Research Article

Peroxisome Proliferator-Activated Receptor-γ Contributes to the Inhibitory Effects of Embelin on Colon Carcinogenesis

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Abstract

Down-regulation of XIAP (X-linked inhibitor of apoptosis protein) sensitizes colon cancer cells to the anticancer effect of peroxisome proliferator-activated receptor-γ (PPARγ) ligands in mice. The aims of this study were to evaluate the effect of embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone), an antagonist of XIAP, on colon cancer, with a particular focus on whether PPARγ is required for embelin to exert its effect. A dominant-negative PPARγ was used to antagonize endogenous PPARγ in HCT116 cells. Cells were treated with or without embelin. Cell proliferation, apoptosis, and nuclear factor-κB (NF-κB) activity were measured. For *in vivo* studies, 1,2-dimethylhydrazine dihydrochloride (DMH) was s.c. injected to induce colon cancer in PPARγ$^{+/+}$ and PPARγ$^{+/-}$ mice. Mice were fed embelin daily for 10 days before DMH injection, and continued for 30 more weeks. Embelin inhibited proliferation and induced apoptosis in HCT116 cells with marked up-regulation of PPARγ. In addition, embelin significantly inhibited the expressions of survivin, cyclin D1, and c-Myc. These effects were partially dependent on PPARγ. PPARγ$^{+/-}$ mice were more susceptible to DMH-induced colon carcinogenesis than PPARγ$^{+/+}$ mice, and embelin significantly reduced the incidence of colon cancer in PPARγ$^{+/+}$ mice but not in PPARγ$^{+/-}$ mice. Embelin inhibited NF-κB activity in PPARγ$^{+/+}$ mice but marginally so in PPARγ$^{+/-}$ mice. Thus, reduced expression of PPARγ significantly sensitizes colonic tissues to the carcinogenic effect of DMH. Embelin inhibits chemical carcinogen-induced colon carcinogenesis, but this effect is partially dependent on the presence of functional PPARγ, indicating that PPARγ is a necessary signaling pathway involved in the antitumor activity of normal organisms. [Cancer Res 2009;69(11):4776–83]

Introduction

We have recently observed that down-regulation of XIAP (X-linked inhibitor of apoptosis protein) coupled with peroxisome proliferator-activated receptor-γ (PPARγ) ligands could synergistically induce apoptosis in colon cancer cells, and such an approach could significantly suppress the growth of colon cancer in mice (1–3). Indeed, XIAP plays a critical role in the resistance of many cancer cells to chemotherapy and radiotherapy (4, 5). Thus, direct inhibition of XIAP may be a promising strategy for the development of novel anticancer agents. Small-molecule inhibitors of XIAP have been tested in many *in vitro* experiments (6, 7).

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) is a potent, nonpeptidic, cell-permeable small molecule inhibitor of XIAP that targets the XIAP BIR3 domain (6). Embelin prevents the development of chemical carcinogen-induced hepatocarcinogenesis in rats (8, 9). The combination of embelin with recombinant human tumor necrosis factor–related apoptosis-inducing ligand could synergistically induce cell death in pancreatic cancer (5). Although the underlying mechanisms remain largely unknown, recent studies have indicated that embelin functions as a strong antioxidant (10) and a potent inhibitor of nuclear factor-κB (NF-κB) in several human cancer cell lines (11). Embelin was found to down-regulate NF-κB–dependent genes involved in tumor cell survival, proliferation, invasion, and angiogenesis, making this agent potentially useful for cancer therapy (11).

Peroxisome proliferator-activated receptor-γ (PPARγ) is a nuclear receptor that acts as a transcription factor. Ligand-induced activation of PPARγ led to the transcription of downstream target genes involved in cell growth, differentiation, and apoptosis in several malignant cell lines, thus, PPARγ plays a crucial role in carcinogenesis (12, 13). Previous studies indicate that activation of PPARγ inhibits growth and induces differentiation and apoptosis of colon cancer cells (14, 15), and that PPARγ ligands suppress colon carcinogenesis *in vivo* (16). Recent studies have shown that activation of PPARγ inhibits multiple steps in the NF-κB signaling pathway, and such an interaction contributes significantly to the antitumor effects of PPARγ (17, 18).

In the current study, we aimed to investigate whether embelin has any anticancer effects in colon cancer, and if so, what are the molecular mechanisms. We focused particularly on whether PPARγ plays any role in the antitumor effect of embelin.

Materials and Methods

Chemicals and antibodies. Embelin was purchased from Advance Scientific & Chemical, Inc. 1,2-Dimethylhydrazine dihydrochloride (DMH) was purchased from Acros Organics. Anti–glyceraldehyde-3-phosphate dehydrogenase was purchased from Abcam, Inc. Phosphorylated p65 (serine 276) was purchased from Cell Signaling Technology, Inc. Cell Death ELISA assay kit, caspase 3 assay kit, and Annexin V/PI staining kit were purchased from Roche Diagnostics. Other primary antibodies and all secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. Human recombinant tumor necrosis factor-α (TNF-α) was purchased from R&D.

Cell culture, transfection, proliferation, and apoptosis assays. Human colon cancer cell line HCT116 was cultured in McCoy's 5A medium as we described (1). Empty vector pcDNA3 for control transfection was purchased from Invitrogen. pcDNA3-PPARγL468A/E471A (dominant-negative PPARγ; dnPPARγ) was kindly provided by Dr. V.K. Chatterjee (University of Cambridge, United Kingdom; ref. 17). These plasmids were

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Table 1. Incidence of colon neoplasms in mice exposed to DMH in the presence or absence of embelin

Group	PPARγ	Treatment (no. of mice examined)	Incidence of colonic neoplasms		
			Total (%) [multiplicity]	Adenocarcinoma (%)	Adenoma (%)
1	+/+	DMH (16)	13 (81.25%) [9.8 ± 4.4]	11 (68.75%)	2 (12.5%)
2	+/+	DMH + embelin (15)	10 (66.7%)* [2.4 ± 1]*	9 (60%)	1 (6.67%)
3	+/−	DMH (15)	15 (100%)* [13 ± 8]*	14 (93.3%)	1 (6.67%)
4	+/−	DMH + embelin (13)	12 (92.3%)† [10.8 ± 6.7]†	11 (84.6%)	1 (7.69%)

NOTE: Animal grouping and incidence of colon neoplasms in mice exposed to DMH in the presence or absence of embelin.
*$P < 0.05$ vs. group 1.
†$P > 0.05$ vs. group 3.

transfected into HCT116 cells with LipofectAMINE 2000 (Invitrogen). Forty-eight hours later, medium was changed to fresh McCoy's 5A containing 800 μg/mL of G418 (Calbiochem). G418-resistant colonies were used for further analyses. Cell proliferation was determined by [^{3}H]thymidine incorporation assay (19). Apoptosis was detected by cell death ELISA assay, Annexin V/PI staining, and caspase 3 activity assay, as reported previously (1, 19, 20).

Luciferase assay. Cells were transfected with pNF-κB-luc (Stratagene), or cotransfected with pNF-κB-luc and pcDNA3 or pcDNA3-PPARγL468A/

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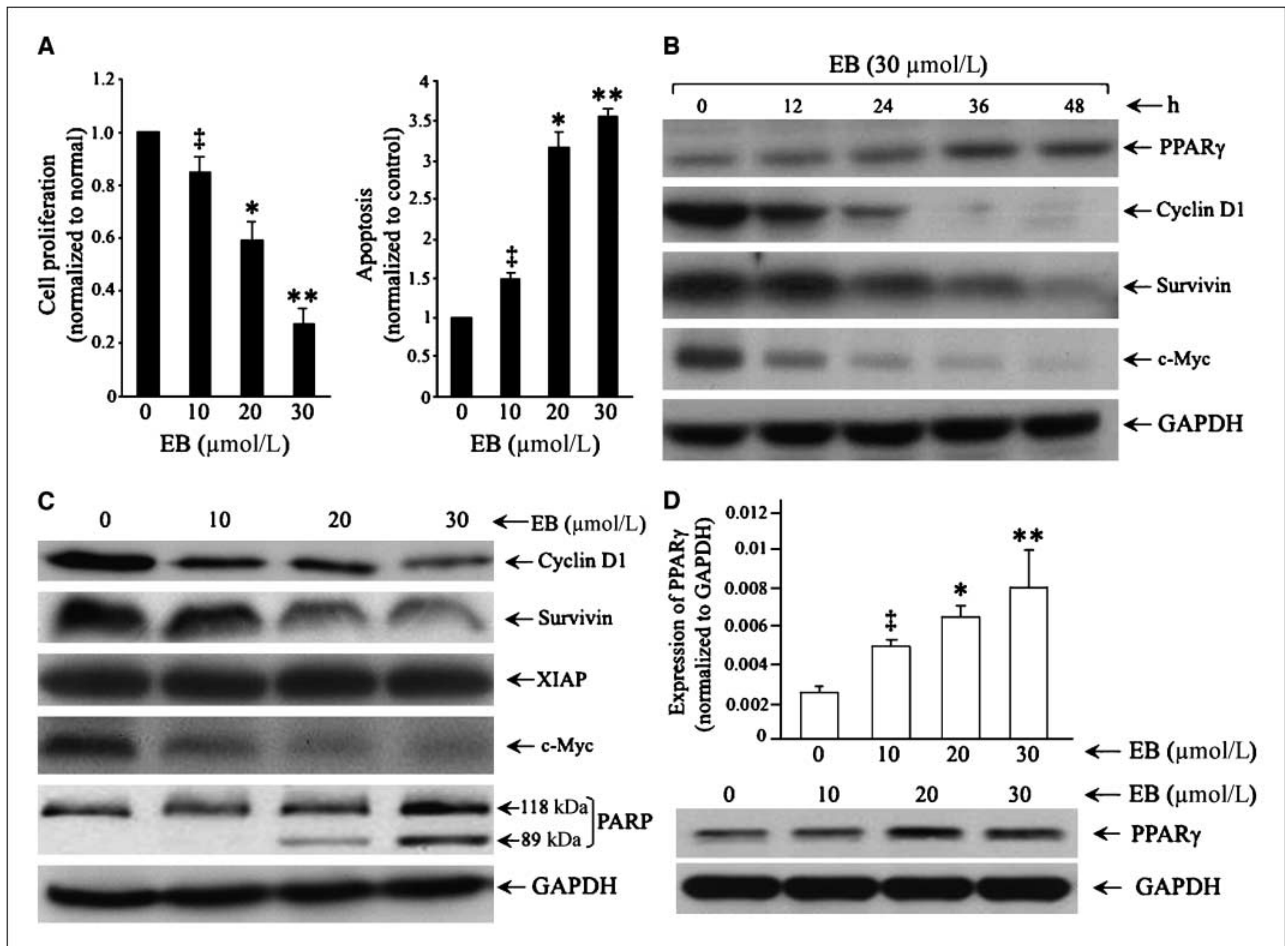

Figure 1. *A,* effects of embelin on the proliferation and apoptosis of HCT116 cells. Cells were treated with the indicated concentrations of embelin for 24 h. Cell proliferation was determined by [^{3}H]thymidine incorporation assay and apoptosis was determined by ELISA assay. Expression of cyclin D1, survivin, XIAP, c-Myc, and PARP were detected by Western blot, and a time-dependent effect (*B*) and a dose-dependent effect (*C*) were shown. *B,* a time course study to examine the effect of embelin on the expression of PPARγ. *D,* in a separate study, the expression of PPARγ was determined by real-time PCR ($n = 4$, *top*) and Western blotting (*bottom*). For Western blot data, representative results of three separate experiments were shown. Quantitative data were normalized to controls and expressed as the mean ± SD of three separate experiments (‡, $P > 0.05$; *, $P < 0.01$; **, $P < 0.001$, compared with the control group; *EB,* 0 μmol/L).

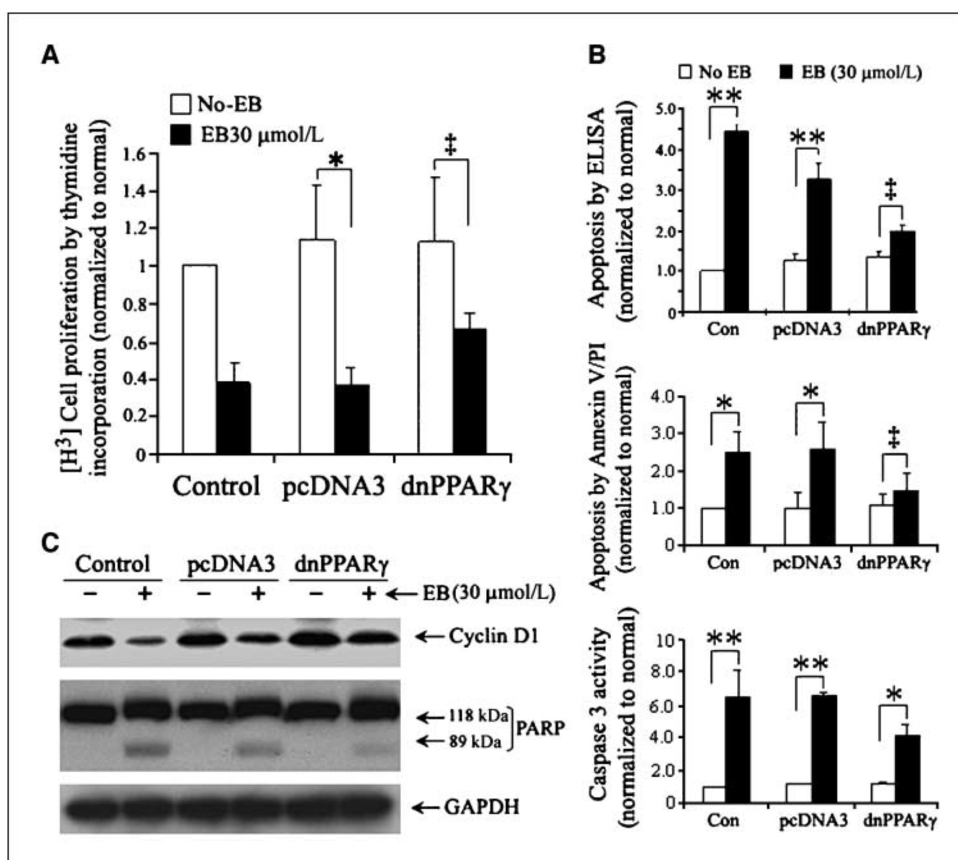

Figure 2. Effect of PPARγ on embelin-induced growth inhibition and apoptosis. HCT116 wild-type, empty vector, and dnPPARγ cells were treated with or without 30 μmol/L of embelin for 24 h. *A,* cell proliferation was determined by [^{3}H]thymidine incorporation assay. *B,* apoptosis was determined by ELISA (*top*), Annexin V/PI staining (*middle*), and caspase 3 activity (*bottom*). All quantitative data were normalized to controls and expressed as the mean ± SD of three separate experiments, each with three replicates. *C,* the expression of cyclin D1 and PARP were detected by Western blotting. Representative results of three separate experiments (‡, $P > 0.05$; *, $P < 0.01$; **, $P < 0.001$).

E471A plasmids. Twenty-four hours after transfection, cells were treated with the indicated concentrations of embelin for 12 h, followed by incubation with or without 1 ng/mL of TNF-α for an additional 24 h. Luciferase activity was determined using the Dual-Glo Luciferase Assay System (Promega), as we previously reported (2).

Real-time quantitative reverse transcriptase PCR and Western blot. Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen). One microgram of total RNA was reverse-transcribed into cDNA. Quantitative reverse transcriptase PCR was performed as we described (21). The results were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean ± SD. Primers used for human PPARγ detection were forward, 5′-GGTGGCCATCCGCATCT-3′; reverse, 5′-TGCTTTTGGCATACTCTGT-GATCT-3′. Western blot was performed as we described (1, 2).

Animals and genotyping. All animal experiments were performed according to protocols approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. PPARγ heterozygous mice (PPARγ$^{+/-}$ mice) were kind gifts from Dr. Frank Gonzalez (National Cancer Institute, Bethesda, MD; ref. 22). PPARγ$^{+/-}$ mice were mated with wild-type C57 mice to produce offspring. Genomic DNA extracted from the ear of each mouse was used to do genotyping by PCR as previously described (22). Both PPARγ$^{+/+}$ and PPARγ$^{+/-}$ mice used in the experiments were littermates with similar genetic backgrounds.

Induction of tumors by DMH and treatment of mice with embelin. Administration of DMH was performed as described previously (23). A total of 59 mice, ages 8 to 12 weeks, were randomly divided into four groups, as detailed in Table 1. Each group consisted of approximately equal numbers of males and females. All animals were s.c. injected with DMH (20 mg/kg body weight/wk) for 15 weeks. In groups 2 and 4, embelin was given to mice 10 days prior to the first DMH injection, and then continued till harvest at week 30. In groups 1 and 3, no embelin but vehicle (DMSO) was added to the diet. Based on the diet consumption, the daily intake of embelin was estimated to be ∼100 mg/d/kg body weight.

Histopathologic evaluation of tumorigenesis. All animals were sacrificed at week 30 following initial DMH injection. Stomach, small intestines, large intestines, liver, and kidneys were carefully inspected for macroscopic lesions. The large bowels were excised and cut open longitudinally and the number of colon tumors was counted and the volume of the largest tumors was measured. After the macroscopic inspection, tumor specimens and their adjacent normal tissues were snap-frozen in liquid nitrogen. A small portion of tumor and adjacent normal tissue were fixed in 10% phosphate-buffered formalin. Histologic diagnosis was performed in H&E-stained slides.

Extraction of nuclear protein and detection for NF-κB activity. Preparation of cytoplasmic and nuclear extracts was performed as we previously described (2). Nuclear extract was assayed for the DNA binding activity of NF-κB by a Nonradioactive Chemiluminescent Transcription Factor Assay kit.

Immunohistochemistry. The tissue expression of p-p65 (phosphorylated NF-κB) and Cox-2 were examined by immunohistochemistry in formalin-fixed paraffin-embedded tissue sections. The tissue sections were incubated with primary antibody followed by incubation with horseradish peroxidase–labeled secondary antibody (Dako). The slides were developed with diaminobenzidine substrate and results were evaluated by a pathologist.

Statistical analysis. The incidences of colonic cancer were analyzed using Fisher's exact test. Student's independent sample *t* test was used for pair-wise comparisons. One-way ANOVA, with Dunnett *t* test, was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Embelin up-regulated the expression of PPARγ, inhibited proliferation, and induced apoptosis in colon cancer cells. Embelin enhanced the expression of PPARγ in HCT116 cells in a time-dependent (Fig. 1*B*) and dose-dependent (Fig. 1*D*) manner.

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The dose-dependent effect of embelin on PPARγ was also observed at the mRNA level by real-time PCR (Fig. 1*D, top*).

In parallel with these changes, embelin significantly reduced cell proliferation and induced apoptosis in this cell type (Fig. 1*A*). These effects were also observed in another colon cancer cell line HT29 cells (data not shown). Inhibition of cell proliferation and induction of apoptosis were associated with decreased expressions of cyclin D1, survivin, and c-Myc both in a time-dependent and dose-dependent manner (Fig. 1*B* and *C*). In addition, an increase in PARP cleavage was observed (*C*). However, embelin did not alter the expression of XIAP (Fig. 1*C*), and the expressions of other IAP family members, including cIAP2 and cIAP1, were not altered either (data not shown).

Blocking PPARγ activity partially abrogated the antiproliferative and proapoptotic effects of embelin on HCT116 cells. Additional studies were focused on elucidating the mechanisms by which embelin inhibited proliferation and induced apoptosis in HCT116 cells. Previously, we observed that XIAP down-regulation and ligand-induced PPARγ activation synergistically inhibit the growth of colon cancer cells (1–3).

We used dnPPARγ to antagonize endogenous PPARγ activity and tested whether blocking PPARγ activity could affect the embelin-induced growth inhibition of colon cancer cells. This construct had been confirmed to efficiently block the function of PPARγ in HCT116 cells (24). As shown in Fig. 2*A*, transfection with pcDNA3 or dnPPARγ did not alter the proliferation of HCT116 cells as compared with controls (cells without any treatment). Following treatment with embelin, cell proliferation was suppressed by 62.1% and 64% in control and pcDNA3 transfected cells, respectively. In contrast, in cells transfected with dnPPARγ followed by embelin treatment, cell proliferation was suppressed by only 34%.

In addition, we examined the effect of dnPPARγ on embelin-induced apoptosis. Apoptosis was examined by three complimentary assays and similar results were obtained (Fig. 2*B*). Treatment of HCT116 wild-type and empty vector cells by 30 μmol/L of embelin for 24 hours led to increased apoptosis, which was associated with decreased cyclin D1 and increased PARP cleavage (Figs. 1*C* and 2*C*). Additional studies revealed that embelin-induced apoptosis, blockade of cyclin D1, and increased PARP cleavage were partially abrogated by dnPPARγ (Fig. 2*B* and *C*).

Loss of PPARγ promoted DMH-induced colonic carcinogenesis and the tumor-preventive effect of embelin was partially dependent on PPARγ. The above results indicated that the antiproliferative and proapoptotic effect of embelin was partially dependent on endogenous PPARγ activation. We therefore investigated whether embelin could alter the incidence of DMH-induced colon carcinogenesis in heterozygous PPARγ-deficient mice. Overall, PPAR$\gamma^{+/-}$ mice showed an increased incidence of colon tumors and enhanced tumor multiplicity compared with PPAR$\gamma^{+/+}$ mice (tumor incidence, 100% versus 81.3%, respectively;

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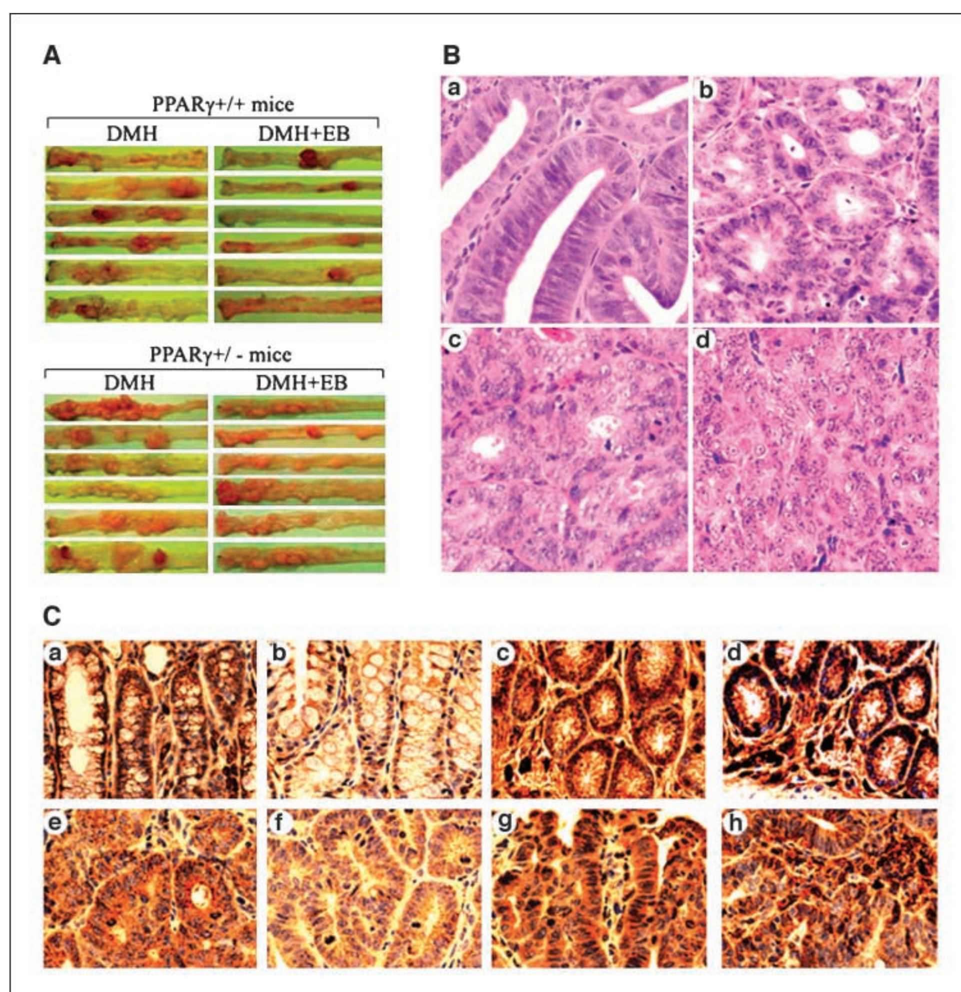

Figure 3. Effect of embelin on DMH-induced colon carcinogenesis and Cox-2 expression in colon tissues. *A,* each strip represents a portion of the colon from separate mice. *B,* microscopically, tumors were adenomas (*a*), well (*b*), moderately (*c*), or poorly differentiated (*d*) adenocarcinomas. *C,* Cox-2 expression was determined by immunohistochemical staining in the adjacent normal (*a, b, c, d*) or tumor (*e, f, g, h*) tissues from PPAR$\gamma^{+/+}$ (*a, b, e, f*) or PPAR$\gamma^{+/-}$ (*c, d, g, h*) mice exposed to DMH alone (*a, e, c, g*) or DMH and embelin (*b, f, d, h*). Original magnifications, ×200.

$P < 0.05$; tumor multiplicity, 13 ± 8 versus 9.8 ± 4.4, respectively; $P < 0.05$; Table 1; Fig. 3A).

The DMH-induced tumor incidence and tumor multiplicity in PPARγ$^{+/+}$ mice receiving embelin were significantly lower than those in PPARγ$^{+/+}$ mice without embelin (tumor incidence, 66.7% versus 81.3%; $P < 0.05$; tumor multiplicity, 2.4 ± 1 versus 9.8 ± 4.4, respectively; $P < 0.05$; Table 1; Fig. 3A). In sharp contrast, tumor incidence and multiplicity in PPARγ$^{+/-}$ mice receiving embelin were not significantly different from those in PPARγ$^{+/-}$ mice without embelin exposure (tumor incidence, 92.3% versus 100%, respectively; $P > 0.05$; tumor multiplicity, 10.8 ± 6.7 versus 13 ± 8, respectively; $P > 0.05$; Table 1; Fig. 3A).

Macroscopically, the neoplastic lesions in both PPARγ$^{+/-}$ and PPARγ$^{+/+}$ mice were similar in the forms of nodular, polypoid, or flat-type tumors, and all confined to the middle and distal colon. No neoplastic lesions were found in the proximal colon, small intestines, stomach, liver, and kidney. Tumors tended to be smaller in PPARγ$^{+/+}$ mice compared with PPARγ$^{+/-}$ mice, and there were more tumors that were >216 mm^3 (width × length × height, mm) in the PPARγ$^{+/-}$ mice, regardless of embelin exposure status (Fig. 3A).

Microscopically, tumors showed a spectrum of progression and various histopathologic types (Fig. 3B). Some tumors comprised of well-formed elongated tubular glands, features characteristic of adenoma (*a*). Some tumors comprised of closely packed but well-formed dilated glands with complex branching lined by dysplastic epithelia, features consistent with an early well-differentiated adenocarcinoma (*b*). The majority of tumors showed glands with complex irregular branching, fusion of glands with frequent nuclear stratification, characteristics of moderately differentiated adenoma (*c*). A small percentage of tumors showed extensively fused glands with clearly visible solid areas of packed tumor cells, characteristic of poorly differentiated adenocarcinomas (*d*).

Overall, the vast majority of tumors in PPARγ$^{+/-}$ mice were adenocarcinomas (93.3%), whereas only two-thirds of tumors in PPARγ$^{+/+}$ mice were adenocarcinomas (68.75%; Table 1). In both groups, >85% of adenocarcinomas were moderately differentiated, ~10% were poorly differentiated, and the rest were either adenomas or well-differentiated adenocarcinomas.

Embelin up-regulated the expression of PPARγ in mouse colon tissues. We then detected the expression of PPARγ in colon tumors and the surrounding nontumorous colonic tissues. As expected, the protein expression of PPARγ in the normal and colon tumors of PPARγ$^{+/+}$ mice was significantly higher than that in PPARγ$^{+/-}$ mice (Fig. 4*A, top*), and there was no significant difference in PPARγ expression between colon tumors and their adjacent normal colonic tissues in both PPARγ$^{+/-}$ mice and PPARγ$^{+/+}$ mice. Following treatment with embelin, PPARγ expression was markedly increased in the tumor tissues of both PPARγ$^{+/+}$ and PPARγ$^{+/-}$ mice (Fig. 4*A, bottom*).

PPARγ deficiency is associated with increased NF-κB activity in colonic tumors. An increase in NF-κB-p50 activity was observed in the tumor tissues of PPARγ$^{+/-}$ mice compared with PPARγ$^{+/+}$ mice following DMH treatment (Fig. 4*B, top*). By immunohistochemistry, the expression of p-p65 in the normal colonic mucosa of both PPARγ$^{+/+}$ and PPARγ$^{+/-}$ mice was very weak (data not shown). However, in cancerous tissues, expression of p-p65 in PPARγ$^{+/-}$ mice was significantly higher than that in PPARγ$^{+/+}$ mice (Fig. 4*B, bottom*).

Following treatment with embelin, NF-κB-p50 transcription activity and the expression of p-p65 were significantly decreased in PPARγ$^{+/+}$ mice (Fig. 4*B, top*). However, in PPARγ$^{+/-}$ mice, the

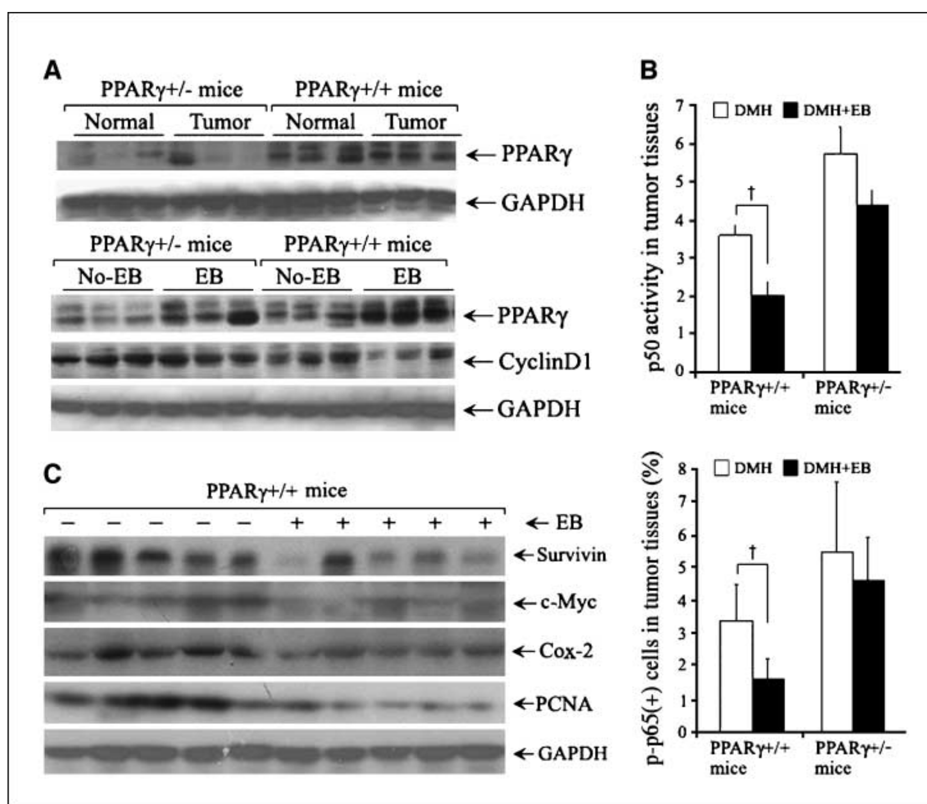

Figure 4. Expression of PPARγ in adjacent normal and tumor tissues from PPARγ$^{+/-}$ and PPARγ$^{+/+}$ mice exposed to DMH and treated with or without embelin. *A,* significantly weaker expression of PPARγ was observed in the adjacent normal and tumor tissues of the colons from PPARγ$^{+/-}$ mice as compared with PPARγ$^{+/+}$ mice (*top*). Effect of embelin on PPARγ expression in DMH-induced colon tumor tissues from PPARγ$^{+/-}$ mice and PPARγ$^{+/+}$ mice (*bottom*). Expression of cyclin D1 was also detected in these tissues. Each lane represents a tumor tissue. *B,* NF-κB p50 activity (*top*) and the number of phosphorylated p65 (*p-p65*) positive cells (*bottom*) in the colon tumor tissues treated with or without embelin were detected by NF-κB activity assay and immunohistochemistry, respectively. Quantitative data were expressed as mean ± SD ($n = 6$; †, $P < 0.05$). *C,* the effect of embelin on Cox-2, c-Myc, survivin, and PCNA was determined by Western blot in the colon cancer tissues from PPARγ$^{+/+}$ mice in the presence or absence of embelin.

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inhibitory effect of embelin on NF-κB activity and p-p65 expression was minimal.

Embelin-induced reduction in NF-κB activity was associated with a down-regulation of cyclin D1 in PPARγ$^{+/+}$ (Fig. 4*A, bottom, lanes 10–12* versus *lanes 7–9*) but only marginally so in PPARγ$^{+/-}$ mice. On the other hand, up-regulation of NF-κB activity in PPARγ$^{+/-}$ mice was associated with an increased cyclin D1 protein expression as compared with PPARγ$^{+/+}$ mice (Fig. 4*A, bottom, lanes 1–3* versus *lanes 7–9*).

Embelin diminished the expression of Cox-2, c-Myc, survivin, and PCNA in PPARγ$^{+/+}$ mice but not in PPARγ$^{+/-}$ mice. In order to elucidate the mechanisms of how embelin exerts its antitumor effect, we examined the expression of Cox-2 and β-catenin in colon tissues, as DMH was known to cause inflammation and embelin was known to possess anti-inflammatory properties. By immunohistochemical staining, there was no difference in the expression of β-catenin between PPARγ$^{+/+}$ mice and PPARγ$^{+/-}$ mice, regardless of embelin exposure. No obvious difference was observed between normal and tumor tissues of each group (data not shown). Cox-2 is richly expressed in all groups of tissues. In the presence of embelin, there was a marked decrease in the expression of Cox-2 in PPARγ$^{+/+}$ mice, but not in PPARγ$^{+/-}$ mice, both in the tumor and adjacent normal tissues (Fig. 3*C*). Consistent with the immunohistochemical staining, embelin decreased the expression of Cox-2 in the colon cancer tissues from PPARγ$^{+/+}$ mice (Fig. 4*C*).

In addition, embelin decreased the expression of cell proliferation markers PCNA and c-Myc, as well as survivin in PPARγ$^{+/+}$ mice (Fig. 4*C*), but not in PPARγ$^{+/-}$ mice (data not shown).

Embelin-induced reduction in NF-κB activity was partially dependent on functional PPARγ. As shown in Fig. 1, embelin time- and dose-dependently enhanced the expression of PPARγ. We then performed additional studies to test the effect of embelin on NF-κB. We performed a NF-κB reporter activity assay. As shown in Fig. 5*A*, embelin had a minimal effect on basal NF-κB activity in HCT116 cells, but it significantly blunted TNF-α–stimulated NF-κB activation. Transfection of cells with dnPPARγ almost reversed the inhibitory effect of embelin on TNF-α–induced NF-κB activation (Fig. 5*A*).

The inhibitory effect of embelin on NF-κB was further evidenced by its effects on some of the NF-κB downstream target genes. We measured Bcl-xL, c-Myc, and four members of the IAP family (cIAP1, cIAP2, XIAP, and survivin). Survivin and c-Myc were significantly inhibited by embelin (Fig. 1*B* and *C*, and Fig. 5*B*), whereas no changes were found in Bcl-xL, cIAP1, and cIAP2. DnPPARγ blunted embelin-induced down-regulation of survivin and c-Myc. No significant changes were observed in XIAP, regardless of the embelin and dnPPARγ treatment (Figs. 1*C* and 5*B, top*). Similar results were observed when PPARγ was inhibited by its specific chemical inhibitor, GW9662. For example, GW9662 significantly attenuated the inhibitory effect of embelin on survivin (Fig. 5*B, bottom*).

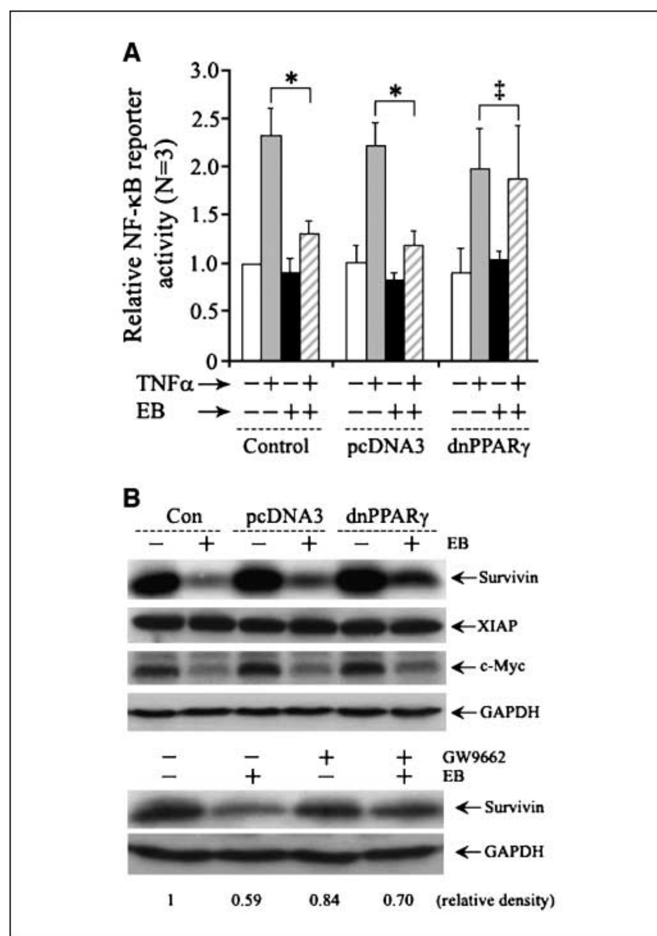

Figure 5. Effect of PPARγ on embelin-induced NF-κB inhibition. HCT116 cells were transfected with pNF-κB-Luc, or cotransfected with pNF-κB-Luc and pcDNA3 or dnPPARγ, followed by treatment with or without embelin (30 μmol/L) and/or TNF-α (1 ng/mL). *A,* luciferase activity was detected and expressed as the mean ± SD of three separate experiments. *B,* expression of the NF-κB downstream target genes in HCT116 wild-type, empty vector, and dnPPARγ cells (*top*) treated with or without 30 μmol/L of embelin was determined by Western blotting (‡, $P > 0.05$; *, $P < 0.01$). Effect of PPARγ blockade on survivin in HCT116 cells treated with or without embelin was determined by Western blot (*bottom*). The PPARγ-specific inhibitor GW9662 was used to block the PPARγ function. The semiquantitative results (arbitrary units) from desitometric scans are shown below the Western blot bands.

Discussion

In this study, we showed that embelin significantly inhibited growth and induced apoptosis in colon cancer cells *in vitro* and effectively suppressed DMH-induced colon carcinogenesis *in vivo*. The novel finding of this study is that the antitumor effect of embelin seems to require the presence of functional PPARγ. Consistent with previous studies by others (10), embelin inhibited NF-κB activity, and such an inhibition was mediated at least in part by PPARγ.

We found that embelin-induced inhibition of cell proliferation and apoptosis were associated with down-regulation of cyclin D1, c-Myc, and survivin. Cyclin D1 plays a critical role in cell cycle progression, especially at the early G_0-G_1 phase (25). As cyclin D1 is under the control of NF-κB, the reduced cyclin D1 expression may also indicate an inhibition of NF-κB by embelin. Similarly, inhibition of survivin and c-Myc was also an indicator for embelin-induced NF-κB inhibition. As c-Myc is important in colon tumorigenesis and the regulation of the cell cycle, angiogenesis, and apoptosis (26–29), inhibition of c-Myc expression by embelin may be an important mechanism responsible for its antitumor effect.

Importantly, our results revealed, for the first time, that the antiproliferative and proapoptotic effects of embelin seem to be associated with increased PPARγ expression. Based on the evidence of the important role of PPARγ in colon cancers (14–16), the finding prompted us to investigate whether the antitumor effect of embelin might be mediated by the activation of PPARγ.

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The ability of PPARγ activation to induce apoptosis and growth inhibition in human cancer cells including colon cancer cell lines is widely recognized (14, 16, 30). Using a dnPPARγ, we found that dnPPARγ dramatically, if not completely, abrogated the antiproliferative and proapoptotic activity of embelin. In addition, dnPPARγ significantly abolished or attenuated the ability of embelin to inhibit the expression of cyclin D1, c-Myc, and survivin, as well as PARP cleavage, suggesting that embelin-mediated inhibition of cell proliferation and apoptosis is mediated by PPARγ. Our result is consistent with previous observations that ligand-induced activation of PPARγ could selectively inhibit the expression of cyclin D1 (31).

To further evaluate the effect of PPARγ on colon carcinogenesis and the chemopreventive potential of embelin, we used DMH, a colon-specific carcinogen (32), to induce colon cancer in mice, in the presence or absence of embelin. Compared with PPARγ$^{+/+}$ mice, PPARγ$^{+/-}$ littermates exhibited a significantly increased susceptibility to DMH-induced colon carcinogenesis. Our results are in good agreement with a previous report (22) that PPARγ haploinsufficiency increased the susceptibility of mice to azoxymethane-induced colon cancer. Clearly, PPARγ plays a critical role in preventing colon carcinogenesis.

The most significant finding of our current study is that embelin markedly reduced the incidence and multiplicity of total tumors in PPARγ$^{+/+}$ but not PPARγ$^{+/-}$ mice, although embelin increased the PPARγ protein expression in both PPARγ$^{+/+}$ and PPARγ$^{+/-}$ groups. These results indicated that the chemopreventive effect of embelin requires endogenous rather than stimulated PPARγ, and the basal expression level of PPARγ may be important. We found that embelin suppressed colon carcinogenesis without affecting the tumor pathology, implying that this agent may act on the very early stage of colon carcinogenesis.

NF-κB signaling is one of the most commonly altered pathways associated with the development of many malignancies including colon cancer (33–35). Activation of PPARγ has been shown to inhibit the activity of NF-κB (36). Thus, both transcription factors may interact to regulate the response of cancer cells to stimuli. We speculated that the preventive effect of embelin on colon carcinogenesis in PPARγ$^{+/+}$ mice may be through inhibiting the NF-κB–mediated transcription. Indeed, reduction of DMH-induced colon carcinogenesis by embelin in PPARγ$^{+/+}$ mice was associated with an inhibited NF-κB-p50 transcription activity and a reduced expression of p-p65. On the other hand, in PPARγ$^{+/-}$ mice which exhibited increased NF-κB activation and p-p65 expression, embelin did not significantly suppress DMH-induced colon carcinogenesis. The inhibitory effect of embelin on NF-κB was also indicated by our finding that embelin suppressed the expressions of several NF-κB target genes including cyclin D1, survivin, Cox-2, and c-Myc. Furthermore, embelin-induced reduction of these target genes was more marked in the presence of functional PPARγ, consistent with the finding that embelin had a more efficient anticancer effect in PPARγ$^{+/+}$ mice.

In conclusion, embelin significantly inhibited the growth of colon cancer cells by reducing cell proliferation and inducing apoptosis. Embelin effectively suppressed DMH-induced colon carcinogenesis in mice when PPARγ is present. Thus, PPARγ plays a critical role in DMH-induced colon carcinogenesis. The antitumor effect of embelin could be attributed to its inhibition on NF-κB activity, which is dependent on PPARγ. Embelin may be a potential chemopreventive agent against colon carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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