Triptolide Induces Pancreatic Cancer Cell Death via Inhibition of Heat Shock Protein 70

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Abstract

Pancreatic cancer is highly resistant to current chemotherapy agents. We therefore examined the effects of triptolide (a diterpenoid triepoxide) on pancreatic cancer growth and local-regional tumor spread using an orthotopic model of pancreatic cancer. We have recently shown that an increased level of HSP70 in pancreatic cancer cells confers resistance to apoptosis and that inhibiting HSP70 induces apoptosis in these cells. In addition, triptolide was recently identified as part of a small molecule screen, as a regulator of the human heat shock response. Therefore, our aims were to examine the effects of triptolide on (*a*) pancreatic cancer cells by assessing viability and apoptosis, (*b*) pancreatic cancer growth and local invasion *in vivo*, and (*c*) HSP70 levels in pancreatic cancer cells. Incubation of PANC-1 and MiaPaCa-2 cells with triptolide (50–200 nmol/L) significantly reduced cell viability, but had no effect on the viability of normal pancreatic ductal cells. Triptolide induced apoptosis (assessed by Annexin V, caspase-3, and terminal nucleotidyl transferase–mediated nick end labeling) and decreased HSP70 mRNA and protein levels in both cell lines. Triptolide (0.2 mg/kg/d for 60 days) administered *in vivo* decreased pancreatic cancer growth and significantly decreased local-regional tumor spread. The control group of mice had extensive local invasion into adjacent organs, including the spleen, liver, kidney, and small intestine. Triptolide causes pancreatic cancer cell death *in vitro* and *in vivo* by induction of apoptosis and its mechanism of action is mediated via the inhibition of HSP70. Triptolide is a potential therapeutic agent that can be used to prevent the progression and metastases of pancreatic cancer. [Cancer Res 2007;67(19):9407–16]

Introduction

Pancreatic cancer is one of the most aggressive of all human malignancies. It has an extremely low 5-year survival rate of <1% (1, 2) and is the fourth leading cause of cancer-related death in the United States. The severity of this malignancy can be appreciated by the fact that in 2005 alone, 32,180 new cases were diagnosed, and almost the same number succumbed to the disease. Efforts to gain information on the pathobiology of pancreatic cancer and to elucidate the molecular mechanisms related to the tumor's invasion and metastases are urgently needed in order to develop innovative and effective treatments.

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doi:10.1158/0008-5472.CAN-07-1077

Resistance to apoptosis is a key factor preventing response to therapies in pancreatic cancer. Pancreatic cancer cells have several survival mechanisms that help them elude cell death. A group of stress proteins known as the heat shock proteins (HSP) have recently been recognized as inhibitors of apoptosis. We have shown that HSP70 mRNA levels are significantly higher in human pancreatic tumor tissue compared with normal tissue from the same patients (3), and Ogata et al. (4) have shown that HSPs were expressed more in poorly differentiated pancreatic adenocarcinomas as compared with well-differentiated ductal adenocarcinomas. Recent studies in our laboratory have shown that HSP70 is up-regulated in pancreatic cancer cells, whereas the inhibition of its expression results in apoptotic cell death (3).

Therefore, our overall aim is to pharmacologically manipulate the levels of HSP70 in order to use its down-regulation as a tool to improve the prognosis of pancreatic cancer. Westerheide et al. (5) recently did a small molecule screen which identified triptolide, a diterpene triepoxide from the Chinese plant *Tripterygium wilfordii*, as an inhibitor of human heat shock gene transcription. Triptolide has been widely used as a natural medicine in China for hundreds of years, particularly in the treatment of autoimmune and inflammatory diseases, including rheumatoid arthritis.

Several reports have indicated that triptolide can inhibit the proliferation of cancer cells *in vitro* and reduce the growth and metastases of tumors *in vivo* (6–10). To date, *in vivo* studies have shown that triptolide inhibits the growth of (*a*) human mammary tumor cells in nude mice (8), (*b*) cholangiocarcinoma cells in hamsters (9), and (*c*) xenografts formed by four different tumor cell lines (melanoma, breast cancer, bladder cancer, and gastric carcinoma; ref. 10). In addition, clinical trials in China using triptolide have shown remission rates of 71% and 87% in mononucleocytic and granulocytic leukemia, respectively (11). However, no previous reports have examined triptolide's ability to induce pancreatic cancer cell death *in vivo*, and its mechanism of action is not well understood. Therefore, the aims of this study were to examine the effect of triptolide on (*a*) pancreatic cancer cell growth and apoptosis *in vitro*, (*b*) pancreatic cancer growth *in vivo*, and (*c*) HSP70 expression in pancreatic cancer cells.

Materials and Methods

Methods. Materials used in this study include triptolide and cytochrome *c* ELISA purchased from Calbiochem, mouse HSP70 antibody from Stressgen, goat-polyclonal actin antibody from Santa Cruz Biotechnology, Inc., WST-8 viability assay from Dojindo Molecular Technologies, DMEM and McCoy's culture media from Invitrogen Corporation, avidin-biotin-peroxidase complex reagent from Vector Laboratories, insulin-transferrin-selenium and bovine pituitary extract from BD Biosciences, Guava Nexin Apoptosis Kit from Guava Technologies Inc., cDNA kit from Applied Biosystems, RNeasy Mini Kit for RNA extraction and Quantitect Sybr-Green PCR kit from Qiagen, Cell Death Detection Kit from Roche, and all other

reagents were purchased from Sigma. Pancreatic cancer cells were a generous gift from Dr. Edward E. Whang (Brigham and Women's Hospital, Harvard Medical School, Boston, MA). Mouse pancreatic ductal cells (primary isolates) were a kind gift from Dr. Anil K. Rustgi (University of Pennsylvania, Philadelphia, PA).

Cell culture. Pancreatic cancer cells (MiaPaCa-2 and PANC-1) were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin. Mouse pancreatic ductal cells were cultured in DMEM/F-12 medium (1:1) with 5 mg/L of D-glucose, 0.1 mg/mL of soybean trypsin inhibitor type I, 5 mL/L of ITS+, 25 μg/mL of bovine pituitary extract, 5 nmol/L of 3,3′,5-triiodo-L-thyronine, 1 μmol/L of dexamethasone, 1.22 mg/mL of nicotinamide, 5% Nu-Serum, 100 ng/mL of cholera toxin, and 1% penicillin-streptomycin (12). All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

The human colon adenocarcinoma HT-29 cells (American Type Culture Collection) were cultured in McCoy's medium, supplemented with 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C.

Treatment of cells with triptolide. Cells were seeded in six-well plates at a density of 2.5×10^5 cells and were then incubated overnight at 37°C for analysis of protein, RNA, or apoptosis. Cells were seeded into 96-well plates using 1×10^4 cells per well for viability measurements. Triptolide was dissolved in DMSO at a concentration of 1 μmol/L, added to cells at the indicated concentrations in serum-free medium, and incubated for varying times at 37°C. Cells treated with medium containing vehicle served as controls.

Determination of cell viability. Cell viability was determined using the Dojindo Cell Counting Kit-8. Cells were seeded into a 96-well plate at 1×10^4 cells per well and allowed to adhere overnight. After treatment with triptolide at various concentrations for 24 and 48 h, 10 μL of the tetrazolium substrate was added to each well of the plate. Plates were incubated at 37°C for 1 h, after which the absorbance at 450 nm was measured. All experiments were done in triplicate and repeated four independent times.

Measurement of Annexin V–positive cells. Phosphatidylserine externalization was analyzed using the Guava Nexin Kit by flow cytometry, as previously described (3).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay for measurement of *in situ* apoptosis. Pancreatic tumor cells were cultured on chamber slides. The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was then carried out according to the manufacturer's instructions (In Situ Cell Death Detection Kit; Roche). Paraffin-embedded pancreatic tumor tissue sections from control and triptolide-treated mice were also processed for TUNEL using the above kit.

Caspase-3 measurements. Caspase-3 activity was analyzed using the Caspase-Glo luminescent-based assays according to the manufacturer's instructions. Cells (1×10^4) were seeded into 96-well white opaque plates and a corresponding optically clear 96-well plate, then allowed to adhere overnight. The next day, cells were treated with varying concentrations of triptolide for varying periods of time. At the end of the incubation time, 100 μL of the appropriate Caspase-Glo reagent was added to each well containing 100 μL of blank, negative control, or treated cells in culture medium. Plates were gently mixed and incubated for 1 h at room temperature. The luminescence was then read in a luminometer. The corresponding 96-well clear plate was used to measure the number of viable cells with the CCK-8 reagent. Caspase activity was normalized to these values.

Cytochrome *c* ELISA. Following treatment with triptolide for 24 h, cells were harvested with trypsin and centrifuged briefly at $800 \times g$. Then, the supernatant was discarded. The cell pellet was resuspended and washed with PBS. Cells were pelleted at $1{,}000 \times g$ for 5 min and the supernatant was discarded. The cell pellet was then resuspended with digitonin cell permeabilization buffer (250 mmol/L sucrose, 137 mmol/L NaCl, 70 mmol/L KCl, 4.3 mmol/L Na_2HPO_4, 1.4 mmol/L K_2HPO_4, 0.2 mg/mL digitonin and 0.1% Hydorol M), vortexed and incubated on ice for 5 min. Cells were then centrifuged at $1{,}000 \times g$ for 5 min at 4°C. The supernatants contained the cytosolic fraction. The remaining pellet was finally resuspended with radioimmunoprecipitation assay cell lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS], vortexed and incubated on ice for 5 min. The lysate was vortexed and centrifuged at $10{,}000 \times g$ for 10 min at 4°C. The supernatant contained the mitochondrial fraction. Both fractions were stored at −80°C until it was analyzed in ELISA. Cytochrome *c* measurements were done using the ELISA kit from Calbiochem according to the manufacturer's instructions.

Quantitative real-time PCR for HSP70. The expression of HSP70 was examined in pancreatic cancer cells after they were treated with triptolide using real-time quantitative PCR. Briefly, RNA was extracted using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions and treated with DNase-I on the column. Total RNA (1 μg) was reverse transcribed. Real-time PCR was done using the Quantitect Sybr green PCR kit (Qiagen) according to the manufacturer's instructions using an Applied Biosystems 7300 real-time PCR system. HSP70 mRNA sequences were retrieved from the National Center for Biotechnology Information web site, and the National Center for Biotechnology Information BLAST server was used to determine primer specificity.

HSP70 forward primer, 5′-ACCAAGCAGACGCAGATCTTC-3′; reverse primer, 5′-CGCCCTCGTACACCTGGAT-3′ (amplicon size, 78 bp). All data were normalized to the housekeeping gene 18S (18S Quantitect Primer Assay; Qiagen).

Measurement of HSP70 levels by Western blotting. Cell lysates were prepared by resuspending cells in lysis buffer [65 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Nonidet P40, 1% sodium deoxycholate, 1 μg/mL aprotinin, and 100 μg/mL phenylmethylsulfonyl fluoride] for 30 min at 4°C and cleared by centrifugation for 30 min at $13{,}000 \times g$. Supernatants were collected and stored at −80°C. Total protein concentration was determined using the Pierce bicinchoninic acid assay. Western blotting for HSP70 was done as previously described using a monoclonal antibody from Stressgen, which binds to the epitope corresponding to the common amino acid residues 436 to 503 of the human and mouse protein (13). Equal protein loading was confirmed by staining with Ponceau S [0.1% Ponceau S (w/v) in 5% acetic acid (v/v)]. Actin expression was used as an internal control.

Development of stable pancreatic cancer cell line. To study pancreatic cancer growth *in vivo*, MiaPaCa-2 cells were stably transfected with the luciferase gene. The luciferase coding sequence was isolated from the pGL-3 vector (Promega) and cloned into the retroviral plasmid pBabe-puro behind the SV40 promoter to form the luciferase-expressing retrovirus (pBabe-puro-Luciferase). pBabe-puro-luciferase and pMDG (encoding the VSV envelope glycoprotein) were transfected (5×10^5 in six-well plates) into the Phoenix packaging cell line using Effectene transfection reagent (Qiagen), according to the manufacturer's instructions. Forty-eight hours after transfection, viral supernatants were collected, filtered through a 0.2-μm filter and transferred onto MiaPaca-2 cells (1×10^5 in six-well plates) for a 5-h infection in the presence of Polybrene (8μg/mL; Sigma). After 5 h, the culture medium was then replaced by fresh medium. After overnight culture, cells were incubated with puromycin (4 μg/mL; Calbiochem) for a period of 2 weeks for selection. Luciferase expression was confirmed using the Dual Luciferase Assay (Promega), and emitted light was directly proportional to cell number.

Orthotopic pancreatic tumors in nude mice. To study the effect of triptolide on pancreatic tumors *in vivo*, we used an orthotopic mouse model. MiaPaca-2 cells were stably transfected with luciferase, as described above. Following harvesting of cells, trypan blue exclusion was used to confirm that cells were >90% viable. Cells were resuspended in Matrigel at a concentration of 1 million cells per 10 μL and kept on ice until injected. Female nude mice (4–6 weeks old from Charles River Laboratories) were anesthetized with ketamine/xylazine (100 mg and 10 mg/kg) and the surgical procedure was carried out according to the guidelines of the University of Minnesota Institutional Animal Care and Use Committee. Briefly, a small left abdominal flank incision was made, and the spleen and pancreas were exteriorized. MiaPaCa-2 tumor cells (1 million cells) were injected into the tail of the pancreas with a Hamilton syringe. The fact that cells were in Matrigel prevented any i.p. leakage because the Matrigel sets at

37°C. Both layers of the abdominal wound were closed with stitches and wound clips.

Six days after tumor transplantation, mice were injected i.p. with D-luciferin (150 mg/kg) and anesthetized. After D-luciferin injection (8–10 min), luminescence was measured using a Xenogen imaging system, which determined that all tumors were of a similar size. Mice were then randomized into two treatment groups: (*a*) control and (*b*) triptolide-treated mice. Mice were given daily i.p. injections of triptolide (0.2 mg/kg) or vehicle (DMSO) for 60 days. Tumor volume was calculated as 0.5 × length × width × depth. Local-regional spread was determined macroscopically at autopsy as well as by a blinded pathologist. Intrapancreatic tumors were rapidly excised and divided into three fragments. One fragment was snap-frozen in liquid nitrogen and stored at −80°C for protein extraction and analysis of HSP70 by Western blot, as described above. Another fragment was stored in RNAlater for RNA extraction and analysis of HSP70 by real-time PCR. A third tumor fragment was fixed in formalin and embedded in paraffin for subsequent H&E staining and TUNEL staining.

HSP70 immunohistochemistry. Antigen retrieval was done on paraffin-embedded pancreatic tumor tissue sections using 10 mmol/L of citrate buffer (pH 6.0). Sections were blocked with 2.5% horse serum and subsequently incubated in blocking buffer containing a rabbit polyclonal antibody against HSP70 according to the manufacturer's instructions (Abcam). Sections were then incubated with the secondary antibody (horseradish peroxidase–labeled horse anti-rabbit) according to the manufacturer's instructions (Vector Lab). 3,3′-Diaminobenzidine was used

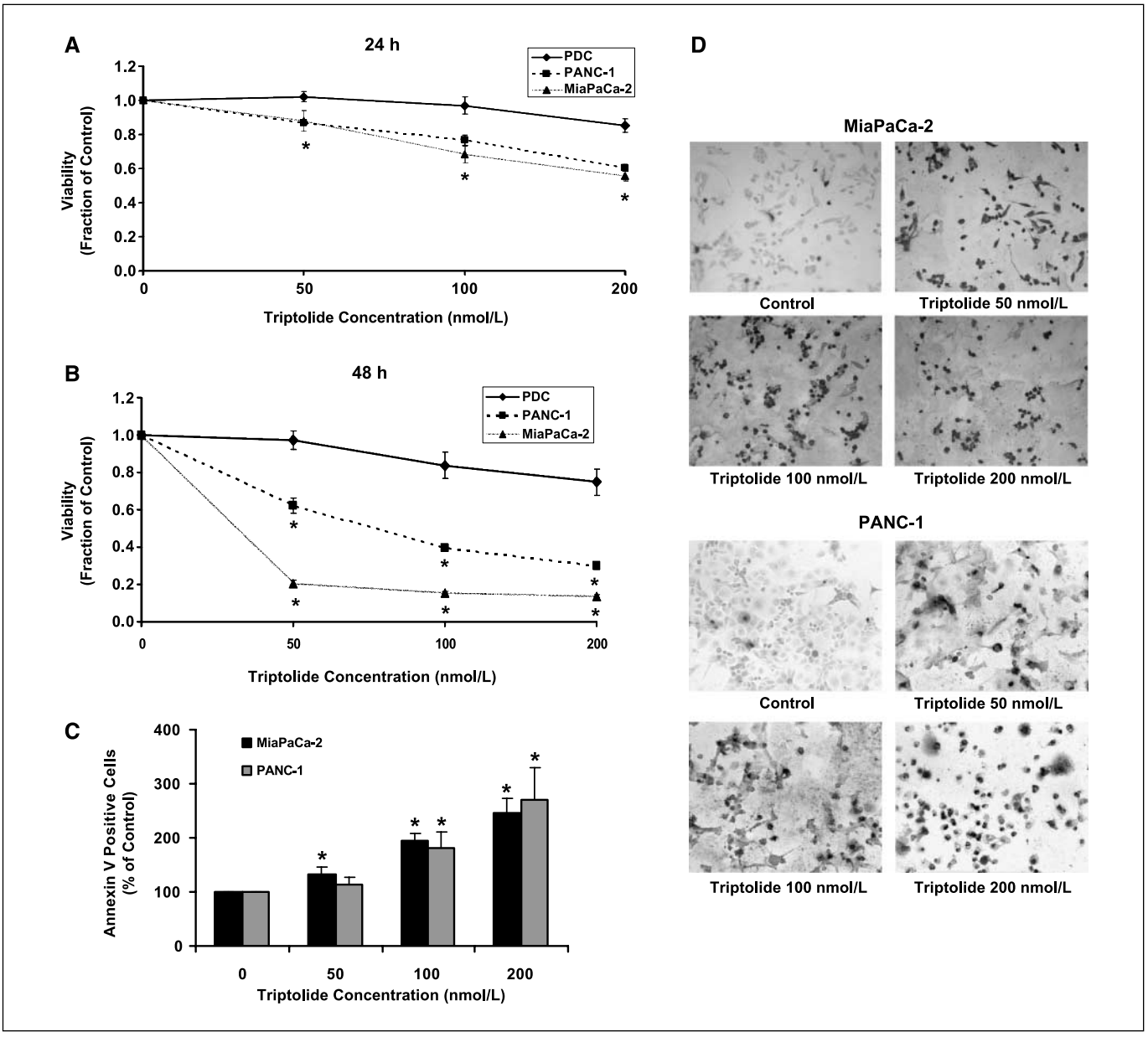


Figure 1. Effect of triptolide on pancreatic cancer cell viability and apoptosis (Annexin V and TUNEL). The presence of 50 to 200 nmol/L of triptolide in the culture medium significantly decreased the viability of both PANC-1 and MiaPaCa-2 cells at both 24 (*A*) and 48 h (*B*) of incubation, as compared with control cells incubated with culture medium alone. In contrast, little effect was observed on normal duct cell viability. *Points,* means; *bars,* SE ($n = 4$; *, $P < 0.05$ for MiaPaCa-2 and PANC-1). *C,* MiaPaCa-2 or PANC-1 cells were incubated with triptolide (50–200 nmol/L) for 24 h and Annexin V levels were assessed by flow cytometry. Triptolide dose-dependently increased Annexin V in both MiaPaCa-2 and PANC-1 cells. *Columns,* means; *bars,* SE ($n = 4$; *, $P < 0.05$). *D,* cells incubated for 48 h with triptolide were examined for *in situ* TUNEL staining. Both the MiaPaCa-2 and PANC-1 cells incubated with triptolide for 48 h show significantly increased TUNEL staining, as compared with control cells incubated with the vehicle alone.

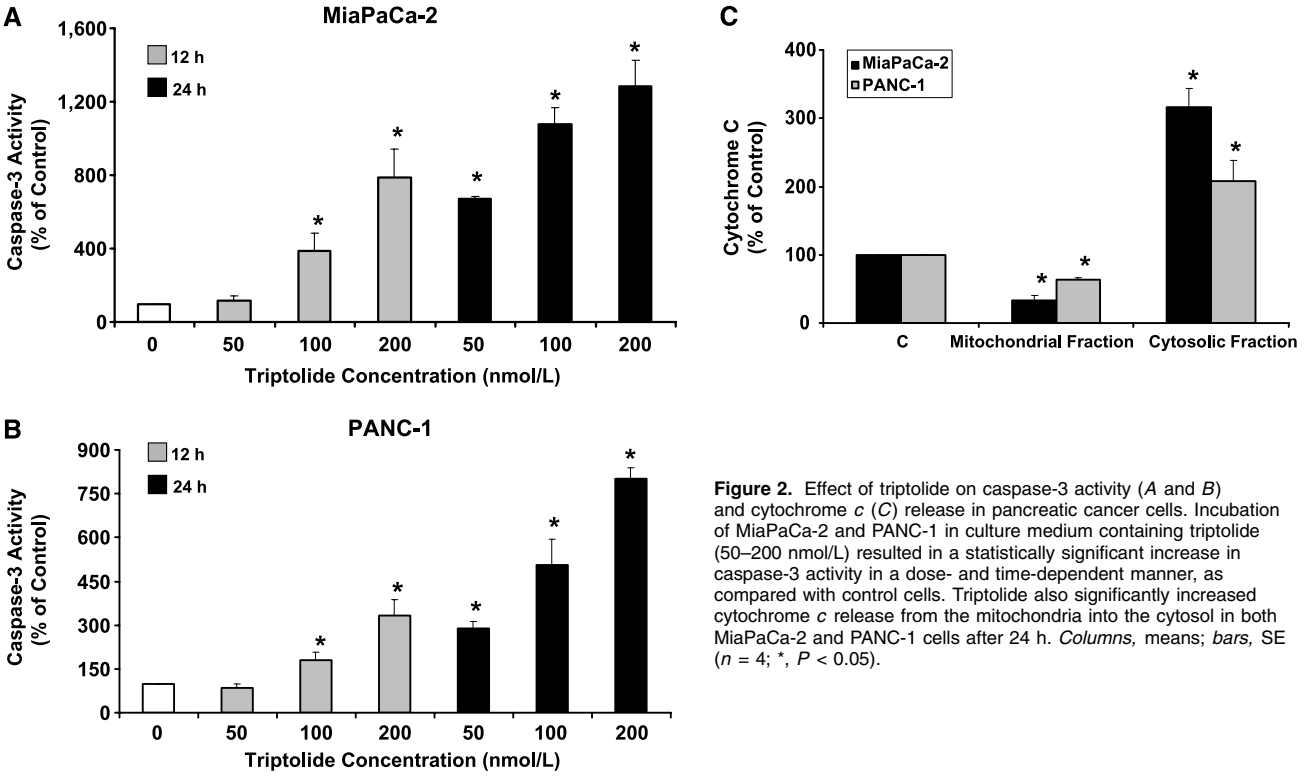


Figure 2. Effect of triptolide on caspase-3 activity (*A* and *B*) and cytochrome *c* (*C*) release in pancreatic cancer cells. Incubation of MiaPaCa-2 and PANC-1 in culture medium containing triptolide (50–200 nmol/L) resulted in a statistically significant increase in caspase-3 activity in a dose- and time-dependent manner, as compared with control cells. Triptolide also significantly increased cytochrome *c* release from the mitochondria into the cytosol in both MiaPaCa-2 and PANC-1 cells after 24 h. *Columns,* means; *bars,* SE ($n = 4$; *, $P < 0.05$).

as a substrate for the peroxidase reaction and methyl green was used as the counterstain.

Statistical analysis. Values are expressed as the mean ± SE. The significance of the difference between the control and each experimental test condition was analyzed by unpaired Student's *t* test and $P < 0.05$ was considered statistically significant. For the *in vivo* experiments, analysis of the effects of triptolide on local-regional tumor spread was done using the χ^2 test and $P < 0.05$ was considered statistically significant.

Results

The Effect of Triptolide on Pancreatic Cancer Cell Viability

The effect of triptolide on the viability of pancreatic cancer cells or normal pancreatic ductal cells was examined after incubation in medium containing triptolide at concentrations of 50 to 200 nmol/L for 24 and 48 h. Cells incubated in the medium without triptolide served as controls. The presence of triptolide significantly reduced both MiaPaCa-2 and PANC-1 cell viability in a dose- and time-dependent manner, whereas little effect was observed on the viability of normal ductal cells (Fig. 1).

The Effect of Triptolide on Apoptosis in Pancreatic Cancer Cells

To assess the mechanism by which triptolide induces cell death, several markers of apoptosis were analyzed, including (*a*) Annexin V, (*b*) TUNEL, (*c*) caspase activity, and (*d*) cytochrome *c* release from the mitochondria.

Annexin V. Phosphatidylserine externalization is a parameter of apoptosis, which can be measured by Annexin V staining. After 24 h, triptolide dose-dependently increased Annexin V in both MiaPaCa-2 and PANC-1 cells (Fig. 1*C*). This result confirms that cell death induced by triptolide in pancreatic cancer cells is facilitated by the induction of apoptosis.

TUNEL. *In situ* apoptosis was measured using TUNEL staining. Both the MiaPaCa-2 and PANC-1 cells incubated with triptolide for 48 h showed significantly increased TUNEL staining, as compared with control cells incubated with the vehicle alone (Fig. 1*D*).

Caspase-3 activity. To determine the effect of triptolide on caspase-3 activity, MiaPaCa-2 and PANC-1 cells were incubated with triptolide (50–200 nmol/L) for 12 and 24 h. Incubation of MiaPaCa-2 and PANC-1 in culture medium containing triptolide resulted in a statistically significant increase in caspase-3 activity in a dose- and time-dependent manner, as compared with control cells (Fig. 2*A* and *B*). An increase in caspase-3 activity was first evident 12 h after incubation with triptolide and continued to increase over time. These results indicate that the observed decrease in pancreatic cancer cell viability after incubation with triptolide was due to caspase-dependent activation of apoptosis.

Cytochrome *c*. Cytochrome *c* is released from the mitochondria in response to an apoptotic stimulus, which activates the caspase cascade and finally leads to apoptosis. Therefore, we examined cytochrome *c* release from the mitochondria of pancreatic cancer cells after incubation with triptolide for 24 h. Triptolide significantly increased cytochrome *c* release from the mitochondria in both MiaPaCa-2 and PANC-1 cells (Fig. 2*C*). Triptolide reduced mitochondrial cytochrome *c* by 50%, suggesting its increased release from the mitochondria. In support of this fact, triptolide increased cytosolic cytochrome *c*. In summary, triptolide is capable of initiating apoptotic cell death in pancreatic cancer

cells via increased cytochrome *c* release from the mitochondria, after which, the caspase cascade is activated.

The Effect of Triptolide on HSP70 Levels in Pancreatic Cancer Cells

Given that triptolide was recently identified (5) as part of a small molecule screen for regulators of the human heat shock gene transcription (5), and considering that our laboratory has previously shown that the inhibition of HSP70 expression causes pancreatic cancer cell death (3), the effect of triptolide on HSP70 expression in MiaPaCa-2 and PANC-1 was analyzed by real-time PCR and Western blot.

Triptolide (50–200 nmol/L) significantly decreased HSP70 mRNA expression by MiaPaCa-2 and PANC-1 at both 12 and 24 h, as compared with control cells not exposed to triptolide (Fig. 3*A*). In accordance with its effect on mRNA expression, triptolide also significantly decreased HSP70 protein levels in both MiaPaCa-2 and PANC-1 (Fig. 3*B* and *C*). Given that triptolide had little effect on normal pancreatic duct cell viability, we assessed its effect on HSP70 levels in these cells. The results show that triptolide had no effect on HSP70 protein expression by normal pancreatic duct cells (Fig. 3*D*). Together, the above data suggests that triptolide causes pancreatic cancer cell death by inhibiting HSP70 expression.

The Effect of Triptolide on Pancreatic Tumor Growth *In vivo*

To examine the effects of triptolide on pancreatic cancer growth and local-regional spread, MiaPaCa-2 cells (stably transfected with

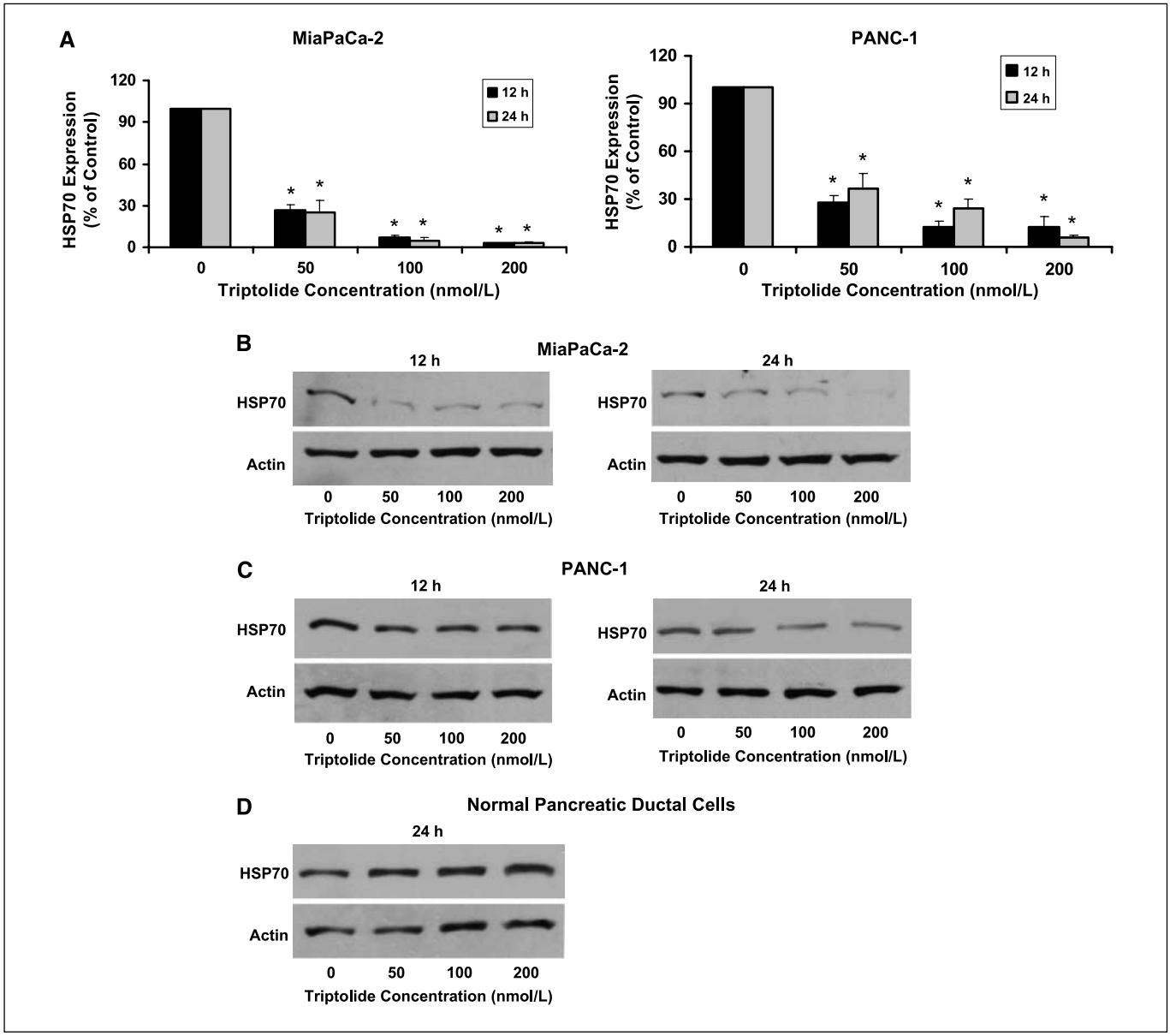


Figure 3. Effect of triptolide on HSP70 mRNA and protein expression in pancreatic cancer cells. *A,* triptolide (50–200 nmol/L) significantly reduced HSP70 mRNA expression (as assessed by real-time PCR) in both MiaPaCa-2 and PANC-1. Expression of HSP70 was normalized against the housekeeping gene 18S. *Columns,* means; *bars,* SE ($n = 3$; *, $P < 0.05$). Triptolide also decreased HSP70 protein levels in MiaPaCa-2 (*B*) and PANC-1 (*C*) at 12 and 24 h (representative Western blots for HSP70 and actin). However, triptolide had no effect on normal pancreatic ductal cells (*D*). Membranes were stripped and reprobed for actin to show equal protein loading ($n = 4$).

Table 1. Analysis of local-regional tumor spread in control versus triptolide-treated mice ($P < 0.001$, $n = 8$, χ^2 analysis)

Organ	Control	Triptolide
Spleen	4/8	0/8
Liver	2/8	1/8
Gall bladder	1/8	0/8
Kidney	2/8	0/8
Intestine	5/8	0/8
Total no. of organs invaded	14	1

luciferase) were injected into the pancreas, as described in Methods. Prior to the commencement of treatment, luminescence was measured using a Xenogen Imaging System, which determined that all tumors were of a similar size. Mice were then randomized into two treatment groups with the same mean tumor size: (*a*) control and (*b*) triptolide-treated mice. After a daily injection of triptolide (0.2 mg/kg) for 60 days, the tumor volume was significantly reduced (Fig. 4*A*), as compared with controls injected with DMSO vehicle. In fact, two mice in the triptolide treatment group had no macroscopically visible tumor, which was confirmed by histologic analysis. Importantly, triptolide had no effect on body weight and mice displayed no signs of toxicity. Furthermore, the results in Table 1 indicate that triptolide also significantly reduced the incidence of local-regional tumor spread. A pathologic evaluation of the local organs listed in Table 1 showed that 87.5% of mice from the control group had local tumor spread, as compared with 12.5% of mice from the triptolide-treated group ($P < 0.001$; χ^2 analysis). Mice in the control group had significantly increased ($P < 0.02$; χ^2 analysis) local spread of tumor cells into the spleen and intestine, as compared with mice from the triptolide-treated group. Examples of local spread in the form of tumor nodules in the spleen, kidney, and bowel can be seen in Fig. 4*B*. Note that arrows point toward tumors.

In order to provide information on the mechanism by which triptolide suppresses the growth of pancreatic tumors *in vivo*, we measured HSP70 levels in pancreatic tumor tissue from both triptolide and control treated mice. Figure 4*C* shows that HSP70 levels were significantly decreased (as assessed by real-time PCR) in tumors from mice treated with triptolide, as compared with mice treated with vehicle. Similar results were obtained with protein levels as assessed by Western blot (Fig. 4*C*). In addition, immunohistochemistry showed decreased HSP70 expression in tumor sections from mice treated with triptolide, as compared with mice treated with vehicle (Fig. 4*C*). This confirms our *in vitro* data in which triptolide induces pancreatic cancer cell death via decreasing HSP70 levels.

In accordance with the *in vitro* data, decreased tumor size following treatment with triptolide was associated with increased TUNEL staining in tumor sections, as compared with sections from control mice (Fig. 4*D*). This data suggests that triptolide-induced cell death *in vivo* is a result of apoptosis.

The Effect of Triptolide on Colon Cancer Cell Viability and HSP70 Expression

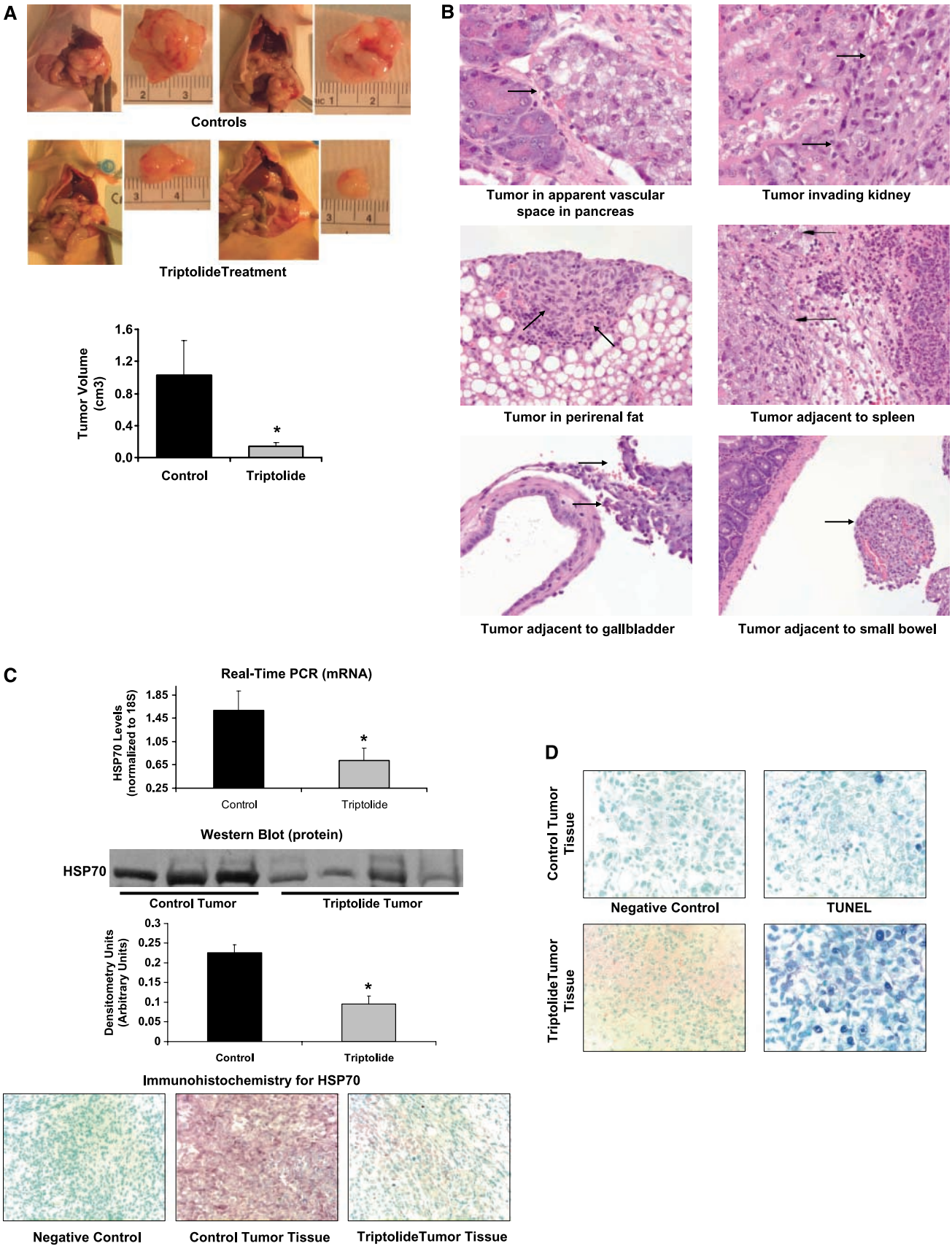
Given that HSP70 has previously been reported to be overexpressed in colon cancer cells, we tested whether or not triptolide could induce colon cancer cell death by the inhibition of HSP70, in a similar fashion to the results in pancreatic cancer, described above. The effect of triptolide on colon cancer cell viability was examined after incubation in medium containing triptolide (50–400 nmol/L) for 24 and 48 h. Cells incubated in medium without triptolide served as controls. The presence of triptolide significantly reduced HT29 cell viability in a time-dependent manner (Fig. 5). The observed decrease in viability was associated with increased Annexin V levels (Fig. 5*B*), and significantly decreased HSP70 mRNA (Fig. 5*C*) and protein levels (Fig. 5*D*). Therefore, triptolide-induced cell death in colon cancer cells is mediated by induction of apoptosis via the inhibition of HSP70. These results further confirm that triptolide's mechanism of action is via inhibition of HSP70.

Discussion

This study has shown that low nanomolar concentrations of triptolide induce pancreatic cancer cell death, without affecting the viability of normal pancreatic duct cells. The mechanism by which triptolide induces pancreatic cancer cell death is by induction of apoptosis via the inhibition of HSP70. In addition, *in vivo* administration of triptolide decreased tumor growth and almost completely prevented local-regional tumor spread in an orthotopic nude mouse model of pancreatic cancer. This decrease in tumor growth was also associated with increased TUNEL and decreased HSP70 levels in the tumor.

In the present study, incubation with triptolide significantly decreased pancreatic cancer cell viability in two pancreatic cancer cell lines, but did not affect that of normal pancreatic ductal cells. Next, we examined whether the apoptosis pathway was responsible for triptolide-induced pancreatic cancer cell death. Triptolide induced apoptosis in pancreatic cancer cells, with significant increases in Annexin V and TUNEL staining. Our data concur with the results of a study by Wang et al. (14), showing that triptolide increased apoptosis in PANC-1 cells. Although the mechanism of action of triptolide has not been fully elucidated, it has been shown to induce caspase activation (15–17). One pathway of caspase activation is the intrinsic or mitochondrial pathway, from which cytochrome *c* is released into the cytosol, which then binds with

Figure 4. The effect of triptolide on pancreatic tumor growth *in vivo*. Nude mice with orthotopic MiaPaCa-2 tumors were given daily triptolide injections (0.2 mg/kg/d) for 60 consecutive days. *A, top,* representative photographs of tumors dissected from the pancreas and corresponding photo of tumor inside the mouse. *Bottom,* graph of triptolide showing significantly reduced pancreatic tumor volume ($n = 8$ animals per treatment group), as compared with controls (injected with vehicle alone). *B,* examples of local-regional invasion into adjacent organs from mice in the control group (H&E-stained sections). *C,* effect of triptolide on HSP70 levels in pancreatic tumor tissue (as assessed by real-time PCR, Western blotting, and immunohistochemistry). Triptolide significantly reduced HSP70 mRNA expression (as assessed by real-time PCR) in tumor tissue. Expression of HSP70 was normalized against the housekeeping gene 18S. *Columns,* means; *bars,* SE ($n = 6$ per treatment group; *, $P < 0.05$). Tumors had significantly less HSP70 protein levels (see representative Western blot and graph of densitometry analysis) following triptolide treatment ($n = 8$ per treatment group; *, $P < 0.05$). Ponceau stain confirmed equal protein loading. Immunohistochemistry confirmed that triptolide treatment reduced HSP70 levels in the tumor tissue (see representative pancreatic tumor tissue sections from control and triptolide treatment groups immunostained for HSP70). The negative control was a tumor section from a control mouse incubated without the primary antibody. Increased purple staining in the control tumor tissue depicts increased HSP70 levels. *D,* representative pancreatic tumor tissue sections stained for TUNEL showed increased TUNEL staining, which is indicative of apoptosis in the triptolide treatment group.



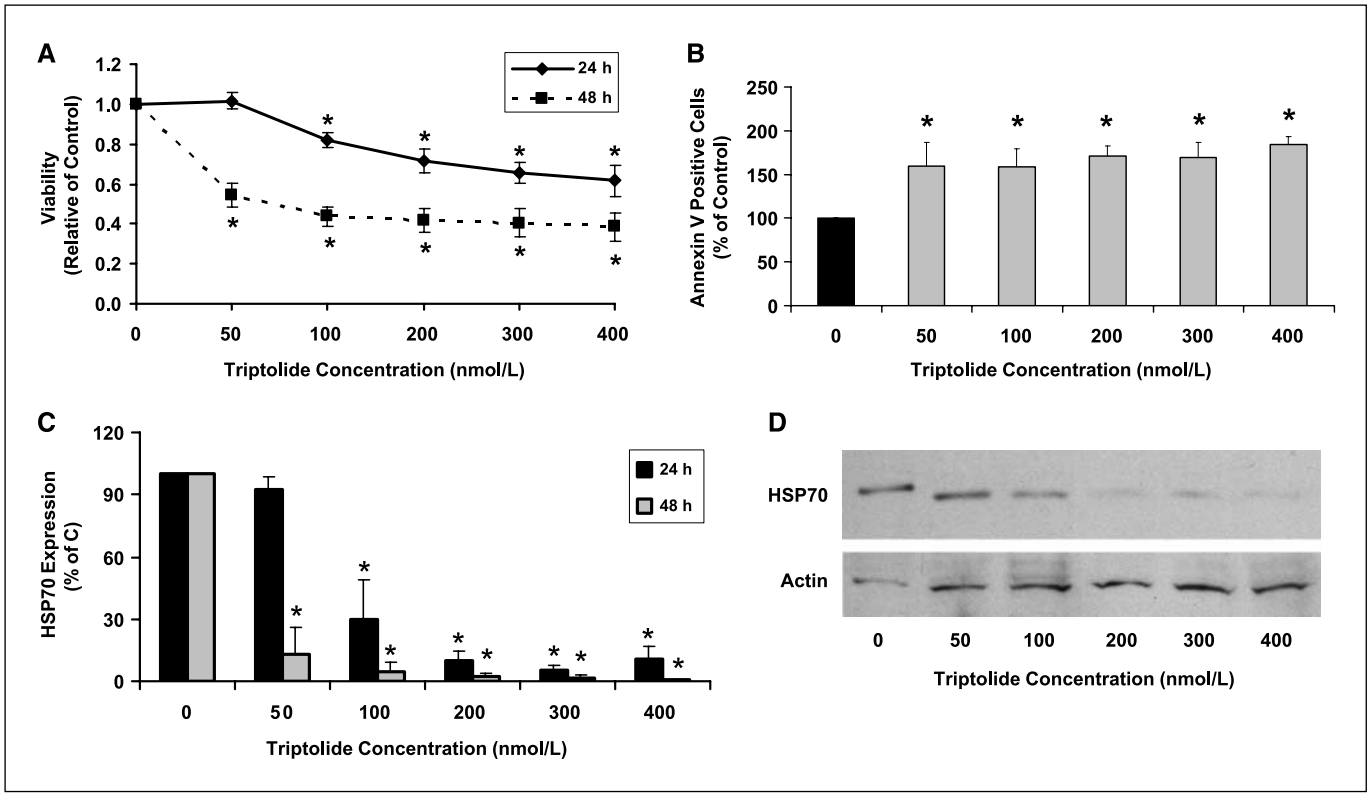


Figure 5. Effect of triptolide (50–400 nmol/L) on colon cancer cells (HT-29). *A,* triptolide significantly reduced HT-29 cell viability in a time- and dose-dependent manner ($n = 4$; *, $P < 0.05$). *B,* triptolide significantly increased Annexin V staining after 48 h ($n = 4$; *, $P < 0.05$). *C,* triptolide significantly reduced HSP70 mRNA expression (as assessed by real-time PCR) in HT-29 cells. Expression of HSP70 was normalized against the housekeeping gene 18S ($n = 3$; *, $P < 0.05$). *D,* triptolide also significantly decreased HSP70 protein levels (as assessed by Western blot) in HT-29 cells. Membranes were stripped and reprobed for actin to show equal protein loading.

apoptosis protease activator factor-1 and activates caspase-9 (18, 19). Activation of the downstream effector caspase-3 follows (18, 19). Our results show that, following incubation with triptolide, caspase-3 activity is significantly increased in two pancreatic cancer cell lines, thus strongly suggesting that the mitochondrial apoptotic pathway is responsible for triptolide-induced cell death. In addition, triptolide induced cytochrome *c* release from the mitochondria in pancreatic cancer cells, suggesting that apoptosis is induced upstream of the mitochondria. Therefore, the mitochondrial apoptosis pathway plays a critical role in triptolide-induced cell death. These results concur with those obtained in leukemia cell lines with triptolide (15).

Cancer research in the last 20 years has identified several proteins that are involved in promoting tumorigenesis via their inhibition of apoptosis, including HSPs (20). HSPs are highly conserved proteins which play a role in protecting cells from adverse environmental, physical, and chemical stresses. HSP70 is the major stress-inducible HSP, which renders cells highly resistant to several chemotherapeutic drugs (21–26). Our laboratory and others have shown that clinical pancreatic tumors have increased levels of HSP70 compared with normal tissue from the same patients (3, 27), whereas Ogata et al. (4) found that more HSPs were expressed in poorly differentiated pancreatic adenocarcinomas compared with well-differentiated ductal adenocarcinomas. Given the above, depleting HSP70 is a means of combating pancreatic cancer. However, only a small number of pharmacologic HSP70 inhibitors have been identified. These include quercetin (a bioflavonoid; refs. 28, 29) and KNK437 (a benzylidene lactam compound; ref. 30). Westerheide et al. (5) recently identified triptolide, a diterpene triepoxide derived from the Chinese plant *T. wilfordii*, as part of a small molecule screen, as an inhibitor of the human heat shock response. However, this group only analyzed triptolide's ability to regulate HSP70 in response to heat shock, and found that in HeLa cells, triptolide prevented the induction of HSP70 mRNA by heat shock (5). However, triptolide's ability to affect basal HSP70 levels has not been previously examined. We show here for the first time that triptolide is a potent inhibitor of both HSP70 mRNA and of protein levels in pancreatic cancer cells. In accordance with triptolide having a minimal effect on the cell viability of normal pancreatic ductal cells, triptolide did not influence HSP70 expression in these cells. This suggests that triptolide possibly interacts with the regulatory elements responsible for increased expression of HSP70 in cancer cells, which may be different from that involved in the overexpression of HSP70 during the heat shock response.

To examine whether the effect of triptolide on HSP70 expression is limited to pancreatic cancer cells exclusively, or if it is a general phenomenon applicable to other cancer cells, we examined the effect of triptolide on the colon cancer cell line HT-29 because high levels of HSP70 expression have been correlated with poor prognosis in colon cancer cells (31). Triptolide significantly reduced HT-29 cell viability, which was associated with an increase in apoptosis. Our data concur with a recent study by Ko et al. (32) showing that triptolide induced HT-29 cell death via increased apoptosis. Similar to the data obtained with pancreatic cancer, triptolide decreased HSP70 levels in HT29 cells. These results

provide further evidence that triptolide is a potent inhibitor of HSP70 in cancer cells. Additional evidence that triptolide-induced cell death is mediated via its effect on HSP70 comes from our recent work which showed that HSP70 inhibition by short interfering RNA significantly decreases pancreatic cancer cell viability as a result of increased apoptosis (3).

The exact site at which HSP70 interacts with the apoptotic cascade is controversial. Some studies have suggested that HSP70 may inhibit apoptosis by acting downstream of mitochondria and cytochrome *c* release (33–35). However, recent studies using cellular death models suggest that HSP70-mediated inhibition of caspase-dependent apoptosis occurs upstream of mitochondrial outer membrane permeabilization and cytochrome *c* release (36–38). Stankiewicz et al. (39) have suggested that HSP70 prevents the activation and translocation of Bax to the mitochondrial membrane, and thus, protects the cell against mitochondrial membrane permeabilization, cytochrome *c* release, and activation of the caspase cascade.

HSP70 can also inhibit caspase-independent apoptosis by inhibiting apoptosis-inhibiting factor (40). Additional mechanisms that have been proposed to explain the antiapoptotic effect of HSP70 include the modulation of the activity of stress kinases such as c-*Jun*-NH_2-kinase (41) and interaction with nuclear factor-κB (42). Some studies have suggested that the ATPase domain of HSP70 and its chaperone function is required for its action at some of these sites (38). Thus, it seems that the site and mechanism by which HSP70 inhibits apoptosis depends, to a large extent, on the cell type. Our data strongly suggests that the possible site of its action is upstream of the mitochondria in pancreatic cancer cells. The exact mechanism by which HSP70 inhibits apoptosis upstream of mitochondria in these cells is currently under investigation in our laboratory.

We also examined the effect of triptolide on pancreatic cancer growth using an orthotopic model of pancreatic cancer derived from the highly malignant pancreatic cancer cell line MiaPaCa-2. Daily triptolide administration decreased pancreatic tumor growth *in vivo*. This decrease in tumor size was associated with decreased HSP70 mRNA and protein expression in tumor tissue (by Western blot and immunohistochemistry), thereby confirming that triptolide decreases pancreatic tumor cell viability via lowering HSP70. In addition, triptolide also significantly reduced the incidence of local-regional tumor spread. In accordance with the *in vitro* data, decreased tumor size following treatment with triptolide was associated with increased TUNEL staining in tumor sections, suggesting that triptolide-induced cell death *in vivo* is a result of apoptosis.

Although our data is encouraging, we cannot completely rule out that triptolide might affect pancreatic cancer cell death by other mechanisms in addition to HSP70 inhibition. However, we believe that one of the major effects of triptolide is mediated via inhibition of HSP70 expression for several reasons:

(*a*) Triptolide is a potent inhibitor of HSP70 transcription. Triptolide significantly decreased HSP70 mRNA and protein levels in two pancreatic cancer cell lines, in tumor tissue, as well as in a colon cancer cell line.

(*b*) Inhibition of HSP70 by triptolide corresponds with the induction of apoptosis. Triptolide induced pancreatic cancer cell death via induction of apoptosis in a similar manner to our previous observations using HSP70-specific short interfering RNA (3).

(*c*) Triptolide does not inhibit HSP70 in normal pancreatic duct cells, and has a minimal effect on their viability. Our results show that triptolide had a minimal effect on normal pancreatic duct cell viability and because these cells have much lower levels of HSP70 than do pancreatic tumor cells (3), this suggests that triptolide promotes cell death in the cancer cells by inhibiting HSP70 levels.

The regulation of HSP70 gene transcription is complex and involves heat shock elements which are promoter sites that have a high affinity for binding to a family of heat shock transcription factors (HSF). HSF1 is well characterized and is required for the regulation of HSP70. The inhibitory mechanism of triptolide on HSP70 transcription might be attributed to the inhibition of the activation of HSF1 or the interaction of HSF1 with heat shock elements. For example, the other HSP70 inhibitor, Quercetin, has been reported to inhibit HSP70 expression by blocking HSF1 and reducing HSP70 mRNA accumulation (28, 43). Although Westerheide et al. (5) have examined the mechanism by which triptolide inhibits the heat shock response, their work may provide some clues for the regulation of HSP70 in cancer cells. They propose that triptolide inhibits the assembly of functional transcriptionally active HSF1 complexes on the HSP70 promoter. Presently, our laboratory is working toward understanding precisely where and how triptolide inhibits HSP70 transcription.

In conclusion, triptolide causes pancreatic cancer cell death *in vitro* and *in vivo* by the induction of apoptosis, and its mechanism of action is mediated via the inhibition of HSP70. In addition, we have provided further support that HSP70 confers resistance to apoptosis in pancreatic cancer cells. Therefore, triptolide is a potential therapeutic agent that can be used to prevent the progression and metastases of pancreatic cancer.

Acknowledgments

Received 3/22/2007; revised 7/17/2007; accepted 7/25/2007.

Grant support: NIH grant DK-58694 (A.K. Saluja).

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We acknowledge the valuable assistance of Drs. Brian Lewis, YaWen Chen, and Jen Morten in establishing the stable luciferase cell line and the orthotopic model of pancreatic cancer.

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