Heat Shock Protein 70 Increases Tumorigenicity and Inhibits Apoptosis in Pancreatic Adenocarcinoma

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Abstract

Pancreatic carcinoma is a malignant disease that responds poorly to chemotherapy because of its resistance to apoptosis. Heat shock proteins (Hsp) are not only cytoprotective but also interfere with the apoptotic cascade. Here, we investigated the role of Hsp70 in regulating apoptosis in pancreatic cancer cells. Hsp70 expression was increased in pancreatic cancer cells compared with normal pancreatic ductal cells. This was confirmed by increased mRNA levels for Hsp70 in human pancreatic cancer tissue compared with neighboring normal tissue from the same patient. Depletion of Hsp70 by quercetin decreased cell viability and induced apoptosis in cancer cells but not in normal pancreatic ductal cells. To show that this is a specific effect of Hsp70 on apoptosis, levels of Hsp70 were knocked down by short interfering RNA treatment, which also induced apoptosis in cancer cells as indicated by Annexin V staining and caspase activation. Daily administration of quercetin to nude mice decreased tumor size as well as Hsp70 levels in tumor tissue. These findings indicate that Hsp70 plays an important role in apoptosis and that selective Hsp70 knockdown can be used to induce apoptosis in pancreatic cancer cells. [Cancer Res 2007;67(2):616–25]

Introduction

Pancreatic cancer is one of the most common malignant diseases in the Western world and has the lowest 5-year survival rate of any cancer (1, 2). Ten percent to 15% of patients undergo potentially curative pancreatic surgery; however, radical surgery does not substantially improve survival (3). Nearly 90% of pancreatic neoplasms are adenocarcinomas arising from the exocrine ductal system (4). It is generally believed that cancer cells have an altered cell physiology comprising abundance of growth signals, insensitivity to all cycle arrest signals, and evasion from programmed cell death (apoptosis). Unresponsiveness to apoptotic stimuli can result in tumor progression and resistance to most oncologic therapies (5).

Cell death can occur in two main forms, apoptosis and necrosis, each with its own morphologic and biochemical manifestations (6–8). Apoptosis is formally defined by the appearance of distinct morphologic changes, including membrane blebbing, chromatin condensation, and fragmentation of the nucleus. Biochemical alterations, such as endonuclease-mediated DNA fragmentation and externalization of phosphatidylserine, underlie the phenotypic changes. Considered a "physiologic cell suicide" program, apoptosis is involved in embryonic development, tissue homeostasis, and immune system function. Disturbances in apoptosis contribute to numerous diseases, including cancer (9–11).

Mediated by a family of cysteine proteases known as caspases, two distinct pathways characterize apoptotic processes. The extrinsic pathway that is triggered by death receptors, such as FAS or the tumor necrosis factor (TNF) receptor with their respective ligands, results in caspase-8 activation. In response to growth factor withdrawal, hypoxia, or DNA damage, the intrinsic pathway is initiated, resulting in cytochrome c release, loss of mitochondrial membrane potential (7), and the apoptosome formation, a complex consisting of cytochrome c, apoptotic protease-activating factor-1 (ApaF-1), and procaspase-9 (9, 12). Both pathways converge at the level of caspase-3, an effector caspase that leads to the typical morphologic and biochemical changes of the apoptotic cell. Tumor cells express several proteins that suppress apoptosis. Functional interactions of those proteins in apoptosis pathways are therefore of particular interest. Among them, the antiapoptotic members of the Bcl-2 protein family, members of the inhibitor of apoptosis protein family, and heat shock proteins (Hsp) play a major role (9, 13).

Hsps are a protein family with a highly conserved structure. They are present in both eukaryotic and prokaryotic cells and act in fundamental cellular processes. Originally, they were discovered in cells after exposure to elevated temperatures and thus were called Hsps. But other cellular stress factors, such as hypoxia, sodium arsenite, and virus transformation, can also induce synthesis of Hsps. Because stress proteins associate with denatured or partially unfolded proteins and protect them from further denaturation, they belong to the superfamily of chaperones (14). Based on their molecular weight and sequence homology, Hsps are classified into different families. A very prominent and well-characterized subgroup of Hsps is the Hsp70 family. It encompasses isoforms ranging from 66 to 78 kDa that are encoded by a multigene family, and heat shock proteins (Hsp) play a major role (9, 13).

Hsp70 is involved in embryonic development, tissue homeostasis, and immune system function. Disturbances in apoptosis contribute to numerous diseases, including cancer (9–11).

A subset of the Hsp70 family, the heat shock cognate proteins (Hsc70, also known as Hsp73), is constitutively expressed under normal physiologic conditions and found within all the major intracellular compartments. Another isoform is stress inducible (also called Hsp72) and distributed predominantly in the cytoplasm, nucleus, and plasma membrane of various tumor cells (19, 20). Besides its essential role in cell survival under stressful conditions, Hsp72 is involved in numerous pathologic conditions, such as neurodegenerative diseases and ischemia (21), inflammatory processes, and immunogenicity (22).
We have shown in the past that inducible Hsp70 is critically involved in the protection against cellular injury in pancreatic acinar cells (23, 24). In the present study, we have studied whether Hsp70 is involved in the resistance to apoptosis of pancreatic cancer cells, a cell type resembling pancreatic duct cells rather than acinar cells. Our approach was to assess the expression of Hsp70 in pancreatic tumor cells and compare it to nontumorous pancreatic ductal cells. Based on those findings, we show induction of apoptosis after inhibition of Hsp70 in tumor cells by quercetin and no apoptosis when the inactive analogue dihydroquercetin is used. Hsp72-specific short interfering RNA (siRNA) also induced apoptosis via a selective down-regulation of the inducible form of Hsp70. These findings identify an essential role of Hsp70 in the resistance to apoptosis of pancreatic cancer cells.

**Materials and Methods**

Quercetin was from Fluka (Buchs, Switzerland); Ac-Asp-Glu-Val-Asp-MCA and Ac-Leu-Glu-His-Asp-MCA were from Peptides International (Louisville, KY); mouse Hsp70 antibody was from Stressgen (Victoria, British Columbia, Canada); goat polyclonal actin antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); WST-8 viability assay was from Dojindo Molecular Technologies (Gaithersburg, MD); LipofectAMINE 2000 reagent and Opti-MEM I medium were from Invitrogen Corp. (Carlsbad, CA); Microprotein kit was from Thermo Electron Corp. (Melbourne, Victoria, Australia); avidin-biotin complex method reagent was from Vector Laboratories (Burlingame, CA); Guava Nexin Apoptosis kit was from Guava Technologies, Inc. (Hayward, CA); cDNA kit and predeveloped assay reagents for Hsp70 and 18S were from Applied Biosystems (Foster City, CA); Cell Death Detection kit was from Roche (Indianapolis, IN); and Hsp72 siRNA was from Dharmacon, Inc. (Lafayette, CO). All other reagents were from Sigma. Pancreatic cancer cells were a kind 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 were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Mouse pancreatic ductal cells were cultured in DMEM/F-12 (1:1) with 5 mg/L D-glucose, 0.1 mg/mL soybean trypsin inhibitor type I, 5 mL/L insulin, transferrin, and selenium, 25 μg/mL bovine pituitary extract, 5 μmol/L 3,3',5-triiodo-t-thyronine, 1 mmol/L dexamethasone, 1.22 mg/mL nicotinamide, 5% Nu-Serum, 100 ng/mL chola toxin, and 1% penicillin-streptomycin. All cells were maintained at 37°C in a humidified air atmosphere with 5% CO2.

Quercetin and dihydroquercetin treatment. Cells were plated in a six-well plate at a density of 5 × 104 and kept overnight. For detecting cell viability, cells were split into 96-well plates using 104 per well. Quercetin or dihydroquercetin (an inactive analogue of quercetin) was dissolved in DMSO and added to the medium with final concentrations from 25 to 100 μmol/L. Cells were incubated at 37°C for 24 h. Cells treated with DMSO alone [-0.2% (ref. 25)] served as controls.

**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% NP40, 1% sodium deoxycholate, 1 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride] for 30 min at 4°C and cleared by centrifugation for 30 min at 13,000 × g. Supernatants were collected and stored at −80°C until used. Total protein concentration was determined using Trace/DMAC Microprotein kit. Western blotting for Hsp70 was done as previously described using a monoclonal antibody (mAb) from Stressgen, which binds to the epitope corresponding to the common amino acid residues 436 to 503 of the human and mouse protein (26). Equal protein loading was confirmed by staining with Poncose S [0.1% Poncose S (w/v) in 5% acetic acid (v/v)]. Actin expression was used as an internal control.

Quantitative real-time PCR for Hsp70. Expression of Hsp70 was examined in human pancreatic tumor tissue and compared with normal tissue from the same patient using real-time quantitative PCR. RNA from normal and tumor tissue from patients undergoing a pancreaticoduodenectomy were obtained from Dr. Hiroomi Tada (University of Massachusetts Medical School, Worcester, MA). RNA was treated with DNase I. Total cellular RNA (1 μg) was reverse transcribed. Predeveloped Taqman gene expression assays (primers and probe labeled with FAM) were used to quantitate the amount of Hsp70. The Taqman endogenous control was a predesigned probe and primer set for human 18S, which was labeled with VIC. All data were normalized to 18S expression.

**Immunolocalization of Hsp70.** Pancreatic tumor cells were plated on chamber slides and kept overnight. Cells were fixed in 4% paraformaldehyde for 30 min and then washed twice with PBS. Immunostaining for Hsp70 was then done as described previously (26).

**Transfection of siRNA.** Double-stranded Hsp72 siRNA duplex was dissolved in a buffer [20 mmol/L KCl, 6 mmol/L HEPES-KOH (pH 7.5), 0.2 mmol/L MgCl2] to a final concentration of 75 mmol/L. The sense strand (siRNA #1) was 5'-GGAGCAUCACGCCCAGACAGTdT-T and the antisense strand was 5'-CUUGUUCGGCGUGAGUCCtTdT-3' (27). An additional siRNA sequence (siRNA #2) for Hsp70 was used to rule out any "off-target" effects (sense strand, 5'-GGGAGAGGGUGAGCCAAAU-3'; antisense strand, 5'-UUGGCGUACACCCCUCCGCUU-3'). Scrambled nonsilencing siRNA [5'-UUCUCGGCAACGUGCUCCGdTdT-3' (sense) and 5'-ACGGUAGACAGCGUGAGAAtdTdT-3' (antisense) from Qiagen] bearing no relevant homology with any relevant human gene served as a control.

For transfection, 105 cells were plated into 24-well plates and incubated overnight. LipofectAMINE 2000 reagent (10 μL) was added to 250 μL Opti-MEM I medium and incubated at room temperature for 5 min. Hsp72 or control siRNA (100 nmol/L) was added and incubated for another 20 min. A third set of transfections was done without any siRNA (mock transfection). The transfection reagent/siRNA complex was added to the wells containing 2 mL DMEM with 10% FBS and incubated for 5 h at 37°C until medium was removed and replaced with fresh medium. Assays were done at 24, 48, and 72 h after transfection.

**Determination of cell viability.** Cell viability was determined by the Dojindo Cell Counting Kit-8. Cells were split into a 96-well plate at 105 per well and allowed to adhere overnight. After treatment with quercetin and dihydroquercetin at various concentrations for 24 h, 10 μL of the tetrazolium substrate were incoated to each well of the plate. Plates were incubated at 37°C for 2 h and the absorbance at 450 nm was measured. All experiments were done in triplicate and repeated at least twice.

To determine the effect of Hsp70-specific siRNA on pancreatic cancer cell viability, the cells were transfected as described above, and 12 h after transfection, the cells were passaged with equal number of cells (104) per well of a 96-well plate. After 72 h post transfection, 10 μL of the tetrazolium substrate was added to each well and absorbance was measured as described above.

**Caspase activity assays.** Caspase-9 and caspase-3 activities were measured by a fluorometric assay in whole-cell lysates using Ac-DEVD-MCA substrate for caspase-3 and Ac-LEHD-MCA substrate for caspase-9. A total of 5 × 104 cells for caspase-3 assay and 106 cells for caspase-9 assay was lysed and sonicated in a buffer composed of 250 mmol/L sucrose, 5 mmol/L MOPS (pH 6.5), and 1 mmol/L MgSO4. Fluorescence was measured in 1 mL of assay buffer [50 mmol/L Tris base (pH 8.1), 150 mmol/L NaCl, 1 mmol/L CaCl2, 10 mmol/L DTT, and 0.1 mg/mL bovine serum albumin] supplemented with 100 μL of protein lysates and incubated with 100 μL of caspase substrate (200 mmol/L). Cleavage of caspase substrate results in the release of methylcholanthrene, which emits a fluorescent signal with wavelengths of 380 nm excitation and 440 nm emission.

**Measurement of Annexin V–positive cells.** Phosphatidylserine externalization was analyzed using the Guava Nexin kit. Briefly, 5 × 104 cells were washed with PBS and adjusted in 1× binding buffer to a concentration of 5 × 106/mL. To 40 μL of cell suspension, 5 μL annexin V–phycocerythrin conjugate and 5 μL 7-aminoactinomycin D were added and incubated for 20 min at room temperature. Samples were analyzed (2,000 events) on a Guava PCA flow cytometer.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay for measurement of in situ apoptosis.** Pancreatic tumor...
cells were cultured on chamber slides. Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 h at 25°C. Permeabilization of cells was achieved by incubation with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was then carried out according to the manufacturer’s instructions (Roche). Signal conversion was achieved using an anti-fluorescein antibody conjugated with alkaline phosphatase, and methyl green was used as a counterstain.

**Induction of s.c. tumors in nude mice.** To determine the effect of quercetin on pancreatic tumor growth in vivo, 4 x 10^6 MiaPaCa-2 cells were injected s.c. into the right mediodorsal flank of 6-week-old male nude mice. Once the tumors reached a measurable size, animals were randomized into a control group (daily injection of vehicle; DMSO in PBS) and quercetin group (daily injection of 50 mg/kg quercetin). Animals were killed with carbon dioxide after 18 days. The tumor size was determined with a caliper, and the tumor volume was calculated by using the formula \((a \times b \times c)/2\), where \(a\) and \(b\) are the shorter and longer diameter of the tumors and \(c\) is the depth. Tumor growth was calculated as percentage increase of initial tumor volume before the start of the treatment. Tumors were removed and frozen in liquid nitrogen. Samples were then homogenized, and total proteins were extracted for analysis of Hsp70 by Western blot as outlined above. All experiments with animals were approved by the animal care and use committee at the University of Massachusetts.

**Statistical analysis.** Values are expressed as the mean ± SE. All experiments with cells were repeated at least three independent times. The significance of the difference between the control and each experimental test condition was analyzed by unpaired Student's t test, and a value of \(P < 0.05\) was considered statistically significant.

**Results**

**Hsp70 is elevated in pancreatic cancer cells.** Using a mAb for the inducible form of Hsp70, we showed the expression of Hsp70 in all tested pancreatic cancer cell lines. Compared with normal nonmalignant pancreatic ductal cells, the levels in all cancer cells were notably higher (Fig. 1A). Actin expression was used to ensure equal protein loading. Real-time PCR confirmed the Western blot data showing increased Hsp70 mRNA expression in Panc-1 cells compared with normal pancreatic duct cells (data not shown). The normalizing gene 18S was similar for both cell types.

Immunocytochemical staining of BxPC-3 cells and normal pancreatic ductal cells revealed higher cytosolic expression of Hsp70 in the tumor cell line (Fig. 1B). Similar results were obtained with the other pancreatic cancer cell lines MiaPaCa-2 and Panc-1 (data not shown). Hsp70-positive staining was already detectable under the 10X objective in BxPC-3 cells. The 40X objective revealed Hsp70 expression in the cytosol and indicated moderate staining of nuclei. No significant staining was detected in healthy pancreatic ductal cells or negative controls. In addition, the above data were supported by the fact that Hsp70 mRNA levels were

![Figure 1. Hsp70 levels in pancreatic cancer cells. A, pancreatic adenocarcinoma cell lines show increased Hsp70 levels compared with normal pancreatic duct cells. Whole-cell lysates were prepared and 10 μg of protein aliquots were loaded per lane on 10% Tris gels and blotted with an antibody against the inducible form of Hsp70 (synonymous to Hsp72). Membranes were stripped and reprobed for actin to show equal protein loading. B, increased Hsp70 expression was shown by immunocytochemistry in BxPC-3 cells (brown stain) compared with normal pancreatic duct cells. A higher magnification (under 40X objective lens) also showed partly stained nuclei. No immunostaining was seen in sections where the primary antibody was omitted. C, Hsp70 expression in human pancreatic tumor specimens. The graph is real-time PCR expression for Hsp70, showing significantly increased (*, \(P < 0.002\); \(n = 7\)) Hsp70 in tumor tissue compared with normal pancreatic tissue from the same patients. Expression of Hsp70 was normalized against 18S.](image-url)
significantly higher in human pancreatic tumor tissue compared with normal tissue from the same patients (Fig. 1C).

**Hsp70 down-regulation by quercetin decreases proliferation of pancreatic cancer cells.** Incubation of pancreatic cancer cells in medium supplemented with various concentrations of the flavonoid quercetin led to a dose-dependent reduction of Hsp70 expression, whereas tumor cells exposed to dihydroquercetin did not show any alterations. Figure 2A and B shows a decrease of Hsp70 levels in MiaPaCa-2 and Panc-1 cells after treatment with different concentrations of quercetin for 24 h. Figure 2C shows that treatment of normal pancreatic duct cells with either quercetin or dihydroquercetin had no effect on Hsp70 levels. Incubation of cells with 0.2% DMSO, used as a vehicle, had no effect on Hsp70 expression. In our experiments, incubation of Panc-1 and MiaPaCa-2 cells with at least 50 μmol/L quercetin led to a notable reduction of at least 50% of Hsp70 as assessed by Western blot (Fig. 2A and B). In BxPC-3 cells, Hsp70 levels were also reduced (data not shown). The higher concentrations of quercetin (i.e., 100 and 200 μmol/L) inhibited Hsp70 expression to a greater extent than the lower concentrations, suggesting a dose-dependent effect on Hsp70 expression. In contrast, incubation with dihydroquercetin (25–200 μmol/L) resulted in no change in Hsp70 in any of the examined pancreatic cancer cell lines (Fig. 2A and B). This leads us to conclude that dihydroquercetin does not down-regulate Hsp70 expression in pancreatic tumor cells.

Next, the effect of quercetin and dihydroquercetin on cell viability was assessed. In all tested tumor cells, the presence of quercetin reduced cell viability in a dose-dependent manner, whereas dihydroquercetin (which does not inhibit Hsp70 expression) had no effect (Fig. 2). Supplementation of medium with 0.2% DMSO had no effect on cell proliferation. Interestingly, both Panc-1 and MiaPaCa-2 had decreased viability by >50% when incubated with 100 μmol/L quercetin. Incubation of normal pancreatic ductal cells with either quercetin or dihydroquercetin had no effect on cell viability (Fig. 2C). Given that normal pancreatic ductal cells had much lower Hsp70 levels when compared with the tumor cells (Fig. 1A), this confirms that quercetin promoted cell death by inhibiting Hsp70 levels.

**Hsp70 inhibition stimulates apoptosis in pancreatic cancer cells.** Apoptosis can be assessed by using several characteristic features of programmed cell death. One variable of apoptosis is phosphatidylserine externalization, which can be measured by

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**Figure 2.** Quercetin decreases Hsp70 expression in pancreatic cancer cells resulting in decreased cell viability. MiaPaCa-2 (A), Panc-1 (B), and normal pancreatic duct cells (C) cells were incubated with various concentrations of quercetin/dihydroquercetin. Top, Western blots for Hsp70; bottom, cell viability data. Protein lysate (10 μg) was used for Western blotting. Equal protein loading was confirmed by staining membranes with Ponceau S and reprobing for actin. DMSO served as a vehicle for quercetin and dihydroquercetin. Final concentrations of DMSO never exceeded 0.2%. Cell viability was assessed by measuring absorbance at 450 nm on a 96-well plate reader. Decreased viability was found after treatment with quercetin for 24 h. Dihydroquercetin did not show any alterations in cell viability. Normal pancreatic duct cells (C) did not show any alterations in cell viability by either flavonoid.
Annexin V staining. The effect of the Hsp70 inhibitor quercetin on Annexin V staining was determined after 24 h of incubation, and a dose-dependent response in both MiaPaCa-2 and Panc-1 cells was observed (Fig. 3A and B). Treatment of quercetin in MiaPaCa-2 cells resulted in an 8-fold increase in Annexin V–positive cells compared with the control group treated with dihydroquercetin. A similar result was obtained with Panc-1 cells (i.e., concentrations of quercetin ranging from 50 to 200 μmol/L induced a significant increase of Annexin V–positive cells).

In situ apoptosis was measured using TUNEL stain. Panc-1 cells incubated with quercetin show a dose-dependent increase in TUNEL staining compared with control cells incubated with the vehicle alone (Fig. 3C). Similar results were obtained with MiaPaCa-2 and BxPC-3 cells (data not shown).

Figure 3. Dose-dependent increase in the number of Annexin V–positive cells (indicating increased apoptosis) following treatment of MiaPaCa-2 (A) and Panc-1 (B) cells with quercetin, but not with dihydroquercetin, for 24 h. Incubation with DMSO (0.2%) alone did not lead to increased apoptosis. Cells (5 × 10^5) were incubated with either quercetin or dihydroquercetin, and 2,000 events were counted by flow cytometry. C, untreated cells; 0 μmol/L, cell that only contains DMSO (0.2%). Columns, mean (n = 3); bars, SE. *, P < 0.05, compared with dihydroquercetin. C, increased apoptosis of Panc-1 treated with quercetin for 48 h as determined by in situ TUNEL staining. Panc-1 cells were treated with varying doses of quercetin, and the extent of apoptosis was evaluated by TUNEL staining 48 h later as described in Materials and Methods. DNA strand breaks usually occur during apoptosis that can be stained by TUNEL method. Red-colored cells, apoptotic cells.
The effect of quercetin and dihydroquercetin on caspase-3 (effector caspase) and caspase-9 (initiator caspase) activation was examined. Quercetin increased caspase-3 and caspase-9 activities in both MiaPaCa-2 and Panc-1 cells, whereas dihydroquercetin had no effect on caspasas (Fig. 4). Treatment of Panc-1 cells with 50 μmol/L quercetin for 24 h led to a >4-fold increase in caspase-3 and a 2-fold increase in caspase-9. MiaPaCa-2 cells incubated with quercetin resulted in a 6.9-fold elevation of caspase-3 and a 3.6-fold increase in caspase-9 compared with incubation with dihydroquercetin. Elevated caspase levels were already apparent after 12 h of treatment with quercetin. The above data suggest that quercetin, which decreases Hsp70 expression, is capable of initiating apoptotic cell death in pancreatic cancer cells.

Hsp70-specific siRNA promotes apoptosis in pancreatic cancer cells. To confirm the effects of Hsp70 expression on apoptosis and exclude any non-Hsp70-related effects of flavonoids, we transfected pancreatic adenocarcinoma cells with double-stranded siRNA oligonucleotides. Cells were harvested at 24, 48, and 72 h after transfection, and Hsp70 expression was analyzed by Western blot (Fig. 5). Whereas Hsp70-specific siRNA (siRNA #1) suppressed Hsp70 synthesis almost completely, control (scrambled) siRNA had no effect on Hsp70 expression. A set of cells transfected with water (mock transfection) showed no decrease in Hsp70 synthesis. Actin expression was unaffected by treatment with either Hsp70 or control siRNA, indicating that nonspecific downregulation of protein expression was not induced by Hsp70 siRNA treatment. To rule out "off-target" effects of the Hsp70 siRNA, we repeated the experiment with an additional Hsp70-specific siRNA (siRNA #2), which confirmed that Hsp70 expression in MiaPaCa-2 cells was inhibited to a similar extent after 72 h (Fig. 6).

Next, we assessed the effect of Hsp70-specific siRNA on cell viability. Inhibition of Hsp70 using siRNA (siRNA #1) significantly decreased cell viability in both MiaPaCa-2 (% of mock: 57.4 ± 2.3) and Panc-1 (% of mock: 54.1 ± 3.1) cells. A similar result was also found with an additional siRNA (siRNA #2) in MiaPaCa-2 (Fig. 6).

We assessed the extent of apoptosis at 24, 48, and 72 h after incubation with siRNA by Annexin V staining and caspase measurement. Transfection of MiaPaCa-2 and Panc-1 cells with Hsp70 siRNA after 48 and 72 h significantly increased Annexin V-positive cells (Fig. 5B and C). In contrast, only a slight enhancement in the number of Annexin V–positive cells was detected after the first 24 h after transfection (at a time when Hsp70 expression had decreased only slightly). An ~2.5-fold increase in Annexin V–positive cells resulted after 72 h of treatment with siRNA specific for Hsp70 in both MiaPaCa-2 and Panc-1 cells. Transfections with scrambled siRNA serving as a control did not show any alterations in Annexin V–stained cells, thus showing that Hsp70 siRNA was responsible for initiating apoptosis in pancreatic tumor cells. To rule out any "off-target" effects of the siRNA, we repeated this experiment with an additional Hsp70-specific siRNA sequence (siRNA #2) with MiaPaCa-2 cells and found similar results (Fig. 6E). This increase in apoptosis was accompanied by increased activity of caspases. Hsp70 siRNA induced a notable increase in caspase-3 activity in
MiaPaCa-2 cells starting at 24 h and in Panc-1 cells at 48 h after transfection (Fig. 6A and B). The highest levels of caspase-3 activity were observed in MiaPaCa-2 cells. Similar results were obtained with an additional siRNA (siRNA #2) in MiaPaca-2 (Fig. 6E). Caspase-9 also showed significant activation during the examined time course (Fig. 6C and D). Application of control siRNA altered neither caspase-3 nor caspase-9 activity compared with mock-transfected cells. Both Annexin V and caspase expression profiles showed that Hsp70-specific oligonucleotides were able to initiate apoptosis in pancreatic cancer cells in vitro.

Inhibition of Hsp70 expression decreases in vivo tumor growth in nude mice. Tumors developed in all animals injected s.c. with MiaPaCa-2 cells. After daily injection of quercetin (50 mg/kg) for 18 consecutive days, the tumor volume was significantly reduced (Fig. 7) compared with controls (injected with DMSO vehicle alone). Western blotting for Hsp70 revealed that these tumors also had decreased Hsp70 following quercetin injection. This confirms our in vitro observation that quercetin induces pancreatic cancer cell apoptosis via decreasing Hsp70.

Discussion

This study has shown that pancreatic cancer cells overexpress Hsp70 when compared with normal pancreatic ductal cells. Inhibition of Hsp70 with both quercetin and siRNA resulted in increased apoptosis of pancreatic cancer cells in vitro. In addition, in vivo administration of quercetin decreased tumor size in a xenograft nude mouse model of pancreatic cancer. This decrease in tumor size was also associated with decreased Hsp70 levels in the tumor.

The finding that pancreatic cancer cells expressed significantly higher Hsp70 levels compared with nonmalignant ductal cells is an important one because it suggests that Hsp70 plays a role in tumor cell resistance to apoptosis. This in vitro finding is in accordance with a report by Gress et al. (28) showing increased Hsp70 mRNA levels in human pancreatic carcinomas (assessed by Northern blot and in situ hybridization). In the present study, real-time PCR confirmed these previous findings, showing increased Hsp70 expression in cancer tissue versus normal tissue from the same pancreatic cancer patient. These findings agree with several reports in the literature showing increased Hsp70 expression in a variety of malignant tumors, such as colorectal, breast, and gastric cancer. The importance of these findings is illustrated by the fact that high levels of Hsp70 expression have been correlated with increased drug resistance in human breast cancer specimens (29), colon cancer cells (30), and human breast cancer cell lines (31).

Quercetin has been reported to inhibit Hsp70 expression by blocking heat shock transcription factor (HSF) 1 and HSF2 and...
reducing Hsp70 mRNA accumulation (32, 33). Quercetin has been used to down-regulate Hsp70 synthesis in various carcinoma cell lines (25, 34–36). In the present study, inhibition of Hsp70 with quercetin significantly decreased pancreatic cancer cell viability (BxPC-3, MiaPaCa-2, and Panc-1) but did not affect that of normal ductal cells, which do not have high levels of endogenous Hsp70. In accordance with quercetin having no effect on cell viability of normal pancreatic ductal cells, quercetin did not influence Hsp70 expression levels in these cells. This shows the complexity of this pathway in normal versus tumor cells. The signaling pathways responsible for up-regulation of Hsp70 in pancreatic tumor cells are unclear. Our additional data show that quercetin does not affect the basal levels of Hsp70 in normal pancreatic duct cells but inhibits the increased levels expressed in cancer cells. This suggests that quercetin is possibly interacting with the pathways responsible for increased expression of Hsp70 in cancer cells and thus causing the cells to die.

To confirm that the quercetin effect on cell viability was specifically due to inhibition of Hsp70 expression, we used the inactive analogue dihydroquercetin, which does not inhibit Hsp70 (37). As a control, dihydroquercetin did not alter cell viability of any tumor cell line. Therefore, the effect observed with quercetin can be ascribed to the reduction in Hsp70. Measurements of lactate dehydrogenase activity in cell culture medium after 12 and 24 h of treatment with quercetin or dihydroquercetin did not reveal any significant increase, indicating that necrosis was not the underlying mechanism for decreasing cell viability with quercetin (data not shown). In contrast, both caspase-3 and caspase-9 activities in MiaPaCa-2 and Panc-1 cells were significantly increased following incubation with quercetin, strongly suggesting that the apoptotic

Figure 6. Selective depletion of Hsp70 by siRNA causes increased caspase-3 and caspase-9 activity. MiaPaCa-2 (A and C) and Panc-1 (B and D) cells were transfected with Hsp70 siRNA, control siRNA, or buffer (mock) and monitored for 24, 48, and 72 h. Caspase activity was assessed using an enzymatic assay on a Hitachi F-2500 fluorescence spectrophotometer. Caspase increment per minute was calculated as ratio to the protein amount in mg. Columns, mean (n = 3); bars, SE. *, P < 0.05, compared with control siRNA was considered as statistically significant. E, top, representative Western blot showing decreased Hsp70 in MiaPaCa-2 using an additional siRNA sequence (siRNA #2); bottom, effect of Hsp70 siRNA #2 on variables of apoptosis (viability, Annexin V, and caspase-3). Data as % of mock (mean ± SE; n ≥ 3). *, P < 0.05, compared with mock was considered as statistically significant.
pathway is responsible for quercetin-induced cell death. In support of this observation, Annexin V labeling and TUNEL staining (markers of apoptosis) were significantly increased following inhibition of Hsp70 in the cancer cell lines. Our data concur with a study by Pandol et al. (38) showing that quercetin increased apoptosis and caspase-3 in MiaPaCa-2 cells.

To rule out any quercetin effect unrelated to Hsp70 inhibition, we used RNA interference for a selective knockout of Hsp70 and assessed apoptosis using the same methodologic approach. In accordance with the above data for quercetin, we were able to show that Hsp70 siRNA resulted in increased apoptosis in pancreatic cancer cell lines, marked by caspase-3 and caspase-9 activation, as well as Annexin V staining. These results clearly support the fact that inhibition of Hsp70 expression results in apoptotic cell death in pancreatic cancer cells and that quercetin-induced cell death is mediated via its effect on Hsp70. However, because the extent of apoptosis is somewhat more with quercetin compared with Hsp70 siRNA, it is possible that quercetin may affect viability by other mechanisms in addition to inhibition of Hsp70. But we believe that one of the major effects of quercetin is mediated via inhibition of Hsp70 due to the following reasons: (a) quercetin significantly decreased Hsp70 in all cell lines tested, (b) quercetin induces apoptosis in pancreatic cancer cells, (c) quercetin inhibits tumor growth in a xenograft model, and (d) dihydroquercetin (inactive analogue of quercetin, which does not inhibit Hsp70) had no effect on Hsp70 and did not induce cell apoptosis.

Apoptosis can occur via two main pathways. The extrinsic pathway is characterized by the activation of death receptors on the cell surface belonging to the TNF/nerve growth factor receptor superfamily and recruitment of the initiator caspases-8 and caspase-10. The intrinsic or mitochondrial pathway is independent of death receptor signaling and involves the release of cytochrome c from the mitochondria with the formation of a complex of cytochrome c, the cytoplasmic Apaf-1, and procaspase-9. Both pathways converge to the activation of downstream effector caspase-3, caspase-6, and caspase-7 (7, 12, 39).

Hsps are highly conserved proteins that play a role in fundamental cellular processes. Hsp70 is one of the main Hsps consisting of both constitutively expressed and stress-inducible members, and it is a molecular chaperone that protects the organism from stress-induced cell injury. Secondly, it has been reported to act on several steps of the apoptotic cascade. The detailed mechanism of the antiapoptotic effect of Hsp70 is yet to be determined. However, it seems to be cell type specific. In a study done by Li et al. (40), overexpression of Hsp70 in U937 lymphoma cells clearly prevented caspase-3 activation, poly(ADP-ribose) polymerase cleavage, and DNA laddering, although cytochrome c release was not blocked. This suggested that Hsp70 may inhibit apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. Another study by Jaattela et al. (13) hypothesized that Hsp70 exerts its antiapoptotic function downstream of caspase-3-like proteases, but it inhibits cytosolic phospholipase A2 activation and causes changes in nuclear morphology that are considered to be late caspase-dependent events. A functional target protein for Hsp70 has been suggested with Apaf-1. After release of cytochrome c from mitochondria, Apaf-1 forms an oligomeric complex with cytochrome c in an ATP/dATP manner. This step is critical for autoproteolytic cleavage of procaspase-9 and subsequent caspase cascade activation. Saleh et al. (41) suggest that Hsp70 is able to bind to Apaf-1 on its caspase recruitment domain (CARD) and competes with procaspase-9 for binding to this domain. After binding of Hsp70 to Apaf-1, a possible conformational change of the complex results in inhibition of Apaf-1 oligomerization. Following this, recruitment of procaspase-9 and subsequently caspase processing is stopped. On the other hand, Beere et al. (42) propose that, after binding of Hsp70, Apaf-1 fails to do a conformational change that would expose CARD for recruitment of procaspase-9. Still, both models show that caspase-9 cannot get activated. The association of cytochrome c and Apaf-1 is still allowed by Hsp70 (41, 42). In addition to Hsp70 interfering with the apoptotic pathway downstream of the mitochondria, several studies have shown that it can also prevent permeabilization of the outer mitochondrial membrane and thus affect the release of cytochrome c.

In this study, we have shown that depletion of Hsp70 in the pancreatic tumor cell lines MiaPaCa-2 and Panc-1 caused activation of caspase-9. These results are consistent with the observations that Hsp70 can prevent caspase-9 recruitment by its ability to interfere with Apaf-1 and to block apoptosome formation. Those data also suggest that, in pancreatic tumor cells, Hsp70 can block apoptosis via the intrinsic branch of the apoptotic cascade because caspase-9 is part of the mitochondrial pathway. Secondly, we showed that Hsp70 acts on its antiapoptotic function upstream of caspase-3. Activity of caspase-9, which triggers the maturation of procaspase-3, was enhanced after Hsp70 knockout done by either quercetin or siRNA. Elevated caspase-3 levels were measured in both MiaPaCa-2 and Panc-1 cells. From our data, we conclude that, in pancreatic carcinoma cell lines, Hsp70 also plays a major role in caspase-dependent apoptosis and that Hsp70 acts upstream of caspase-3.

However, the connection between Hsp70 and the oncogenic potential of tumor cells is likely to be more complex than described above. Apoptosis also occurs by caspase-independent mechanisms in which Hsp70 may interact (43, 44). Presumably, one of the...
targeted proteins is the apoptosis-inducing factor (AIF), which is also released from the mitochondria but triggers apoptosis independently of caspases. To this end, Ravagnan et al. (44) have shown that Hsp70 was able to antagonize the apoptogenic effects of AIF both in a cell-free system and in intact cells.

We have also examined the effect of quercetin on pancreatic cancer growth using a nude mouse model of pancreatic cancer derived from the highly malignant pancreatic cancer cell line MiaPaCa-2. Daily quercetin administration decreasedexpression of Hsp70 in tumor tissue, thereby confirming that quercetin decreases pancreatic tumor cell viability via lowering Hsp70.

In conclusion, we have identified a major role of Hsp70 in the resistance of pancreatic cancer cells to apoptosis. Furthermore, our studies suggest that the bioflavonoid quercetin and other pharmacologica-l agents that inhibit Hsp70 are possible therapeutic agents in the treatment of pancreatic cancer. Taken together, these results serve to reinforce our belief that further in vivo and in vitro experiments need to be done using quercetin and/or Hsp70 siRNA in combination with chemotherapeutic drugs. Agents that can down-regulate Hsp70 expression may allow new strategies in cancer therapy and may improve the dismal prognosis of pancreatic cancer.

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References


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