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Dietary Feeding of Silibinin Inhibits Advance Human Prostate Carcinoma Growth in Athymic Nude Mice and Increases Plasma Insulin-like Growth Factor-binding Protein-3 Levels[1]

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ABSTRACT

We have reported recently the anticancer effect of flavonoid antioxidant silymarin, the major part of milk thistle extract, against advanced human prostate carcinoma DU145 cells (X. Zi *et al.*, Cancer Res., *58:* 1920–1929, 1998) and later identified that silibinin is the main active component in silymarin responsible for its effect in cell culture studies. On the basis of these observations, here we assessed *in vivo* growth inhibitory potential of silibinin against advanced human prostate cancer (PCA). Dietary feeding of silibinin at 0.05 and 0.1% doses (w/w) for 60 days, 24 h after s.c. DU145 tumor xenograft implantation in athymic male nude mice, significantly inhibited tumor volume by 35 and 58% ($P < 0.05$), and wet weight of tumor by 29 and 40% ($P < 0.05$), respectively. In a second experiment where mice were fed with these test diets for 3 weeks before tumor xenograft implantation and continued on these diets for a total of 63 days, tumor volume and wet weight of tumor were reduced by 53–64% ($P < 0.001$–0.05) and 31–52% ($P < 0.05$), respectively. In both studies, animals did not show weight loss or reduced food consumption. These *in vivo* anticancer effects of silibinin were associated with an increased accumulation (up to 5.8 fold; $P < 0.05$) of human insulin-like growth factor-binding protein-3 in mouse plasma. In additional studies assessing biological availability of silibinin in nude mice and its antiproliferative activity at such doses in DU145 cells in culture, silibinin levels in plasma and prostate were found to be in the range of 7–13 μg/ml and 3.7–4.6 μg/g, respectively. At these biologically achievable silibinin concentrations, increased IGFBP-3 level in DU145 cell culture medium and a strong DU145 cell growth inhibition were observed that were irreversible in the absence of silibinin in culture medium. These findings extend and translate our observations on *in vitro* anticancer effect of silibinin/silymarin to an *in vivo* preclinical PCA model, which may form the basis for a Phase I clinical trial in PCA patients.

INTRODUCTION

Silibinin, a naturally occurring polyphenolic flavonoid, constitutes a major biologically active portion of the plant extract, milk thistle (*Silybum marianum*) that is widely consumed as a dietary supplement in the United States and Europe, accounting for ~8 million dollar sale in United States market in 1999 (1, 2). Silibinin and its crude form silymarin are clinically used to treat certain liver complications, and are devoid of any toxic effects even at acute or chronic administrations of the drug in both animal and human studies (2). Our cell culture as well as *in vivo* studies have established the antineoplastic effects of both silymarin and silibinin against different human carcinoma cells including PCA[3] (3–8). However, both efficacy and associated molecular mechanism of silibinin remain to be established in *in vivo* preclinical PCA models. In the present study, we investigated the *in vivo* growth inhibitory efficacy of silibinin against advance human prostate carcinoma DU145 tumor xenografts in nude mice.

Growth factors and associated receptors have been implicated as causative epigenetic events that stimulate proliferation and enhance the possibility of malignant transformation of epithelial cells (Ref. 9 and references therein). For example, several studies indicate that mitogenic/cell survival activity of IGFs are tightly controlled by the presence of IGFBPs and influenced by the balance of these factors in cellular microenvironment (10). IGFBP-3, a high affinity major IGF-binding protein in plasma, exists in ternary complex with IGF-I or IGF-II and an acid-labile unit. In case of inhibition of mitogenic activity of IGFs, soluble IGFBP-3 has been shown to sequester IGFs and prevent their interaction with cell surface-associated IGFBPs and/or IGF receptors (11). Prostate-specific antigen has shown to decrease IGFBP-3 affinity for IGF and potentiate IGF action that significantly contributes to normal as well as malignant prostate growth (12). Although IGFBP-3 is a potent inhibitor of IGF activity, it also has IGF-independent antiproliferative effects on cell growth (13). The tumor suppressor p53-mediated IGFBP-3 expression has also been reported in apoptosis induction in response to cellular stress (13). It has been suggested that IGFBP-3 may serve to protect against potentially carcinogenic effects of growth hormones and IGFs (13, 14). Furthermore, it is important to emphasize here that in recent studies, IGFs/IGFBP-3 plasma levels are being monitored as a potential end point surrogate biomarker for PCA risk (14), and that recently, we showed that silibinin up-regulates IGFBP-3 expression and inhibits proliferation of human prostate carcinoma PC-3 cells (15).

Taken together, based on above studies, we reasoned that serum level of IGFBP-3 might be one of the useful surrogate end point biomarkers to be evaluated as a potential mechanism of inhibitory efficacy of silibinin against advance human PCA growth in nude mice. Overall, present study addressed the questions whether: (*a*) silibinin prevents or inhibits the growth of advance prostate carcinoma *in vivo*; (*b*) its *in vivo* anticancer effect is correlated with up-regulation of IGFBP-3; (*c*) it is toxic in long-term animal studies; and (*d*) pharmacologically achievable concentrations have any biological significance.

MATERIALS AND METHODS

Cell Line and Reagents. DU145 human prostate carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 (Life Technologies, Inc.) with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 95% air and 5% CO_2 atmosphere. DU145 cells grown as mono-

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[3] The abbreviations used are: PCA, prostate cancer; IGF, insulin-like growth factor; IGFBP-3, insulin-like growth factor-binding protein 3; HPLC, high performance liquid chromatography.

layer were harvested by brief incubation with 0.25% trypsin-EDTA solution (Life Technologies, Inc.) and used for xenograft implantation in nude mice.

Animals and Diet. Athymic (*nu/nu*) male nude mice were obtained from the National Cancer Institute (Bethesda, MD) and housed in our nude mice animal care facility at standard laboratory conditions (in laminar airflow cabinets under pathogen-free conditions with a 12 h light/12 h dark schedule) and fed autoclaved Harlan Teklad Sterilizable rodent diet (W) and water *ad libitum*. For all of the animal studies, γ-irradiated sterile AIN-93M purified rodent diet pellets containing no silibinin (control diet), 0.05% (w/w) silibinin (test diet 1), or 0.1% (w/w) silibinin (test diet 2), were used. These diets were prepared commercially by Dyets Inc., Bethlehem, PA. Silibinin used in the diet was from Sigma Co., and its purity was checked as 100% as described recently (16).

Experimental Design for Tumor Xenograft Study. To determine the effect of silibinin on prostate tumor growth, DU145 tumors were grown s.c. in nude mice. Nude mice were randomly divided into two sets of experiments, each having 30 animals divided into three groups. In first set of experiment, ~2 million DU145 cells were suspended in 0.05 ml of medium and mixed with 0.05 ml of Matrigel brand (basement membrane matrix; Collaborative Biomedical Products, Bedford, MA), and were s.c. injected in the right flank of each mouse to initiate tumor growth. After 24 h, mice were transferred from regular diet to AIN-93M purified diet (group I, control diet; group II, 0.05% silibinin diet; and group III, 0.1% silibinin diet) for 60 days. In the second set of experiments, mice in three groups were fed with AIN-93M purified diet (group I, control diet; group II, 0.05% silibinin diet; and group III, 0.1% silibinin diet) for 3 weeks and then implanted with DU145 prostate carcinoma cells as described above. The defined diet feeding in three groups continued for an additional 6 weeks. In both of the experiments, food consumption and animal body weight were monitored twice weekly throughout the study. Once the tumor xenografts started growing, their sizes were measured twice weekly in two dimensions. The tumor volume was calculated by the formula: 0.5236 $L_1(L_2)^2$, where L_1 is long diameter, and L_2 is short diameter. At the termination of these studies, tumors were excised and weighed to record wet tumor weight in each case.

IGFBP-3 Assay in Plasma and Cell Culture Medium. At the termination of tumor experiments, blood was collected intracardiacally from mice in heparinized tubes, and plasma was separated for IGFBP-3 (as well as silibinin) analysis. Quantikine human IGFBP-3 immunoassay kit from R & D Systems, Inc. (Minneapolis, MN) was used to determine the IGFBP-3 concentration secreted from DU145 tumors in mouse plasma following step-by-step manufacturer's protocol. The principle of the assay was based on quantitative sandwich enzyme immunoassay using precoated monoclonal antibody specific for IGFBP-3 onto a microplate for solid-phase ELISA. Briefly, 100 μl of plasma was used in the assay, and finally the absorbance of the developed color was determined using a microplate reader set to 450 nm with correction wavelength at 540 nm. IGFBP-3 concentration was extrapolated from the standard curve generated using recombinant human IGFBP-3 in the assay.

For the analysis of IGFBP-3 secreted from DU145 cells in culture medium, cells were grown at standard culture conditions in 10% fetal bovine serum-supplemented RPMI 1640. At 60–70% confluency, cells were treated either with DMSO (final concentration in all treatment groups 0.05% v/v) or 15, 30, and 50 μM silibinin in serum-free medium containing 1 mg/ml BSA and 50 μg/ml ascorbic acid for 24 and 48 h. At the end of treatment medium was harvested for IGFBP-3 assay as detailed above, and cells were collected and counted after a brief trypsinization. Final data are presented as $A_{450-540\ nm}$ for equal number of live cells (10^6) in each treatment group. We observed that known concentrations of recombinant human IGFBP-3 added in mouse plasma and cell culture medium yielded a linear standard curve similar to that generated in the present study, but efficiency of detection was ~40 and 60%, respectively; however, there was no cross-reactivity of mouse plasma alone toward human IGFBP-3 reagent used in the kit (data not shown). IGFBP-3 concentrations shown in the figures are without any extrapolation for the efficiency of the detection.

HPLC Analysis of Plasma and Prostate Samples for Silibinin Levels. At the end of tumor studies, all of the mice in each group were sacrificed and plasma samples harvested from the blood as detailed above, and stored at −80°C until additional processing. Prostate glands were also collected from all of the groups of mice, quickly weighed, and then stored at −80°C for silibinin analysis. Prostate (100 mg) samples from the mice in each group were thoroughly homogenized in 3 volumes of 50 mM Tris-HCl (pH 7.4) at 4°C using a Polytron PT-10 homogenizer (VWR Scientific, Plainfield, NJ). Homogenates were centrifuged at 1500 rpm for 5 min at 4°C, and resultant clear supernatants were used for the additional processing. Briefly, 100 μl of each sample (plasma or prostate tissue homogenate) was mixed with 10 μl of ascorbate-EDTA solution (20% ascorbic acid and 0.01% EDTA dissolved in a 0.4 M sodium phosphate buffer, final pH 3.6), and a mixture of β-glucuronidase (250 units) and sulfatase (20 units). The reaction mixture was then incubated at 37°C for 45 min and extracted twice with ethyl acetate. The combined ethyl acetate extracts were pooled and evaporated to dryness in a vacuum centrifuge concentrator. The residues obtained were redissolved in 20 μl of 10% acetonitrile aqueous solution and subjected to centrifugation. The resultant clear supernatants were brought to 1 ml in an aqueous solution of 2% acetonitrile and 46% methanol, and 100 μl injected on the HPLC system. A modified protocol described previously (16) was used for HPLC analysis.

The HPLC system used was a Beckman Gold System (Beckman-Coulter, Fullerton, CA) consisting of a 126 pump module, 508 auto sampler, and 166 UV/Vis detector with UV absorption measured at 288 nm. The isocratic mobile phase consisted of 46% methanol and 54% 100 mM sodium phosphate (pH 3.0), and had a flow rate of 0.75 ml/min. Separation was done on a Hypersil ODS C18 (3 μm) 150 mm × 4.6 mm column fitted with a Hypersil C18 guard cartridge (Alltech Associates Inc., Deerfield, IL). Resolution of the silibinin diastereomers was seen with retention times of 15.5 and 17.3 min. Final silibinin concentrations were calculated based on the sum of the area of each diastereomer peak and comparison to a standard curve. The standard curve was linear over a range of 15–5000 ng/ml, and the limit of detection was 15 ng/ml.

Study of Pharmacologically Achievable Doses of Silibinin on DU145 Cell Growth. DU145 cells were grown in RPMI 1640 supplemented with serum and antibiotics at standard culture condition as detailed above. Pharmacologically achievable silibinin concentration from animal study was in the range of 15–30 μM, and additionally a higher dose (50 μM) of silibinin was also used in the present study. In the first set of the experiment, cells at ~30% confluency were treated with either DMSO alone (0.05%, v/v) or 15, 30, and 50 μM silibinin (final concentration in medium) in fresh medium everyday for 7 days, and cells were harvested every 24 h after defined treatments and counted with a hemocytometer. In the second set of the experiment (a washout study) our aim was to assess the growth inhibitory nature (static/reversible/irreversible) of silibinin once it is removed from the medium after its treatment for certain time. In this experiment, cells were treated with the same doses of silibinin each day for 4 days and then treatment medium was replaced with fresh medium without silibinin at the end of the 4 days of treatment, and cells were harvested at days 5, 6, 7, and 8, and counted. In another subset of a similar experiment, cells were treated with the same doses of silibinin each day for 4 days and then treatment medium was replaced with fresh medium without silibinin at the end of the 4 days of treatment. However, in this case, fresh medium without silibinin was added each day from day 5 to 7, and cells were harvested at days 5, 6, 7, and 8, and counted. Each treatment and time point had two independent plates, and each sample was counted in duplicate. The experiment was repeated with similar results.

Statistical Analysis. The data were analyzed using the Jandel Scientific SigmaStat 2.0 software. For all of the measurements, one-way ANOVA followed by Turky test was used to assess the statistical significance of difference between control and silibinin-treated groups in animal and cell culture studies. A statistically significant difference was considered to be present at $P < 0.05$.

RESULTS

Silibinin Inhibits Human Prostate Carcinoma DU145 Tumor Xenograft Growth in Nude Mice. Consistent with our recent cell culture studies showing strong growth inhibition of DU145 cells by silibinin and silymarin, in the present study, dietary feeding of silibinin at 0.05% or 0.1% (w/w) dose to nude mice showed a dose-dependent inhibition of tumor xenograft growth in terms of tumor volume as well as wet weight of tumors. In the first set of the experiment, where dietary feeding of silibinin (0.05% or 0.1%) started 24 h after s.c. injection of DU145 cells, tumor volume was inhibited by 35 and 58% ($P < 0.05$; Fig. 1*A*), and wet weight of tumor was

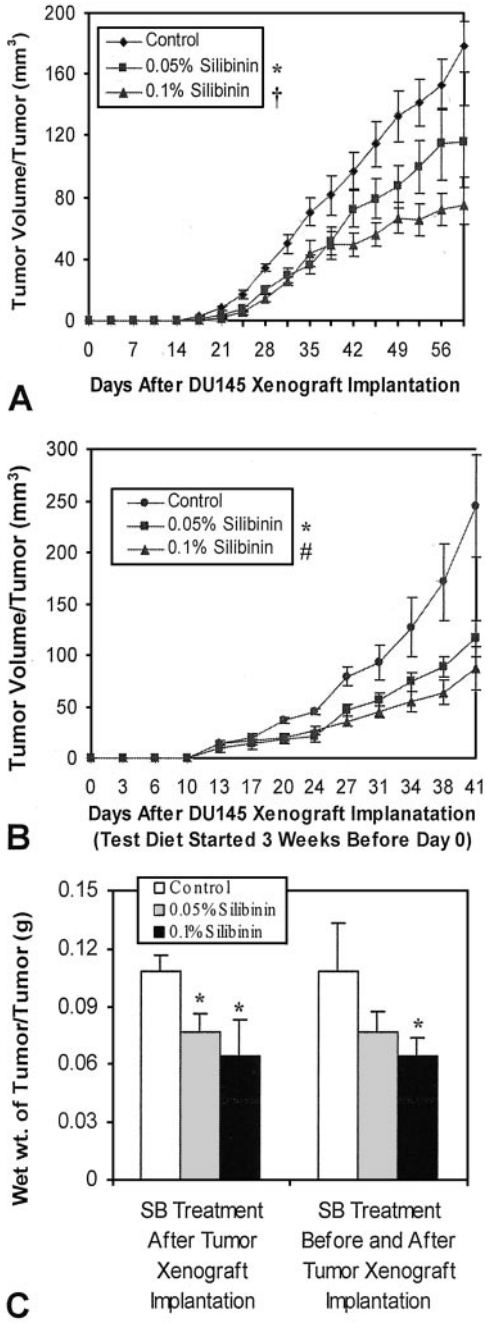

Fig. 1. Effect of dietary feeding of silibinin on DU145 tumor xenograft growth in athymic male nude mice. Approximately 2 million DU145 cells were s.c. injected in the right flank of each mouse to initiate ectopic prostate tumor growth as described in "Materials and Methods." *A*, dietary feeding of silibinin started after 24 h of tumor cells injection; *B*, dietary feeding of silibinin started before and after tumor cell injection. Mice were fed *ad libitum* with AIN-93M purified diet in three different groups as: group I, control diet; group II, 0.05% silibinin in diet; and group III, 0.1% silibinin in diet, for 60 days. Once tumor xenografts started growing, their sizes were measured twice weekly in two dimensions, throughout the study. The tumor volume was calculated by the formula: $0.5236\ L_1(L_2)^2$, where L_1 is long diameter and L_2 is the short diameter. Tumor volume (mm^3) is represented as mean of 8–10 mice in each group. *C*, effect on wet weight of the tumor. At the termination of the study, tumors were excised from each mouse in different groups and weighed. Wet weight of tumors is represented as mean of 8–10 tumors from individual mouse in each group. *SB*, silibinin; *, $P < 0.05$; #, $P < 0.01$; †, $P < 0.001$ at the termination of the experiments; *bars*, ± SE.

decreased by 29 and 40% ($P < 0.05$; Fig. 1*C*), respectively, at the end of experiment. In this experimental protocol, whereas initial effects of silibinin feeding were moderate and comparable at both dose levels, a clear dose-dependent inhibitory efficacy of silibinin started to appear around 6 weeks of feeding, which remained so up to the end of the study (Fig. 1*A*).

In the second set of the experiment we studied whether silibinin feeding before tumor implantation in mice has any additional inhibitory effect on the growth of advanced PCA tumor xenograft. In this study, where mice were first fed with the 0.05 or 0.1% silibinin-containing diet for 3 weeks before the implantation of DU145 cells and continued with same diet for a total 63 days, tumor volume was reduced by 53 and 64% ($P < 0.05$; Fig. 1*B*) and wet weight of tumor was decreased by 31 and 52% ($P < 0.05$; Fig. 1*C*), respectively, at the end of experiment. These results are indicative that silibinin has an additional growth inhibitory effect against PCA when mice were pretreated with the test compound before the implantation of tumor xenograft.

Dietary Consumption of Silibinin Does Not Show Any Apparent Sign of Toxicity in Nude Mice. In achieving chemopreventive prophylaxis or therapeutic effect of a compound under test, the toxicity and/or side effects are also important determinants to be assessed before any clinical trial in humans and subsequent use. Accordingly, in our nude mice tumor studies, we also assessed any toxicity and/or untoward effects of dietary silibinin feeding throughout the study. In this regard, dietary feeding of silibinin did not show any adverse effect to the mice as monitored by diet consumption, body weight gain, and prostate weight. As shown in Fig. 2, dietary feeding of silibinin at 0.05 or 0.1% (w/w) dose for 60–63 days did not show any reduction in food consumption (Fig. 2, *A* and *B*). When these food consumption levels were extrapolated with silibinin intake, on an average throughout the study, its consumption was found to be 1.78–1.79 and 3.29–3.84 mg/mouse/day (approximately equivalent to 65 and 130 mg/kg body weight) at 0.05 and 0.1% dose levels, respectively (Fig. 2*C*).

Body weight gain profile is considered as one of the parameters extensively used in cancer chemoprevention/therapy studies, which is closely associated with the adverse side effects of the drug. Consistent with the favorable effect of silibinin on food consumption in nude mice, silibinin did not show any decrease in body weight, and its dietary feeding did not affect the body weight gain profile when compared with control group of mice throughout the study (Fig. 3, *A* and *B*). Because our aim was to establish anticancer effect of silibinin against PCA, we also estimated the prostate weight at the termination of experiments to evaluate the adverse effect of the compound, if any, such as hypertrophy or hyperplasia. Our results did not show any increase in the prostate weight of the mice fed with dietary silibinin as compared with their control groups (Fig. 3*C*). In fact, silibinin feeding caused a slight decrease in prostate weight, although it was not significant in all of the treatment groups except at lower dose in first set of experiment (Fig. 3*C*).

Dietary Feeding of Silibinin Induces Human IGFBP-3 Level in Mouse Plasma. On the basis of our cell culture study where silibinin has shown an antiproliferative effect against androgen-independent human prostate carcinoma PC-3 cells, which was associated with up-regulation of IGFBP-3 (15), we also assessed whether the same mechanism is operative in *in vivo* condition in inhibiting DU145 tumor xenograft growth in nude mice. For this, using quantikine ELISA, we measured the human IGFBP-3 level in mice plasma secreted by DU145 tumor xenograft after silibinin treatment at the end of experiment. A standard curve was made using recombinant human IGFBP-3 (supplied with kit) in the assay to extrapolate the plasma level of IGFBP-3 (Fig. 4*A*). Dietary feeding of silibinin at 0.05 or 0.1% (w/w) dose for 60 days resulted in 2.33 ± 0.46 and 3.38 ± 0.92 ng IGFBP-3/ml plasma as compared with 0.58 ± 0.14 (ng/ml) in control group, respectively (Fig. 4*B*), which accounted for 4- and 5.8-fold ($P < 0.05$) increases as compared with control value, respectively. This observation provides the first evidence for an *in vivo*

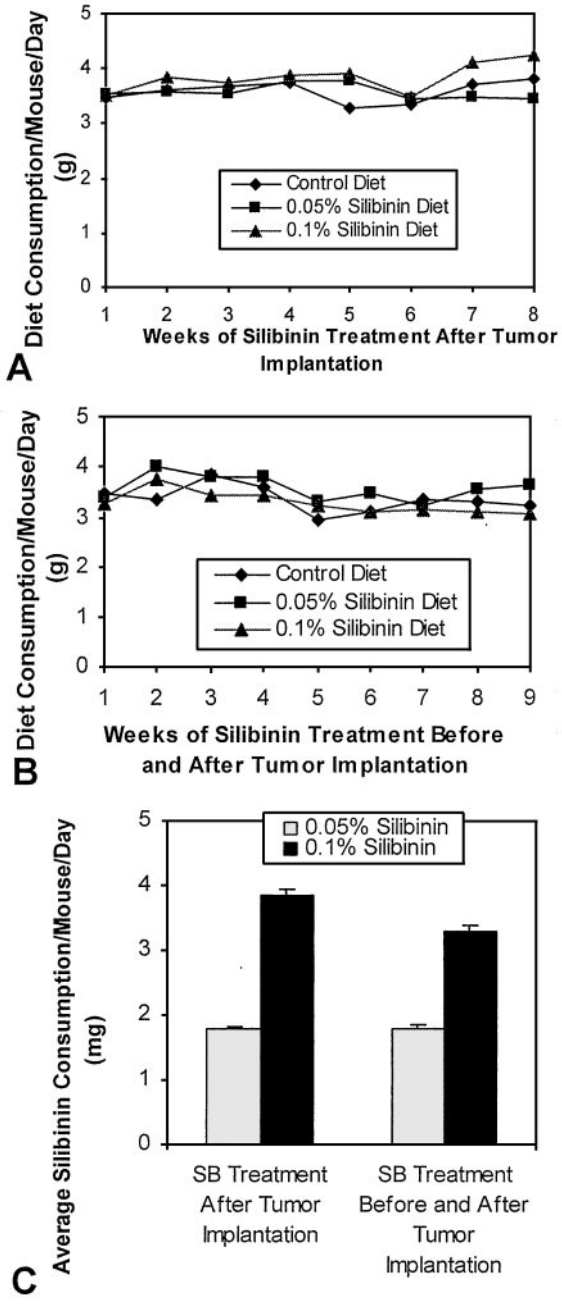

Fig. 2. Effect of silibinin on daily diet and corresponding silibinin consumption in nude mice during DU145 tumor xenograft studies. In the experiments detailed in Fig. 1, daily food intake was recorded throughout the feeding regimen in each group, and corresponding silibinin consumption levels were calculated. *A* and *B*, diet consumption/mouse/day (*g*) is plotted as a function of time (*week*) for 10 mice in each group from the experiments described in Fig. 1. *C*, silibinin consumption/mouse/day (*mg*) is extrapolated from the diet intake during total experimental duration in both sets of the experiment. *SB*, silibinin; *bars*, ± SE.

association between IGFBP-3 up-regulation and inhibition of PCA tumor xenograft growth by silibinin.

Inhibition of Tumor Xenograft Growth by Silibinin Is at Pharmacologically Achievable Doses Effective in Cell Culture. After establishing the anticancer effect of silibinin against human PCA tumor xenograft growth in nude mice, we next asked the question what are the pharmacological levels of silibinin in nude mice studies and whether such levels are effective in inhibiting DU145 cell growth in culture. To assess the levels of silibinin in plasma and prostate of the silibinin-fed nude mice, first a standard HPLC profile of silibinin was developed and its retention time was determined (data not shown). On the basis of this HPLC profile of silibinin, a linear detection range of silibinin was next established (data not shown). The silibinin concentrations in plasma and prostate were then calculated under the linear range of detection using area under curve of silibinin peak in HPLC profiles of these samples. As shown in Fig. 5*A*, compared with the control samples showing undetectable levels of silibinin, 0.05 or 0.1% silibinin feeding in diet (w/w), resulted in 7.1 ± 0.05 and 12.8 ± 0.09 μg silibinin/ml plasma (corresponding to 15 and 27 μM) in first, and 6.7 ± 0.3 and 10.2 ± 0.5 μg silibinin/ml plasma (corresponding to 14 and 21 μM) in the second set of tumor experiment, respectively (Fig. 5*A*). Because of this similar trend in dose-related bioavailability of silibinin in both protocols, the silibinin level in prostate was determine only in first set of tumor study. Both dietary doses of silibinin showed similar trend (as in plasma) in *in situ*

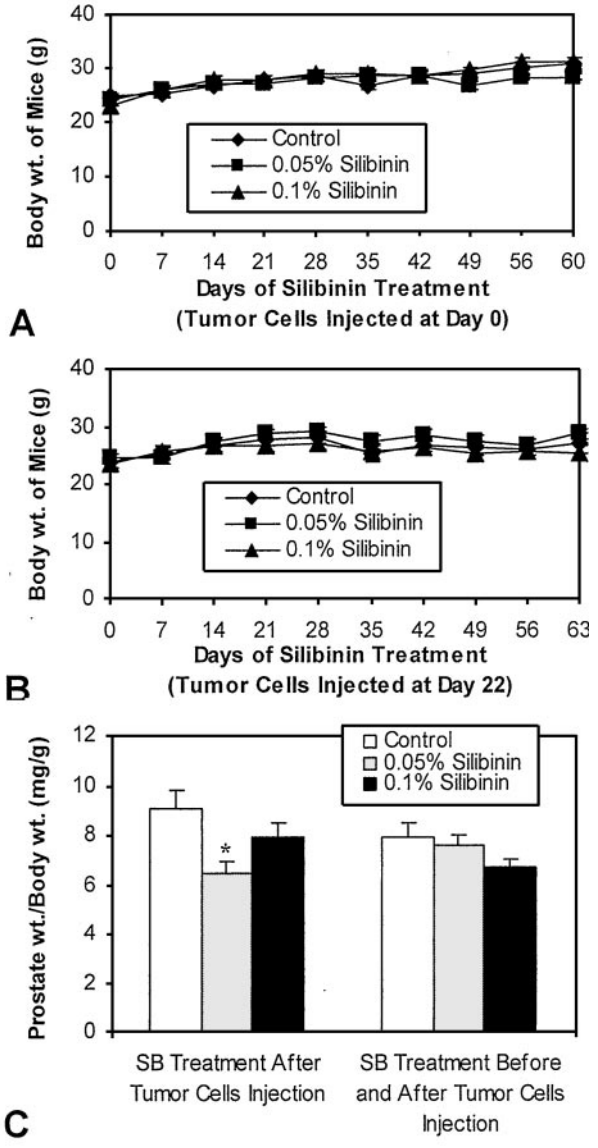

Fig. 3. Effect of dietary feeding of silibinin on body and prostate weights of nude mice during DU145 tumor xenograft study. In the experiments detailed in Fig. 1, body weight of each mouse in different groups was recorded twice a week throughout the experiment, and at the termination of the studies, prostate weight of each mouse in different groups was also recorded. *A* and *B*, body weights of mice are represented as mean of 10 mice in each group as a function of time in weeks from the experiments described in Fig. 1. *C*, prostate weight (*mg*) is represented as a ratio of body weight (*g*) of 10 individual mice from each group as mean in both sets of the experiment. *SB*, silibinin; ∗, $P < 0.05$; *bars*, ± SE.

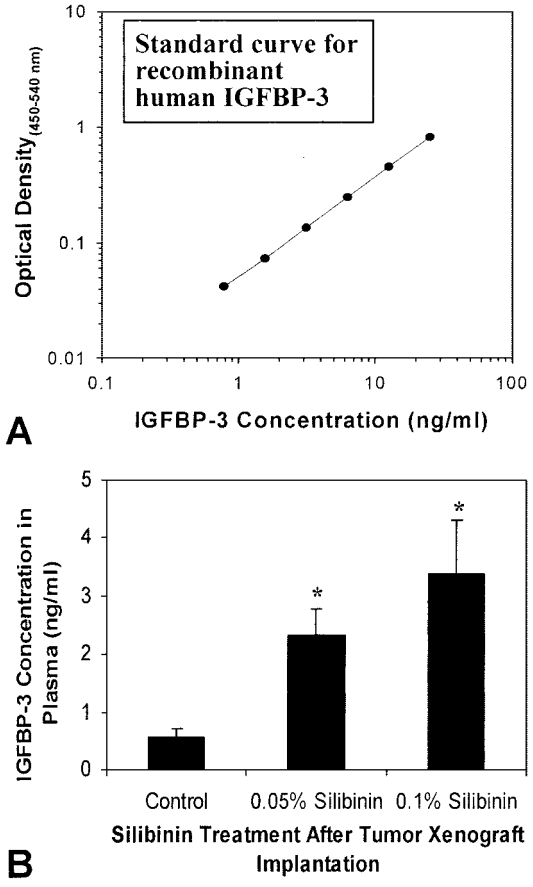

Fig. 4. Effect of dietary feeding of silibinin on plasma level of IGFBP-3 in nude mice DU145 tumor xenograft study. In the first set of experiment detailed in Fig. 1, at the termination of the study, plasma concentration of human IGFBP-3 was estimated using quantikine ELISA, as mentioned in "Materials and Methods." *A*, standard curve using recombinant human IGFBP-3 to extrapolate the plasma level of human IGFBP-3. Absorbance of the developed color in reaction was determined using a microplate reader set to 450 nm with correction wavelength at 540 nm, and a standard curve was established with known concentrations of IGFBP-3 at logarithmic scale. *B*, IGFBP-3 concentrations (ng/ml) in plasma samples were calculated by four-parameter logistic curve-fit generated standard curve and are represented as mean of 7–8 plasma samples from individual mouse in each treatment group. *, $P < 0.05$; *bars*, ± SE.

silibinin concentration in prostate accounting for 3.7 and 4.6 μg silibinin/g prostate tissue (corresponding to ~6 and 10 μmoles silibinin/g tissue), respectively (Fig. 5*A*).

The levels of silibinin achievable in the plasma after its dietary feeding were additionally assessed for their biological relevance in DU145 cell cultures. In this study, treatment of DU145 cells for 4 days with 15 and 30 μM doses of silibinin, which are approximately equivalent to 7.1 ± 0.05 and 12.8 ± 0.09 μg silibinin/ml plasma, or with a higher dose of silibinin (50 μM), resulted in 10–25, 29–57, and 55–80% cell growth inhibition at each dose level, respectively (Fig. 5*B*). Next, we asked the question of what happens to the silibinin-caused dose-dependent inhibition of DU145 cell growth when silibinin is removed from the medium after a certain time of treatment. To address this question, we did wash out experiments where after 4 days of silibinin treatments at above doses, cell growth was determined from 5–8 days during which time cells were either treated with fresh medium without silibinin everyday (Fig. 5*C*) or left in the fresh medium without silibinin for the rest of the days (data not shown). The results of these experiments showed that growth inhibition achieved at 4 days of silibinin treatment remained static during 5–8 days even in the absence of silibinin. This response remained consistent in both subsets of the experiments where growth inhibition rates were 25% in 15 μM silibinin dose, 57% in 30 μM silibinin dose, and 80–81% in 50 μM silibinin dose during 5–8 days of cell growth determination (Fig. 5*C*; data not shown).

Pharmacologically Achievable Doses of Silibinin Increases IGFBP-3 Level in DU145 Cell Culture Medium. Consistent with inducing effect of silibinin on IGFBP-3 secretion from DU145 tumors in nude mice study, pharmacologically achievable doses of silibinin (15 and 30 μM or a higher dose, 50 μM) resulted in significant increase in IGFBP-3 levels in DU145 cell culture medium. After 24 h of treatment, these silibinin doses increased IGFBP-3 level in medium by 1.1-, 1.3- ($P < 0.05$), and 1.4- ($P < 0.05$) fold as compared with control, respectively (Fig. 5*D*). Treatment with 15 and 30 μM of silibinin doses for 48 h resulted in 1.3- ($P < 0.05$) and 1.6- ($P < 0.01$) fold increase in IGFBP-3 levels secreted from cells in culture medium over that of control value (Fig. 5*D*).

DISCUSSION

Overall, the major findings of the present study are that (*a*) silibinin significantly inhibits advanced human prostate carcinoma growth in an *in vivo* preclinical PCA model, (*b*) which is accompanied by a strong increase in plasma level of human IGFBP-3, (*c*) without any apparent signs of toxicity in athymic nude mice; and that (*d*) plasma levels of silibinin in the nude mice study exert strong inhibition of DU145 cell growth in culture. The additional cancer chemopreventive effect of silibinin was also evident by the results in which mice were fed with silibinin for 3 weeks before the implantation of the tumor xenograft.

In recent years, naturally occurring plant products are getting increased attention toward the prevention and/or intervention of the (*a*) early stages of carcinogenesis and (*b*) neoplastic progression before invasive malignant disease occurs. On the basis of this idea, certain foods, including many vegetables, fruits, and grains, as well as phytochemicals of diversified pharmacological efficacy have been shown to offer significant protection against various cancers (17). Furthermore, there is an increased focus on providing scientific basis to use these agents in prevention strategy for the people at high risk for cancer. In this regard, our extensive investigations with silymarin/silibinin have shown promising results against different epithelial cancers including PCA (3–8). Furthermore, in the present study, we provide first *in vivo* evidence for the efficacy of silibinin against advanced prostate carcinoma growth in athymic nude mice. Translating the observed *in vivo* efficacy of silibinin in inhibiting PCA xenograft growth in nude mice to its practical implications in human PCA, it is important to emphasize here that the observed inhibitory effects of silibinin were not complete in either of the two studies. However, because the major task here is to control its growth even in terms of slowing it down, one could argue that the inhibition observed by silibinin is of significant merit in terms of moving forward with this agent and conduct additional preclinical studies in PCA models followed by clinical trials in human PCA patients.

Previous reports have shown that both silymarin and silibinin are devoid of any toxicity and untoward effects in animal studies, and that they are used clinically as antihepatotoxic agents (2) and consumed extensively as a dietary supplement around the world including the United States (1). Consistent with these reports, in our study, dietary feeding of silibinin (up to 0.1%, w/w) did not show any adverse effect on diet consumption, animal body weight gain, and prostate weight. This finding not only additionally support the notion that silibinin and silymarin, as well as the milk thistle extract (consumed as dietary supplement) that constitute them, are devoid of toxicity, but also suggests that the doses of silibinin higher than those used in the present study should be evaluated for their inhibitory effect on PCA

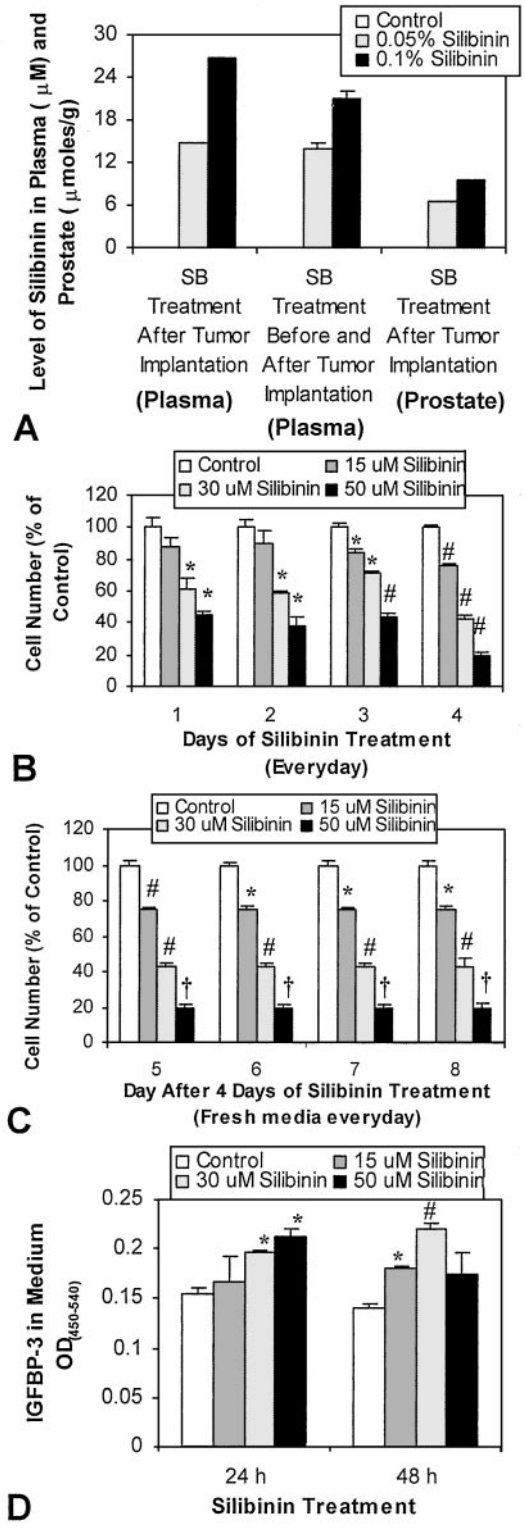

Fig. 5. Pharmacologically achievable silibinin concentrations in plasma and prostate, and their effect on DU145 cells growth and IGFBP-3 secretion in medium. *A,* silibinin levels in plasma (μM) and prostate (μmoles/g) were detected by HPLC analysis as described in "Materials and Methods." In each case, the data shown are mean of 8–10 plasma or prostate samples; the SE *bars* not evident in some cases are because those values were too small. *B* and *C,* DU145 cells were grown in RPMI 1640, and at ~30% confluency treated with either DMSO alone or 15, 30, and 50 μM silibinin (final concentration in medium) at standard culture condition as detailed in "Materials and Methods." *B,* in first set of experiments, cells were treated with fresh medium every day for 7 days, and cells were harvested 24 h after each treatment and counted with hemocytometer. *C,* in the second set of experiments (wash-out) cells were treated with same doses of silibinin each day for 4 days, and treatment medium was replaced with fresh medium without silibinin each day from day 5 to 7, and cells were harvested at days 5, 6, 7 and 8, and counted, as detailed in "Materials and Methods." Data shown are mean of two independent plates; each sample was counted in duplicate, and the experiment was repeated with similar results. *D,* DU145 cells were treated with DMSO alone or 15, 30, and 50 μM doses of silibinin (final concentration in medium) for 24 and 48 h, and medium was harvested for IGFBP-3 levels using quantikine ELISA as described in "Materials and Methods." Values are presented as mean of $A_{450-540\ nm}$ of duplicate samples for 10^6 live cells in each treatment group. *SB,* silibinin; *, $P < 0.05$; #, $P < 0.01$; †, $P < 0.001$; *bars,* ± SE.

growth. On the basis of a dose-dependent inhibitory effect of silibinin observed in the present study, there is a strong possibility that an additional increase in silibinin dose might achieve better and may complete PCA growth inhibition. Such studies might also help establish any toxicity and untoward effects associated with silibinin at higher doses, as well as maximum tolerated dose of silibinin, in long-term animal treatment protocols. In this regard, it is also important to emphasize here that depending on which Neutraceutical company's product is being used, on an average, the maximum suggested milk thistle extract consumption as a dietary supplement is somewhere between 0.5 and 2.0 g/day taken p.o. Extrapolating it on an average food intake of 2000 calories, these doses translate into 250-1000 ppm or 0.025–0.1% of silibinin, an equivalent dose range used in the present study. Accordingly, we suggest that a significantly strong PCA xenograft growth inhibitory effect of silibinin at 0.05 and 0.1% dietary dose levels without any noticeable toxicity, observed in the present study, could have a direct practical and translational relevance to human PCA patients. This suggestion could be specifically useful in the patients with androgen-independent PCA where most treatments fail and median survival time of the patient is reduced to less than a year. There is a possibility that in such cases chemopreventive agents such as silibinin could have immediate clinical importance in that they may prolong the survival and quality of life of the patient by preventing or retarding the progression of PCA.

Recent epidemiological studies have shown a close association between increasing plasma level of IGF-1 and PCA risk, as well as an inverse association with plasma IGFBP-3 levels (18). Recently, other studies have also demonstrated that higher circulating IGF-1 level and/or lower IGFBP-3 level are strongly and positively correlated with increased risk of PCA, and could be an excellent determinant of PCA progression in humans (14, 19). The importance of IGF-1 signaling and IGFBPs in deregulated cellular growth has also been established in prostate carcinoma cells and transgenic mice (20). It has also been reported that the antiapoptotic signaling is constitutively active, which in turn up-regulates phosphatidylinositol 3′-kinase activity in cancerous prostate epithelial cells (21). Together, these studies suggested that a mitogenic as well as cell survival (antiapoptotic) signaling via IGFBP-IGF-1/IGF-1R pathway is constitutively activated in human PCA cells, and that it is in part responsible for the growth and metastatic potential of PCA at an advanced and androgen-independent stage of this malignancy. This suggestion is also consistent with a series of studies in recent years showing that receptor tyrosine kinases and associated growth factors that interact in an autocrine manner to provide androgen-independent growth advantage to human PCA cells as well as cancer tissue are constitutively active (9, 22). Consistent with these studies, in a recent report we showed that one of the mechanisms of silibinin's antiproliferative effect in advanced and androgen-independent human PCA PC-3 cells is the strong induction of IGFBP-3 levels (15). In the present study, we additionally extended this cell culture observation to assess whether the observed *in vivo* PCA xenograft inhibitory efficacy of silibinin in nude mice is associated with an induction of IGFBP-3 levels in plasma. Indeed, we found this to be the case suggesting that the inhibition of IGF-1R signaling by a strong induction in IGFBP-3 might be one of the major *in vivo* mechanisms of silibinin-caused

PCA xenograft growth inhibition in nude mice. To support this conclusion additional studies are needed to trace the effect of silibinin on IGF-1-IGF-1R-phosphatidylinositol 3′-kinase signaling under *in vivo* condition. Additionally, IGF-1-independent association between PCA risk and IGFBP-3 level has also been observed where IGFBP-3 has been shown to induce apoptosis independent of its IGF-sequestering action (23). Whether, *in vivo* inhibitory effect of silibinin on PCA xenograft growth is in part via such a mechanism also remains to be established. However, based on the data of the present study, we suggest that in a silibinin chemoprevention trial in human PCA patients, the circulating level of IGFBP-3 in plasma may serve as a prognostic biomarker as a surrogate measure of success of silibinin treatment. In such studies, the physiological manifestation of IGFBP-3 in a population seems to be an important target in correlating increased/decreased incidence of PCA.

One important component in cancer chemoprevention research that is receiving increased attention is what the pharmacological levels are of the agent being tested and whether such levels have any biological efficacy. Accordingly, one of our other aims in this study was to address this issue. First we determined the levels of silibinin in both plasma and prostate tissue samples from both of the tumor experiments, and then evaluated the biological significance of plasma concentrations of silibinin in DU145 cells in culture. The pharmacologically achievable levels (15–30 μM) of silibinin showed dose- as well as time-dependent inhibition of DU145 cells growth in culture. Furthermore, the wash out experiments, where silibinin was removed from the medium after 4 days of treatment, did not show any change in the rate of cell proliferation as compared with control. This result suggested that the antineoplastic effect of silibinin is static and that after its removal from the medium, it does not exceed the deregulated PCA cell proliferation when compared with control. This observation also indicated that it could be mostly the epigenetic cell signaling events that are being modulated by silibinin in DU145 cells to produce its antiproliferative effect against these cancer cells; a possibility supported by several studies in recent years by us in PCA cell culture (3–8, 15, 22).

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