Chemopreventive effects of silymarin and silibinin on N-butyl-N-(4-hydroxybutyl) nitrosamine–induced urinary bladder carcinogenesis in male ICR mice

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Abstract

Effective strategies are lacking for the management of urinary bladder cancer for which smoking is a potential risk factor. Herein, we evaluated chemoprevention of urinary bladder cancer by natural chemopreventive agents, silymarin and silibinin, in a preclinical animal (ICR mouse) model of bladder cancer induced by tobacco smoke carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine (OH-BBN). Mice were fed p.o. with saline or OH-BBN (0.05%, w/v) in drinking water for 6 weeks or with silymarin or silibinin (200 mg/kg body weight for both) starting 1 week before OH-BBN exposure for 51 weeks. Silymarin and silibinin strongly arrested OH-BBN–induced tumor progression at the stage of mucosal dysplasia with a striking reduction in papillary nodular dysplasia as well as invasive carcinoma. Some silymarin- or silibinin-treated mice developed no urothelial lesions in spite of OH-BBN exposure. Immunohistochemical analyses at study conclusion revealed that silymarin and silibinin decreased cell proliferation by 42% ($P < 0.001$) and 44% ($P < 0.001$) and increased apoptosis by 4-fold ($P < 0.05$) and 6-fold ($P < 0.05$) in OH-BBN–induced urothelium, respectively. Antiproliferative and apoptotic effects of silymarin and silibinin were associated with decreases in (a) cyclin D1 protein level and extracellular signal–regulated kinase-1/2 phosphorylation and in (b) protein levels of survivin and nuclear phospho-p65 (Ser^{276} and Ser^{536}), respectively. Together, these results suggest that silymarin and silibinin inhibit chemically induced urinary bladder tumor growth and progression possibly by inhibiting cell proliferation and enhancing apoptosis. [Mol Cancer Ther 2007;6(12):3248–55]

Introduction

Bladder cancer is the fourth and eighth most common cancer in men and women in the United States, respectively. According to the estimates by the American Cancer Society for the year 2007, there will be 67,160 new bladder cancer cases and 13,750 associated deaths in the United States alone (1). Several epidemiologic studies have shown that there is an association between specific risk factors and urothelial tumors (2). More than 50% of bladder carcinoma is thought to be associated with cigarette smoking or exposure to chemical carcinogens (aromatic amines and nitrosamines) in the environment (3). Accordingly, one of the widely used murine models to study bladder cancer growth and progression is the one induced by N-butyl-N-(4-hydroxybutyl) nitrosamine (OH-BBN; Fig. 1A) administered either by oral gavage or in drinking water (4). In this model, the tumor development progresses from dysplasia (preinvasive) to invasive carcinoma stage and shows the progressive stages of bladder cancer as observed in humans (4, 5).

In the last few years, there have been several studies directed toward the identification of dietary or nondietary natural components for the prevention and intervention of various human malignancies including bladder cancer (6, 7). One such naturally occurring agent is silibinin (Fig. 1B), a major flavonolignan component in silymarin, which is a mixture of flavonoids present in milk thistle; the chemical structures of these isomers were recently reported by us (8). Several studies by us and others have shown the cancer-preventive and therapeutic efficacy of silymarin and silibinin in different animal tumor models and cancer cells in culture (9). Studies conducted by our group have also shown that silibinin possesses strong anticancer efficacy against human bladder cancer *in vitro* in which it inhibited the growth and proliferation of human bladder transitional-cell carcinoma cells by causing cell cycle arrest and induction of apoptosis (10–12). Under *in vivo* conditions, a study by another group (13) has shown that dietary administration of silymarin at the pre-initiation or post-initiation phase of OH-BBN–induced urinary bladder carcinogenesis in male ICR mice decreases the incidence of bladder neoplasms and preneoplastic lesions. In the present study, we compared the chemopreventive efficacy

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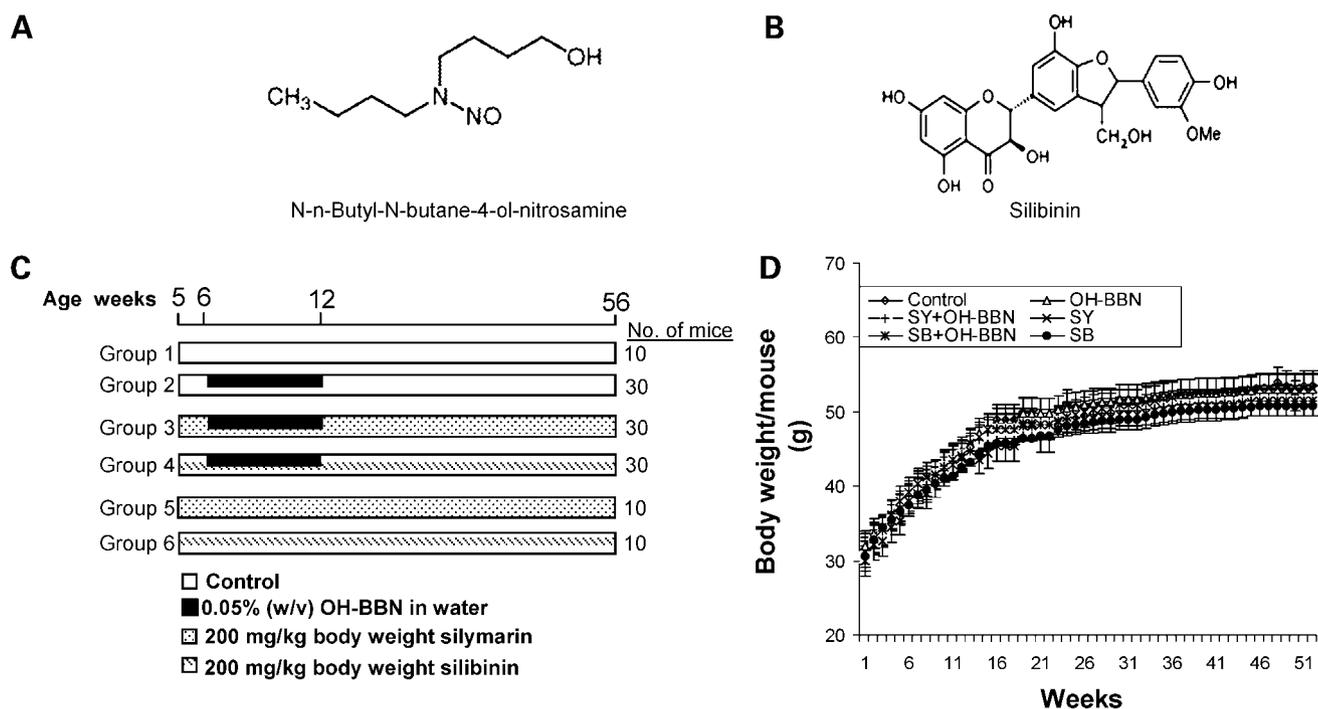
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Figure 1. Experimental design to study the effect of silymarin and silibinin on OH-BBN–induced bladder carcinogenesis in male ICR mice. Chemical structure of OH-BBN (**A**) and chemical structure of silibinin (**B**). **C,** 5-wk-old male ICR mice were randomly divided into six groups. Bladder cancer was induced in animals of groups 2 to 4 (30 mice in each group) by administration of OH-BBN (0.05%, w/v) in the drinking water for 6 wk. The drinking water containing carcinogen was changed twice a week. Mice in groups 3 and 4 were also gavaged with silymarin or silibinin (200 mg/kg body weight) in sterile saline, 5 d/wk for a period of 51 wk, starting 1 wk before OH-BBN exposure. Mice in groups 1, 5, and 6 (10 mice in each group) were gavaged with sterile saline or silymarin/silibinin (200 mg/kg body weight) in sterile saline for the same time period. **D,** in the experiment detailed in **C**, body weight of each mouse was recorded weekly throughout the experiment. *Points,* mean body weight per mouse of all the mice from each group, plotted as a function of time (weeks) for each group; *bars,* SE. *SY,* silymarin; *SB,* silibinin.

of silymarin and silibinin on tumor growth and progression in the OH-BBN–induced bladder cancer model. The uniqueness of this study is that these agents were not only p.o. administered in the pre-initiation phase but also continually administered throughout the course of a long-term study conducted for 51 weeks. Additionally, we have analyzed the potential *in vivo* molecular targets for the chemopreventive effectiveness of silymarin and silibinin against urinary bladder carcinogenesis in OH-BBN–induced bladder cancer model.

Materials and Methods

Animals and Treatment Protocol

OH-BBN was purchased from TCI America. Silymarin and silibinin were from Sigma Chemical Co., and the purity of silibinin was checked as >98% as previously described (14). Five-week-old male ICR mice (The Jackson Laboratory) were randomly divided into six groups (Fig. 1C). Bladder cancer was induced in the animals of groups 2 to 4 (30 mice in each group) by administration of OH-BBN (0.05%, w/v) in the drinking water for 6 weeks. The drinking water

containing carcinogen was changed twice a week. Mice in groups 3 and 4 were also gavaged with silymarin or silibinin (200 mg/kg body weight) in sterile saline, respectively, 5 d/wk for 51 weeks, starting 1 week before OH-BBN exposure. The selection of this dose was based on recently completed studies in other models showing efficacy at this dose in xenograft studies.[5] Mice in groups 1, 5, and 6 (10 mice in each group) were gavaged with sterile saline or silymarin/silibinin (200 mg/kg body weight) in sterile saline, respectively, for the same time period. All groups were maintained on a control diet (Purina chow) for the 51 weeks. Body weight and diet consumption were monitored weekly during the entire experiment. Animals were housed under standard laboratory conditions in the Laboratory Animal Care facility at the University of Colorado Health Sciences Center. Animal care was in accordance with institutional guidelines, and all animal treatments were done under an institutional protocol that was approved by the animal care and use committee.

Necropsy

At the end of 51 weeks of study, animals were sacrificed by lethal ketamine injection. At necropsy, urinary bladders were inflated with 10% buffered formalin and tied by a ligature around the neck of each bladder to maintain proper distention. Once the bladders were fixed, they were untied,

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[5] Unpublished observations.

processed by routine tissue processing methods, and embedded in paraffin. Serial tissue sections (5 µm) were cut, processed, and stained with H&E for histopathologic evaluation. Five randomly chosen bladder samples from each group were snap frozen in liquid nitrogen and stored at −80°C.

Immunohistochemical Analysis

Paraffin-embedded, 5-µm-thick sections were deparaffinized and stained with primary antibodies anti–proliferating cell nuclear antigen (PCNA; DAKO) and anti-survivin (Novus) followed by 3,3-diaminobenzidine staining as recently published (15). The immunostained cells were quantified by counting the brown cells and the total number of cells at five randomly selected fields at ×400 magnification. The proliferation index and survivin-positive cells were determined as (number of positively stained cells × 100) / total number of cells counted.

In situ Apoptosis Detection

Paraffin-embedded, 5-µm-thick sections were also used to identify apoptotic cells by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining using Dead End Colorometric TUNEL System (Promega Corporation) as earlier reported (14). The
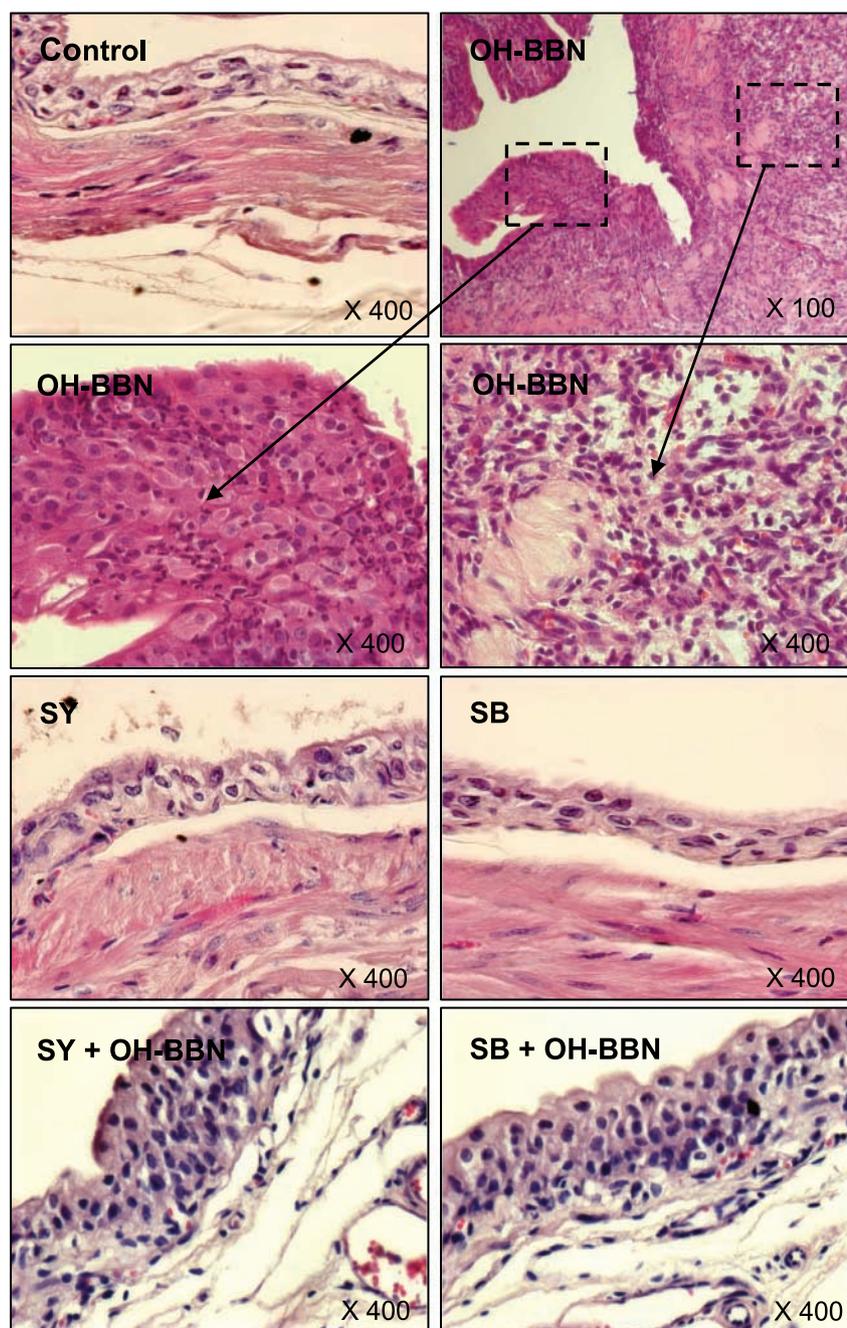

Figure 2. Histopathology of the urothelium of mice in OH-BBN – induced bladder carcinogenesis. At the end of the study detailed in Fig. 1C, urinary bladders were processed for H&E staining, and a representative picture is shown for each group. Normal urothelial mucosa, characterized by epithelium of <3 layers without any anaplasia, is shown in control, silymarin, and silibinin groups (×400). OH-BBN – treated mouse urothelium showing localized cellular proliferation, with invasive carcinoma infiltrating the submucosa or muscle layer with undifferentiated features (×100; subsets, ×400). Silymarin + OH-BBN and silibinin + OH-BBN groups show epithelium of ≥3 layers with diffused mucosal dysplasia.
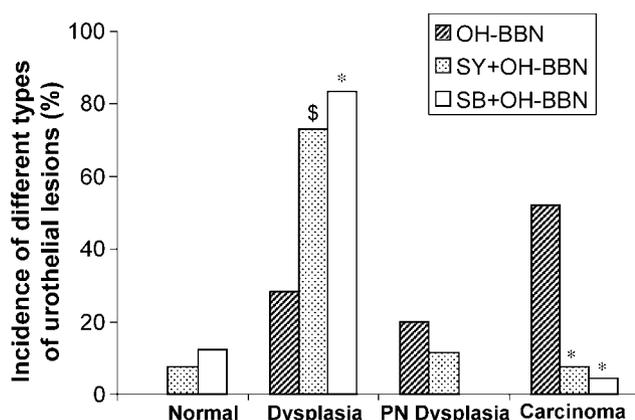
Figure 3. Silymarin and silibinin feeding inhibits the neoplastic progression of OH-BBN–induced bladder carcinogenesis in male ICR mice. Urothelial tissue samples obtained from the experimental groups as detailed in Fig. 1C were randomly analyzed in a double-blinded manner to evaluate the histopathology of urothelium. Urothelium was classified as normal urothelial mucosa, characterized by epithelium of <3 layers without any anaplasia; mucosal dysplasia, characterized by epithelium of ≥3 layers with moderate to severe anaplasia with diffused proliferation; papillary/nodular (*PN*) dysplasia, characterized by moderate or severe anaplastic epithelial lesion of localized cellular proliferation resulting in nodular or papillary forms; urothelial carcinoma, characterized by invasive carcinoma infiltrating the submucosa or muscle layer with transitional-cell carcinoma or undifferentiated features. χ^2 analysis and Fisher exact test were used to compare the incidence of dysplasia, papillary/nodular dysplasia, and carcinoma in OH-BBN and silymarin + OH-BBN or silibinin + OH-BBN groups; $P < 0.05$ was considered significant. *, $P < 0.001$; \$, $P < 0.01$, versus OH-BBN group.

apoptosis was evaluated by counting the TUNEL-positive cells (brown stained) as well as the total number of cells in five randomly selected fields at ×400 magnification. The apoptotic index was calculated as (number of apoptotic cells × 100) / total number of cells.

Immunoblot Analysis of Tissue Lysates

Total tissue lysate and nuclear lysate were prepared as earlier published (15). Protein concentration in tissue lysates was determined with Bio-Rad detergent-compatible protein assay kit (Bio-Rad Laboratories) by the Lowry method and 50 to 70 μg of protein were then subjected to immunoblot analysis as previously described (15). Membranes were probed with different primary antibodies for PCNA, cyclin D1, phospho–extracellular signal–regulated kinase (ERK)-1/2, total ERK1/2, cleaved caspase-3, cleaved poly(ADP-ribose) polymerase, survivin, phospho–nuclear factor κB (NF-κB) p65 (Ser[276]), phospho–NF-κB p65 (Ser[536]), and total NF-κB p65 (Cell Signaling). The secondary antibodies used were antirabbit IgG (Cell Signaling Technology) or antimouse IgG (Amersham Corp.). Protein loading was confirmed by stripping membranes and probing with anti–β-actin or anti–histone H1 antibody.

Statistical Analysis

All statistical analyses were carried out with Sigma Stat software version 2.03 (Jandel Scientific), and $P < 0.05$ was considered significant. χ^2 analysis and Fisher exact test were used to compare the incidence of dysplasia, papillary/nodular dysplasia, and carcinoma in OH-BBN and silymarin + OH-BBN or silibinin + OH-BBN groups. For other data, the significance of difference between OH-BBN and silymarin + OH-BBN or silibinin + OH-BBN groups was calculated by one-way ANOVA. Double-blinded examination was conducted by the authors A.T. and K.R. to evaluate the histopathologic changes in the urinary bladder, and the results are reported as mean of both these observations. All the microscopic histopathologic and immunohistochemical analyses were done with Zeiss Axioscope 2 microscope (Carl Zeiss, Inc.) and photomicrographs were captured with Carl Zeiss AxioCam MrC5 camera with Axiovision Rel 4.5 software.

Results

General Observations

At the time of necropsy, all animals were examined for gross pathology, and we did not observe any signs of edema or abnormality in nontarget organs. P.o. administration of silymarin/silibinin (200 mg/kg body weight in sterile saline, 5 d/wk, starting 1 week before OH-BBN administration) did not show any change in diet consumption during the 51 weeks of treatment (data not shown). There were no differences between the mean body weights of mice in all the groups during the experimental period (Fig. 1D).

Silymarin and Silibinin Feeding Decreases the Incidence of OH-BBN–Induced Invasive Urinary Lesions in Mice

A detailed histopathologic analysis of the neoplastic progression in the OH-BBN–induced urinary bladder was done (Fig. 2). H&E-stained sections were microscopically examined and classified as (*a*) normal urothelial mucosa, characterized by epithelium of <3 layers without any anaplasia; (*b*) mucosal dysplasia, characterized by epithelium of ≥3 layers with moderate to severe anaplasia with diffused proliferation; (*c*) papillary/nodular dysplasia, characterized by moderate or severe anaplastic epithelial lesion of localized cellular proliferation resulting in nodular or papillary forms; and (*d*) urothelial carcinoma, characterized by invasive carcinoma infiltrating the submucosa or muscle layer with transitional-cell carcinoma or undifferentiated features.

Six-week administration of OH-BBN (0.05%, w/v) to ICR male mice resulted in the induction of mucosal dysplasia, papillary/nodular dysplasia, and highly aggressive carcinoma of the urinary bladder at the end of the 51-week study (Fig. 2). Groups not induced by OH-BBN showed normal histologic characteristics (Fig. 2). When mice were fed with silymarin or silibinin at a dose of 200 mg/kg body weight beginning 7 days before OH-BBN administration and continued throughout the duration of experiment, 8% and 13% ($P < 0.001$, for both) of mice did not show any urothelial lesions in silymarin-treated and silibinin-treated groups, respectively (Fig. 3). Interestingly, silymarin and silibinin showed 2.5-fold ($P < 0.01$) and 3-fold ($P < 0.001$) higher incidence of mucosal dysplasia with a concomitant decrease in papillary/nodular dysplasia and invasive carcinoma, respectively (Fig. 3). OH-BBN alone showed areas of intense dysplasia with cellular atypia and hyperchromatic

nuclei in 48% of mice (quantitative data not shown), and ~20% of mice displayed characteristics of papillary/nodular dysplasia, which was reduced to 11% by silymarin treatment and was absolutely absent in silibinin-treated mice followed by OH-BBN challenge. As shown in Fig. 3, 52% of the OH-BBN–treated mice developed invasive carcinoma, 8% of which showed undifferentiated features whereas 4% displayed papillary features. This incidence of invasive carcinoma was strongly reduced from 52% in OH-BBN group to 7.7% and 4.1% ($P < 0.001$, for both) by silymarin and silibinin treatments, respectively (Fig. 3). Taken together, these results clearly show that both silymarin and silibinin caused the arrest of tumor progression at the preoplastic stage (dysplasia) with marked reduction in advanced dysplasia and invasive carcinoma in OH-BBN–induced urinary bladder urothelium.

Silymarin and Silibinin Decrease Urothelial Cell Proliferation, Cyclin D1 Level, and ERK1/2 Phosphorylation in OH-BBN–Treated Mice

To assess the *in vivo* effect of silymarin and silibinin feeding on the proliferation index in the urothelium of OH-BBN–treated mice, the tissue samples were analyzed first for PCNA immunostaining. Qualitative microscopic examination of PCNA-stained sections showed a substantial decrease in PCNA-positive cells in the urinary bladder tissue samples from silymarin + OH-BBN or silibinin + OH-BBN mice compared with OH-BBN controls (Fig. 4A). The quantification of PCNA staining showed 33 ± 5% and 32 ± 4% PCNA-positive cells in silymarin + OH-BBN and silibinin + OH-BBN groups, respectively, as compared with 57 ± 3% PCNA-positive cells in OH-BBN controls, accounting for a decrease in proliferation index by 42% and 44% ($P < 0.001$, for both), respectively (Fig. 4B). Immunoblot analysis of bladder tissue also showed a decreased expression of PCNA protein in these groups of mice compared with carcinogen alone (Fig. 4C).

Based on results showing an inhibition of cell proliferation by silymarin and silibinin in OH-BBN–induced urothelium, we rationalized that these agents might have modulatory effects on mitogenic signaling to regulate cell proliferation in OH-BBN–treated urothelium. Accordingly, expression levels of cyclin D1 and ERK1/2 phosphorylation were next analyzed by immunoblotting. Indeed, urothelium tissue samples from silymarin + OH-BBN or
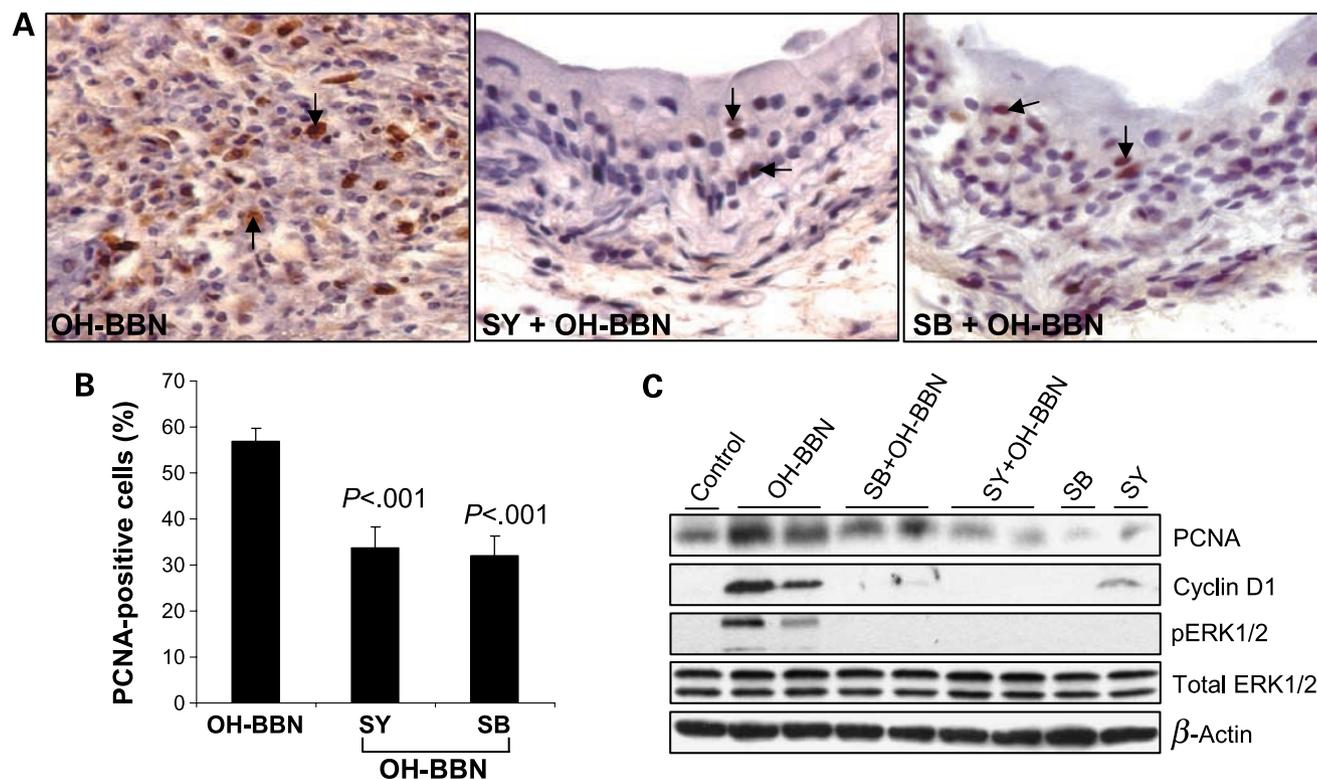

Figure 4. Effect of silymarin and silibinin feeding on OH-BBN–induced urothelial cell proliferation and mitogenic signaling. Immunohistochemical and immunoblot analyses of urothelial tissue samples obtained from the experiment detailed in Fig. 1C were done to study the molecular biomarkers and events associated with proliferation. **A,** immunohistochemical staining for PCNA (magnification, ×400) in urothelium was done as detailed in Materials and Methods. *Arrows,* PCNA-positive cells. **B,** the proliferating cells were quantified by counting PCNA-positive cells over total cells in five randomly selected fields at ×400 magnification from 10 different samples in each group. The proliferation index was determined as (number of positively stained cells × 100) / total number of cells counted. *Columns,* mean proliferation index in each group; *bars,* SE. **C,** bladder tissue samples were randomly taken from each group and analyzed for PCNA, cyclin D1, and phosphorylated and total ERK1/2 protein levels by immunoblotting. Membrane was stripped and reprobed with β-actin as loading control.
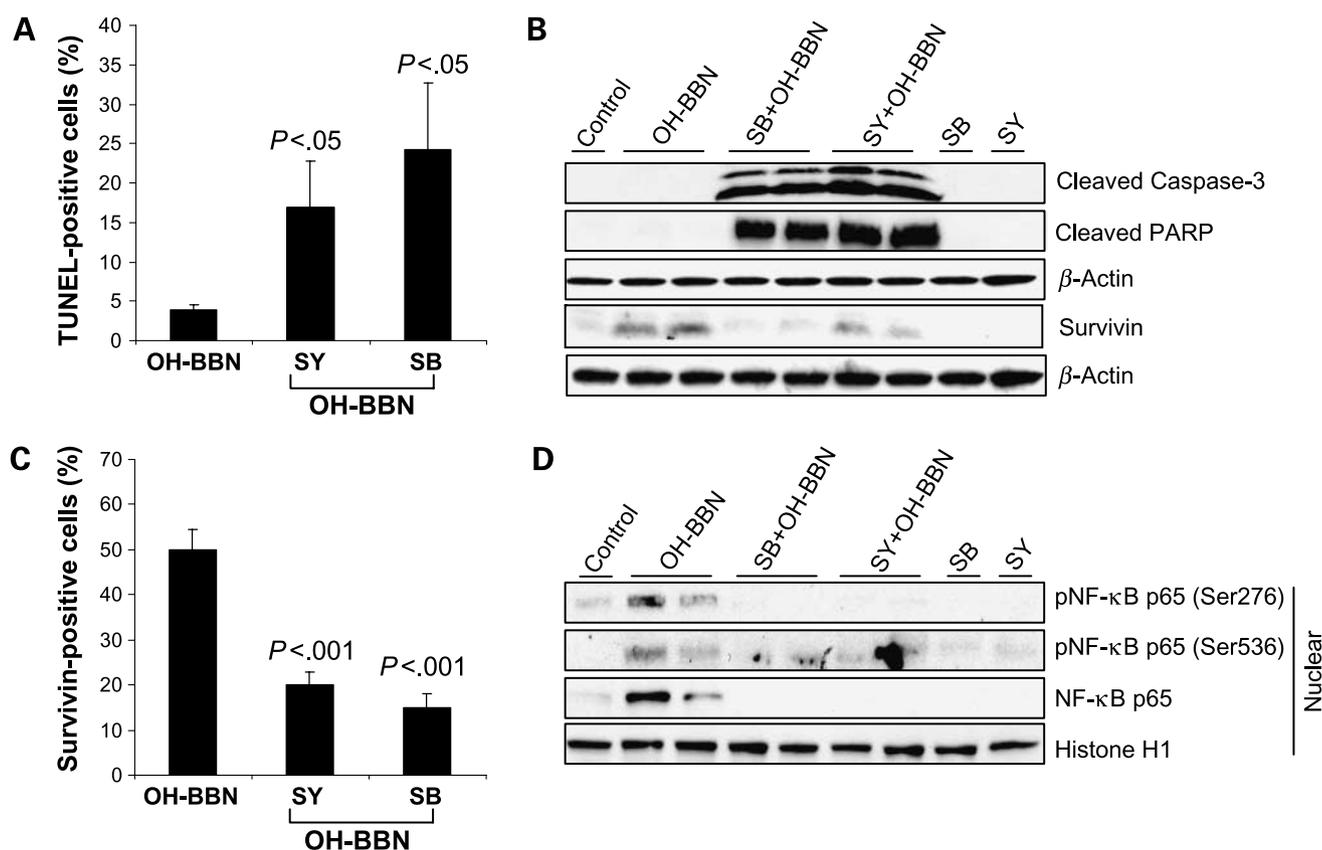
Figure 5. Effect of silymarin and silibinin on apoptosis and associated molecular events in OH-BBN–induced urothelium. Immunohistochemical and immunoblot analyses of urothelial tissue samples obtained from the experiment detailed in Fig. 1C were done to study the molecular biomarkers and events associated with apoptosis. **A,** apoptosis was analyzed by TUNEL staining in urothelium as detailed in Materials and Methods. The apoptotic index was determined as (number of TUNEL-positive cells × 100) / total number of cells counted. *Columns,* mean in each group; *bars,* SE. **B,** bladder tissue samples were randomly taken from each group and analyzed for cleaved caspase-3, cleaved poly(ADP-ribose) polymerase (*PARP*), and survivin protein levels by immunoblotting. Each membrane was stripped and reprobed with β-actin as loading control. **C,** immunohistochemical staining for survivin in urothelium was done as detailed in Materials and Methods. The survivin-positive cells were determined as [number of positively stained (brown) cells × 100] / total number of cells counted. *Columns,* mean in each group; *bars,* SE. **D,** nuclear extract was prepared from bladder tissue samples randomly taken from each group and analyzed for phosphorylated and total NF-κB p65 protein levels by immunoblotting. Membrane was stripped and reprobed with histone H1 as loading control.

silibinin + OH-BBN groups showed a strong decrease in the levels of cyclin D1 and phosphorylation of ERK1/2 compared with a strong induction of cyclin D1 protein level and ERK1/2 phosphorylation in OH-BBN group without any changes in total ERK1/2 levels (Fig. 4C). These observations suggest a role of ERK1/2 signaling leading to up-regulation of cyclin D1 and PCNA in OH-BBN–induced bladder carcinogenesis, and indicate that silymarin and silibinin could modulate these events for bladder cancer chemoprevention.

Silymarin and Silibinin Increase the Apoptotic Index in the Urothelium of OH-BBN–Treated Mice

In vivo apoptotic response of silymarin and silibinin feeding in OH-BBN–induced bladder tumorigenesis was investigated by TUNEL staining. Microscopic examination of the bladder tissue sections showed an increased number of TUNEL-positive cells in silymarin + OH-BBN and

silibinin + OH-BBN groups of mice as compared with OH-BBN group (Fig. 5A). The number of TUNEL-positive apoptotic cells was $17 \pm 6\%$ and $24 \pm 8\%$ in silymarin + OH-BBN and silibinin + OH-BBN groups, respectively, compared with $4 \pm 0.5\%$ in the OH-BBN group, accounting for ~4- and 6-fold increase ($P < 0.05$, for both) in the apoptotic index by these two agents, respectively. The immunoblot analysis of bladder tissue lysates also showed a strong reactivity for cleaved caspase-3 as well as cleaved poly(ADP-ribose) polymerase in silymarin + OH-BBN and silibinin + OH-BBN groups compared with OH-BBN alone (Fig. 5B).

Silymarin and Silibinin Decrease OH-BBN–Induced Survivin Expression and Activation of NF-κB p65 in Urothelium

The high levels of cleaved caspase-3 in the urothelium from silymarin + OH-BBN and silibinin + OH-BBN groups of mice also prompted us to analyze the levels of survivin in

urothelium because cleaved caspase-3 could also be directly modulated by interaction with survivin (16). Expression of survivin was observed in the bladder urothelium of all OH-BBN–treated mice. However, immunohistochemical analysis showed that there were less survivin-positive cells in silymarin + OH-BBN (20 ± 3%) and silibinin + OH-BBN (15 ± 3%) groups of mice as compared with OH-BBN alone (50 ± 4%), which accounted for 60% and 70% (*P* < 0.001, for both) decrease in survivin-positive cells (Fig. 5C). This result was further confirmed by immunoblot analysis of bladder tissue lysates, which also showed a strong decrease in the expression of survivin protein in both silymarin + OH-BBN and silibinin + OH-BBN groups of mice compared with OH-BBN group (Fig. 5B).

Next, we analyzed nuclear levels of NF-κB, which is associated with cellular proliferation and suppression of apoptosis (17, 18). The immunoblot analysis for nuclear phospho–NF-κB p65 (Ser^{276} and Ser^{536}) in urothelium showed a strong decrease in silymarin + OH-BBN and silibinin + OH-BBN groups as compared with OH-BBN group (Fig. 5D). Histone H1 probing of these blots, used as loading control, did not show any considerable change among different groups (Fig. 5D). NF-κB signaling can also target survivin expression, which is absent in most normal tissues and shows highly selective expression in cancers including bladder cancer (19). These observations suggest a potential role of NF-κB signaling and survivin in OH-BBN–induced bladder carcinogenesis in mice, which could be targeted for bladder cancer chemoprevention by silymarin and silibinin.

Discussion

The findings of the present study clearly show that silymarin as well as silibinin effectively inhibits urinary bladder carcinogenesis in mice initiated by OH-BBN by reducing the incidence of invasive bladder lesions; this was accompanied by *in vivo* antiproliferative and proapoptotic effects. For the first time, we also observed that silymarin and silibinin could inhibit *in vivo* ERK1/2 and NF-κB signaling in OH-BBN–induced bladder urothelium during bladder carcinogenesis as potential mechanisms to suppress tumor progression. Additionally, the levels of cyclin D1 and survivin were correlated with disease progression and could serve as potential molecular biomarkers for bladder cancer chemoprevention.

The carcinogenesis process in the OH-BBN–induced murine bladder model has been well studied (4). Bladder mucosa after OH-BBN administration undergoes several pathologic changes from normal urothelium through mucosal dysplasia, papillary/nodular dysplasia, and finally to invasive carcinoma (4). In the present study, mice fed with silymarin/silibinin beginning 7 days before OH-BBN administration and continued for 51 weeks exhibited arrested tumor progression at preinvasive lesions and a strongly decreased incidence of invasive lesions without any adverse health effects. Immunohistochemical and immunoblot analyses revealed the inhibition of OH-

BBN–activated urothelial cell proliferation by silymarin and silibinin. Further, these agents also induced apoptotic cell death in the urothelium of OH-BBN–treated mice. The observed strong incidence of dysplasia in both silymarin- and silibinin-fed groups of mice challenged with OH-BBN compared with OH-BBN alone further supports the most effective role of these agents in causing prevention of chemical carcinogen–induced bladder tumorigenesis in this mouse model specifically during tumor progression stage, which certainly has more clinical relevance. The observed alterations in the molecular pathways favoring the antiproliferative and proapoptotic effects of these agents in the urothelium at the end of the tumorigenesis experiment also support the notion that both silymarin and silibinin are more effective during tumor progression stage.

The genetic events such as carcinogen-induced DNA adducts and gene mutations, which affect cell proliferation and growth-regulating mechanisms, are required for tumorigenesis to occur *in vivo* (20). Increased numbers of proliferating cells in an organ would be expected to increase susceptibility to tumor induction (20). Cyclins are positive regulators of cell cycle progression and function by forming complexes and activating cyclin-dependent kinases, which are essential for cell cycle transition (21). Overexpression of cyclin D1 is observed in a variety of cancers, and antisense blocking of its action results in reversal of malignant cells (22, 23). Papillary/nodular dysplasia with extensive cyclin D1 overexpression is more likely to progress to advanced lesions, such as papillomas or carcinomas, in murine bladder carcinogenesis (13). In our study, immunohistochemical and immunoblot analyses for PCNA and cyclin D1 revealed overexpression of both these molecules in OH-BBN–induced bladder urothelium, which strongly decreased with both silymarin and silibinin treatments. Further, mitogenic signaling involving ERK1/2 activation is shown to regulate the expression of both these proteins in many normal as well as neoplastic cell types, and could also be causally linked to neoplastic transformation of normal cells (24, 25). In this regard, silymarin and silibinin decreased ERK1/2 signaling in OH-BBN–induced bladder urothelial tissue. Therefore, it is likely that both these agents inhibit ERK1/2 signaling, causing a decrease in cyclin D1 and PCNA expression thereby mediating their chemopreventive effects against chemically induced bladder carcinogenesis.

Survivin is overexpressed to inhibit apoptosis and promote cell proliferation in a wide variety of cancers including urinary bladder cancer (16, 26–28), and thus plays a key role in cancer progression. Because of the large difference in its expression between normal and malignant tissues and its role in cancer progression, survivin is currently undergoing intensive investigation as a potential tumor marker (28, 29). Survivin is a member of a class of inhibitors of apoptosis and seems to block apoptosis by interfering with the caspase activation pathway. Our studies have shown that silibinin down-regulates the expression of survivin in human bladder transitional-cell papilloma RT4 cells *in vitro* (10) and in tumor xenograft (30). In the present study, survivin was significantly less expressed in normal

bladder urothelium but highly expressed in OH-BBN–induced bladder urothelium with different stages of carcinogenesis, which was strongly decreased by silymarin or silibinin treatment. Both agents also increased apoptotic cells in urothelium, which was confirmed by strong levels of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase. These observations suggest that survivin is a potential target for silymarin and silibinin, which is consistent with their apoptotic effect in OH-BBN–induced bladder urothelium during the prevention of bladder carcinogenesis.

The transcription factor NF-κB has been linked with cell proliferation, invasion, angiogenesis, metastasis, suppression of apoptosis, and chemoresistance in multiple types of tumor (17). Therefore, the likely role of NF-κB signaling in OH-BBN–induced bladder carcinogenesis and its modulation by silymarin and silibinin were also investigated by us. The nuclear level of NF-κB p65 and its serine phosphorylation levels corresponding to NF-κB transcriptional activity were higher in OH-BBN–induced urothelium compared with normal urothelium. This observation suggests that an up-regulation of NF-κB signaling may play a role in carcinogen-induced bladder tumorigenesis and could be targeted by chemopreventive agents like silymarin and silibinin, as observed in the present study, to suppress neoplastic growth and progression of bladder cancer. Consistent with this, our results also showed that silymarin and silibinin decrease the expression of important NF-κB–regulated proteins *in vivo*, such as cyclin D1 and survivin, which are implicated in bladder carcinogenesis.

In summary, the findings in the present study show that p.o. feeding of silymarin and silibinin inhibits tumor growth and progression through different stages of dysplasia and carcinoma, as well as reduces the severity of the cancerous lesions in the chemically induced mouse bladder tumorigenesis model. These chemopreventive effects were accompanied by a decrease in cell proliferation and an increase in apoptosis involving down-regulation of ERK1/2 and NF-κB signaling, as well as decreased expression of cyclin D1 and survivin. Together, these pathologic and mechanistic insights for the chemopreventive efficacy of silymarin and silibinin suggest their potential use against human bladder cancer.

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