louis, MO). MMC was obtained from Bristol-Myers Squibb Co. (Stamford, CT). Cycloheximide and actinomycin D were purchased from Sigma-Aldrich. *N*-Acetyl-Leu-Leu-norleucinal (ALLN), MG132, and U0126 were purchased from Calbiochem-Novabiochem (San Diego, CA). Emodin, actinomycin D, ALLN, MG132, and U0126 were dissolved in dimethyl sulfoxide (DMSO). Cycloheximide and MMC were dissolved in Milli-Q-purified water (Millipore, Billerica, MA).

The specific phospho-ERK1/2 (Thr^{202}/Tyr^{204}) antibody was purchased from Cell Signaling Technology, Inc (Danvers, MA). Rabbit polyclonal antibodies against Rad51(His92), ERK2(Lys23), hemagglutinin(Phe7), and actin(Ile19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines and Culture. Human lung squamous cell carcinoma H1703 (CRL-5889) and A549 (CCL-185) cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 complete medium supplemented with sodium bicarbonate [2.2% (w/v)], L-glutamine [0.03% (w/v)], penicillin (100 U/ml), streptomycin (100 μg/ml), and fetal calf serum (10%).

Western Blot Analysis. After the different treatments, the cells were rinsed twice with ice-cold phosphate-buffered saline and lysed in whole-cell extraction buffer [20 mM HEPES, pH 7.6, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na_3VO_4, 50 mM NaF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride]. Equal amounts of protein from each set of experiments were subjected to Western blot analysis as described previously (Ko et al., 2008a). The relative protein blot intensities were determined using a computing densitometer equipped with the ImageQuant analysis program (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The relative fold under each blot was calculated by averaging the results of three independent experiments and was normalized by arbitrarily setting the densitometry of control cells to 1.

Combination Index Analysis. The cytotoxicity induced by the combined treatment with emodin and MMC was compared with the cytotoxicity induced by each drug using the combination index (CI), where $CI < 0.9$, $CI = 0.9$ to 1.1, and $CI > 1.1$ indicate synergistic, additive, and antagonistic effects, respectively (Peters et al., 2000). The combination index analysis was performed using CalcuSyn software (Biosoft, Oxford, UK). The CI values at a fraction affected of 0.5, 0.75, and 0.9 were averaged for each experiment, and the values were used to calculate the mean between the three independent experiments.

Transfection with Small Interfering RNA. The sense-strand sequences of small interfering RNA (siRNA) duplexes used for Rad51 and the scrambled sequences (as a control) were 5′-UGUAGCAUAUGCUCGAGCG-3′ and 5′-GCGCGCUUUGUAGGATTCG-3′ (Dharmacon RNA Technologies, Lafayette, CO), respectively. H1703 and A549 cells were transfected with siRNA duplexes (200 nM) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 24 h.

Transfection of MKK1/2-CA Plasmids into NSCLC Cells. Plasmid transfection of MKK1-CA (a constitutively active form of MKK1, ΔN3/S218E/S222D) and MKK2-CA (a constitutively active form of MKK2, ΔN4/S222E/S226D) was achieved as described previously (Ko et al., 2008b). Exponentially growing human lung cancer cells (10^6) were plated for 18 h before exposure to emodin and MMC for 24 h in RPMI 1640 complete medium. To determine the effects of MKK1/2-ERK1/2 signaling on Rad51 expression, MKK1/2-CA expression vectors were transfected into H1703 and A549 cells using Lipofectamine (Invitrogen) before combined treatment with emodin and MMC.

Reverse Transcriptase-Polymerase Chain Reaction. RNA was isolated from cultured cells using TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with 2 μg of total RNA using random hexamers following the Moloney murine leukemia virus reverse transcriptase cDNA synthesis system (Invitrogen). The final cDNA was used for subsequent polymerase chain reactions. Rad51 was amplified by using the primers 5′-CTTTGGCCCACAACCCATTTC-3′ (forward) and 5′-ATGGCCTTTCCTTCACCTCCAC-3′ (reverse) in conjunction with a thermal cycling program consisting of 26 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 60 s. Glycer-

aldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. The GAPDH primers were 5′-CTACATGGTTTACATGTTCC-3′ (forward) and 5′-GTGAGCTTCCCGTTCAGCTCA-3′ (reverse). The samples were analyzed in triplicate, and the levels were normalized to those of GAPDH in each sample.

Cell Viability Assays. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma-Aldrich) assays were used to evaluate the inhibitory effects of emodin and MMC on cell viability as described previously (Ko et al., 2008a). In brief, cells were seeded on 96-well plates with RPMI containing 10% fetal calf serum in a final volume of 0.2 ml, incubated for 18 h, and then treated with the drugs for 24 h. After drug treatment, the MTT solution was added to each well and incubated for 3 h before the medium was removed. DMSO was then added and the plates were shaken for 15 min at room temperature. Cell viability was determined by measuring the absorbance at 562 nm in a microplate reader (Bio-Rad Technologies, Hercules, CA).

Trypan Blue Dye Exclusion Assay. Cells were treated with emodin and/or MMC for 24 h. In each preparation, the cell viability of H1703 and A549 cells was determined by the trypan blue dye exclusion assay; this dye excluded living cells and only penetrated dead cells. The proportion of dead cells was determined by using a hemocytometer (Chang Bioscience Inc., Castro Valley, CA) to count the number of stained cells.

Statistical Analysis. For each protocol, three or four independent experiments were performed. Results were expressed as the mean ± S.E.M. Statistical calculations were performed by using SigmaPlot 2000 (Systat Software, San Jose, CA). Differences in measured variables between experimental and control groups were assessed by the unpaired t test. $P < 0.05$ was considered statistically significant.

Results

Emodin Decreases the Levels of Phosphorylated ERK1/2 and Rad51 Induced by MMC. Human NSCLC cell lines, H1703 and A549, were exposed to various concentrations of emodin and MMC for 24 h. Phosphorylation of ERK1/2 was determined by Western blot analysis using antibodies specific to phospho-ERK1/2. Exposure to MMC increased the levels of phosphorylated ERK1/2 in a dose-dependent manner. In contrast, emodin treatment decreased the basal levels of phospho-ERK1/2 and decreased MMC-elicited phosphorylation of ERK1/2 (Fig. 1, A and B), whereas there was no change in the endogenous protein levels of ERK1/2. The protein level of Rad51 was up-regulated in MMC-treated H1703 and A549 cells (Fig. 1, A and B). However, emodin decreased MMC-induced Rad51 protein expression (Fig. 1).

Influence of Emodin and MMC on Cellular Rad51 mRNA Expression. To elucidate whether the observed MMC stimulation of Rad51 protein expression occurred at the transcriptional level, various concentrations of MMC were added to H1703 and A549 cells for 24 h. Total RNA was isolated and subjected to RT-PCR analysis for Rad51. MMC up-regulated Rad51 mRNA levels in H1703 and A549 cells (Fig. 2A). In contrast, Rad51 mRNA was gradually down-regulated by various concentrations of emodin (Fig. 2B). It is interesting that emodin (60 μM) in combination with MMC (1 μg/ml) produced a lower expression level of Rad51 mRNA in comparison with untreated H1703 or A549 cells (Fig. 2B). To examine whether emodin could decrease the stability of Rad51 mRNA, H1703 and A549 cells were treated with actinomycin D (an inhibitor of RNA synthesis) and emodin/MMC for 3 to 9 h. Figure 2C shows that the mRNA stability of Rad51 was not affected after treatment with MMC. However, emodin increased the instability of Rad51 mRNA in these two NSCLC cell lines treated with MMC.

Influence of Emodin Treatment on MMC-Enhanced Rad51 Protein Stability. To investigate whether the changes in the expression of Rad51 protein by treatment with emodin in combination with MMC was also regulated at the post-translational level, cycloheximide (an inhibitor of de novo protein synthesis) was added with MMC for 3 to 9 h. Rad51 protein levels were progressively reduced over time in the presence of cycloheximide. MMC treatment significantly prevented Rad51 degradation after cycloheximide treatment compared with DMSO-treated cells (Fig. 3A). Therefore, Rad51 was more stable after MMC treatment. However, in H1703 cells, in the presence of cycloheximide for 9 h, approximately 65% of Rad51 remained in cells treated with MMC, whereas only 25% of Rad51 remained in cells treated with MMC combined with emodin (Fig. 3B), indicating that Rad51 was less stable after cotreatment with emodin and MMC compared with MMC alone. Taken together, these results suggest that the down-regulation of Rad51 protein expression levels by cotreatment with emodin and MMC was caused by the decrease in its protein and mRNA stability.

Association of Rad51 Protein Instability with 26S Proteasome-Mediated Proteolysis of Rad51 in Emodin- and MMC-Cotreated NSCLC Cells. To investigate the role of the proteasome-mediated degradation of Rad51 induced by emodin and MMC, the 26S proteasome inhibitors MG132 and ALLN were added for the final 6 h before harvesting in emodin- and MMC-cotreated H1703 and A549 cells. As shown in Fig. 3C, both MG132 and ALLN restored the decreased Rad51 protein levels induced by cotreatment with emodin and MMC. These data suggest that the emodin-enhanced Rad51 protein instability in cells treated with MMC was via a 26S proteasome-mediated proteolysis mechanism.

Influence of ERK1/2 Activation on the Regulation of Rad51 Protein Expression and Stability. To determine whether the ERK1/2 signaling pathway was involved in the regulation of Rad51 protein expression, H1703 and A549 cells were transiently transfected with a plasmid carrying MKK1-CA or MKK2-CA, which are constitutively active forms of MKK1 or MKK2, respectively. Consistent with our previous studies, transfection of MKK1-CA or MKK2-CA vectors increased the phosphorylation of ERK1/2 and protein expression levels of Rad51 (Fig. 4A). In addition, expression of MKK1/2-CA rescued the decrease of Rad51 protein levels and ERK1/2 phosphorylation in cells cotreated with emodin and MMC (Fig. 4A). Furthermore, we investigated the effects of the MKK1-ERK1/2 signaling pathway on the stability of Rad51 by cycloheximide chase analysis. Transfection with the MKK1-CA vector increased Rad51 protein stability in H1703 and A549 cells cotreated with emodin and MMC compared with the protein stability in pcDNA3-transfected control cells (Fig. 4B), consistent with a role for the ERK1/2 signaling pathway as the upstream signal for the regulation of Rad51 protein stability. Furthermore, H1703 and A549 cells were treated with various concentrations of MMC and the MKK1/2 inhibitor U0126 (5 μM) for 24 h. As shown in Fig. 4C, MMC-induced ERK1/2 phosphorylation and Rad51 protein levels were significantly decreased after treatment with U0126. In addition, the stability of the Rad51 protein in U0126- and MMC-treated cells were analyzed by cycloheximide chase analysis. As shown in Fig. 4D, U0126

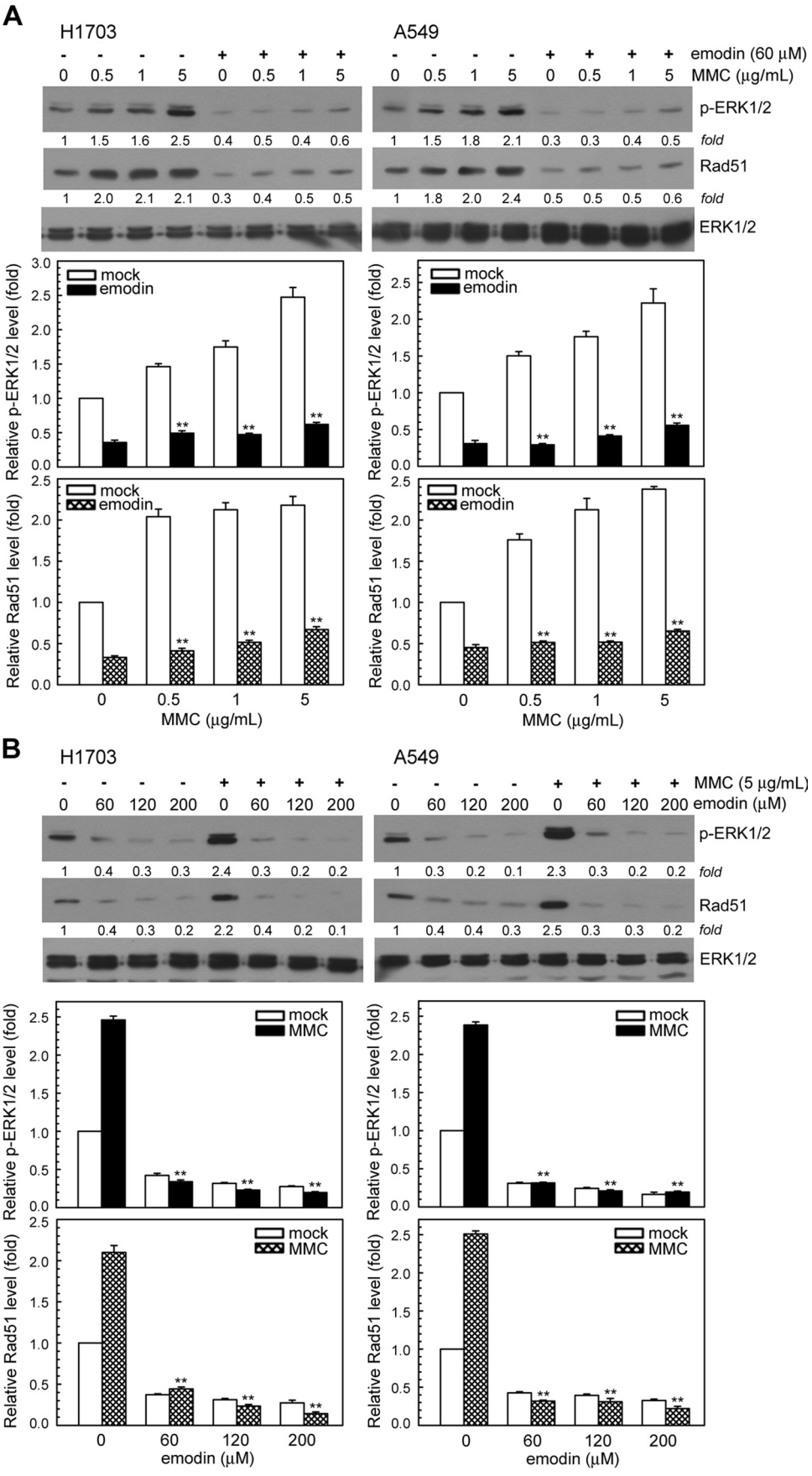

Fig. 1. Emodin decreases basal or MMC-induced phospho-ERK1/2 and Rad51 protein levels in human lung cancer cells. A, H1703 and A549 cells (10^6) were cultured in complete medium for 18 h and then exposed to MMC (0.5–5 μg/ml) and emodin (60 μM) for 24 h in complete medium. B, cells were exposed to various concentrations of emodin (60–200 μM) and MMC (5 μg/ml) for 24 h. After treatment, cell extracts were examined by Western blot for the determination of phospho-ERK1/2, Rad51, and ERK1/2 protein expression levels. Relative fold under each blot was calculated by averaging the results of three independent experiments and was normalized by arbitrarily setting the densitometry of control cells to 1. The mean ± S.E. from four independent experiments is shown. **, $P < 0.01$, using the Student's t test for the comparison between cells treated with emodin/MMC alone and those treated with a combination of MMC and emodin.

treatment markedly decreased the protein stability of Rad51 induced by MMC.

Emodin Potentiates Growth Inhibition Induced by MMC in NSCLC Cells. We investigated the combined effects of emodin and MMC on cell viability by the MTT assay. For these studies, cells were treated with emodin (60–200 μM), MMC (0.5–5 μg/ml), or a combination of the two drugs for 24 h. Viable cells were evaluated by the MTT and trypan blue exclusion assays. We found that treatment with emodin plus MMC for 24 h resulted in a greater loss of cell viability

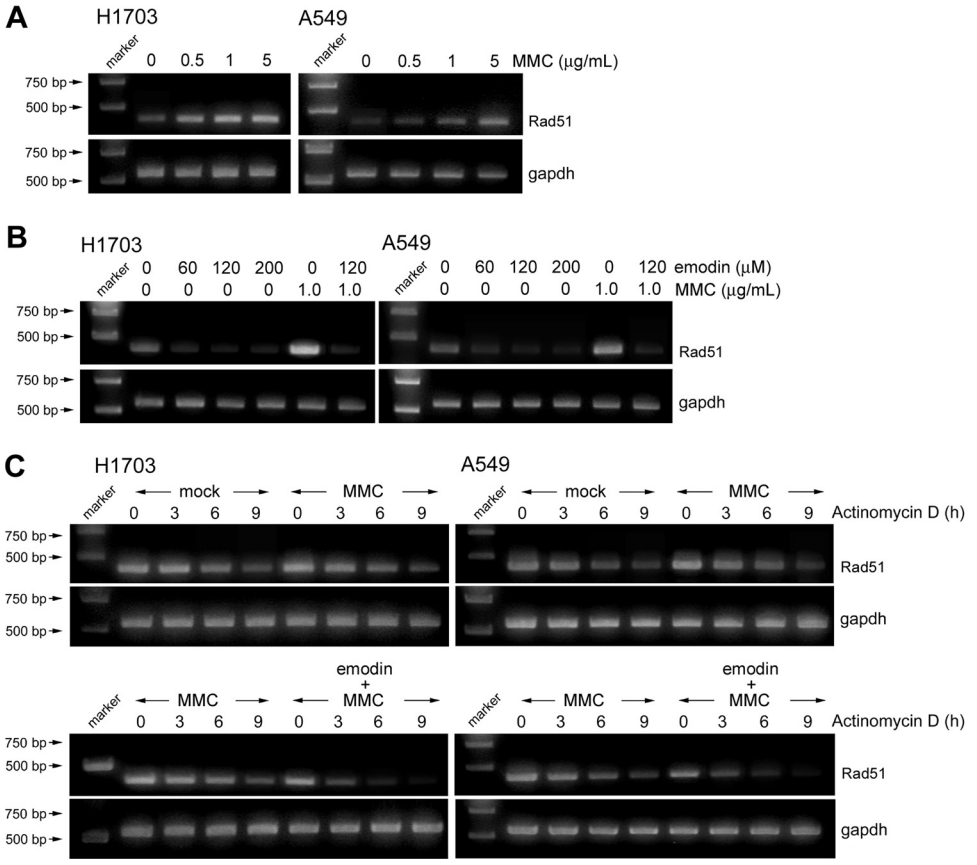

Fig. 2. Emodin decreases Rad51 mRNA in MMC-treated H1703 and A549 cells. A and B, H1703 and A549 cells (10^6) were cultured in complete medium for 18 h and then treated with MMC (0.5–5 μg/ml) and/or emodin (60–120 μM) for 24 h. C, cells were exposed to MMC (1 μg/ml) alone or to MMC in combination with emodin (120 μM) for 9 h, followed by the addition of actinomycin D (2 μg/ml) for 3 to 9 h. After treatment, total RNA was isolated and subjected to RT-PCR for Rad51.

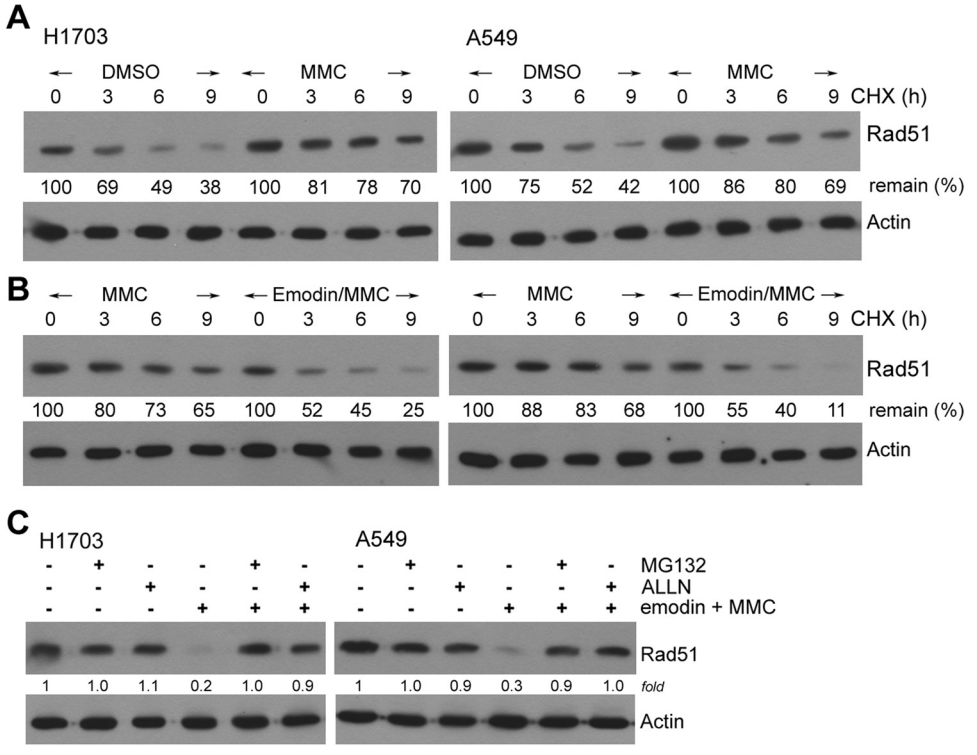

Fig. 3. Emodin decreases Rad51 protein stability in MMC-treated H1703 and A549 cells. A and B, H1703 and A549 cells were treated with MMC (1 μg/ml) and/or emodin (60 μM) for 9 h followed by treatment with cycloheximide (CHX, 60 μg/ml) for 3 to 9 h. C, emodin and MMC cotreatment triggers 26S proteasome-mediated proteolysis of Rad51. Emodin (60 μM) and MMC (0.5 μg/ml) were added to H1703 and A549 cells for 18 h. Cells were then treated with MG132 (10 μM) or ALLN (10 μM) for 6 h. Whole-cell extracts were collected for Western blot analysis.

than either emodin or MMC alone in H1703 and A549 cells (Fig. 5A). The combined effects of the drugs were analyzed as described under *Materials and Methods*. CI values significantly less than 1 indicate synergy; values close to 1 indicate an additive effect; and values significantly greater than 1 indicate an antagonistic effect of the two agents. As seen in Fig. 5B, MMC and emodin synergistically inhibited cell viability of H1703 and A549 cells; for H1703 and A549 cells, the CI values were 0.05 and 0.40, respectively. Moreover, assessment of cell death using the trypan blue exclusion assay revealed that emodin in combination with MMC produced synergistic cell death effects (Fig. 5C). In Fig. 5D, it is shown that MMC could significantly suppress lung cancer cell growth in H1703 and A549 cells and that emodin could enhance this growth suppression. In summary, synergism with the combined treatment of MMC and emodin was observed in lung cancer cell lines, which showed sensitivity to both drugs as individual agents.

Knockdown of Rad51 Sensitizes NSCLC Cells to Cytotoxicity Induced by MMC and Emodin. To verify the involvement of Rad51 in the cytotoxicity induced by emodin and/or MMC, protein expression of Rad51 was knocked down using specific siRNA duplexes. As shown in Fig. 6A, Rad51 siRNA inhibited Rad51 levels (a 70–80% reduction based on densitometry), and MMC-induced Rad51 levels in H1703 and A549 cells. It is interesting that knockdown of Rad51 expression had no effect on the activation of ERK1/2, implying that ERK1/2 may be the upstream signal for inducing Rad51 expression after MMC exposure. Suppression of Rad51 protein expression by siRad51 RNA resulted in the significant enhancement of H1703 and A549 cytotoxicity caused by emodin and/or MMC compared with si-control transfected cells (Fig. 6B, black and white bars, respectively). Cell growth was determined by trypan blue exclusion. Figure 6C shows that the knockdown Rad51 expression enhances MMC-induced cell growth arrest.

Emodin and MMC-Induced Synergistic Cytotoxic Effects Were Abrogated in Lung Cancer Cells with Rad51 Overexpression. To strengthen the role of Rad51 on protection from cytotoxicity induced by MMC and emodin, a

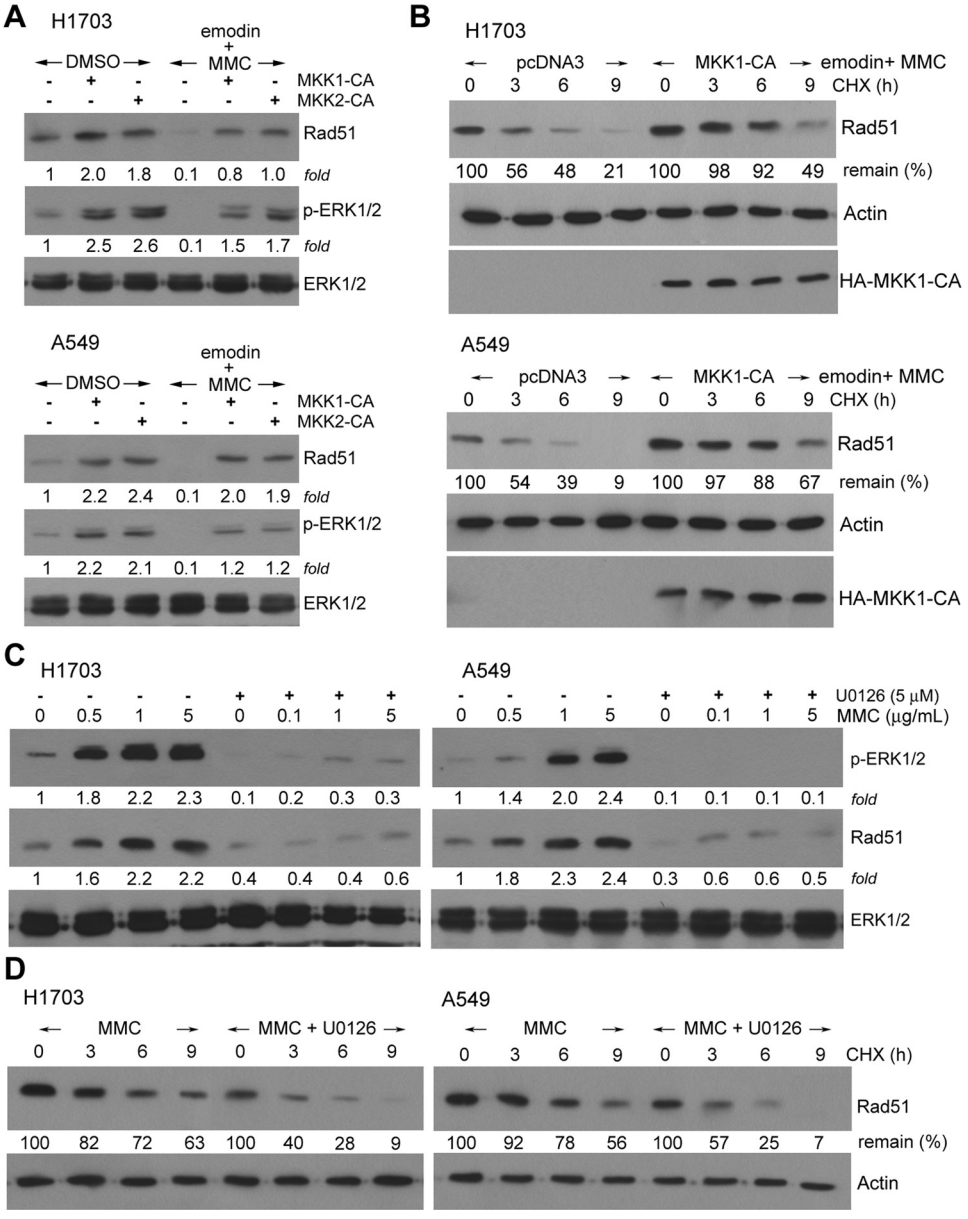

Fig. 4. Overexpression of MKK1/2-CA restores emodin-suppressed ERK1/2 activation, Rad51 expression, and Rad51 protein stability induced by MMC. A, MKK1-CA expression vectors were transfected into H1703 and A549 cells using Lipofectamine. After expression for 24 h, the cells were treated with emodin (60 μM) and MMC (1 μg/ml) as described in Fig. 1. B, MKK1-CA expression vectors were transfected into H1703 and A549 cells using Lipofectamine. After expression for 24 h, the cells were treated with emodin (60 μM) and MMC (1 μg/ml) for 9 h, followed by the addition of cycloheximide (60 μg/ml) for 3 to 9 h. C, H1703 and A549 cells were cotreated with various concentration of MMC and U0126 (5 μM) for 24 h. Western blot analysis was performed using specific antibodies against Rad51, phospho-ERK1/2, and ERK1/2. D, cells were treated with MMC (1 μg/ml) and/or U0126 (5 μM) for 9 h, followed by the addition of cycloheximide (60 μg/ml) for 3 to 9 h. Western blot analysis was performed using specific antibodies against Rad51.

Flag-Rad51 vector was transfected into H1703 and A549 cells. As shown in Fig. 6, D and E, transfection of the Flag-Rad51 vector did not affect ERK1/2 inactivation by emodin or the increase in ERK1/2 phosphorylation by MMC but prevented the cell death induced by emodin alone or by emodin in combination with MMC in H1703 and A549 cells.

Influence of the MKK1/2-ERK1/2 Signal Pathway on Cytotoxicity Induced by Emodin and MMC Cotreatment. To evaluate the effects of ERK1/2 on the synergistic cytotoxicity induced by emodin-MMC cotreatment, H1703 and A549 cells were transfected with the MKK1/2-CA vectors followed by treatment with emodin and MMC before assessment by the MTT assay. Transfection with the MKK1/2-CA vectors enhanced cell survival and ERK1/2 phosphorylation that was suppressed previously by cotreatment with emodin-MMC (Figs. 4A and 7A). On the other hand, U0126 (an MKK1/2 inhibitor) markedly enhanced the cytotoxicity of H1703 and A549 cells treated with MMC (Fig. 7B). These results were consistent with the synergistic cytotoxic effects of emodin and the chemotherapeutic agents resulting from the suppression of ERK1/2 activation.

Discussion

In this study, emodin combined with MMC exhibited a more synergistic cytotoxic effect on NSCLC cells, which was correlated with the down-regulation of Rad51 and the inactivation of ERK1/2. To our knowledge, our study is the first to demonstrate that emodin decreases the MMC-up-regulated expression of Rad51 in NSCLC. Rad51 plays a protective role in emodin and MMC-induced synergistic cytotoxicity.

The mammalian Rad51 protein is a key recombination protein that promotes the pairing and exchange of strands between homologous DNA molecules during HRR (Richardson, 2005). It functions as a helical nucleoprotein filament, which constitutes the core of the HRR reaction (West, 2003). Rad51 interacts with many accessory proteins and is involved in the repair of DNA cross-links that are caused by agents such as MMC. Knockdown of Pir51, a protein that interacts with Rad51, significantly increased the sensitivity of the HeLa cell line to MMC (Henson et al., 2006). A defect in Rad51C, a Rad51 paralog, also resulted in an increase of spontaneous and MMC-induced cytotoxicity and chromosomal aberrations (Godthelp et al., 2002). Moreover, treat-

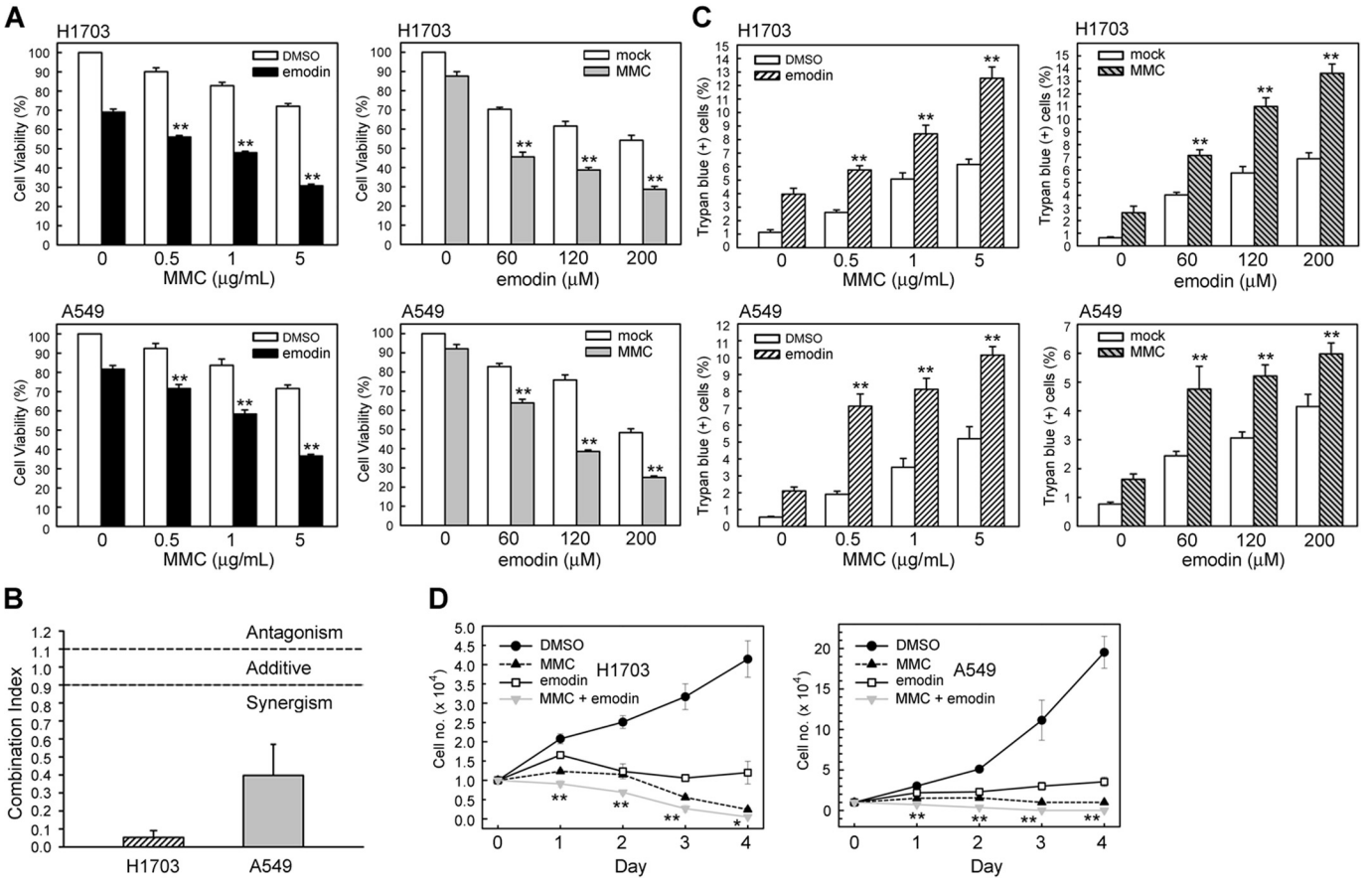

Fig. 5. Emodin cotreated with MMC synergistically enhances cytotoxicity. A, left, H1703 or A549 cells were treated with various concentrations of MMC (0.5–5 μg/ml) and emodin (60 μM) for 24 h. Right, cells were treated with emodin (60–200 μM) and MMC (0.5 μg/ml) for 24 h. Cytotoxicity was determined by the MTT assay. B, the mean CI values for the emodin-MMC combination treatment in H1703 and A549 cells. CI values were averaged for each experiment, and the values were used to calculate the mean between experiments, as described under *Materials and Methods*. Points and columns, mean values obtained from four independent experiments; bars, S.E. C, at the end of treatment as in A, unattached and attached cells were collected and stained with trypan blue dye. The numbers of stained cells (dead) were manually counted. Columns, percentage of trypan blue-positive cells, representing the population of dead cells; bar, S.E. from three independent experiments. *, $P < 0.05$, and **, $P < 0.01$ using the Student's t test for the comparison between cells treated with emodin/MMC alone and cells cotreated with MMC-emodin. D, cells were treated with MMC (2 μg/ml) and/or emodin (60 μM) for 1 to 4 days, after which the number of surviving cells was counted after staining with trypan blue (in triplicate). **, $P <$ 0.01 using the Student's t test for comparison between cells treated with emodin or MMC alone and those cotreated with emodin-MMC.

ment with caffeine, a protein kinase inhibitor, decreased the characteristic association of Rad51 with chromatin and the nuclear matrix caused by MMC (Mladenov et al., 2006). In this study, down-regulation of Rad51 enhanced emodin and/or MMC-induced cytotoxicity in human lung cancer cells; in contrast, overexpression of Rad51 rescued the cytotoxic effect. Therefore, Rad51 plays a protective role in emodin- and/or MMC-induced cytotoxicity.

MMC is an antibiotic produced by *Streptomyces caespitosus* and has both antiproliferative and antimetabolic properties. It functions as an alkylating agent that causes cross-linking of DNA and inhibits the synthesis of RNA and

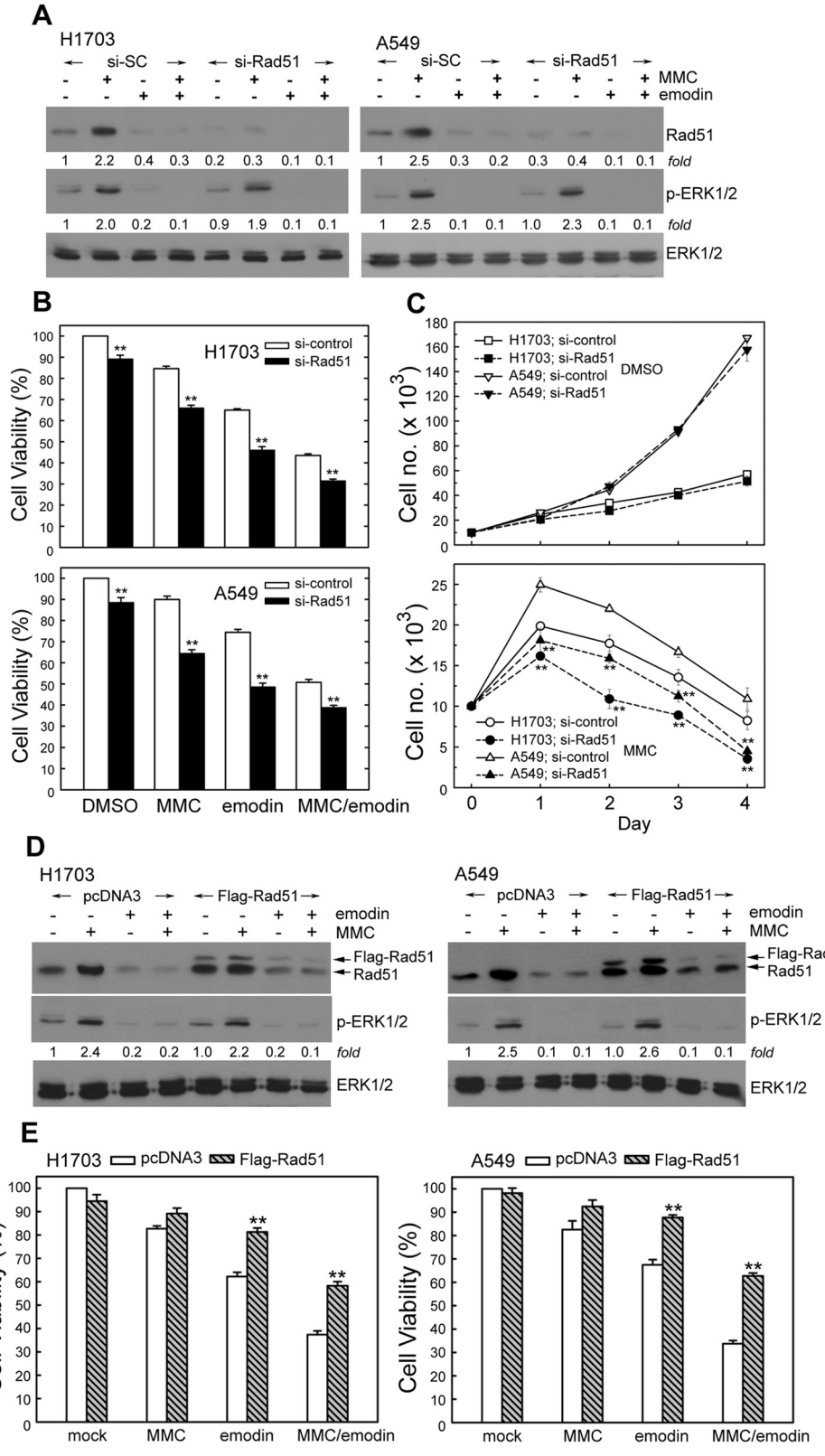

Fig. 6. Knockdown of Rad51 expression by siRNA transfection enhances cell death induced by MMC and/or emodin. A, H1703 and A549 cells were transfected with siRNA duplexes (200 nM) specific to Rad51 or scrambled control in complete medium for 24 h before treatment with MMC (0.5 μg/ml) and/or emodin (60 μM) in complete medium for 24 h. Whole-cell extracts were collected for Western blot analysis using specific antibodies against Rad51, phospho-ERK1/2, and ERK1/2. B, after treatment, cytotoxicity was determined by the MTT assay. C, after cells were transfected with si-Rad51 or si-scrambled RNA, the cells (10^4 cells/dish) were seeded onto 60-mm dishes. After 18 h, the cells were treated with or without MMC (0.1 μg/ml) for 1 to 4 days. The surviving cells were counted after staining with trypan blue for each of these days in triplicate. The results (mean ± S.E.M.) were from four independent experiments. **, $P < 0.01$ using the Student's t test for the comparison between cells treated with MMC and/or emodin in si-Rad51 RNA or si-scrambled RNA transfected cells. D, H1703 and A549 cells were transfected with the Flag-Rad51 vector for 24 h, and the cells were then treated with MMC (1 μg/ml) and/or emodin (120 μM) for 24 h. Whole-cell extracts were collected for Western blot analysis using specific antibodies against Rad51. E, after treatment as in D, the cytotoxicity was determined with use of the MTT assay.

protein. Treatment with MMC arrested the cells in the S-phase and induced the appearance of Rad51 nuclear foci. However, these effects were abrogated by caffeine, which inhibits ataxia telangiectasia mutated and ataxia telangiectasia mutated- and Rad3-related kinases (Mladenov et al., 2007). The increased expression of transcripts encoding DNA repair-associated proteins was also evident in Fanconi anemia cells after treatment with MMC, including the BLM helicase and the nucleotide excision repair proteins XPC and DDB2 (Martinez et al., 2008). Our studies have shown that MMC enhances the protein expression levels and stability of Rad51 in an MKK-ERK activation-dependent manner. The detailed mechanism of how MKK-ERK controls the post-translational modification of Rad51 protein expression in NSCLC cell lines is currently under investigation.

Emodin, an active constituent isolated from the root of *R. palmatum L.*, is used for its antitumor, antibacterial, immunosuppressive, hepatoprotective, antiviral, antiulcerogenic, and chemopreventive activities (Huang et al., 1991, 1992; Lin et al., 1996; Wang and Chung, 1997; Kuo et al., 2001; Hsiang and Ho, 2008). Moreover, emodin inhibits cell growth in several types of tumor cells, (Zhang et al., 1998; Chen et al., 2002; Jing et al., 2002; Srinivas et al., 2003), and it regulates genes related to the control of cell proliferation, cell apoptosis, oncogenesis, and cancer cell invasion and metastasis (Cha et al., 2005; Kim et al., 2005; Huang et al., 2006; Kwak et al., 2006). Our previous studies indicated that emodin decreases the protein and mRNA expression levels of excision repair cross-complementing 1 gene and Rad51 by increasing the protein and mRNA instability in human lung cancer cells (Ko et al., 2009). It is noteworthy that excision repair cross-complementing 1 gene and Rad51 can protect NSCLC cells from cytotoxicity induced by emodin. Consistent with this study, overexpression of Rad51 could rescue the cell viability that was decreased by emodin alone or was cotreated with MMC in NSCLC cells.

It has been shown that Rad51 also plays a vital role in the resistance to SN-38 (the active metabolite of irinotecan) and that flavopiridol, a synthetic flavone, induces a p53-dependent suppression of Rad51 that results in the induction of apoptosis (Ambrosini et al., 2008). In this study, we used two NSCLC cell lines, A549 and H1703, to examine the role of Rad51 in regulating the MMC and emodin-induced synergistic cytotoxicity. A549 cell line has wild-type p53, but H1703 has mutational inactivation of p53 (Brognard and Dennis, 2002). However, in both of these two lung cancer cell lines, emodin decreased the MMC-elicited phosphorylated ERK1/2 and Rad51 levels. Down-regulation of Rad51 enhanced emodin or/and MMC-induced cytotoxicity; in contrast, overexpression of Rad51 rescued the cytotoxic effect induced by emodin and MMC in both cell lines. In the case of flavopiridol, flavopiridol could differentially regulate Rad51 depending on the p53 status of the cells. The suppression of Rad51 with SN-38 and flavopiridol was observed in the cell lines that are wild-type for p53 but not in the cells that are mutant for p53 (Ambrosini et al., 2008). Therefore, we suggest that the combination effect of MMC and emodin in these two cell lines, H1703 and A549, may not be associated with the p53 status of the cells.

Emodin affected the expression of genes involved in transcription, apoptosis, tumor metastasis, and chemotherapy resistance in small cell lung cancer NCI-H446 cells (Fu et al., 2007). For example, the histone deacetylase 1 was down-regulated by emodin. The dynamic balance between histone deacetylation and acetylation plays a significant role in the regulation of genes transcription. On the other hand, emodin has been shown to inhibit CK2 activity (Yim et al., 1999). Previous studies indicated that CK2 targets several proteins that involve in the basal class II transcriptional machinery, including TATA-binding protein (Maldonado and Allende, 1999) and TFIIF (Rossignol et al., 1999). In this study, emodin could suppress endogenous and MMC-induced Rad51 mRNA levels. According to these finding, we suggest that emodin may regulate the Rad51 transcription through CK2

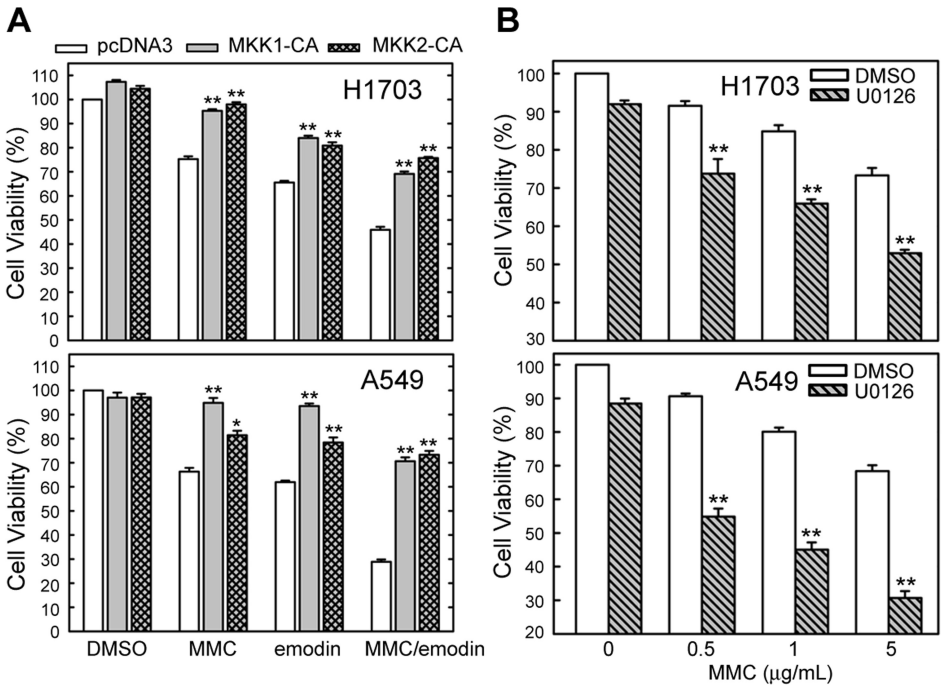

Fig. 7. The influence of the MKK1/2-ERK1/2 signaling pathway on cell viability in H1703 and A549 cells treated with MMC and/or emodin. A, overexpression of MKK1-CA rescue the levels of cytotoxicity induced by MMC and emodin. Cells were transfected with MKK1-CA vectors for 24 h. MMC (5 μg/ml) and/or emodin (120 μM) were then added, and the cells were incubated for 24 h. The cytotoxicity affected by MKK1-CA vector transfection was determined by the MTT assay. The results (mean ± S.E.M.) were obtained from four independent experiments. *, $P < 0.05$, and **, $P < 0.01$ using the Student's t test for the comparisons between cells transfected with the MKK1-CA or pcDNA3 vectors. B, H1703 and A549 cells were exposed to U0126 (5 μM), an MKK1/2 inhibitor, and MMC (0.5–5 μg/ml) for 24 h. After treatment, cell viability was determined by the MTT assay. **, $P < 0.01$ using the Student's t test for comparisons between cells treated with MMC/DMSO alone or with U0126 cotreatment.

inhibition. However, the detailed mechanism of emodin controlling the Rad51 gene expression and how the CK2 is involved in this regulation in NSCLC cell lines will be included in our next investigation.

Emodin blocks the phosphorylation of HER2/neu and ERK1/2 in the prostate cancer PC3 cell line (Zhou et al., 2006) and enhances the sensitivity of cancer cells to chemotherapeutic agents (Yi et al., 2004). In this study, emodin decreased the cellular and MMC-induced levels of phospho-ERK1/2 in lung cancer cells. In addition, the inactivation of ERK1/2 is required for MMC and emodin-induced cytotoxicity. Previous studies have shown that emodin is a strong reactive oxygen species-producing agent (Jing et al., 2006) and a genotoxic compound that is able to induce DNA damage (Wang et al., 2006). Recently, studies have shown that emodin potentiates the antitumor effects of gemcitabine in vivo by down-regulating the expression of survivin and β-catenin in pancreatic cancer cell lines (Guo et al., 2009). Moreover, cotreatment with emodin and cisplatin remarkably elevates the levels of reactive oxygen species and enhances the chemosensitivity of DU-145 cells, a multidrug-resistant prostate carcinoma cell line, compared with cisplatin-only treatment, but exerts little effect on nontumor cells (Huang et al., 2008). Emodin could suppress high glucose-induced cell proliferation in rat mesangial cells by inhibiting the p38 mitogen-activated protein kinase pathway (Liu et al., 2009). In the present study, we found that emodin significantly enhanced MMC-induced cytotoxic effect and suppressed Rad51 expression in cultured NSCLC cells by inhibiting the MKK-ERK pathway.

In conclusion, we show that emodin enhanced the MMC-induced cytotoxicity, and this effect is mediated by ERK1/2 inactivation and Rad51 down-regulation in lung cancer cells. These findings should aid in the understanding of the molecular mechanisms of the action of emodin and provide a basis for the therapeutic use of this compound.

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