Lupeol, a fruit and vegetable based triterpene, induces apoptotic death of human pancreatic adenocarcinoma cells via inhibition of Ras signaling pathway

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Pancreatic cancer is an exceptionally aggressive disease, the treatment of which has largely been unsuccessful due to higher resistance offered by pancreatic cancer cells to conventional approaches such as surgery, radiation and/or chemotherapy. The aberration of Ras oncoprotein has been linked to the induction of multiple signaling pathways and to the resistance offered by pancreatic cancer cells to apoptosis. Therefore, there is a need for development of new and effective chemotherapeutic agents which can target multiple pathways to induce responsiveness of pancreatic cancer cells to death signals. In this study, human pancreatic adenocarcinoma cells AsPC-1 were used to investigate the effect of Lupeol on cell growth and its effects on the modulation of multiple Ras-induced signaling pathways. Lupeol caused a dose-dependent inhibition of cell growth as assessed by MTT assay and induction of apoptosis as assessed by flow cytometry, fluorescence microscopy and western blotting. Lupeol treatment to cells was found to cause a dose-dependent inhibition of cell growth as assessed by MTT assay and induction of apoptosis as assessed by flow cytometry, fluorescence microscopy and western blotting. Lupeol treatment to cells was found to significantly reduce the expression of Ras oncoprotein and modulate the protein expression of various signaling molecules involved in PKCα/ODC, PI3K/Akt and MAPKs pathways along with a significant reduction in the activation of NFκB signaling pathway. Our data suggest that Lupeol can adopt a multi-prong strategy to target multiple signaling pathways leading to induction of apoptosis and inhibition of growth of pancreatic cancer cells. Lupeol could be a potential agent against pancreatic cancer, however, further in-depth in vitro studies are warranted.

Introduction

Pancreatic cancer is the most fatal of all cancers and the fifth most common cause of cancer-related deaths among both men and women in the western countries. It is estimated that 32 180 cases of pancreatic cancer will be diagnosed in the United States in 2005 and almost the same number of pancreatic cancer-related deaths are projected (1). Since the mortality from pancreatic cancer compares strikingly with its incidence, it has become a significant public health concern (1). Long-term survival of patients with organ-confined disease is only 15%, and in the majority of cases with invasive metastasis survival is only 4%. Despite these disappointing statistics and the increase in incidence of pancreatic cancer over the past several decades, the molecular and biochemical determinants of the disease remain poorly understood and no effective therapeutic regimens to significantly ameliorate the clinical course or prognosis of this disease is apparent (1).

Thus, pancreatic cancer presents a clinical and experimental challenge because of its relative resistance to conventional modes of therapies such as chemotherapy and radiation and only 4% of such patients are reported to survive after surgery (1). It is therefore necessary to intensify our efforts for a better understanding of this disease and for the development of novel approaches for its prevention and treatment. In more than 90% of pancreatic cancers, the ras oncogene has been shown to be mutated [(2,3) and references therein]. This mutation is considered an early genetic event in the development of pancreatic cancer and results in constitutive activation of an intracellular pathway leading to cellular proliferation. Several ras-induced signaling pathways, such as the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B pathway) mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NFκB) pathways, have been linked to chemoresistance of the pancreatic carcinoma cells (3–6). These findings suggest that Ras oncoprotein could be an important target for developing agents against pancreatic cancer.

Epidemiological studies have found a lower risk of pancreatic cancer in populations with higher consumption of fresh fruits, vitamin C and dietary fiber (7,8). In addition, consumption of white wine has been reported to have an inverse relation with the risk of pancreatic cancer (9). These data suggest that some dietary substances could be effective in reducing pancreatic cancer incidence. We argued that those agents, which have the ability to intervene at more than one critical pathway in pancreatic carcinogenic process, will have greater advantage over other single-target agents. Lupeol, a triterpene (Figure 1A) is found in various edible plants such as olive, fig, mango, strawberry, red grapes and medicinal plants used by native people in North America, Japan, China, Latin America and Caribbean islands (10–12). Lupeol has been shown to exhibit strong anti-inflammatory, anti-arthritic, anti-mutagenic and anti-malarial activity in in vitro and in vivo systems (10). Lupeol has been shown to act as a potent inhibitor of protein kinases and serine proteases and of DNA topoisomerase II, a target for anticancer chemotherapy (13,14). Lupeol has been reported to induce differentiation and inhibit the growth of melanoma and leukemia cells (15–17). Recently, we have shown that Lupeol inhibits tumor promotion in two-stage mouse skin carcinogenesis by

Abbreviations: DMSO, dimethyl sulfoxide; MAPKs, mitogen-activated protein kinases; NFκB, nuclear factor kappa B; ODC, ornithine decarboxylase; PARP, poly(ADP-ribose)polymerase; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; PKCs, protein kinase Cα; PMSF, phenylmethylsulfonyl fluoride; TdT, terminal deoxynucleotidyl transferase.

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modulating various signaling pathways (18). In the present study, employing human pancreatic adenocarcinoma cell line AsPC-1, we show that Lupeol is a potent multi-target anticancer agent that induces apoptotic cell death of pancreatic cancer cells via modulation of Ras-induced protein kinase Cα (PKCα)/ornithine decarboxylase (ODC), PI3K/Akt, MAPKs and NFκB signaling pathways. We suggest that Lupeol may be an effective agent that should be tested for its in vivo efficacy against pancreatic cancer.

Materials and methods

Cell culture and reagents

Human pancreatic adenocarcinoma cells AsPC-1 were a gift from Dr Nihal Ahmad, Department of Dermatology, University of Wisconsin-Madison. The cells were cultured in RPMI 1640 (ATCC, Manassas VA) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Antibodies were procured from BD Transduction laboratories (San Jose, CA), Santacruz Biotechnology (Santa Cruz, CA) and Upstate (Charlottesville, VA). Lupeol was purchased from Sigma Chemical Company (St Louis, MO).

Treatment of cells

Lupeol stock (30 mM) was prepared by dissolving Lupeol in warm alcohol and diluting in dimethyl sulfoxide (DMSO) in a 1:1 ratio. Working solutions were prepared by diluting the stock solution with DMSO to get desired final concentrations. For dose-dependent studies, the cells (50% confluent) were treated with Lupeol (30, 40, 50 μM) for 48 h in complete cell medium. Cells that served as controls were incubated with the vehicle (alcohol + DMSO) alone. The final concentration of DMSO and alcohol was 0.25 and 0.075%, respectively, in all treatment protocols.

Cell viability assay

The effect of Lupeol on the viability of AsPC-1 cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay. The cells were plated at 1 × 10^5 cells per well in 200 μl of complete culture medium and treated with 10, 20, 30, 40, 50, 75, 100, 125 μM concentrations of Lupeol in 96-well microtiter plates. Lupeol stock solutions prepared at 30 mM concentration were mixed with fresh medium to achieve the desired final concentration. Each concentration of Lupeol was repeated in 10 wells. After incubation for 24, 48 and 72 h at 37°C in a humidified incubator, cell viability was determined. MTT [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well and incubated for 2 h after which the plate was centrifuged at 1800 r.p.m. (500× g) for 5 min at 4°C. The supernatant 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 of Lupeol on growth inhibition was assessed as percent cell viability where vehicle-treated cells were taken as 100% viable.

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. This kit uses a dual-staining protocol in which the cells are stained with annexin and propidium iodide (PI). Cell populations that potentially may be detected are as follows: viable cells will be non-fluorescent; cells in the metabolically active stages of apoptosis will stain with annexin (green fluorescence) but not with PI (red fluorescence) and necrotic cells with PI staining. In addition, cells undergoing late stage apoptosis bind both annexin and PI. Briefly, AsPC-1 cells were grown to ~50% confluence on slides and then treated with Lupeol (30, 40, 50 μM) for 48 h. Apoptosis and necrosis was detected by using a Zeiss Axiovert 100 microscope. Briefly, the samples were excited at 330–380 nm, and the image was observed and photographed under a combination of a 400 nm dichroic mirror and then 420 nm high-pass filter.

Quantification of apoptosis

Apopotosis was quantified by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick and labeling (TUNEL) method. Briefly, the cells were grown to a density of 1 × 10^6 cells in 100-mm culture dishes and then treated with Lupeol (30, 40, 50 μM) for 48 h. The cells were trypsinized, washed with PBS and processed for labeling with fluorescein-tagged deoxyuridine triphosphate-nucleotide and PI by use of an APO-DIRECT apoptosis kit obtained from Phoenix Flow Systems (San Diego, CA) and following the manufacturer’s protocol. The labeled cells were then analyzed by flow cytometry.

Total cell lysate and nuclear lysate preparation

The total cell lysate was prepared in cold buffer [50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 100 mM Na3VO4, 0.5% NP-40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4] with freshly added protease inhibitor cocktail (Protease inhibitor Cocktail Set III; Calbiochem, La Jolla, CA) in ice. For the preparation of nuclear fractions, the cells were washed with cold PBS and suspended in 0.4 ml of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 200 mM NaCl, 1 mM EDTA, 20 mM NaF, 100 mM Na3VO4, 0.5% NP-40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4) with freshly added protease and phosphatase inhibitors.
0.5 mM PMSF, Protease Inhibitor Cocktail Set III) in a microfuge tube. The cells were incubated on ice for 15 min, after which 12.5 μl of 10% Nonidet P-40 was added and the contents were mixed on a vortex and then centrifuged for 1 min at 4°C at 14,000 g. The nuclear pellet was resuspended in 25 μl of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, Protease Inhibitor Cocktail Set III) and incubated on ice for 30 min with intermittent mixing. The tube was centrifuged for 5 min at 14,000 g at 4°C and the supernatant (nuclear extract) was stored at -80°C. The protein concentration was determined by BCA assay (Pierce, Rockford, IL) as per the manufacturer’s protocol.

**Western blot analysis**

For western blot analysis, 40 μg of the protein was resolved over 8–12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot containing the transferred protein was kept in blockingbuffer (5% nonfat dry milk, 1% Tween-20; in 20 mM TBS, pH 7.6) on a shaker for 1 h at room temperature followed by incubation with appropriate primary antibody in blocking buffer for 1 h to overnight at 4°C. This was followed by incubation with anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham Life Sciences) and autoradiography using XAR-5 film obtained from Eastman Kodak (Rochester, NY). Immunoblots were scanned by HP PrecisionScan Pro 3.13 (Hewlett-Packard, Palo Alto, CA). Densitometry measurements of the scanned bands were performed using digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT). Data were normalized to β-actin and expressed as mean values ± SE of three separate sets of experiments.

**Electrophoretic mobility shift assay**

To detect NFκB-DNA binding, electrophoretic mobility shift assay (EMSA) was performed using lightshift™ chemiluminescent kit (Pierce, Rockford, IL) by following the manufacturer’s protocol. To start with, DNA was biotin labeled using the biotin 3′ end labeling kit (Pierce, Rockford, IL). Briefly, in a 50 μl reaction buffer, 5 pmol of double-stranded NFκB oligonucleotide 5′-ATG TGA GGC AAC CAC C-3′; 3′-TCA ACT CCC CTG AAA GGG TCC G-5′, 10 μl of 10 mM Tris (pH 7.4), 10 μl of 1 mM DTT, 50 ng of poly(dI-dC) (5 mg/ml), 0.25% NP-40, 5 μl of 5× binding buffer was added, subjected to gel electrophoresis on a native polyacrylamide gel and transferred to a nylon membrane. When the transfer was complete, DNA was cross-linked to the membrane at 120 mJ/cm² using a UV cross-linker equipped with 254 nm bulbs. The biotin end-labeled DNA was detected using streptavidin–horseradish peroxidase conjugate and a chemiluminescent kit (Pierce, Rockford, IL). Densitometry measurements of the transferred protein was kept in blocking buffer (5% nonfat dry milk, 1% Tween-20; in 20 mM TBS, pH 7.6) on a shaker for 1 h at room temperature followed by incubation with appropriate primary antibody in blocking buffer for 1 h to overnight at 4°C. This was followed by incubation with anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham Life Sciences) and autoradiography using XAR-5 film obtained from Eastman Kodak (Rochester, NY). Immunoblots were scanned by HP PrecisionScan Pro 3.13 (Hewlett-Packard, Palo Alto, CA). Densitometry measurements of the scanned bands were performed using digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT). Data were normalized to β-actin and expressed as mean values ± SE of three separate sets of experiments.

**Results**

Lupeol induces growth inhibition and apoptosis in AsPC-1 cells

We first evaluated the effect of Lupeol on cell viability by MTT assay. As shown in Figure 1B, treatment of AsPC-1 cells with Lupeol (10–125 μM) resulted in a dose-dependent inhibition of cell growth when assessed at 24, 48 or 72 h post-treatment (Figure 1B). The IC50 value of Lupeol on growth inhibition was estimated to be 35 μM at 48 h. Based on these observations, we selected a dose of 30, 40 and 50 μM and a time period of 48 h post-Lupeol treatment for further mechanistic studies.

We next determined whether Lupeol-mediated loss of cell viability in AsPC-1 cells is a result of induction of apoptosis. The induction of apoptosis by Lupeol (30–50 μM) was evident from the morphology of cells, as assessed by fluorescence microscopy after labeling the cells with annexin and PI (Figure 2, upper panel). As shown by representative pictures (Figure 2, upper panel), Lupeol treatment resulted in induction of apoptosis in a dose-dependent manner. These data also 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 labeled with bromo-deoxyuridine triphosphate and PI. As shown by the data in Figure 2 (bottom panel), compared with control, Lupeol treatment resulted in 0.3, 8.6 and 22.0% TUNEL-positive cells at 30, 40 and 50 μM at 48 h post-treatment, respectively. Consistent with the fluorescence microscopy data, TUNEL assay by flow cytometry revealed that treatment of AsPC-1 cells with Lupeol resulted in a dose-dependent induction of apoptosis.

**Lupeol induces PARP cleavage and increases Bax protein expression in AsPC-1 cells**

Since poly(ADP-ribosylation) is a post-translational modification of proteins which plays a crucial role in DNA repair and cell death, poly(ADP-ribose)polymerase (PARP), a DNA nick sensor, serves as one of the best-known biomarkers of apoptosis (19). During apoptosis, PARP protein is cleaved into an 85 kDa C-terminal fragment, with a reduced catalytic activity, and a 24 kDa N-terminal peptide, which retains the DNA binding domains. Next, we assessed the effect of Lupeol (30–50 μM) treatment of cells on PARP cleavage. The PARP cleavage analysis showed that the full-size PARP (116 kDa) protein was cleaved to yield an 85 kDa fragment after treatment of cells with Lupeol at 30, 40 and 50 μM concentrations at 48 h post-treatment (Figure 3). The expression of Bax (pro-apoptotic protein) and Bcl-2 (anti-apoptotic protein) have been reported to play a crucial role in apoptotic response mediated by many agents (20,21). We next determined the effect of Lupeol treatment of cells on the protein levels of Bax and Bcl-2. The immunoblot analysis exhibited a significant increase in the protein expression of Bax in Lupeol-treated cells (Figure 3). However, the protein expression of Bcl-2 did not exhibit any change by Lupeol treatment at any dose.

**Lupeol induces activation of caspases in AsPC-1 cells**

Caspases receive the upstream death signals and are the final executors of apoptosis (22,23). In most cancer cells, caspases are present in the pro-forms (inactive) and require cleavage of protein to become active to participate in the process of apoptosis. Through this process, the pro-forms of caspases are lost and the expression of their active forms (cleaved products) increases during apoptosis (22,23). We next performed immunoblot analysis of the members of caspases in the cell lysates obtained from Lupeol-treated cells. We have used antibodies that specifically recognize the pro-forms and cleaved products (active forms) of caspases. As shown in Figure 4, Lupeol-treated cells did not exhibit any change in the expression level of procaspase-3, however, the expression of active caspase-3 was found to be increased in such cells (Figure 4). Further Lupeol treatment induced the expression of caspases-8 and -9 in a dose-dependent manner which is evident from the decrease in the protein expression of their pro-forms (inactive forms) with a concomitant increase in their active forms as compared with vehicle-treated control (Figure 4).
Lupeol decreases the expression of Ras, PKCα, ODC proteins and inhibits PI3K/Akt in AsPC-1 cells

Many cancers including pancreatic cancer exhibit aberrant Ras signaling that leads to chronic up-regulation of the Ras pathway and accumulation of Ras oncoprotein in transformed cells (24). PKCα has been implicated in Ras signaling and transformed cells have been reported to have high levels of PKCα (25). In various cancer types, Ras-induced PKCα has been reported to activate ODC, an oncogene which is highly activated during the cellular proliferation (25–27). Next, we determined the effect of Lupeol treatment of cells on the expression of Ras, PKCα and ODC proteins. It is evident from the data of immunoblot analysis that treatment of Lupeol significantly reduced the protein expression of Ras in a dose-dependent manner (Figure 5). Similarly, Lupeol caused a significant dose-dependent decrease in the phosphorylation of Akt protein in cells (Figure 5). The treatment of Lupeol did not cause any change in the protein levels of total Akt (Figure 5). These results suggest the inhibitory potential of Lupeol against PI3K/Akt pathway, which is highly
activated during the development and progression of pancreatic cancer.

**Lupeol inhibits the expression of MAPK proteins p38 and Erk1/2 in AsPC-1 cells**

Constitutive levels of Erk1/2 protein have been reported to be very low in pancreatic cancer cells and studies have linked the dysregulation of MAPKs with tumor cell survival in various cancer types including pancreatic adenocarcinoma (3,29,30). Therefore, we determined whether MAPKs contributed to Lupeol-induced apoptosis in our model. We addressed this question by determining the effect of Lupeol treatment on expression levels of Erk1/2, JNK and p38 MAPK proteins. As shown in Figure 5, exposure of cells to Lupeol resulted in a dose-dependent activation (phosphorylation) of Erk1/2. Lupeol treatment caused a dose-dependent decrease in the level of phospho-p38 MAPK (Figure 5). Unlike ERK1/2 and p38, however, Lupeol treatment did not alter the level of JNK1 protein (data not shown). Taken together, these observations indicate that activation of Erk1/2 protein and decrease in expression levels of phospho-p38 protein might be contributing towards apoptosis of cells induced by Lupeol.

**Lupeol inhibits phosphorylation of IκBα and NF-κB/p65 in AsPC-1 cells**

A number of studies clarified the role of NFκB in tumor cell survival by inhibiting apoptosis (31). The activation of NFκB...
pathway in pancreatic cancer cells has been linked to their resistance to conventional chemotherapy (32). The phosphorylation of inhibitory protein IkBα is a critical step in the pathway of NFκB activation that results in the translocation of NFκB molecule to nucleus from cytosol, which ultimately leads to the survival of tumor cells. The translocation of NFκB to nucleus is preceded by the phosphorylation of its p65 subunit and therefore is an important event. Next, we investigated the effect of Lupeol treatment on the phosphorylation of IkBα and NFκB/p65 in cells. As shown by immunoblot analysis (Figure 6A), Lupeol treatment resulted in a dose-dependent inhibition of phosphorylation of NFκB/p65 protein in these cells. The inhibition of phosphorylation of NF-κB/p65 molecule was concomitant with a decrease in the phosphorylation of IkBα protein (Figure 6A).

**NFκB–DNA binding is reduced in Lupeol-treated AsPC-1 cells**

The translocation of NFκB to nucleus is marked by its binding with DNA to activate the expression of various genes leading to the tumor cell survival (31). We next performed EMSA to investigate the effect of Lupeol treatment on NFκB–DNA binding activity. As shown in Figure 5B, untreated and vehicle control cells exhibited a constitutively increased NFκB–DNA binding activity; however, in cells treated with Lupeol, a significant decrease in NFκB–DNA binding activity was observed (Figure 6B). The effect of Lupeol on NFκB–DNA binding in cells was concomitant with the effect of Lupeol on phosphorylation of NF-κB/p65 subunit (Figure 6A). As is evident from results of supershift assay using anti-p65 antibodies, Lupeol was observed to specifically decrease the levels of p65 subunit of NF-κB in the nucleus of cells (Figure 6C).

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**Fig. 6.** (A) Effect of Lupeol treatment on the phosphorylation of IkBα protein and p65-subunit of NFκB transcriptional factor in AsPC-1 cells. The cells were treated with vehicle (DMSO + alcohol) only or specified concentrations of Lupeol for 48 h, harvested and total cell lysates were prepared. Equal loading was confirmed by stripping immunoblots and reprobing it for β-actin. Values given above each blot represents the relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results. The details are described under Materials and methods.

(B) Effect of Lupeol treatment on the binding of NFκB transcriptional factor with DNA in AsPC-1 cells as assessed by EMSA. After 48 h of treatment with Lupeol, the cells were harvested, nuclear lysates were prepared and DNA binding was determined by EMSA as described under Materials and methods. I, II, and III refer to internal experimental controls, where I represents biotin–EBNA (Epstein–Barr virus nuclear antigen) control DNA, II represents biotin–EBNA control DNA and EBNA extract, and III represents biotin–EBNA control DNA and EBNA extract plus 200-fold molar excess of EBNA DNA. In control number I, no protein extract for DNA to bind resulted in an unshifted band. In control number II, sufficient target protein leads to DNA–protein binding resulting in shift detected by comparison to band at position I. Control number II demonstrated that the signal shift observed could be prevented by competition from excess unlabelled DNA. The data shown here are representative of three independent experiments with similar results. The details are described under Materials and methods. The data shown here are representative of three independent experiments with similar results, ns represents non-specific binding.

(C) Effect of Lupeol treatment on the binding of p65-subunit of NFκB transcriptional factor with DNA in AsPC-1 cells. The cells were treated with vehicle only or specified concentrations of Lupeol for 48 h, and then processed for nuclear lysate. NFκB/DNA binding activity was determined by Supershift assay using anti-p65 antibody and EMSA kit from Pierce (Rockford, IL). The details are described under Materials and methods. The data shown here are representative of three independent experiments with similar results, ns represents non-specific binding.
Discussion

The aggressive nature of pancreatic adenocarcinoma is related to several abnormalities in growth factors and their receptors that affect the downstream signal transduction pathways involved in the control of growth and differentiation (4). These perturbations confer a tremendous survival and growth advantage to pancreatic cancer cells. The acquisition of an apoptosis-resistant phenotype by pancreatic cancer cells may also upset the success of conventional radio- and chemotherapy (32). In addition to perturbations in cell cycle control, resistance to apoptosis may promote tumor growth and metastasis. Apoptosis resistance mechanisms seem to vary among different tumor types and have only begun to be characterized at the molecular level.

Elucidation of molecular events during pancreatic cancer development has led to several distinct therapeutic advances and many novel agents and inhibitors including monoclonal antibodies have been developed and are undergoing clinical trials (4,32–34). However, many of these agents are not effective alone and have to be combined for maximum efficacy leading to undesirable and sometimes fatal side effects. One promising approach could be identification of non-nutrient dietary constituents that can target multiple pathways without causing any undesirable toxicity. Here, we describe the usefulness of Lupeol, a novel non-nutrient dietary agent against human pancreatic cancer cells. In the current study, we demonstrate that Lupeol treatment induces a dose-dependent death of human pancreatic cancer cells. From our data, it is evident that the induction of cell death is mediated through apoptosis. We also found that Lupeol-induced apoptosis of pancreatic cells is mediated through the activation of caspases-3, -8 and -9. The observation that expression levels of procaspase-3 did not exhibit any significant change upon Lupeol treatment could be explained through a possible involvement of a feedback mechanism, which restores depleted procaspase-3 levels at intracellular level. The only other pro-apoptotic effect of lupeol reported so far is on mouse melanoma and human leukemia cells (15–17).

Transformed cells have been shown to possess a dysregulated apoptotic machinery (35). Several pro-apoptotic proteins are either lost or diminished and many anti-apoptotic proteins are activated during the development of cancer (35). As is evident from our data, induction of pro-apoptotic Bax protein by Lupeol indicates that Lupeol may rectify the errors in apoptotic machinery of pancreatic cancer cells. Although many studies have shown that natural agents decrease the expression of anti-apoptotic protein Bcl-2 with a concomitant increase in the expression of pro-apoptotic Bax protein and shift the balance towards apoptosis, we did not observe any change in the Bcl-2 protein expression in Lupeol-treated pancreatic cancer cells. However, with an increase in Bax expression the shift is indeed towards apoptosis. Since our data suggest that Lupeol is a multi-target agent, other mechanisms of apoptosis could not be ruled out.

Other contributing molecular changes in pancreatic adenocarcinoma include activation of oncopogenes and inactivation of tumor suppressor genes (2–5). The ras oncogene is thought to play an important role in the growth of pancreatic cancer, because an activated (mutated) ras gene is found in ~90% of human pancreatic cancers (2–5). The ras mutation results in the accumulation of Ras oncoprotein, and constitutive activation of various intracellular signaling pathways, leading to cellular proliferation (2–6). Targeting the ras gene has been shown to inhibit the growth of metastatic pancreatic cells (4). In the present study, we observed that treatment of human pancreatic cancer cells with Lupeol decreases the protein expression of Ras in a dose-dependent manner.

Ras protein is reported to regulate various downstream signaling pathways such as the PI3K/Akt pathway. The growth-promoting potential of the PI3K/Akt pathway and its anti-apoptotic properties are closely linked to the resistance of cancer cells to a broad spectrum of apoptotic stimuli and several studies have demonstrated that treatment with the PI3K inhibitors substantially enhances apoptosis (4–6,36,37). Studies have shown that inhibitors of PI3K/Akt induce dose-dependent apoptosis of pancreatic cancer cells and inhibit tumor growth of pancreatic cancer xenografts (37,38). In the current study, we show that treatment of pancreatic cancer cells with Lupeol reduced the expression of PI3K and Akt proteins in a dose-dependent manner. This is consistent with our earlier report where we demonstrated that Lupeol inhibited the PI3K/Akt signaling pathway in vivo during two-stage mouse skin tumorigenesis (18). These results suggest that Lupeol has an efficiency to modulate the signaling pathways which are known to promote pancreatic cancer cell growth and survival.

The effects mediated by aberrantly activated Ras in human cancers are likely to be very complex and are currently not completely understood. Both Ras and PI3K/Akt signaling pathways have been reported to regulate the expression of MAPKs during the progression of various cancer types including pancreatic cancer (3,4,29,30). Chronic stimulation of the Ras/Raf/MEK/ERK pathway by oncogenic Ras is thought to promote cellular transformation and this pathway has been reported to be activated in various cancer types, however, Erk1/2 activation does not always correlate with the expression of mutant ras or other oncogenes (3). Constitutive levels of Erk1/2 proteins have been reported to be very low in pancreatic cancer cells despite Ras activation and Erk1/2 inhibition in pancreatic cancer cells have been linked with the resistance to apoptosis [(3, 30) and references therein]. As evident from our data, the correlation of significant cell death with increased expression of Erk1/2 proteins in Lupeol-treated cells is evidence that low levels of Erk1/2 protein have an association with tumor cell survival in pancreatic cancer. Our data are supported by recent studies showing that chemotherapeutic agent-induced apoptosis in pancreatic cancer cells is through Erk1/2 activation. It has been demonstrated that p38 MAPK activation triggers an anti-apoptotic response to tumor cells, although there have also been some contrasting reports that p38 MAPK activation (phosphorylation) is central to the pro-apoptotic effects in some cancer cell lines (39,40). Recent reports have demonstrated a link between the Ras oncoprotein and p38 MAPK activation in transformed cells (41). It is evident from our data that Lupeol treatment significantly decreases the expression of phospho-p38 MAPK in cells leading to their apoptotic death.

Ras oncoprotein has been shown to contribute to constitutive activation of NFκB signaling in pancreatic cancer through the activation of PI3K/Akt and MAPK pathways (32). Wang et al. reported that p65 subunit of NFκB was constitutively activated in ~67% of pancreatic adenocarcinomas and a positive correlation exists between the activation of NFκB and Ras oncoprotein during the proliferation of pancreatic cancer cells (42). NFκB plays an important role in tumor resistance.
to apoptosis and studies have shown that pancreatic cancer cells resistant to apoptosis exhibit a high basal NFkB activity (31,43). Interestingly, we found that treatment of Lupeol inhibited expression of NFkB and phosphorylation of IkBa protein in pancreatic cancer cells. Because Lupeol inhibits IkBa phosphorylation, our study suggests that the effect of Lupeol on NFkB/65 is through inhibition of phosphorylation of IkBa which in turn is regulated by Ras and PI3K/Akt pathways.

The Ras oncoprotein is also reported to regulate PKCa (23,24). PKCa has been reported to be involved in the regulation of apoptosis and in animal models, tumorigenicity of pancreatic cancer cells has been directly related to increased expression of PKCa protein (25,26). Numerous studies have shown that PKC levels are high in pancreatic cancer patients and increased PKC levels offer resistance to apoptosis of pancreatic cancer cells (32). In this scenario, Lupeol with an efficacy of targeting PKCa protein seems to be a promising agent against the growth of pancreatic cancer cells in a dose-dependent manner.

Ras-induced PKCa is known to induce the ODC protein, the first and the rate-limiting enzyme in the biosynthesis of polyamines expression during the development of various cancer types (26,27,44). Elevated levels of ODC gene products are consistently detected in transformed cell lines, virtually all-animal tumors and in certain tissues predisposed to tumorigenesis (24,25). Because tumor formation can be prevented by the agents that block induction of ODC, several ODC inhibitors have been tested against various types of cancers [(18) and references therein]. These data demonstrate that Lupeol is a potent inhibitor of ODC expression and is consistent with our earlier report where we showed that Lupeol inhibits ODC activity and expression in vivo (18).

Based on the outcome of this study, we suggest Lupeol could provide a multi-prong beneficial strategy for targeting multiple signaling pathways leading to apoptosis and inhibition of growth of pancreatic cancer cells. The schematic representation of the action of Lupeol is depicted in Figure 7. This may be explained by modulation of NFkB mediated by Ras-induced pathways such as PKCa/ODC, PI3K/Akt and MAPK signaling. Since pancreatic cancer is resistant to conventional chemotherapeutic regimens, Lupeol could be a potential agent to overcome this resistance. Further in-depth in vivo studies are warranted to verify this suggestion and are presently under investigation in our laboratory.

**Supplementary material**

Supplementary material can be found at: http://carcin.oxfordjournals.org/

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