A Novel Dietary Triterpene Lupeol Induces Fas-Mediated Apoptotic Death of Androgen-Sensitive Prostate Cancer Cells and Inhibits Tumor Growth in a Xenograft Model

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Abstract

In prostate cancer, a fine balance between cell proliferation and apoptotic death is lost, resulting in increased cellular mass and tumor progression. One approach to redress this imbalance and control this malignancy is its preventive intervention through the use of dietary natural agents. Here, we investigated the growth-inhibitory effect and associated mechanisms of Lupeol, a triterpene present in fruits and vegetables, in androgen-sensitive human prostate cancer cells. Lupeol treatment resulted in significant inhibition of cell viability in a dose-dependent manner and caused apoptotic death of prostate cancer cells. Lupeol was found to induce the cleavage of poly(ADP-ribose) polymerase protein and degradation of acinus protein with a significant increase in the expression of FADD protein. Among all death receptor targets examined, Lupeol specifically caused a significant increase in the expression of Fas receptor. The small interfering RNA–mediated silencing of the Fas gene and inhibition of caspase-6, caspase-8, and caspase-9 by their specific inhibitors confirmed that Lupeol specifically activates the Fas receptor–mediated apoptotic pathway in androgen-sensitive prostate cancer cells. The treatment of cells with a combination of anti-Fas monoclonal antibody and Lupeol resulted in higher cell death compared with the additive effect of the two compounds alone, suggesting a synergistic effect. Lupeol treatment resulted in a significant inhibition in growth of tumors with concomitant reduction in prostate-specific antigen secretion in athymic nude mice implanted with CWR22Rv1 cells. Because early clinical prostate cancer growth is an androgen-dependent response, the results of the present study suggest that Lupeol may have a potential to be an effective agent against prostate cancer. (Cancer Res 2005; 65(23): 11203-13)

Introduction

Prostate cancer is the most common invasive malignancy in males in the United States. It is estimated that 323,090 new prostate cancer cases will be diagnosed, and ~30,350 prostate cancer–related deaths are predicted during the year 2005 alone in the United States (1). Alterations in the molecules regulating apoptotic pathways have been implicated to play a key role in human prostate cancer development (2). Compelling evidence accumulated in recent years suggest that the tumorigenic growth of the prostate gland depends on the evasion of the normal homeostatic control mechanisms, where cell proliferation exceeds cell death (2). This has led to the identification of the apoptotic profile of prostate cancer cells as an important feature of tumor pathology that may influence prognosis, therapeutic response, and disease progression. The therapeutic significance of apoptosis in the treatment of prostate cancer stems from evidence showing that prostate cancer cells in androgen-dependent tumors undergo apoptosis in response to androgen deprivation (3, 4).

In recent years, there is an intense activity to identify effective therapeutic modalities and novel preventive approaches for prostate cancer. In the recent past, agents obtained from herbs and plants have gained considerable attention for the prevention and/or treatment of certain cancer types, including prostate cancer (5–7). The in vitro and in vivo studies have shown that several chemopreventive agents found in dietary plants, such as green tea, activate cell death signals and induce apoptosis in precancerous or cancer cells, including prostate cancer cells, resulting in the inhibition of cancer development and/or progression (8–13). Epidemiologic and case-control studies suggest that these preclinical cell culture and animal data may have relevance to prostate cancer patients (14, 15). Many other dietary agents in cell culture and animal studies are showing promise against prostate cancer (16, 17).

Lupeol (20R,28R)-29,30,35-trihydroxy-3,24,26-tri-O-methyl-24,28,29,30,35-pentahydroxyolean-28-en-3β-ol (Lupeol; Fig. 1), a triterpene found in fruits (such as olive, mango, strawberry, grapes, and figs), in many vegetables, and in several medicinal plants, is used in the treatment of various ailments worldwide by native people (18–20). Lupeol possesses strong antioxidant, anti-inflammatory, antiarthritic, antimutagenic, and antimalarial activity in vitro and in vivo systems; acts as a potent inhibitor of protein kinases and serine proteases; and inhibits the activity of DNA topoisomerase II, a target for anticancer chemotherapy (21–27). It has also been shown that Lupeol induces differentiation and inhibits the cell growth of mouse melanoma and human leukemia cells (28, 29). Recently, we have shown that Lupeol exhibits significant antitumor-promoting activity in a two-stage model of mouse skin carcinogenesis (30).

In view of the wide ranging pharmacologic activities of Lupeol, here, we show that Lupeol activates Fas receptor–mediated apoptotic machinery of LNCaP cells and inhibits the tumorigenesis of prostate cancer cells in an animal model. Because early clinical prostate cancer growth is an androgen-dependent response, we suggest that Lupeol has potential to be an effective agent against androgen-dependent human prostate cancer.

Materials and Methods

Cell culture. Human prostate epithelial cells (PrEC) and PrEC medium were obtained from Cambrex Bioscience (Walkersville, MD). Androgen-sensitive human prostate cancer cells LNCaP and CWR22Rv1 and fetal...
bovine serum (FBS) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s MEM supplemented with 10% FBS supplemented with 1% penicillin-streptomycin (Cellgro Mediatech, Inc., Herndon, VA).

**Treatment of cells.** A stock solution of Lupeol (30 mmol/L) was prepared by dissolving it in warm alcohol and diluted in DMSO in a 1:1 ratio. For dose-dependent studies, the cells (50% confluent) were treated with Lupeol (1-30 μmol/L) for 48 hours in complete cell medium. The final concentrations of DMSO and alcohol were 0.25% and 0.075%, respectively, in all treatment protocols. After 48 hours of treatment with Lupeol, the cells were harvested, and cell lysates were prepared and stored at −80°C for later use.

**Cell viability assay.** The effect of Lupeol on the viability of PrEC, LNCaP, and CWR22Rv1 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were plated at 1 × 10^4 per well in 200 μL of complete culture medium and treated with 1 to 30 μmol/L concentrations of Lupeol in 96-well microtiter plates. After incubation for 48 hours at 37°C in a humidified incubator, cell viability was determined. MTT (5 mg/mL in PBS) was added to each well and incubated for 2 hours, after which the plate was centrifuged at 1,800 rpm for 5 minutes at 4°C. After careful removal of the medium, 0.1 mL of buffered DMSO was added to each well, and plates were shaken. The absorbance was recorded at the wavelength of 540 nm. The effect of Lupeol on growth inhibition was assessed as percent cell viability where vehicle-treated cells were taken as 100% viable.

**Western blot analysis.** The total cell lysates were prepared, and Western blot analysis was done as described earlier (12, 30).

**Detection of apoptosis and necrosis by fluorescence microscopy.** The Annexin V-FLUOS apoptosis detection kit (Roche Applied Science, Indianapolis, IN) was used for the detection of apoptotic and necrotic cells. Briefly, prostate cancer cells were grown to about 50% confluence on slides. After incubating cells with Lupeol (1-30 μmol/L) for 48 hours, cells were processed for labeling with fluorescein-tagged dUTP nucleotide and propidium iodide by use of an APO-DIRECT apoptosis kit (Phoenix Flow Systems, San Diego, CA). The labeled cells were then analyzed by flow cytometry.

**Treatment of cells with anti-Fas antibodies.** To evaluate the combined effect anti-Fas monoclonal antibody (mAb) and Lupeol, cells were treated with different concentrations of anti-Fas mAb followed by treatment with low dose of Lupeol (20 μmol/L). Cells were harvested for viability assay and cell lysate preparation. The treatment of cells was designed in a way that cells would be treated with Lupeol either 1 hour after mAb treatment (pretreatment) or 0 hour (cotreatment) after antibody treatment.

**Small interfering RNA–mediated Fas gene silencing in LNCaP cells.** The small interfering RNA (siRNA) against Fas was procured from Qiagen (Germantown, MD). The sequence of selected region to be targeted by siRNA for Fas was AAGACCTGTGTGCAGCATTTA (404-424 nucleotide region), and a nonsilencing siRNA with a target region AATTCTCC- GAACGTGTCTCGT was used as a negative control. Transfection was done using Dharmafect Transfection kit (Dharmacon, Lafayette, CO) according to the manufacturer’s protocol. Briefly, LNCaP cells were transfected with 50 nmol/L of siRNAs directed against the Fas and a nonsilencing siRNA and incubated for 6 hours. All siRNAs were labeled with a fluorescent tag to confirm the transfection. After incubation, the cells were washed with PBS and treated with Lupeol (30 μmol/L for LNCaP cells and 20 μmol/L for CWR22Rv1 cells) or anti-Fas mAb (1 μg/mL) or combination in fresh medium follows by an incubation of 48 hours. After 48 hours, cells were harvested and processed for cell lysate preparation.

**Inhibition of caspases by specific inhibitors.** Specific inhibitors of caspase-8 and caspase-6 (Ac-AEVD-CHO) and caspase-9 (Ac-LEHD-CHO) were procured from Axcora Corp. (San Diego, CA). LNCaP cells were incubated with caspase inhibitors (100 μmol/L) for 1 hour, washed with PBS, and treated with Lupeol for 48 hours, and cell viability was determined. In addition, effect of Lupeol on Fas-silenced cells pretreated with caspase inhibitors was also examined.

**Tumorigenicity studies in athymic nude mice.** For these studies, we employed CWR22Rv1 cells because they develop rapid tumors with high secretion of prostate-specific antigen (PSA) in the host. A total of 1 × 10^6 cells suspended in 50 μL of media and 50 μL of Matrigel (BD Biosciences,}

**Figure 1.** Effect of Lupeol on viability of normal human PrEC and androgen-sensitive prostate cancer cells (LNCaP and CWR22Rv1). Cells were treated with specified concentrations of Lupeol for 48 hours, and cell viability was determined by MTT assay. Each concentration of Lupeol (1-30 μmol/L) was repeated in 10 wells. Percent viable cells, where vehicle-treated cells were regarded as 100% viable. Points, mean of percent viable cells of three independent experiments; bars, SE. C and V, control and vehicle-treated (alcohol + DMSO) cells, respectively. Details are described in Materials and Methods. Inset, structure of Lupeol, a triterpene found in fruits and vegetables.
Bedford, MA) were inoculated s.c. into the right flank of sixteen 6-week-old mice by using a 27-gauge needle and divided into two groups. Whereas one group of animals received an i.p. injection of Lupeol (1 mg/animal) in 0.2 mL of corn oil, the second group received corn oil alone and served as the control group. These treatments were repeated thrice a week. Every week, tumor growth was estimated in terms of volume of tumors (mm$^3$) as a function of time (days).

Prostate-specific antigen levels in serum of athymic nude mice. To measure the concentration of human PSA in the blood of mice with xenografts, 0.2 mL of whole blood was drawn from mandibular vein. After centrifuging to separate the serum from the blood cells, the serum samples were analyzed for PSA using an ELISA kit as per manufacturer’s protocol (Anogen, Mississauga, Ontario, Canada).

Statistical analyses. Student’s $t$ test for independent analysis was applied to evaluate differences between treated and untreated cells with respect to the expression of various proteins. A Kaplan-Meier survival analysis with the corresponding log-rank analysis and a linear regression analysis was used to measure the rate of mean tumor volume growth as a function of time using S-plus Software (Insightful, Seattle, WA). $P < 0.05$ was considered statistically significant.

![Figure 2](https://example.com/figure2.png)

Figure 2. Lupeol induces apoptosis in LNCaP cells as assessed by (A) fluorescence microscopy and (B) flow cytometry. A, representative micrographs of LNCaP cells undergoing apoptosis induced by treatment with Lupeol as assessed by fluorescence microscopy. Cells were treated with vehicle alone or specified concentration of Lupeol for 48 hours. Apoptosis and necrosis was detected by a Zeiss Axiovert 100 microscope. Samples were excited at 330 to 380 nm, and the image was observed and photographed under a combination of a 400-nm dichoric mirror and then 420-nm high-pass filter. Cell populations that potentially are detected are as follows: cells in the metabolically active stages (green), necrotic cells (red), and cells undergoing late-stage apoptosis (both green and red). B, quantitative representation of Lupeol-induced apoptosis in LNCaP cells as assessed by flow cytometry. Cells were treated with Lupeol (1-30 μmol/L for 48 hours), labeled with dUTP using terminal deoxynucleotide transferase and propidium iodide using an apoptosis. Cells showing dUTP fluorescence above that of control population (line in each histogram) are considered as apoptotic cells. Representative of three independent experiments with similar results. Percent apoptotic cells. Details are described in Materials and Methods.
Results

Effect of Lupeol on cell viability of PrEC, LNCaP, and CWR22Rv1 cells. The treatment of PrEC cells with Lupeol (1-30 μmol/L) for 48 hours was without significant effect on cell viability (Fig. 1). However, treatment of LNCaP and CWR22Rv1 cells with similar doses of Lupeol, in a dose-dependent manner, significantly decreased cell viability (Fig. 1). The IC₅₀ for Lupeol was estimated to be 21 and 18.5 μmol/L for LNCaP and CWR22Rv1 cells, respectively. Based on these observations, we selected a dose of 1 to 30 μmol/L and a time period of 48 hours after Lupeol treatment for further mechanistic studies.

Lupeol induces apoptosis in LNCaP cells. We next determined whether Lupeol-mediated inhibition of cell viability in prostate cancer cells is a result of induction of apoptosis. The induction of apoptosis of LNCaP cells by Lupeol (1-30 μmol/L) was evident from the morphology of cells as assessed by fluorescence microscopy (Fig. 2A). As shown by representative pictures (Fig. 2A), Lupeol treatment resulted in induction of apoptosis in a dose-dependent manner. These data show that Lupeol treatment also resulted in necrosis of these cells, which may be a secondary event in the apoptotic process. We next quantified the extent of apoptosis by flow cytometric analysis of Lupeol-treated cells. As shown by the data in Fig. 2B, compared with control, Lupeol treatment resulted in 1.4%, 1.5%, 4%, 16%, and 37% TUNEL-positive cells at 1, 5, 10, 20, and 30 μmol/L, respectively. Consistent with the fluorescence microscopy data, TUNEL assay by flow cytometry revealed that treatment of LNCaP cells with Lupeol resulted in a dose-dependent induction of apoptosis.

Effect of Lupeol on the expression of poly(ADP-ribose) polymerase and acinus proteins in LNCaP cells. Several proteins, including poly(ADP-ribose) polymerase (PARP) and acinus, play an important role in the condensation and degradation of chromatin of the cells going through the apoptotic death (31). Therefore, the cleavage of PARP and acinus proteins is considered as an important biomarker of apoptosis. Next, we measured the cleavage of PARP protein and the expression of acinus protein in LNCaP cells pretreated with Lupeol. Lupeol treatment of cells caused a significant degradation of PARP¹⁶ in cells in a dose-dependent manner (Fig. 3A). Similarly, a significant amount of the cleaved product detected as PARP²⁸ was found in cells treated with Lupeol (Fig. 3A). Next, we measured the cleavage of acinus protein that was measured in terms of loss of the expression of inactive acinus. The expression of inactive acinus protein was observed to be significantly diminished in Lupeol-treated cells (Fig. 3A). These data suggest that Lupeol induces apoptosis of prostate cancer cells by activating PARP and acinus cleavage, resulting in the degradation of cellular chromatin.

Effect of Lupeol on the expression of mitochondria-dependent apoptotic proteins. To determine the pathway adopted by Lupeol to cause apoptosis of LNCaP cells, we first measured the expression of mitochondria-associated proteins (i.e., Bcl-2 and Bax), known to play a role in apoptosis process. As shown in Fig. 3A, the treatment of cells with Lupeol did not cause any significant change in the expression of Bcl-2 and Bax (Fig. 3A). Caspases play an important role as mediators of apoptotic signals from upstream molecules, and their activation is considered as a hallmark of apoptosis, although for some agents, induction of apoptosis is also mediated via caspase-independent pathways (32). We found that treatment of cells with Lupeol resulted in a significant decrease in the expression of procaspase-6, procaspase-8, and procaspase-9; however, no change was observed in the status of procaspase-3 protein (Fig. 3A). These results suggest that Lupeol-induced apoptosis of prostate cancer cells is mediated through caspase-8 and caspase-9 and is executed through caspase-6.

Effect of Lupeol on viability of prostate cancer cells pretreated with caspase inhibitors. To confirm further that caspase-8, caspase-9, and caspase-6 are involved in Lupeol-induced apoptosis, prostate cancer cells were treated with specific inhibitors of abovementioned caspases. As shown in Fig. 3B, treatment of LNCaP to LNCaP cells pretreated with caspase inhibitors (Lupeol + caspase inhibitor) caused lesser cell death than in cells treated with Lupeol alone (Fig. 3B).

Effect of Lupeol on the expression of death domain receptor–dependent apoptotic proteins in LNCaP cells. Because the expression of mitochondrial proteins Bcl-2 and Bax were not affected by Lupeol, we speculated that Lupeol-induced apoptosis might be occurring through an alternative apoptotic pathway, such as death receptor–dependent apoptotic pathway, because activation of caspase-8 and caspase-9 (considered as integral components of death receptor–dependent apoptotic pathway) was evident. Next, we determined the effect of treatment of LNCaP cells with Lupeol on the expression of (a) death receptor proteins [i.e., Fas, death receptor 3 (DR-3), and DR-5], (b) death receptor ligands [i.e., Fas ligand (Fasl), Tweak, and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)], and (c) death adaptor proteins (i.e., FADD and TRADD) in cells treated with Lupeol. As shown in Fig. 3C, LNCaP cells treated with Lupeol exhibited no change in the expression of FasL, Tweak, TRAIL, DR-3, DR-5, and TRADD proteins (Fig. 3C). In sharp contrast, the treatment of cells with Lupeol was observed to cause a significant dose-dependent induction in the expression of Fas receptor (Fig. 3C). Furthermore, the expression of Fas receptor–associated FADD protein was observed to be significantly increased in Lupeol-treated cells (Fig. 3C). These data suggest that Lupeol induces apoptosis of prostate cancer cells through Fas-mediated apoptotic pathway.

Effect of low dose of Lupeol in combination with anti-Fas monoclonal antibody on the growth of PrEC and LNCaP cells. To further strengthen our hypothesis that Lupeol-induced

Figure 3. Effect of Lupeol treatment on PARP cleavage and on protein expression of acinus, Bcl-2, Bax, and procaspase-3, procaspase-6, procaspase-8, and procaspase-9 in LNCaP cells. A. cells were treated with vehicle (DMSO + alcohol) only or specified concentrations of Lupeol for 48 hours and harvested, and total cellular lysates were prepared. PARP cleavage and the expression of acinus, Bcl-2, Bax, and procaspase-3, procaspase-6, procaspase-8, and procaspase-9 were determined by Western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for v-actin. Representative immunoblots of three independent experiments with similar results. Details are described in Materials and Methods. C and V, control and vehicle-treated (alcohol + DMSO) cells, respectively. B, effect of Lupeol treatment on viability of prostate cancer cells pretreated with caspase inhibitors. Cells were treated with specified concentrations of caspase inhibitors alone, Lupeol alone, or with combination (caspase inhibitor + Lupeol) for 48 hours, and the cell viability was determined by MTT assay. Percent viable cells, where vehicle-treated cells were regarded as 100% viable. Columns, mean of percent viable cells of three independent experiments; bars, SE. Details are described in Materials and Methods. C, effect of Lupeol treatment on death receptor–mediated apoptotic pathway in LNCaP cells. Cells were treated with vehicle (DMSO + alcohol) only or specified concentrations of Lupeol for 48 hours and harvested, and total cellular lysates were prepared. Expression of death receptors (Fas, DR-3, and DR-5), death receptor ligands (Fasl, Tweak, and TRAIL), and death adaptor proteins (FADD and TRADD) were determined by Western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for v-actin. Representative immunoblots of three independent experiments with similar results. Details are described in Materials and Methods. C and V, control and vehicle-treated (alcohol + DMSO) cells, respectively.
apoptosis is through Fas receptor, we did a combinatorial study of Lupeol and Fas-mAb. Anti-Fas mAb has also been reported to activate the Fas receptor and induce apoptosis in various cell types (33–35). Because Lupeol was also observed to induce the expression of Fas receptor in LNCaP cells, we next evaluated the effect of low dose (20 μmol/L) of Lupeol in combination with anti-Fas mAb. To determine the optimum dose at which anti-Fas mAb causes almost 50% cell death, a cell viability test was done in a time-dependent manner (Fig. 4A). The treatment of cells with anti-Fas mAb caused a dose-dependent and time-dependent inhibition
of cell viability (Fig. 4A). The treatment of cells with anti-Fas mAb at a concentration of 1 μg/mL was observed to cause almost 50% cell death at 48 hours after treatment (Fig. 4A). To evaluate the effect of combination (Lupeol + anti-Fas mAb) on viability of cells, 1 μg/mL of anti-Fas mAb and 20 μmol/L of Lupeol were used. Cells were cotreated as well as pretreated with anti-Fas mAb followed by Lupeol treatment. The treatment of Lupeol to cells pretreated or cotreated with anti-Fas mAb (1 μg/mL) was observed to cause significantly higher cell death compared with Lupeol alone or anti-Fas mAb alone treated cells (Fig. 4B). The effect of Lupeol on cell viability was almost similar in both anti-Fas mAb pretreated and cotreated cells. Furthermore, the effect of combination (Lupeol and anti-Fas mAb) on cell viability was much higher than the additive effect of Lupeol alone or anti-Fas mAb alone (Fig. 4B). However, PrEC cells when treated with the combination of Lupeol and anti-Fas mAb did not exhibit any significant effect on the viability at the similar dose (data not shown). Because the death of prostate cancer cells was very high with the treatment of...
combination (anti-Fas mAb + Lupeol) with no significant effect on the viability of PrEC cells, we next measured the effect of Lupeol on expression levels of Fas receptor protein in cells pretreated with low and optimum doses of anti-Fas mAb (0.5-1 μg/mL). The expression of Fas protein was found to be highly increased in LNCaP cells treated with the combination (anti-Fas mAb + Lupeol) compared with Lupeol alone–treated and anti-Fas mAb alone–treated cells (Fig. 4C). However, PrEC cells treated with Lupeol alone, anti-Fas mAb alone, and combination (Lupeol + anti-Fas mAb) did not exhibit any increase in the expression levels of Fas receptor (Fig. 4D). These data suggest a synergistic effect of Lupeol with anti-Fas mAb–treated prostate cancer cells and provides evidence that Lupeol-induced cell death is mediated through Fas receptor.

Effect of Lupeol on small interfering RNA–mediated silencing of Fas receptor in LNCaP and CWR22Rv1 cells. We next determined whether Lupeol-induced cell death occurs exclusively through the Fas receptor protein by taking advantage of siRNA technique. LNCaP cells were transfected with different doses of Fas-siRNA and Fas protein expression was assessed (data not shown). Cells treated with Fas-siRNA exhibited a significant decrease in the expression of Fas receptor protein at a dose of 50 nM of siRNA, and this dose of siRNA was used for further studies. Next, we determined the effect of treatment of Lupeol (30 μM/L) on the growth of Fas-silenced cells. As shown in Fig. 5, treatment of cells with Lupeol caused only 25% cell death in Fas-silenced cells compared with Fas-positive cells where cell death

![Graph A](image_url)

![Graph B](image_url)

![Graph C](image_url)

Figure 5. Effect of Lupeol treatment on the growth of prostate cancer cells transfected with Fas-siRNA and on the expression of Fas receptor in Fas-silenced prostate cancer cells. A, cells were treated with specified concentrations of Lupeol alone, anti-Fas mAb or nonsense siRNA (negative control). B, cells were treated with specified concentrations of Lupeol alone, specific caspase inhibitor alone or nonsense siRNA (negative control). C, cells were treated with specified concentrations of Lupeol alone, anti-Fas mAb or nonsense siRNA (negative control). Lupeol (30 μM) was used as Lupeol + Fas-siRNA and combination (Lupeol + anti-Fas mAb, Lupeol + Fas siRNA, and combination (Lupeol + Fas siRNA, and combination (anti-Fas mAb + Fas siRNA). Incubation of cells with Fas-siRNA was for 6 hours followed by treatment with Lupeol. After 48 hours, cell viability was determined by MTT assay. Percent viable cells, where vehicle-treated cells were regarded as 100% viable. Columns, mean of percent viable cells of three independent experiments; bars, SE. Details are described in Materials and Methods. B, cells were treated with specified concentrations of Lupeol alone, specific caspase inhibitor alone or nonsense siRNA (negative control). Lupeol (30 μM) was used as Lupeol + Fas-siRNA and combination (Lupeol + Fas-siRNA, and combination (anti-Fas mAb + Fas siRNA). Incubation of cells with Fas-siRNA was for 6 hours followed by treatment with Lupeol. After 48 hours, cell viability was determined by MTT assay. Percent viable cells, where vehicle-treated cells were regarded as 100% viable. Columns, mean of percent viable cells of three independent experiments; bars, SE. Details are described in Materials and Methods. C, cells were treated with specified concentrations of Lupeol alone, anti-Fas mAb or nonsense siRNA (negative control). Lupeol (30 μM) was used as Lupeol + Fas-siRNA and combination (Lupeol + Fas-siRNA, and combination (anti-Fas mAb + Fas siRNA). Incubation of cells with Fas-siRNA was for 6 hours followed by treatment with Lupeol. After 48 hours, cells were harvested, and total cell lysates were prepared. We used 20 μM/L of Lupeol against CWR22Rv1 cells, because IC50 of Lupeol was observed to be lesser in CWR22Rv1 than in LNCaP cells. Expression of Fas receptor protein in total cell lysate was determined by Western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for β-actin. Representative immunoblots of three independent experiments with similar results. Details are described in Materials and Methods.
was observed to be 70% (Fig. 5A). These data were consistent with the observation that anti-Fas mAb caused only 15% cell death in Fas-silenced cells compared with Fas-positive cells which exhibited 50% loss of viability (Fig. 5). One possible explanation for cell death in Fas-siRNA treatment groups is that some of the cells might have escaped from Fas-siRNA transfection. Similar results were observed when Fas-silenced CWR22Rv1 cells were treated with Lupeol or anti-Fas mAb (data not shown).

To strengthen our argument that Lupeol-induced apoptosis of LNCaP cells is mediated through Fas receptor and caspase-8, caspase-9, and caspase-6, we next determined the effect of Lupeol on the viability of Fas-silenced cells in the presence of caspase inhibitors. The death-inducing effect of Lupeol in LNCaP cells was almost completely blocked/or inhibited in Fas-silenced cells pretreated with Lupeol than in cells treated with Lupeol alone (Fig. 5B). Because caspase-8 and caspase-9 are known to be required for Fas-mediated death signaling process, our data provide evidence that Lupeol-induced apoptosis of LNCaP cells is indeed mediated through Fas receptor death signaling pathway.

Fas-silenced LNCaP and CWR22Rv1 cells treated with Lupeol or anti-Fas mAb exhibited a reduced expression level of Fas receptor protein compared with Fas-positive cells (Fig. 5C). From these data, it was evident that the reduced levels of Fas receptor protein in Fas-silenced prostate cancer cells corresponded with their increased viability and were not affected significantly with the treatment of Lupeol or anti-Fas mAb (Fig. 5A). These data supports our hypothesis that Lupeol-induced apoptosis is through the Fas receptor.

**Effect of Lupeol on tumorigenicity of CWR22Rv1 cells in an athymic nude mouse model.** Because Lupeol was observed to be effective in inhibiting the growth of androgen-dependent prostate cancer cells in vitro, we next investigated whether these results could be translated into an in vivo xenograft model. Lupeol treatment did not cause any loss in the body weight, food intake, or exhibited apparent signs of toxicity in animals. Implantation of CWR22Rv1 cells onto nude mice produced visible tumors in mice with a mean latent period of 12 days. The average volume of tumors in control mice increased as a function of time and reached a preset end point of 1,000 mm³ in 5.5 weeks after inoculation. However, at this time, the average tumor volume was only 400 mm³ in mice treated with Lupeol (Fig. 6A). According to the linear regression analysis of data, tumors were found to grow an average of 39.6 mm³/day in the control group and 3.9 mm³/day in the Lupeol-treated group. Next, we evaluated whether or not treatment of Lupeol to animals leads to enhancement of delay in the growth of tumors in nude mice. The observed differences for tumor growth between the control and Lupeol-treated group was evident as early as third week after inoculation and continued throughout the treatment protocol.

**Effect of Lupeol on the expression levels of prostate-specific antigen and androgen receptor in LNCaP cells.** Because we observed that Lupeol reduces the human PSA levels in the serum of nude mice implanted with xenografts, we evaluated the protein expression of PSA and androgen receptor in LNCaP cells. Interestingly, a decrease in the expression levels of PSA and androgen receptor by Lupeol treatment was also observed under in vitro conditions in LNCaP cells (Fig. 6D).

**Discussion**

Common therapies for prostate cancer, such as androgen withdrawal, cytotoxic chemotherapy or radiotherapy, etc., which are relatively nonselective and highly toxic to normal tissues, are rarely curative due to the recurrence of this disease and apoptotic resistance offered by prostate cancer cells (33). The high recurrence of apoptotic resistant hormone refractory prostate cancer is responsible for ~28,000 deaths per year in the United States alone (34, 35). Apoptosis plays an important role in the renewal of the normal prostatic epithelium and in neoplastic prostate, and a reduced apoptotic rate has been associated with the progression of locally invasive prostate cancers to metastatic disease (2, 11). Because restoring apoptosis has been suggested as a possible therapeutic strategy, a great deal of research has been devoted to understanding the abnormalities in the cellular machinery that causes resistance to apoptosis in prostate cancer cells (11). In an effort to develop effective strategies to overcome the apoptotic resistance offered by prostate cancer cells, novel agents that possess high therapeutic potential with less systemic toxicity are being evaluated worldwide. With the advent of chemopreventive approaches for the treatment of cancer, there is widespread interest in the possibility that this approach may eventually have effect as well as could improve the quality of life of prostate cancer patients. In this regard, many diet-based polyphenolic agents with high anticancer efficacy and no or acceptable toxicity to normal tissues are suggested as possible candidates for use by prostate cancer patients. Due to low immunogenicity and high stability, natural agents that induce apoptosis may provide opportunity for minimal acquired drug resistance, decreased mutagenesis, and reduced toxicity. In addition, epidemiologic studies, together with extensive basic laboratory findings, support the potential role of phytochemicals at least as an adjuvant to conventional therapy, in the prevention and treatment of prostate cancer (14, 15). In the current study, we provide evidence that Lupeol, a diet-based agent, could ameliorate inefficiency of prostate cancer cells to undergo apoptosis and inhibit the human prostate cancer growth in vitro and in vivo.

We evaluated the growth-inhibitory response of Lupeol in human PrEC and prostate cancer cells. A striking observation from this data was that prostate cancer cells were highly sensitive to Lupeol-mediated loss of viability, and no such effect was observed in PrEC cells. These observations suggested a selective response of Lupeol to prostate cancer cells compared with PrEC. Our data is significant because in recent years, emphasis is on natural diet-based agents capable of selective/preferential elimination of cancer cells by inhibiting cell cycle progression and/or causing apoptosis.

Recent studies have shown that the prostate regression in animal models is linked to expression of Fas receptor (also known as CD95/APO-1), a cell surface protein that is also expressed in a...
variety of normal cells, including prostate epithelial cells, and in neoplastic cells (36–39). Fas-mediated signaling has been reported to play a significant role in the hormonal regulation of the normal and differentiated prostatic epithelium and mutations in the Fas gene have been shown to be closely associated with the pathogenesis of prostatic intraepithelial neoplasia and concurrent carcinomas (40, 41). In the current study, we observed that Lupeol-induced apoptosis of prostate cancer cells was concomitant to the induction of Fas receptor and its adaptor protein (i.e., FADD). We propose that Lupeol-induced death of prostate cancer cells is through death receptor–dependent apoptotic pathways, because no change in the expression of mitochondrial-dependent apoptotic signaling molecules, such as BCL-2 and Bax, was observed. Further among various death receptor pathways, Lupeol was observed to adopt Fas-associated apoptotic pathway, which is evident from our data that treatment of Lupeol did not cause change in death receptor proteins, such as TNFRI, DR-3, and DR-5, and death receptor adaptor protein TRADD. The end result of Fas receptor activation process is an unmasking of the proteolytic activity of caspase-8, which is then recruited to Fas-associated and trigger self-activation of the cascade of caspases (38). We observed that Lupeol-induced Fas signaling in prostate cancer cells was mediated through initiator caspase-8 and caspase-9 and by effector caspase-6, whereas no change was observed in the expression of caspase-3.

Triggering of Fas signaling by Fasl or agonistic antibodies (anti-Fas mAb) results in rapid induction of apoptosis in susceptible cells (33, 34, 42). Recent studies have shown that higher doses of agonistic anti-Fas mAb kill Fas-positive tumor cells; however, they could cause severe hepatotoxicity, which may restrict clinical application of the anti-Fas mAb (42). We suggest that combination treatment of prostate cancer cells with low (subtoxic) doses of anti-Fas mAb and Lupeol could be more effective than the anti-Fas mAb alone. In this study, we observed that low doses of anti-Fas mAb in

Figure 6. Effect of Lupeol on tumorigenicity in vivo and on PSA levels in vitro and in vivo. A total of $1 \times 10^6$ CWR22Rv1 cells were suspended in 50 μL of media and 50 μL of Matrigel. For s.c. tumor growth, the cell suspension was inoculated s.c. into the right flank on 6-week-old male athymic mice by using a 27-gauge needle.

A, effect of lupeol treatment on the growth of tumors from CWR22Rv1 cells implanted in nude mice. Growth was measured in terms of average volume of tumors as a function of time. Points, mean; bars, SD. *, $P < 0.05$. B, number of mice remaining with tumor volumes <1,000 mm$^3$ after treatment with corn oil alone or Lupeol for indicated weeks. *, $P < 0.05$. C, effect of Lupeol on human PSA levels in serum of athymic nude mice implanted with CWR22Rv1 cells. Points, mean; bars, SD. *, $P < 0.05$. D, effect of Lupeol on the expression levels of PSA and androgen receptor in LNCaP cells. Cells were treated with vehicle (DMSO + alcohol) only or specified concentrations of Lupeol for 48 hours and harvested, and total cell lysates were prepared. Expression of PSA protein and androgen receptor was determined by Western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for β-actin. Representative immunoblots of three independent experiments with similar results. Details are described in Materials and Methods. C and V, control and vehicle-treated (alcohol + DMSO) cells, respectively.

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the combination of Lupeol resulted in a significant synergistic cytotoxic effect on prostate cancer cells. This study showed that combination treatment of prostate cancer cells with anti-Fas mAb and Lupeol can overcome the resistance of prostate cancer cells to apoptosis and that the up-regulation of Fas expression by Lupeol may play a role in enhanced cytotoxicity. The striking observation was that in the combination (Lupeol + anti-Fas mAb)-treated cells, the expression of Fas receptor was significantly higher than Lupeol alone--or anti-Fas mAb alone--treated cells, suggesting the importance of Fas receptor in Lupeol-induced apoptosis of prostate cancer cells.

Our argument that Lupeol-induced apoptosis of prostate cancer cells is through Fas activation mediated by caspase-8, caspase-9, and caspase-6 is further strengthened by the data that the effect of Lupeol on viability of prostate cancer cells was highly reduced when Fas receptor was silenced by Fas-specific siRNA and the activity of abovementioned caspasess was inhibited by their specific inhibitors. These data show that Lupeol is a highly potent activator of prostate cancer cells. These data show that Lupeol is a highly potent activator of prostate cancer cells.

Finally, i.p. administration of Lupeol to mice showed inhibitory effects against the growth of prostate cancer cell-derived tumors.

There was a notable reduction in serum PSA levels in Lupeol-treated mice. However, the extent of reduction in serum PSA levels by Lupeol was found not to parallel the extent of inhibition of tumor growth in nude mice. This phenomenon is not unprecedented as several studies have shown that prostate cancer tumor growth is not always parallel to the changes in PSA levels in vivo (44, 45). These in vivo growth inhibitory effects of Lupeol can be explained by the biochemical mechanisms observed in the present study.

Taken together, our present findings showed the in vitro as well as in vivo anticancer efficacy of Lupeol, with mechanistic rationale (Fas-mediated apoptosis induction), against androgen-sensitive human prostate cancer cells without any cytotoxicity to non-neoplastic prostate epithelial cells. These observations warrant further in vivo efficacy studies in models that mimic progressive forms of human prostatic disease as well as estimation of pharmacologically achievable doses having biological significance in in vitro studies. The positive outcomes of such an in vitro study could form a strong basis for the development of Lupeol as a novel agent for human prostate cancer prevention and/or intervention.

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