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Suppression of cFLIP by Lupeol, a Dietary Triterpene, Is Sufficient to Overcome Resistance to TRAIL-Mediated Apoptosis in Chemoresistant Human Pancreatic Cancer Cells

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Abstract

Overexpression of cellular FLICE-like inhibitory protein (cFLIP) is reported to confer chemoresistance in pancreatic cancer (PaC) cells. This study was designed to investigate the effect of lupeol, a dietary triterpene, on (a) apoptosis of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) therapy–resistant PaC cells overexpressing cFLIP and (b) growth of human pancreatic tumor xenografts *in vivo*. The effect of lupeol treatment on proliferation and TRAIL/caspase-8/cFLIP machinery in PaC cells was investigated. Next, cFLIP-overexpressing and cFLIP-suppressed cells were tested for sensitivity to recombinant TRAIL therapy in the presence of lupeol. Further, athymic nude mice implanted with AsPC-1 cells were treated with lupeol (40 mg/kg) thrice a week and surrogate biomarkers were evaluated in tumors. Lupeol alone treatment of cells caused (a) decrease in proliferation, (b) induction of caspase-8 and poly(ADP-ribose) polymerase cleavage, and (c) down-regulation of transcriptional activation and expression of cFLIP. Lupeol was observed to increase the TRAIL protein level in cells. Lupeol significantly decreased the viability of AsPC-1 cells both in cFLIP-suppressed cells and in cFLIP-overexpressing cells. Lupeol significantly sensitized chemoresistant PaC cells to undergo apoptosis by recombinant TRAIL. Finally, lupeol significantly reduced the growth of human PaC tumors propagated in athymic nude mice and caused modulation of cFLIP and TRAIL protein levels in tumors. Our findings showed the anticancer efficacy of lupeol with mechanistic rationale against highly chemoresistant human PaC cells. We suggest that lupeol, alone or as an adjuvant to current therapies, could be useful for the management of human PaC.

Introduction

In the United States alone, 37,680 newly diagnosed pancreatic cancer (PaC) cases will be detected and 34,290 deaths are anticipated, making PaC the fourth leading cause of cancer deaths in adults (1). It has one of the worst prognoses with an overall 5-year survival rate of <5%, and most patients succumb to death within the first 2 years (2). Advanced PaC tumors are highly aggressive and resistant to conventional therapies such as radiation and chemotherapy (3). Advanced PaC cells are known to respond poorly to treatment with death-inducing chemotherapeutic agents due to the alteration in cellular apoptotic machinery

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(4). Therefore, efforts are being made to explore the possibility of cellular apoptotic machinery as a potential target for new therapeutic agents for treating PaC patients (5).

Recently, death receptor ligands, especially tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), have received a great deal of attention as novel therapeutic agents due to their high potential for selective induction of apoptosis in transformed cells (6–8). TRAIL-induced apoptosis in cancer cells is known to be mediated by caspase-8 (9). Recent studies have shown that, despite its potent tumoricidal activity, PaC remains refractory toward TRAIL treatment (10–12). The phenomenon of chemoresistance exhibited by PaC cells to TRAIL therapy is reported to be associated with the down-regulation of the activity of caspase-8 protein, which relays the apoptotic signals inside a cell (13–16). It is noteworthy that in chemoresistant cells, the activity of caspase-8 is reported to be inhibited by its inhibitory protein, cellular FLICE-like inhibitory protein (cFLIP; refs. 17–19). cFLIP has been shown to be highly expressed in TRAIL-resistant PaC cells as compared with TRAIL-sensitive cells (13,20–22). Further, down-regulation of cFLIP has been shown to render highly resistant PaC cells sensitive to TRAIL therapy (20–22). cFLIP-induced chemoresistance is not restricted to TRAIL alone but also extends to other chemotherapeutic agents such as gemcitabine that are used to treat PaC (ref. 6 and references therein). Combination therapies with cocktail chemotherapeutic agents (cisplatin, camptothecin, and celecoxib) are reported to cause a synergistic apoptotic effect with TRAIL; however, these therapies pose adverse undesirable side effects in PaC patients (23). There is an unmet need to overcome the cFLIP-mediated chemoresistance of PaC tumor cells by adopting novel approaches by the use of naturally occurring nontoxic dietary agents. Therefore, the search for novel, effective, and diet-based agents with anti-PaC activity is of paramount importance.

Lupeol [Lup-20(29)-en-3 β -ol; Fig. 1A] is a triterpene found in fruits such as olive, mango, strawberry, grapes, and figs; in many vegetables; and in several medicinal plants (ref. 24 and references therein). Lupeol is found as an active constituent of various medicinal plants used by native people in the treatment of various skin ailments in North America, Japan, China, Latin America, and Caribbean islands (ref. 12 and references therein). Lupeol possesses strong antioxidant, anti-inflammatory, antiarthritic, antimutagenic, and antimalarial activities in *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 known target for anticancer chemotherapy (25–28). Recently, we have shown that lupeol induces apoptosis in highly metastatic human PaC cells AsPC-1 (29).

Here, we show that lupeol significantly inhibits the growth and tumorigenicity of chemoresistant AsPC-1 cells through modulation of TRAIL/cFLIP under *in vitro* conditions and in an athymic nude mouse model. We suggest that lupeol alone or as an adjuvant to therapeutic agents could be developed as a potential agent for treating human PaC patients.

Materials and Methods

Cell culture

Human PaC cell lines AsPC-1, BxPC-3, and PANC-1 were obtained from American Type Culture Collection (ATCC) and grown in appropriate media (ATCC) supplemented with 10% fetal bovine serum (ATCC) and 1% penicillin-streptomycin (Cellgro Mediatech, Inc.) under standard cell culture conditions of 5% CO₂ at 37°C in an incubator.

Lupeol

Lupeol ($\geq 95\%$ purity) was procured from Sigma. A stock solution of lupeol (30 mmol/L) was prepared in warm alcohol and diluted in DMSO in a 1:1 ratio. The final concentrations of DMSO and alcohol were 0.25% and 0.075%, respectively, in all treatment protocols.

Cell viability assay

The effect lupeol on the viability of cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay. The cells were plated at 1×10^4 per well in 200 μ L of complete culture medium. The next day, cells were treated with lupeol (5–50 μ mol/L) for 48 h. Each concentration was repeated in 10 wells. After incubation for a specified time at 37°C in a humidified incubator, MTT (5 mg/mL in PBS) was added to each well and incubated for 2 h; then the plate was centrifuged at $500 \times g$ for 5 min at 4°C. The MTT solution was removed from the wells by aspiration. 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 on a microplate reader at the wavelength of 540 nm. The effect on cell growth inhibition was assessed as percent cell viability wherein vehicle-treated cells were taken as 100% viable.

[3 H]Thymidine incorporation assay

AsPC-1 cells (60% confluent) grown in 24-well culture plates were subjected to lupeol treatment for 48 h, the last 16 h of which were in the presence of [3 H]thymidine (0.25 μ Ci/mL). Cells were then washed twice with PBS at room temperature and then with ice-cold 5% trichloroacetic acid. The cells were next incubated with trichloroacetic acid solution on ice for 30 min and, subsequently, the acid-insoluble fraction was dissolved in 1 mL of 1 mol/L NaOH. Incorporated [3 H]thymidine was quantified by liquid scintillation counting.

Treatment of cells

For dose-dependent studies, the cells (50% confluent) were treated with lupeol (10–50 μ mol/L) for 48 h in complete media. Vehicle-treated cells served as controls. After 48 h of treatment with lupeol, the cells were harvested and cell lysates were prepared and stored at -80°C for later use.

Treatment of cells with recombinant TRAIL

Recombinant TRAIL (rTRAIL; 20 μ mol/L) was procured from Sigma Chemical Co. and stored at -80°C . Cells were treated with 100 nmol/L of rTRAIL alone and in combination with lupeol (20 μ mol/L) and were tested for viability and apoptosis. Briefly, cells were pretreated with either lupeol or vehicle (DMSO-ethyl alcohol) for 42 h followed by an additional 6-h incubation in the presence of TRAIL. At 48 h after lupeol or vehicle treatment, cells were tested for viability, proliferation, and apoptosis.

Western blot analysis

Cell and tissue lysates were prepared in cold lysis buffer [0.05 mmol/L Tris-HCl, 0.15 mmol/L NaCl, 1 mol/L EGTA, 1 mol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4 , 0.5% NP40, 1% Triton X-100, 1 mol/L phenyl methylsulfonyl fluoride (pH 7.4)] with freshly added Protease Inhibitor Cocktail Set III (Calbiochem). The lysate was collected and cleared by centrifugation, and the supernatant was aliquoted and stored at -80°C . The protein content in the lysates was measured by BCA protein assay (Pierce) as per vendor's protocol. For Western blot analysis, 25 to 40 μ g of protein were resolved over 12% Tris-glycine polyacrylamide gels (Novex) under nonreduced conditions, transferred onto nitrocellulose membranes, and subsequently incubated in blocking buffer (5% nonfat dry milk/1% Tween 20, in 20 mmol/L TBS, pH 7.6) for 2 h. The blots were incubated with

appropriate primary antibody, washed, and incubated with appropriate horseradish peroxidase (HRP)–conjugated secondary antibody (Amersham Biosciences). The blots were detected with chemiluminescence (ECL kit, Amersham Biosciences) followed by autoradiography, using XAR-5 film (Eastman Kodak). Equal loading of protein was confirmed by stripping the blots and reprobing with β -actin (Sigma). Densitometry measurements of the scanned bands were done using digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation). Data were normalized to β -actin and expressed as the mean of three tumor specimens $\pm$ SE.

Detection of apoptosis and necrosis by fluorescence microscopy

The Annexin V-FLUOS apoptosis detection kit (Roche Applied Science) was used for the detection of apoptotic and necrotic cells. This kit uses a dual-staining protocol in which the cells are stained with Annexin and propidium iodide. Cell populations that potentially may be detected are as follows: Viable cells will be nonfluorescent; cells in the metabolically active stages of apoptosis will stain with Annexin (green fluorescence) but not with propidium iodide (red fluorescence); and necrotic cells will stain with propidium iodide. In addition, cells undergoing late-stage apoptosis bind both Annexin and propidium iodide. Briefly, AsPC-1 cells were grown on slides to ~50% confluence and then treated with either lupeol (20–40 μ mol/L) or rTRAIL (100 nmol/L). Apoptotic and necrotic cells were detected with a Nikon OPTIPHOT-150 microscope connected to a SPOT Insight charge-coupled device camera (Diagnostic Instruments, Inc.). Briefly, the samples were excited at 330 to 380 nm, and the image was observed and photographed under a combination of a 400-nm dichroic mirror and a 420-nm high-pass filter.

cFLIP siRNA transfection

Transfections were done by using the Amaxa nucleofection kit as per vendor's protocol. The siRNA against cFLIP was procured from Santa Cruz Biotechnology. Briefly, 1×10^6 cells were transfected with 50 to 100 nmol/L of siRNA directed against cFLIP. Control cells were transfected with scrambled siRNA (50–100 nmol/L). After overnight incubation, transfected cells were treated with lupeol (20–40 μ mol/L) alone, rTRAIL alone (100 nmol/L), or a combination of lupeol and rTRAIL. At 48 h posttreatment, cells were tested for viability by MTT assay and for apoptotic markers by immunoblotting.

pCMV6-cFLIP transfection

The pCMV6-cFLIP expression plasmid was procured from OriGene Technologies, Inc. Briefly, 2×10^6 cells were transfected with 2 μ g of cFLIP cDNA. Control cells transfected with 2 μ g of empty vector (pCMV6) were run in parallel. After overnight incubation, transfected cells were treated with lupeol (20–40 μ mol/L) alone, rTRAIL alone (100 nmol/L), or a combination of lupeol and rTRAIL. At 48 h posttreatment, cells were tested for viability by MTT assay and for apoptotic markers by immunoblotting.

Transcriptional activity of cFLIP

The human cFLIP reporter plasmid (pGL3-cFLIP-luc) was a kind gift from Dr. Peter Erb (University of Basel, Basel, Switzerland). *E. coli* bacteria with plasmids were used for transformation using an agar medium and Maxiprep kit (Qiagen). Cells plated at a density of 5×10^4 per well were transfected with the plasmids (1 μ g/million cells) for 24 h. *Renilla* luciferase (50 ng/million cells, pRL-TK; Promega) was used as an internal control. In addition, the same amounts of empty vectors were transfected in controls cells. At 12 h posttransfection, fresh medium was added with lupeol (10–20 μ mol/L) and incubated for 24 h. The cells were then harvested, and transcriptional activity was measured in terms of luciferase activity in quadruplicates by using the dual-luciferase reporter assay system

(Promega). Relative luciferase activity was calculated with the values from vector alone group with or without the values from the lupeol-treated group.

Tumorigenicity studies in athymic nude mice

A total of 1×10^6 cells suspended in 50 μ L of media and 50 μ L of Matrigel (BD Biosciences) were inoculated s.c. into the right flank of 16 athymic nude mice (6 wk old) by using a 27-gauge needle and then randomly divided into two equal groups. One group of animals received an i.p. injection of lupeol (40 mg/kg body weight in 0.1 mL corn oil), and the second group of animals received corn oil alone and served as the control group. The dose of lupeol was selected on the basis of our previous studies (30,31). These treatments were repeated thrice a week on Mondays, Wednesdays, and Fridays. Once tumors started growing, their sizes were measured weekly and tumor volumes calculated by the formula $0.5238L_1L_2H$, where L_1 is the long diameter, L_2 is the short diameter, and H is the height of the tumor. At the termination of experiment, animals were sacrificed and tumor tissues and visceral organs were harvested. From the harvested tissues, lysates were prepared and paraffin tumor sections were prepared on slides. The lysates were stored at -80°C and paraffin tumor sections were immediately processed for immunohistochemical analysis.

Immunohistochemical analysis

Immunohistochemical staining was done as described earlier (30,32). Paraffin sections (to be evaluated for cFLIP and TRAIL) were pretreated with citrate buffer (pH 6) for 10 min in a microwave for antigen retrieval. After antigen retrieval, paraffin-embedded sections (5 μ m) were dewaxed, rehydrated, and treated to inhibit endogenous peroxidase activity. Sections were washed in water and PBS and blocked in blocking buffer (2% goat serum/5% bovine serum albumin in PBS) for 30 min followed by incubation with appropriate primary antibody (cFLIP and TRAIL) at the dilution of 1:50 for 12 h at 4°C . A negative control was included, in which sections were incubated with normal mouse IgG1 replacing the primary monoclonal antibody. After incubation in the primary antibody, sections were washed twice in PBS to remove unbound antibody, followed by incubation for 2 h at room temperature with appropriate HRP-conjugated secondary antibody. Immunoreactive complexes were detected with 3,3'-diaminobenzidine (DAKO). Slides were then counter-stained in hematoxylin, mounted in crystal mount, and coverslipped in 50:50 xylene/Permount. Sections were visualized with a Nikon OPTIPHOT-150 microscope connected to a SPOT Insight charge-coupled device camera (Diagnostic Instruments).

Statistical analyses

Student's *t* test for independent analysis was applied to evaluate differences between the treated and untreated groups with respect to the expression of various proteins. S-plus software (Insightful) was used to measure the rate of mean tumor volume growth as a function of time. $P < 0.05$ was considered statistically significant.

Results

Effect of lupeol on cell growth and viability

To evaluate the effect of lupeol on the growth of human PaC cells, we selected BxPC-3, AsPC-1, and PANC-1 cells. The choice of these cells was based on the fact that these cells show differential resistance pattern to conventional chemotherapeutic regimens. Treatment of AsPC-1 and PANC-1 cells with lupeol resulted in a dose-dependent growth inhibition with an IC_{50} of 27.5 and 10 $\mu\text{mol/L}$, respectively (Fig. 1A). In contrast, BxPC-3 cells showed considerable resistance to lupeol-induced cell growth inhibitory effects with $\text{IC}_{50} > 80 \mu\text{mol/L}$ (Fig. 1A). These results suggested that the cell line AsPC-1, which is highly

resistant to currently available chemotherapeutic drugs, remarkably showed sensitivity to lupeol treatment. Therefore, to understand the underlying mechanism(s) of lupeol-induced cell death, we selected AsPC-1 cells for further studies.

Effect of lupeol on AsPC-1 cell proliferation

Recently, we showed significant growth inhibitory effects of lupeol on AsPC-1 cells in a time- and dose-dependent manner (29). It is well known that proliferating cells exhibit increased [³H]thymidine incorporation into DNA, which arises from increased growth factor expression and activity in cancer cells (33,34). Next, we determined the effect of lupeol treatment (20–40 μmol/L) on the rate of proliferation of AsPC-1 cells by measuring the rate of uptake of thymidine by dividing cells. We observed that pretreatment of cells with lupeol significantly decreased thymidine incorporation in a dose-dependent manner, further confirming the antiproliferative efficacy of lupeol (Fig. 1B).

Effect of lupeol on caspase-8/cFLIP/TRAIL apoptotic machinery in AsPC-1 cells

Caspase-8 is an integral protein in death receptor–associated apoptosis (35–37). Caspase-8 expression is reported to be lost by the epigenetic phenomenon (36,38,39). Chemoresistant cancerous cells have defective caspase-8 function due to their inability to bind to the apoptosis executing complex and because of the presence of competitive inhibitors such as cFLIP (13–16,40). Lupeol treatment of AsPC-1 cells caused a significant decrease in the level of procaspase-8 protein (Fig. 1C). Further, lupeol treatment of cells also caused an increase in the expression level of active-caspase-8 (Fig. 1C).

Recent studies have shown that cFLIP impedes TRAIL-α-, Fas ligand-, and TRAIL-induced apoptosis by binding to Fas-associated death domain (FADD) and/or caspase-8, thus resulting in the prevention of death-inducing signaling complex (DISC) formation in PaC cells (35,41). The chemoresistance offered by PaC cells is mostly attributed to the down-regulation cFLIP and inactivation of caspase-8 (13–16,38–43). Because lupeol was observed to decrease the growth of highly chemoresistant PaC cells and activate caspase-8, we asked whether this effect is associated with cFLIP. Next, we determined the effect of lupeol treatment on the level of cFLIP protein, which is highly expressed in malignant PaC cells. Interestingly, lupeol treatment was found to significantly decrease the expression levels of cFLIP protein in a dose-dependent manner (Fig. 1C). It is noteworthy that the effect of lupeol on cFLIP levels well correlated with its effect on the viability and proliferation of AsPC-1 cells (Fig. 1A and B).

TRAIL ligand is known to be produced by tumor cells; however, with the advancement of the disease, these cells become nonresponsive to endogenous TRAIL due to the up-regulation of cFLIP (40–43). Similarly, advanced PaC tumors are reported to exhibit high resistance to endogenous as well as exogenous TRAIL treatment due to the up-regulation of cFLIP (11,12). We found that lupeol treatment resulted in an increase in the TRAIL protein level in AsPC-1 cells (Fig. 1D). The induction in TRAIL levels correlated with the effect on cell viability and proliferation (Fig. 1A and B). Next, we investigated the effect of lupeol treatment on death receptor (DR)-4 and DR5, the receptors of TRAIL. We did not observe any significant change in the expression levels of DR and DR5 in lupeol-treated AsPC-1 cells (Fig. 1D).

Effect of lupeol and rTRAIL on viability of AsPC-1 cells

AsPC-1 cells are known to be highly chemoresistant to TRAIL therapy (11,12). Next, we asked if lupeol treatment sensitizes AsPC-1 cells to TRAIL treatment and whether cFLIP was involved in the observed effect. rTRAIL treatment (100 nmol/L) did not cause any effect on the viability of AsPC-1 cells; however, cells that were pretreated with lupeol for 42

h at sublethal doses (20 $\mu\text{mol/L}$) and subsequently treated with rTRAIL (100 nmol/L) for 6 hours exhibited highly reduced viability (Fig. 2A). As evident from fluorescent microscopy analysis, we observed that the rTRAIL-induced growth inhibitory effect on lupeol-pretreated cells was as a result of induction of apoptosis (Fig. 2B). Next, we investigated the effect of TRAIL treatment on the proliferation potential of cells pretreated with lupeol. Lupeol-pretreated cells, on exposure to rTRAIL treatment, were observed to exhibit decreased proliferating potential as was assessed from the rate of uptake of [^3H]thymidine by dividing cells (Fig. 2C). These data suggest that lupeol possesses the potential to sensitize highly chemoresistant AsPC-1 cells to TRAIL therapy.

Effect of lupeol and rTRAIL on viability and apoptosis of cFLIP-knocked down AsPC-1 cells

Because lupeol was observed to decrease the viability of AsPC-1 cells, decrease the expression level of cFLIP, and increase the level of active caspase-8, we asked whether the apoptosis-inducing effects of lupeol are mediated through cFLIP. For this purpose, knockdown of cFLIP was achieved by transfecting cells with a siRNA directed against cFLIP. The expression level of cFLIP was significantly suppressed (50–90%) at the doses of 50 to 100 nmol/L siRNA at 24 h posttransfection; however, at a higher dose (100 nmol/L), cell viability was significantly reduced (data not shown). Thus, a dose of 50 nmol/L siRNA and a time point of 48 hours for lupeol treatment were selected for further experiments. The scrambled siRNA-transfected cells served as control. Next, we determined the effect of lupeol treatment (20 $\mu\text{mol/L}$) in siRNA-transfected cells. cFLIP-suppressed cells showed increased sensitivity toward lupeol treatment (Fig. 3A). Lupeol alone treatment (20 $\mu\text{mol/L}$) reduced the viability by 65% in scrambled siRNA-transfected cells and by 85% in cFLIP-knocked down cells (Fig. 3A). Next, these cells were evaluated for cFLIP expression level. TRAIL treatment did not result in any significant modulation in the viability of cells (Fig. 3B). Lupeol treatment caused a decrease of cFLIP expression level in scrambled siRNA-transfected as well as cFLIP siRNA-transfected cells; however the down-regulation of cFLIP was significantly more pronounced in cFLIP-silenced cells. To investigate whether lupeol-induced reduction in the viability of cFLIP-suppressed cells was a result of increased apoptosis, we evaluated these cells for poly(ADP-ribose) polymerase (PARP) cleavage as a measure of apoptosis. As is evident from the immunoblots representing the PARP cleavage (Fig. 3B), lupeol-induced PARP cleavage (thus reflecting apoptosis) was highly increased in cFLIP-knocked down cells (Fig. 3B), which we observed was concomitant with a significant decrease in the expression level of cFLIP (Fig. 3B).

We next determined the effect of rTRAIL treatment on cFLIP-silenced AsPC-1 cells. rTRAIL treatment did not exhibit any significant effect on scrambled siRNA-transfected cells, which served as control but exhibited 40% reduction in the viability of cFLIP-silenced cells (Fig. 3C). Further, the viability of lupeolpretreated cFLIP-silenced cells was highly reduced (95%) when exposed to rTRAIL treatment for 6 hours (Fig. 3C). These data suggest the significance of cFLIP in observed apoptosis and confirmed the apoptotic potential of lupeol alone or in combination with known chemotherapeutic agents in PaC cells.

Effect of lupeol and rTRAIL on viability and apoptosis of cFLIP-overexpressing AsPC-1 cells

Further, to validate that cFLIP indeed plays a role in lupeol-induced effects, we next asked whether the apoptosis-inducing effects of lupeol could be achieved in cells with forced cFLIP expression. AsPC-1 cells transfected with cFLIP-overexpressing plasmid (pCMV6-cFLIP) were found to exhibit significantly higher expression level of cFLIP as compared with control cells (transfected with pCMV6 vector alone) at 48 h posttransfection (data not shown). AsPC-1 cells forced to overexpress cFLIP were treated with lupeol and rTRAIL

alone and in combination. A test group was included wherein cells were pretreated with lupeol and, after 42 h, were exposed to rTRAIL treatment for 6 hours. rTRAIL treatment was observed to exert no significant effect on the viability of either control cells or cFLIP-overexpressing cells (Fig. 4A). Although lupeol treatment significantly reduced the viability of control cells, forced overexpression of cFLIP in AsPC-1 reduced the extent of lupeol-induced cell viability. However, the inhibitory effect on viability was significant, suggesting the potential of lupeol to abolish the cFLIP-conferred chemoresistance (Fig. 4A). Next, these cells were evaluated for cFLIP expression level. Lupeol treatment caused a decrease in cFLIP expression level in vector alone-transfected as well as cFLIP-overexpressing cells; however, the effect of down-regulation of cFLIP was more pronounced in control cells (Fig. 4B). Next, we asked whether lupeol could sensitize the cFLIP-overexpressing PaC cells to rTRAIL therapy. Interestingly, lupeol treatment was observed to significantly sensitize both control and cFLIP-overexpressing cells to rTRAIL therapy; however, the effect was higher in control cells (Fig. 4C). These data suggest that lupeol possesses the potential to decrease the viability of chemoresistant PaC cells irrespective of the level of cFLIP expression and even sensitize them to chemotherapy.

Effect of lupeol treatment on transcriptional activation of cFLIP in AsPC-1 cells

Because lupeol was observed to decrease the level of cFLIP protein, we next determined the effect of lupeol treatment on the transcriptional activation of cFLIP. Lupeol was tested for cFLIP promoter activity in AsPC-1 cells transfected with pGL3-cFLIP-Luc. Lupeol treatment was observed to significantly decrease the promoter activity of cFLIP, suggesting that the effect of lupeol on cFLIP occurred at transcriptional level (Fig. 4D).

Effect of lupeol on tumorigenicity of AsPC-1 cells in an athymic nude mouse model

Because lupeol was observed to be effective in inhibiting the growth of highly aggressive and chemoresistant AsPC-1 cells *in vitro*, we next determined whether these results could be translated into an *in vivo* xenograft model. Lupeol treatment neither caused any loss in the body weight or food intake nor exhibited apparent signs of toxicity in animals. Implantation of AsPC-1 cells into nude mice produced visible tumors in mice with a mean latent period of 7 days. The average volume of tumors in control mice increased as a function of time and reached a preset end point of 1,300 mm³ in 40 days postinoculation. However, at this time the average tumor volume was only 720 mm³ in mice treated with lupeol (Fig. 5A). Tumors were found to grow an average of 28 mm³ per day in the control group and 13 mm³ per day in the lupeol-treated group. Next, we evaluated whether or not the treatment of lupeol to animals caused a delay in the growth of tumors in nude mice. The observed differences for tumor development in lupeol-treated mice as compared with control mice were statistically significant with $P < 0.01$ (Fig. 5B). Approximately 75% of mice that received lupeol treatment did not cross the preset end point of the tumor volume of 1,300 mm³ even at the end of 7th week (Fig. 5B). Tumors from three animals from the control and treated groups were excised at the 49th day posttreatment when 100% of control (corn oil-treated) animals reached the tumor volume of ~ 1,300 mm³. The rest of the animals in treated group remained on the protocol until they crossed the preset end point (i.e., tumor volume of 1,300 mm³). From these data we conclude that lupeol is an effective agent that has the potential to inhibit the tumorigenicity of PaC cells *in vivo*.

Lupeol administration induces apoptosis of tumor cells in athymic nude mice

We next asked whether the observed effect of lupeol is the end result of growth arrest followed by apoptosis of tumor cells. We investigated the effect of lupeol administration on apoptotic markers (PARP cleavage and activation of caspase-8) in tumor tissues harvested from control and lupeol-treated mice. Lupeol administration was observed to induce the expression level of PARP85 (the cleaved product) and cleaved caspase-8 and decrease the

expression level of PARP116 and procaspase-8 (Fig. 5C). Because TRAIL and cFLIP were observed to be modulated by lupeol treatment under *in vitro* conditions, we next determined the effect of lupeol administration on the expression levels of these proteins in tumor tissues and calculated their ratio. Lupeol administration was observed to significantly decrease the expression level of cFLIP protein and marginally increase the expression level of TRAIL protein (Fig. 6A and C). These studies were confirmed by performing immunohistochemical analysis of TRAIL and cFLIP proteins in tumor sections of both groups of animals. In corn oil-treated animals, cFLIP-positive staining was significantly higher than in tumor tissues of animals receiving lupeol, and vice versa for TRAIL (Fig. 6B and D). Collectively, these data suggest that lupeol administration induces apoptosis leading to the reduced tumorigenicity of PaC tumor cells under *in vivo* conditions.

Discussion

PaC is one of the most aggressive malignant tumors with a low survival rate (1,2). PaC tumor development and progression, as well as resistance to most oncologic therapies such as cisplatin and gemcitabine therapy, result mainly from lack of response to apoptotic stimuli (3). PaC cells with acquired resistance to apoptosis and alterations in apoptotic machinery are often resistant to conventional chemotherapy (3–5). Therefore, novel therapeutic strategies that overcome the resistant mechanisms in PaC would be crucial in improving the survival rate of the patients. In recent years, the idea to directly trigger apoptosis in PaC cells by stimulating death receptor-mediated apoptosis by natural agents has gained considerable attention (29,44,45). The major finding of the current study is that lupeol, a dietary triterpene, could ameliorate the inefficiency of PaC cells to undergo apoptosis, sensitize the chemoresistant PaC cells to TRAIL therapy, and inhibit the human PaC proliferation by down-regulating the antiapoptotic protein cFLIP under *in vitro* and *in vivo* conditions.

Realizing the cell heterogeneity of solid tumors including PaC, which may determine the cell response to chemotherapeutic agents, we tested the effect of lupeol on several PaC cells such as AsPC1, BxPC-3, and PANC-1 of different origin and differentiation and their differing sensitivity to TRAIL to examine whether the effect of lupeol is dependent or independent of the cancer cell type. Interestingly, the BxPC-1 cell line, which shows greatest sensitivity to TRAIL, was less responsive to lupeol treatment compared with PANC-1 and AsPC-1 cells, which show either an intermediate or complete resistance to TRAIL treatment, respectively (11,20). Treatment of TRAIL-resistant AsPC-1 cells with lupeol resulted in dose-dependent growth inhibition with an IC₅₀ of 27.5 µmol/L. A striking observation from our data was that cells of variable cFLIP-expression were all highly sensitive to lupeol-mediated loss of viability. In addition, these data provide evidence that lupeol has the potential to kill the tumor cells and inhibit the proliferation of advanced stage characteristics such as AsPC-1 cells, which are generally resistant to conventional chemotherapeutic agents.

Among the death ligands, TRAIL-mediated therapy is showing the most promise as a potential cancer therapeutic agent due to its apparent specificity for tumor cells and lack of normal cell toxicity (6–8,46). Previous studies have shown that TRAIL is abundantly expressed in most normal tissues without causing significant apoptosis; however, rTRAIL seems to selectively induce apoptosis in transformed cells (47). However, PaC cells have been found to express the molecules of the TRAIL signaling machinery, but most of the PaC cells remain resistant toward endogenous TRAIL as well as rTRAIL therapy. However, PaC, being one of the most resistant types of cancer to various available therapeutic agents, also shows resistance to TRAIL-based therapy and thus limiting its use in PaC. Resistance to TRAIL-induced apoptosis can emerge in TRAIL-sensitive cells exposed to TRAIL, with the

emergence of resistant clones (13). An interesting finding of the current study is that AsPC-1 cells, which are highly chemoresistant, attain sensitivity to TRAIL therapy when pre-treated with lupeol. These data suggest the possibility of exploring lupeol in future as an adjuvant to chemotherapeutic agents for the treatment of chemoresistant PaC. Interestingly, we observed that lupeol treatment also caused an increased level of endogenous TRAIL in PaC cells as well as in PaC tumors. It is noteworthy that lupeol was observed to increase the protein level of TRAIL at the lowest dose (10 μ mol/L), suggesting the efficacy of lupeol to trigger the process of apoptosis at low concentrations. The proapoptotic role of lupeol could be explained by its efficiency to act in a double-edge sword mechanistic way: (a) by sensitizing PaC cells for TRAIL therapy by correcting the alterations in apoptotic machinery and (b) by increasing the TRAIL protein pool in PaC cells.

The basis of resistance offered by PaC cells to TRAIL is not fully understood. A broad body of evidence suggests that modulation of DISC-recruited molecules such as FADD, caspase-8, and cFLIP determine the sensitivity of death receptor-expressing cells to TRAIL (13,14,38–43). Caspases initiate and execute cell death and are highly regulated by a variety of different inhibitors and activators. For example, activation of procaspase-8 requires association with its cofactor FADD (38–43). Previous studies have shown that resistance to TRAIL-induced apoptosis correlates with a loss of caspase-8 expression in cancerous cells (14). We observed that lupeol significantly activates caspase-8 in PaC cells and in PaC-originated tumors implanted as xenografts in athymic nude mice. Our data are significant because lupeol-induced caspase-8 activation was observed to be accompanied by increased apoptosis, which is evident from the cleavage of apoptotic marker PARP under *in vitro* as well as *in vivo* conditions. Our data are in agreement with other studies showing that reintroduction or activation of caspase-8 by oligonucleotides induces apoptosis and sensitizes cancerous cells to TRAIL therapy (14–16).

Nonformation of DISC complex due to the inability of caspase-8 binding is reported to be the major contributor of chemoresistance in PaC cells (35–37). Studies have shown that the inability of caspase-8 to form DISC complex is due to the strong presence of inhibitor proteins such as cFLIP in chemoresistant cells (refs. 39–43 and references therein). Previous studies have shown that cFLIP interrupts apoptotic signaling by interacting with FADD and caspase-8 and by blocking the activity of caspase-8, suggesting that the intracellular level of cFLIP may determine the sensitivity of tumor cells to a variety of proapoptotic stimuli (refs. 39–43 and references therein). Elevated expression of cFLIP has been found in various types of tumor cells that are often resistant to death receptor-mediated apoptosis (42). Those tumors include colorectal carcinoma, gastric carcinoma, pancreatic carcinoma, Hodgkin's lymphoma, B-cell chronic lymphocytic leukemia, melanoma, ovarian carcinoma, cervical carcinoma, bladder urothelial carcinoma, and prostate carcinoma (17–19). The expression of cFLIP has been proved to be one of the major determinants of resistance to death ligands such as Fas ligand and TRAIL, and numerous reports have shown that down-regulation of cFLIP results in sensitizing various types of resistant tumor cells including PaC (refs. 8,20 and references therein). Conversely, forced expression of cFLIP is known to render cells resistant to Fas and/or TRAIL (ref. 8 and references therein). Taken together, these studies imply that cFLIP may be an attractive therapeutic target against PaC whose malignancy and resistance have been shown to be strongly dependent on cFLIP overexpression (42,43). The promising approach is limiting cFLIP protein level; once achieved, it could sensitize cancer cells to death ligand-induced cell death. To date, several kinds of small molecules such as DNA-damaging agents, RNA synthesis inhibitors, protein synthesis inhibitors, topoisomerase I inhibitors, and histone deacetylase inhibitors have been known to lower cFLIP expression and sensitize resistant tumor cells to death receptor-mediated apoptosis (ref. 8 and references therein). The biggest problem with the above-mentioned agents is that these are not specific for cFLIP and exhibit their antitumor effects at higher doses, which are

reported to produce potential side effects in humans (ref. 8 and references therein). In this context, we provide compelling evidence showing that lupeol treatment significantly reduces the expression level of cFLIP protein in AsPC-1 cells at sublethal doses. Interestingly, when PaC-originated tumor xeno-grafts were tested for cFLIP protein level, those retrieved from lupeol-treated animals exhibited significantly reduced cFLIP level. These data are significant because down-regulation of cFLIP was observed to be concomitant with increased apoptosis in lupeol-treated PaC cells as well as PaC-originated tumors. It is noteworthy that lupeol was observed to induce apoptosis even in conditions when cFLIP was overexpressed in AsPC-1 cells. In addition, such cells also showed increased sensitivity to rTRAIL therapy. These data are significant because under PaC tumor milieu, where clones of tumor cells with variable cFLIP expression are present, an agent targeting cFLIP is desired. On the basis of our data, we suggest that lupeol could be such an agent.

Recent studies have shown that blocking the transcriptional activation of cFLIP could be a better approach to reduce cFLIP levels in tumor cells (ref. 8 and references therein). However, it should be noted that the agents directly targeting FLIP at the mRNA and protein levels have not yet been developed. Interestingly, lupeol treatment was observed to inhibit the transcriptional activation of cFLIP in PaC cells, suggesting that the effect of lupeol on cFLIP is at transcriptional level. Although the mechanism by which cFLIP expression is regulated at transcriptional level is not well understood, recent studies have shown that phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and nuclear factor κ B (NF κ B) contribute toward the transcriptional activation of cFLIP in cancer cells (ref. 48 and references therein). It is interesting to note that the NF κ B and PI3K/Akt signaling pathways are activated in response to Ras activation, which is frequent in PaC patients (ref. 29 and references therein). Having reported earlier that lupeol treatment modulates the Ras/ PI3K/NF κ B signaling axis in PaC cells, the effect of lupeol treatment on the transcriptional activation of cFLIP could be explained on the possibility of involvement of Ras/PI3K/NF κ B signaling axis (29). The outcome of current study corroborates with recent studies showing that the down-regulation of transcriptional activation of cFLIP sensitized tumor cells to chemotherapeutic agents (49).

Because lupeol treatment significantly inhibited the growth of highly chemoresistant PaC cells under *in vitro* conditions, we asked whether these data could be translated under *in vivo* situations. An i.p. administration of lupeol to athymic nude mice showed inhibitory effects against the growth of PaC cell-derived tumors. These *in vivo* growth inhibitory effects of lupeol could be explained by biochemical mechanisms.

To summarize, our present findings showed the *in vitro* as well as *in vivo* anticancer efficacy of lupeol, with mechanistic rationale (TRAIL-mediated apoptosis caused by decreasing total cFLIP expression and increasing endogenous TRAIL), against chemoresistant human PaC cells. These observations warrant further *in vivo* efficacy studies in models that mimic progressive forms of human PaC as well as estimation of pharmacologically achievable doses exhibiting biological significance in *in vitro* studies. The positive outcomes of such an *in vivo* study could form a strong basis for the development of lupeol as a novel agent for human PaC prevention and/or intervention alone or as an adjuvant to known therapeutic agents such as rTRAIL for the treatment of PaC.

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Figure 1.

Effect of lupeol on cell viability, proliferation, activation of caspase-8, and the expression level of cFLIP, TRAIL, and death receptors in AsPC-1 cells. **A**, viability of human PaC AsPC-1, BxPC-3, and PANC-1 cells treated with lupeol (5–50 μmol/L for 48 h) as measured by MTT assay. Each concentration of lupeol was repeated in 10 wells. The values are represented as percent viable cells, with vehicle (alcohol + DMSO)-treated cells regarded as 100% viable. *Points*, mean of three independent experiments; *bars*, SE. **B**, histogram showing the rate of [³H]thymidine uptake in AsPC-1 cells treated with lupeol. Cells were subjected to lupeol treatment (20–40 μmol/L) for 48 h, the last 16 h of which was in the presence of [³H]thymidine (0.5 μCi/mL). *Columns*, mean of three independent experiments; *bars*, SE. *, *P* < 0.05. *Inset*, structure of lupeol [Lup-20(29)-en-3β-ol]. **C**, effect of lupeol (0–50 μmol/L) treatment on the expression levels of procaspase-8 and cleaved caspase-8 and on the protein expression of cFLIP. **D**, effect of lupeol (0–50 μmol/L) treatment on the expression levels of TRAIL, DR4, and DR5 proteins as determined by immunoblot analysis. Representative of three independent experiments with similar results. The details are described in Materials and Methods. V, vehicle (alcohol + DMSO).

Figure 2.

Effect of lupeol and rTRAIL treatment, alone and in combination (Lupeol + rTRAIL), on cell viability, proliferation, and apoptosis of AsPC-1 cells. **A**, histogram representing the viability of cells treated with specified concentrations of lupeol (20–30 $\mu\text{mol/L}$) and rTRAIL (100 nmol/L). Cells were treated with lupeol for 24 h and were then exposed to rTRAIL for 6 h. After TRAIL treatment, cell viability was assessed by MTT assay. Each concentration of lupeol was repeated in 10 wells. The values are represented as percent viable cells, with vehicle (alcohol + DMSO)–treated cells regarded as 100% viable. *Columns*, mean of three independent experiments; *bars*, SE. **B**, representative photomicrographs showing induction of apoptosis in AsPC-1 cells treated with lupeol and rTRAIL alone and in combination. Green and red fluorescence indicate Annexin V and propidium iodide staining. Representative of three independent experiments with similar results. **C**, histogram showing the rate of [^3H]thymidine uptake in AsPC-1 cells treated with lupeol and rTRAIL alone and in combination. Cells were subjected to lupeol treatment (20 $\mu\text{mol/L}$) for 48 h, the last 16 h of which was in the presence of [^3H]thymidine (0.5 $\mu\text{Ci/mL}$). *Columns*, mean of three independent experiments; *bars*, SE. *, $P < 0.01$. The details are described in Materials and Methods.

Figure 3.

Effect of lupeol treatment on cell viability and on the expression of cFLIP and PARP cleavage in cFLIP-silenced AsPC-1 cells. **A**, histogram representing the effect of lupeol on the growth of cFLIP-silenced AsPC-1 cells as determined by MTT assay. Cells were transfected with cFLIP siRNA or scrambled siRNA (control) by electroporation. Twelve hours posttransfection, cells were treated with specified concentrations of lupeol and cell viability was determined. The values represent percent viable cells, with vehicle-treated cells regarded as 100% viable. *Columns*, mean percent viable cells of three independent experiments; *bars*, SE. The details are described in Materials and Methods. **B**, representative immunoblots of cFLIP and PARP protein cleavage. Cells were transfected with cFLIP

siRNA or scrambled siRNA (control) by electroporation. At 12 h posttransfection, cells were treated with specified concentrations of lupeol. After 48 h, cells were harvested and cell lysates analyzed for cFLIP and PARP cleavage. Equal loading was confirmed by stripping immunoblots and reprobing them for β -actin. *C*, histogram representing the effect of rTRAIL and lupeol alone and in combination on the growth of cFLIP-silenced AsPC-1 cells. Cells were transfected with cFLIP siRNA or scrambled siRNA (control) by electroporation. Twelve hours posttransfection, cells were treated with specified concentrations of rTRAIL, lupeol, or their combination. After 48 h, cell viability was determined by MTT assay. The values represent percent viable cells, with vehicle-treated cells regarded as 100% viable. *Columns*, mean percent viable cells of three independent experiments; *bars*, SE. The details are described in Materials and Methods.

Figure 4.

Effect of lupeol treatment on cell viability and on the expression of cFLIP and PARP cleavage in AsPC-1 cells transfected with cFLIP-overexpressing plasmid. **A**, histogram representing the effect of lupeol on the growth of cFLIP-overexpressing AsPC-1 cells. Cells were transfected with pCMV6-cFLIP or pCMV6 vector alone (control) by electroporation. Twelve hours posttransfection, cells were treated with specified concentrations of lupeol and cell viability was determined by MTT assay. The values represent percent viable cells, with vehicle-treated cells regarded as 100% viable. *Columns*, mean percent viable cells of three independent experiments; *bars*, SE. The details are described in Materials and Methods. **B**, representative immunoblots of cFLIP and PARP protein cleavage in cFLIP-overexpressing AsPC-1 cells. Cells were transfected with pCMV6-cFLIP or pCMV6 vector alone (control) by electroporation. Twelve hours posttransfection, cells were treated with specified concentrations of lupeol; cell lysates were prepared and analyzed for cFLIP and PARP cleavage 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. **C**, histogram representing the effect of rTRAIL and lupeol alone and in combination on the growth of cFLIP-overexpressing AsPC-1 cells. Cells were transfected with pCMV6-cFLIP or pCMV6 vector alone (control) by electroporation. Twelve hours posttransfection, cells were treated with specified concentrations of rTRAIL and lupeol alone and in combination and cell viability was determined by MTT assay. The values represent percent viable cells, with vehicle-treated cells regarded as 100% viable. *Columns*, mean percent viable cells of three independent experiments; *bars*, SE. The details are described in Materials and Methods. **D**, histogram representing the effect of lupeol treatment on the transcriptional activation of cFLIP in AsPC-1 cells. Cells were transfected with pGL3-cFLIP-luc for 24 h. *Renilla* luciferase was used as an internal control. In addition, controls were transfected with the same amount of empty vectors. Twelve hours posttransfection, fresh medium was added with lupeol and incubated for 24 h. The cells

were then harvested and transcriptional activity was measured in quadruplicates as described in Materials and Methods.

Figure 5.

Effect of lupeol administration on the tumorigenicity of AsPC-1 cells and the expression level of known apoptotic markers PARP and caspase-8 under *in vivo* conditions. **A**, graphical representation of data showing the effect of lupeol treatment on the growth of tumors from AsPC-1 cells implanted in athymic nude mice. The growth was measured in terms of average volume of tumors as a function of time. *Points*, mean; *bars*, SD. *, $P < 0.05$, versus the control group. **B**, graphical representation of the data depicting the number of mice remaining with tumor volumes $< 1,300 \text{ mm}^3$ after treatment with corn oil alone or lupeol for the indicated weeks. **C**, effect of lupeol administration on the expression levels of PARP cleavage, procaspase-8, and active caspase-8 in AsPC-1 cell-derived tumors excised

at the 49th day posttreatment as determined by immunoblot analysis. Equal loading was confirmed by stripping the membrane and reprobing them for β -actin. Representative immunoblot of three samples from each group. The details are described in Materials and Methods.

Figure 6. Effect of lupeol administration on the expression level of cFLIP and TRAIL proteins in AsPC-1 cell-derived tumors in athymic nude mice. **A**, effect of lupeol treatment on the expression level of TRAIL in AsPC-1 cell-derived tumors excised at the 49th day posttreatment as determined by immunoblot analysis. Equal loading was confirmed by stripping the membrane and reprobing them for β -actin. Representative immunoblot of three samples from each group. Values above the immunoblots represent mean relative densities of the bands normalized to β -actin $\pm$ SE. **B**, representative photomicrographs (magnification, $\times 200$) showing immunohistochemical staining for TRAIL in tumor sections of corn oil-treated and lupeol-treated mice. *Arrows*, regions exhibiting immunoreactivity for TRAIL protein. The immunostaining data were confirmed in two or more specimens of each group. **C**, effect of lupeol treatment on the expression levels of cFLIP in AsPC-1 cell-derived tumors excised at the 49th day posttreatment as determined by immunoblot analysis. Equal loading was confirmed by stripping the membrane and reprobing them for β -actin. Representative immunoblot of three samples from each group. Values above the immunoblots represent mean relative densities of the bands normalized to β -actin; *bars*, SE. **D**, representative photomicrographs (magnification, $\times 200$) showing immunohistochemical staining for cFLIP in tumor sections of corn oil-treated and lupeol-treated mice. *Arrows*, regions exhibiting immunoreactivity for cFLIP protein. The immunostaining data were confirmed in three specimens from each group. The details are described in Materials and Methods.