Heat shock protein 27 inhibition of the caspase cascade in human breast cancer cells

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HEAT SHOCK PROTEIN 27 INHIBITION OF THE CASPASE CASCADE IN HUMAN BREAST CANCER CELLS

by

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Bachelor of Arts
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1997

Master of Science
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ABSTRACT

Heat Shock Protein 27 Inhibition of the Caspase Cascade in Human Breast Cancer Cells

by

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Cells may die via a programmed cell death called apoptosis. Apoptosis can be triggered by many death signals, some of which cause cytochrome c to be released from the mitochondria initiating the caspase cascade. When there is an over-expression of Heat Shock Protein 27 (HSP27), the cell does not obey the death signals that it receives. In this study, the molecular mechanism for the HSP 27 inhibition of the caspase cascade is described in human breast cancer cells that were engineered to constitutively express HSP 27. HSP 27 directly bound to cytochrome c and inhibited the activation of caspase 9 and caspase 3. In the presence of an excess amount of cytochrome c, the caspase cascade was reactivated. Granzyme B activated caspase 3 even in the presence of HSP 27. This
suggests the conclusion that HSP 27 is a novel inhibitor of apoptosis by binding to cytochrome c.
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CHAPTER I

INTRODUCTION

Cancer

The growth of normal cells is regulated to meet the needs of the cell, whereas cancer cells continually replicate without any regulation. With more and more invading cancer cells, function of normal cellular activity and tissues is inhibited (1,2). The transformation of a normal cell into a cancer cell must involve changes in cellular processes that are central to normal cells. These changes in the cancer cells might be due to chromosomal structure and are completely inherited at the cellular level to the daughter cells (1). A cancer cell with these mutations will never give rise to another normal cell (2,3). Cancerous cells can also have a faulty mechanism that prevents them from undergoing programmed cell death (apoptosis). Without the mechanism for cell death, the cancer cell will keep dividing to form tumors in the tissue. In some breast cancers there is an elevated level of Heat Shock Protein 27 (HSP 27). Breast cancers that over-express HSP 27 show a much higher recurrence of breast cancer (4-6). These elevated levels of HSP 27 also lead to a poor prognosis for the patient (4,5).

Heat Shock Protein 27

Heat Shock Protein 27 (HSP 27) is expressed when cells undergo stress. In normal cells, HSP 27 is expressed at very low (basal) levels. When the cell is exposed to
a heat shock or cytotoxic chemicals, HSP 27 levels are increased (7). HSP 27 can be used as a marker for more aggressive cancers because HSP 27 can provide resistance to chemotherapeutic drugs (4). One of the functions of HSP 27 is as a molecular chaperone preventing unfolding and degradation of proteins (8-11). HSP 27 has also been reported to protect cells from undergoing apoptosis by inhibiting caspase activation (12).

HSP 27 is also an estrogen responsive protein. Estrogen responsive means that if estrogen is added, the levels of HSP 27 will increase. Once estrogen is added to cancerous cells, HSP 27 will be produced allowing the cancer cell to survive when a chemotherapeutic drug is applied. An elevated level of HSP 27 in tumor cells results in less cellular death compared to basal levels of HSP 27 when cells are stimulated to die (5). HSP 27 can also protect estrogen receptor negative cells from cytotoxic drugs and heat shock (5,7). Therefore, based on these data, HSP 27 inhibits apoptosis by an undefined mechanism.

Apoptosis

Cells live and divide for a certain number of times and then will die by programmed cell death (apoptosis). Apoptosis is an active form of cell death. The characteristics of apoptosis include plasma membrane blebbing, nuclear condensation, loss of cell volume and DNA fragmentation (13-15). Another feature of apoptosis includes the absence of tissue inflammation and swelling (15,16). Apoptosis is critical during development and homeostasis and any alteration to the apoptotic pathway leads to a number of diseases. Diseases associated with a faulty apoptotic pathway include
cancer, autoimmune disorders such as Alzheimer's disease, Parkinson's disease and spinal muscular atrophy (15,16).

There are several pathways that lead to apoptosis in cells. The major receptor apoptotic pathways are the TNFα and Fas L (Figure 1, page 7). The TNFα pathway results when TNFα binds to the TNFα 1 receptor, leading to activate procaspase 8 into caspase 8. Once caspase 8 is activated it will cleave other procaspase 8 molecules as well as other procaspase enzymes. The Fas pathway also activates caspase 8 and activates apoptosis through the cleaving of the other caspases. Another apoptotic activating enzyme is granzyme B. Granzyme B is a serine protease that is released from T cells with another protein called perforin. Perforin forms a pore for granzyme B that can be used to enter the cell (17,18). Once the pore is formed, granzyme B enters the cell and starts to cleave at specific aspartic acid residue target sites (18,19). Granzyme B will cleave interleukin-1B-converting enzyme (ICE) and other apoptotic cysteine proteases such as caspases 8, 3, 4, and 5 (18,20). Once these caspases are activated, the cell is committed to die via apoptosis. The final apoptotic pathway, and the most relevant one to this study, are the actions of cytotoxic drugs on the mitochondria. When a cytotoxic drug is applied to cells, it somehow signals the mitochondria to release cytochrome c. The bcl-2 family of proteins form pores for the release of cytochrome c from the mitochondria (20, 21). Once cytochrome c is released, cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1), procaspase 9 and dATP to form the apoptosome (21). The formation of this apoptosome initiates the caspase cascade.
Caspases and the Caspase Cascade

Caspases are enzymes that degrade other proteins. These cysteine proteases cleave at aspartic residues and derive their name from their function and substrate specificity (15,22). To date there are identified 11 caspases which are subdivided into 3 groups. The ICE subfamily includes caspases 1, 4, 5 and 11, the CPP32 subfamily consists of caspases 3, 6, 7, 8, 9 and 10, and caspase 2 stands alone (15). This classification can be broken down into initiator caspases which have long prodomains like caspases 8 and 9 or effector caspases with short prodomains like caspases 3, 6, and 7 (23-26). The effector caspases are responsible for apoptosis. These enzymes cleave cellular substrates such as poly (ADP-ribose) polymerase, and structural proteins like lamina and actin. They also degrade protein kinase Cδ that is involved in the cell cycle (15,23,24,27). Caspase 3 is also responsible for cleaving the inhibitor protein for the caspase-activated endonuclease (ICAD). Without the inhibitor protein CAD (caspase activated DNase) cleaves DNA at the linker regions between nucleosomes which results in DNA fragmentation of approximately 200 base pairs (28).

Caspases are synthesized as zymogens, and thus are in an inactive form in the cell. Once the cell receives an apoptotic signal, a procaspase will be cleaved to a functional caspase. A functional caspase can activate other caspases or cleave specific target proteins. There is a specific pathway that the caspases follow when a cell is treated with a cytotoxic drug: the so-called caspase cascade.

The caspase cascade is induced through the mitochondria (Figure 2, page 8). An apoptotic-inducing drug, such as sodium butyrate, releases cytochrome c from the mitochondria. Cytochrome c forms the apoptosome with procaspase9, dATP and Apaf-1
The apoptosome then cleaves procaspase 9 activating it to caspase 9. Caspase 9 then cleaves other procaspase 9 molecules or it can activate procaspase 3 to caspase 3, procaspase 6 to caspase 6, or procaspase 7 to caspase 7 (23,29,30). Once caspase 6 and 7 are activated they will cleave their substrates. When caspase 3 is activated, it will cleave all the other caspases including caspases 1 and 8. Once caspase 3 is activated, cell death is inevitable. Caspase 3 is known as the "executioner" of the cell. Another caspase cascade occurs when activated caspase 8 activates caspase 3 and all the other caspases (23,26). The caspase cascade of interest to this study is through the mitochondria. This is because sodium butyrate and other apoptotic drugs cause a release of cytochrome c but do not interact with any other apoptotic pathways and do not activate caspase 8.

Sodium butyrate will release cytochrome c from the mitochondria but will not activate any other form of apoptosis or necrosis (20). Using a specific assay that measured DNA degradation between the nucleosomes, Sindhu Padmanabhan showed in her thesis that human breast cancer cells used in this thesis undergo apoptosis (31). Using this result, it was determined to investigate the role of the caspase cascade in the HSP 27 mediated inhibition of apoptosis.

Inhibitors of Apoptosis

Inhibitors of apoptosis can halt caspase activation. The baculovirus protein p35 is an inhibitor of all activated caspases (32). CrmA protein from the cowpox virus inhibits caspase 8 activation (32). The protein FLIP inhibits the Fas death inducing signal complex (DISC). Mammalian inhibitors of apoptosis homologs B & C (MIHB and MIHC) inhibit the TNF DISC (33). These DISC inhibitors prevent the activation of
caspase 8. The X-linked inhibitor of apoptosis (XIAP) inhibits activated caspase 3, 7 and 9 (34). HSP 27 has no sequence homology with any of these known inhibitors but still prevents the cell from undergoing apoptosis. HSP 27 might be inhibiting one of the caspases or it might be working up stream of the caspases.

Significance of the Study

The main goal of this study is to understand where HSP 27 is inhibiting the caspase cascade in breast cancer cells. From previous work it was shown that sodium butyrate will induce apoptosis in these cells and an over-expression of HSP 27 will inhibit apoptosis. This project will determine if HSP 27 inhibits either caspase 3, 8, or 9 or if it is acting prior to the activation of these caspases. The hypothesis for this study is that HSP 27 is inhibiting the caspase cascade by the inactivation of caspase 3. The techniques that will be used to determine if the hypothesis is correct, are colormetric assays, kinetic assays and western blot analysis.
Figure 1  Receptor Mediated Apoptosis. The FAS ligand will bind to the FAS receptor. This will activate FAS associated death domain (FADD) which will activate procaspase 8 to caspase 8. Caspase 8 will then activate procaspase 3 to active caspase 3. The protein FLIP will inhibit FADD from becoming active which will inhibit the formation of active caspase 8. The other pathway is the Tumor Necrosis Factor alpha (TNF-α) I pathway. TNF-α will bind to its receptor and activate TNF-α receptor associated death domain (FADD). FADD will then activate procaspase 8 to active caspase 8 and the caspase 3 will become active once again. Mammalian inhibitors of apoptosis homologs B & C (MIHB and MIHC) will inhibit TRADD so caspase 8 will not become active.
Figure 2  Caspase Cascade Pathway. When a cytotoxic drug releases cytochrome c, it will bind to procaspase 9, dATP, and Apaf-1 to form the apoptosome. The apoptosome will then cleave procaspase 9 to form active caspase 9. Caspase 9 will then cleave procaspase 3 to active caspase 3. Once caspase 3 is active it will cleave other caspases, structural proteins, kinases, ICAD (which results in DNA degradation), and cell cycle enzymes.
CHAPTER 2

MATERIALS AND METHODS

Cell Lines

The cell lines that were used included DC 4, and DB 46. These cell lines were derived from an estrogen receptor-negative human breast cancer cell line MBA-MD-231. The constitutive expression vector pB27 was used in constructing DB 46 cells whereas the control vector pHB-Apr-1-neo was used in constructing DC 4 cells (35). All cell lines were grown in Minimal Essential Media (MEM). MEM was supplemented with 10% fetal bovine serum, 25mM HEPES buffer, 100IU penicillin/ml, 100μg/ml streptomycin, 2mM L-glutamine (Biowhittaker Inc.), and 600μg/ml G418 sulfate (Gibco BRL). Cells were kept at 37°C and in a 5% CO2: 95% air environment. Twenty-four hours before an experiment, the cells were placed in MEM lacking G418 (35).

Drugs and Proteins

Vitamin E Succinate (VES), cyclohexamide (CHX) and sodium butyrate (NaB) (all from Sigma Chemical Co.) were prepared at 10mg/ml, 100μM and 100mM, respectively, in deionized water and filter sterilized (0.2μ filter). HSP 27 was purchased from StressGen (Vancouver, BC) and resuspended in a buffer containing 20mM Tris-HCL pH 7.5, 100mM NaCl, 0.1mM EDTA, and 1mM DTT. Granzyme B ((MES pH 6.0 with 400mM NaCl) (32,000 mOD min⁻¹/ml, Assayed with Ellman's to detect the HSBL
with 400mM NaCl) (32,000 mOD min⁻¹/ml, Assayed with Ellman's to detect the HSBL leaving group from 90μM Boc-Ala-Ala-Asp-Sbzl in 0.1M HEPES pH 7.5 with 0.5M NaCl at 410nm) was a kind gift from Dr. Dorothy Hudry, University of Nevada, Reno.

**Cell Survival**

DC 4 (-HSP 27) and DB 46 (+HSP 27) cells were exposed to either 10mM NaB, 100mM CHX or 10mg/ml VES for one hour. The cells were then washed in PBS, resuspended in supplemented MEM, and 2000 cells were plated in 5ml of supplemented MEM in 60mm tissue culture dishes. Cells were incubated at 37°C in a 5% CO₂: 95% air incubator for fourteen days. After the incubation period, the media was removed and the cells were stained with crystal violet (5% Crystal violet: 95% ethanol), dried, and counted for colonies containing more than 50 cells (cell survived). Results are presented as a percent survival.

**Caspase 3 Time Course**

DC 4 (-HSP 27) and DB 46 (+HSP 27) cells were induced to undergo apoptosis with continuous exposure to 10mM NaB and assayed at the indicated times. After 0, 2, 4, and 6 hours, 5 x 10⁶ cells were washed with phosphate buffer saline (PBS) and centrifuged. Lysis buffer was added at 1x10⁶ cells/ 25μl cells and the cells were incubated on ice for ten minutes. The cell suspensions were then centrifuged at 14,000 x g for fifteen minutes at 4°C. Fifty microliters of lysate was added to 50μl of assay buffer in a 96-well plate. Five microliters of the substrate (Asp-Glu-Val-Asp-pNA) were added to each well. Following incubation at 37°C for one hour, the absorbance at 510 nm was
measured using a microplate reader (Dynatech MR5000). Results are presented as relative caspase 3 activity. Lysis buffer, assay buffer and substrate were used from a caspase 3 assay kit (Clone Tech.).

Caspase 9 Time Course

DC 4 (-HSP 27) and DB 46 (+HSP 27) cells were induced to undergo apoptosis at the indicated times with 10mM NaB. Five million cells were washed with phosphate buffer saline and then resuspended in 1x10^6/ 25 µl cells of lysis buffer. Cells were incubated on ice for ten minutes and then centrifuged at 14,000 x g for fifteen minutes at 4°C. Fifty microliters of lysate were added to 50 µl of assay buffer and then 5 µl of caspase 9 substrate were added (Leu-Glu-His-Asp-pNA). Following an incubation at 37°C for one hour, the absorbance was read at 510nm on a microplate reader (Dynatech MR 5000). Results are presented as relative caspase 9 activity. The lysis buffer, assay buffer and substrate were obtained from Medical and Biological Laboratories Co. LTD.

Caspase 3, 8 and 9 Kinetics

Caspase 3 (Calbiochem) instructions were followed. Briefly, equimolar concentrations of caspase 3 and HSP 27 were added to wells in a 96 well plate, diluted to a volume of 90 µl, and incubated at room temperature for ten minutes. Ten microliters of the substrate (DEVD-pNA) was added to wells and the plate was read every minute for 45 minutes at 510nm on a microplate reader (Dynatech MR 5000). Slopes of each line were compared and data reported as relative percent caspase activity.
Caspase 8 assay instructions were followed (Calbiochem). Briefly, equal molar concentrations of caspase 8 and HSP 27 were added to wells in a 96 well plate and diluted to a volume of 90μl, and incubated at room temperature for ten minutes. Ten microliters of the substrate (IETD-pNA) was added to the wells and the plate was read for every minute for 45 minutes at 510nm on a microplate reader (Dynatech MR 5000). Slopes of each line were compared and data is presented as relative percent caspase activity.

Caspase 9 assay instructions were followed (Alexis). Equal molar concentrations of caspase 9 and HSP 27 or caspase 9 inhibitor were added to wells in a 96 well plate and brought up to a volume of 90μl, and incubated at room temperature for ten minutes. Ten microliters of the substrate (LEHD-pNA) was added to the wells and the plate was analyzed for optical density every minute for 45 minutes at 510nm. Slopes of each line were compared and data is relative percent caspase activity.

Immunoprecipitations

DC 4 cell (-HSP 27) extracts were prepared as follows: 5 x 10^6 cells were washed in PBS and incubated in 600μl cytochrome c buffer (220mM mannitol, 68mM sucrose, 50mM PIPES-KOH, pH 7.4, 50mM KCl, 5mM EGTA, 2mM MgCl₂, 1mM dithiothreitol (DTT) and 1 pill of protease inhibitors (Complete Cocktail, Boehringer Mannheim)) (36) for thirty minutes at room temperature. Cells were then homogenized for 40 strokes using a dounce homogenizer and a B pestle. Cells were then centrifuged for fifteen minutes at 14,000 x g. To the supernatant extract, 5μl of HSP 27 (1μg/μl) and 5μl cytochrome c (1μg/μl) was added. The lysate was allowed to incubate for ten minutes at
room temperature. The lysate was then divided into two samples. To one sample HSP 27 antibody (StressGen) (1:200) was added and cytochrome c antibody (Pharmingen) (1:200) was added to the other sample. Samples were incubated on a lab quake for sixty minutes at 4°C. To all samples 20μl of A/G protein and agarose was added and samples were incubated for forty-five minutes on a lab quake at 4°C. Samples were centrifuged at 14,000 x g for 15 minutes, the supernatant discarded, and the pellet resuspended in denaturing/reducing sample buffer (125mM Tris pH 7.4, 4% SDS, 10% glycerol, .005% Bromophenol Blue, 2% B-mercaptoethanol). Samples were electrophoresed on a 10.0% separating gel at 100V for 45 minutes. The samples were then blotted (Bio Rad) for eighty minutes at 100mV onto nitrocellulose paper. The nitrocellulose paper was transferred into blocking solution (0.5% Blotto, 0.05% BSA in 1X TrisBorateSaline-Tween20 (TBST)) for 24 hours at 4°C. The primary antibodies (1:1000) were dissolved in 1X TBST, added to each paper, and allowed to shake for sixty minutes. The blots were then washed three times in 1X TBST for ten minutes. The secondary goat anti mouse antibody (Pharmingen) was dissolved in 1X TBST (1:2500) and added to the membranes. This was then shaken for one hour at room temperature, and three more washes with 1X TBST were performed. Finally, 1ml of the chemilumensence substrate was added to each membrane and exposed to x ray film for thirty seconds. The results are shown on the western blots.

**Cytochrome c Assays**

Five million DC 4 cells (-HSP 27) were harvested and washed in PBS. Six hundred microliters of cytochrome c buffer were added to 2.5 x 10⁶ cells (220mM
mannitol, 68mM sucrose, 50mM PIPES-KOH, pH 7.4, 50mM KCl, 5mM EGTA, 2mM MgCl2, 1mM dithiothreitol (DTT) with 1 of the pill protease inhibitors (Complete Cocktail, Boehringer Mannheim) (36). Following incubation for ten minutes on ice the cells were homogenized with a dounce homogenizer with a B pestle for forty strokes. The cell lysate was then aliquoted into two 1.5ml microfuge tubes and centrifuged at 14,000 x g for fifteen minutes at 4°C (36). Cell lysate was used with the caspase 3 assay kit (Calbiochem). The volume of cell lysate (75μl) and substrate (10μl) remained constant and the amounts of 1mg/ml cytochrome c (Sigma) and assay buffer was varied for a final volume of 100μl. Various concentrations of HSP 27 (StressGen) and cytochrome c (Sigma) were used in the experiments. Slopes of the line for thirty minutes were compared to the control and data is expressed as relative caspase 3 activity.

A caspase 9 inhibitor (Leu-Glu-His-Asp-CHO) (Alexis) was used in two concentrations (5μl and 10μl) to determine if caspase 9 was being activated in the caspase cascade. The above procedure was used and caspase 9 inhibitor was added prior to the start of the assay. The assay was conducted for thirty minutes and slopes of the lines were compared to the control.

Granzyme B

Five million DC 4 cells (-HSP 27) were washed in PBS, and 600μl of cytochrome c buffer/2.5 x 10^6 cells was added. Following an incubation on ice for ten minutes, the cells were homogenized with a dounce homogenizer for forty strokes with a B pestle. The cell lysate was then put into two 1.5ml microfuge tubes and centrifuged at 14,000 x g for fifteen minutes at 4°C. Cell lysate was used with the caspase 3 assay kit as previously
described (Calbiochem). The amount of lysate and substrate remained the same with additions of cytochrome c or granzyme B (either alone or in combination with equal molar amounts of HSP 27) being made 10 minutes prior to the assay. Assay buffer was used to bring the total volume up to 100μl. The kinetic assay was run for thirty minutes. The slopes of the lines were compared to the control and data is reported as relative caspase activity.

Statistical Analysis

Results are reported as standard error of the means ± S.E.M. Statistical analysis was performed with a statistical software package (Student version of Minitab). Statistical significance was determined by one-way ANOVA for repeated measurements followed by a student t-test.
CHAPTER 3

RESULTS

HSP 27 Can Provide Long Term Protection from Different Cytotoxic Drugs

To determine if HSP 27 could prevent cell death (apoptosis) over long periods, a clonogenic cell survival assay was conducted, which measures viable cells fourteen days after exposure to cytotoxic drugs as described in chapter 2 (Materials and Methods). DC 4 (-HSP 27) and DB 46 (+HSP 27) cells were each treated with 10mM sodium butyrate (NaB), or 100µM cyclohexamide (CHX) or 10mg/ml vitamin E succinate (figure 3, page 22). DC 4 cells (-HSP 27) cell survival was about 66.9% with 100µM cyclohexamide compared to an 89.7% survival in the DB 46 cells (+HSP 27). With 10mM sodium butyrate DC 4 cells only had a 67.1% survival compared to 96.3% survival in the DB 46 cells. With 10mg/ml vitamin E succinate DC 4 survival was 73.6% compared to 98.9% in the DB 46 cells. Over-expression of HSP 27 protected cells from three different inducers of apoptosis during a two-week interval.

HSP 27 Inhibits Caspase 3 Activity

To determine if HSP 27 inhibits caspase 3 activation, a time course study was conducted as described in Material and Methods. Both DC 4 (-HSP 27) and DB 46 (+HSP 27) cells were induced to undergo apoptosis with 10mM sodium butyrate (NaB) and assayed every two hours to determine if caspase 3 activation increases in one or both cell

16
lines (figure 4, page 23). There was no increase in caspase 3 activation for the first four hours in either cell line. In the sixth hour the DC 4 (-HSP 27) cells showed a 2.5 fold increase in caspase 3 activation whereas DB 46 (+HSP 27) cells showed no increase in caspase 3 activation. Longer time courses were conducted for up to forty-eight hours in continuous exposure to NaB but there was no increase in caspase 3 activity in the HSP 27 transfected cell line (data not shown). Therefore, HSP 27 appeared to inhibit caspase 3 activation.

HSP 27 does not Directly Inhibit Caspase 3, Caspase 8, and Caspase 9

To determine if HSP 27 can directly inhibit caspase 3 or caspase 8, kinetic assays were conducted as described in chapter 2. There was no difference between the control and HSP 27 values. The effects of equimolar amounts HSP27 showed no inhibitory function on the activity of either caspases 3 or 8 compared to the control (figure 5, page 24). In addition, HSP 27 did not inhibit caspase 9 (figure 6, page 25). The control value was 100% and the HSP 27 plus caspase 9 value was 127% with standard deviations of 20%. Therefore there was no statistical difference between the two. When a caspase 9 competitive inhibitor is present, activity dropped to about 36%. The competitive inhibitor will not totally inhibit caspase 9 activity. These results lead to the assumption that HSP 27 does not directly inhibit caspase activity.
HSP 27 Inhibits Caspase 9 Activation

To determine if HSP 27 inhibits caspase 9 activation a time course was conducted as described in the chapter 2. DC 4 (-HSP 27) and DB 46 (+HSP 27) cells were induced to undergo apoptosis with 10mM sodium butyrate (NaB) and assayed at 0, 15, 30 and 45 minutes following sodium butyrate exposure. Within fifteen minutes caspase 9 was active with a 50% increase in the DC 4 (-HSP 27) cells while the DB 46 (+HSP 27) cells did not increase with any significance (figure 7, page 26). Longer caspase 9 time courses up to six hours were also performed but there was no increase in caspase 9 activation in the DB 46 (+HSP27) cells (data not shown). This leads to the conclusion that HSP 27 is inhibiting caspase 9 activation.

HSP 27 Binds Directly to Cytochrome c

To determine if a direct interaction between HSP 27 and cytochrome c was occurring, HSP 27 and cytochrome c immunoprecipitations (IP) were conducted as outlined in chapter 2. Lane one of the IP is the standard marker for cytochrome c (figure 8, page 27). Lane two shows that the antibody for cytochrome c will bind to the cytochrome c. Lane three shows that there is a little cross reactivity between cytochrome c and the HSP 27 antibody. This might be due to the fact that the HSP 27 antibody can recognize a denatured protein and bind to it. Lane four shows that when a cell free lysate is used, there is a small amount of free cytochrome c in the lysate and not all in the mitochondria. This could be due to rupturing the mitochondria membranes during the preparation of the cell lysate. This will cause a little cytochrome c to be in the cell lysate and not all in the mitochondria. In lane five once again there is a slight cross reactivity
between the HSP 27 antibody and the free cytochrome c in the cell lysate. With the addition of cytochrome c in the cell lysate and probed with the HSP 27 antibody, there is a greater amount of cross reactivity. This once again can be due to the fact that the HSP 27 antibody may bind to denatured proteins. Lane 7 shows that when HSP 27, cytochrome c and the cytochrome c antibody is used there is a strong signal indicating that HSP 27 and cytochrome c form a complex. In lane 8, cytochrome c and HSP 27 are added together and precipitated with the HSP 27 antibody. Once again there was still a strong signal that indicates HSP 27 and cytochrome c form a complex. Therefore it was concluded that HSP 27 and cytochrome c form a complex in vitro.

Additions of Cytochrome c Causes an Increase in Caspase 3 Activity in Cell Free Extracts

A cell free extract system was utilized to determine the ability of HSP 27 to inhibit the caspase cascade. A dose response to cytochrome c was performed in a cell free extract as described in chapter 2. With increasing amounts of cytochrome c there is also an increase in caspase 3 activity (figure 9, page 28). With the addition of 0.5μg of cytochrome c there is about a 1-fold increase in caspase 3 activity. With 1μg of cytochrome c there is about a 4.5 fold increase, with 2.5μg of cytochrome c it is about an 11 fold increase and at 5μg there is about a 19 fold increase in caspase 3 activity. However, with 10μg of cytochrome c the dose response was only about 12 fold. At this highest dose of cytochrome c tested, there was reduction of caspase 3 activity. This reduction could be due to the changing of the ionic strength or it could be because too much cytochrome c would form aggregates and not be available in the formation of the
apoptosome. The dose response seen here has also been seen in other experiments (data not shown). It is concluded that cytochrome c can activate the caspase cascade in this cell-free extract.

Caspase 9 is in the Caspase Cascade

Caspase 9 is activated during the addition of cytochrome c. This was demonstrated by adding a caspase 9 inhibitor and measuring caspase 3 activity (figure 10, page 29). This demonstrated that if caspase 9 is inhibited, then caspase 3 activity will be reduced. With the presence of cytochrome c there is an increase in caspase 3 activity which was reduced by the caspase 9 inhibitor. When the inhibitor is added it lowers the caspase 3 activation by about 65%. Since the caspase 9 inhibitor did not completely inhibit the caspase 9 activation other caspases might also be activating caspase 3. However, it should be noted that the caspase 9 inhibitor was not 100% effective against activated caspase 9 (Fig 6, page 25). This is due to the fact that the inhibitor is a competitive inhibitor and is concentration dependent. A conclusion can be made that a majority of the caspase 3 activation results from active caspase 9.

HSP 27 Inhibits Cytochrome c Activation of Caspase 3 in a Cell Free Lysate

To confirm that HSP 27 binds to cytochrome c, a dose response to cytochrome c was conducted with or without the addition of 5μg of HSP 27 to the cell free extract. The addition of 2.5μg of cytochrome c resulted in a 2-fold increase in caspase 3 activity. However, in the presence of HSP 27, 2.5μg of cytochrome c failed to increase the caspase 3 activity. With 5μg of cytochrome c, there was about a 12 fold increase in caspase 3 activity. Again, in the presence of 5μg of HSP 27, there was an inhibition of
caspase 3 activity. With the addition of 10μg of cytochrome c, there was an 11 fold increase in caspase 3 activity. In the presence of HSP 27, 10μg of cytochrome c resulted in only a 3-fold increase in caspase 3 activity over the control (figure 11, page 30). This suggests, that with an excess of cytochrome c it may be possible to over-come the inhibitory effects of HSP 27 and restart the apoptotic cascade. This trend has been seen in other experiments (data not shown).

Granzyme B can Overcome HSP 27 Inhibition of Caspase 3 Activation in Cell Free Extracts

To help confirm that HSP 27 acts at the level of cytochrome c, cell free extracts were prepared as described in Chapter 2. To the extracts were added either 2μg of cytochrome c or 1μg granzyme B. To other extracts were added 2μg cytochrome c with 2μg HSP 27 or 1μg granzyme B and 1μg HSP 27. In the presence of 2μg of cytochrome c there was about a 21-fold increase in caspase 3 activation (figure 12, page 31). With the addition of HSP 27 and cytochrome c there was about a 2-fold increase in caspase 3 activation compared to the control (figure 12, page 31). The ability of HSP 27 to inhibit cytochrome c activation of caspase 3 is also shown in figure 9 (page 28). In the presence of 1μg of granzyme B there was about a 6 fold increase in caspase 3 activation. With the addition of granzyme B and HSP 27 there was about a 7 fold increase in caspase 3 activity. Since HSP 27 did not decrease the caspase 3 activity with granzyme B, granzyme B may be activating caspase 3 in a cytochrome c independent pathway. The results are from a single experiment.
Figure 3  HSP 27 inhibits cell death. Percent survival of DB 46 (+HSP 27) and DC 4 (-HSP 27) cells 14 days after thr exposure of cyclohexamide (CHX), Sodium Butyrate (NaB) or Vitamin E succinate (VES). Error Bars represent standard error of the means. Results are from one experiment done in triplicate. (*: p=0.05)
Figure 4  HSP 27 inhibits caspase 3 activation. Time course for caspase 3 activity in DB 46 (+HSP 27) and DC 4 (-HSP 27) at 0, 2, 4, 6, 8 hours following induction of apoptosis by 10mM sodium butyrate (NaB). Error bars represent standard error of the means; results are reported as the average of three experiments. (*: p=0.05 and + = too few values to determine significance)
Figure 5  HSP 27 does not directly inhibit caspase activity. Percent of inhibition of caspase 8 and 3 by equal molar amounts of HSP 27. Error bars represent the standard error of the means. Data was averaged from two experiments run in triplicate. There was no statistically significant differences in values between the control and the HSP 27 containing caspase assay.
Figure 6  HSP 27 does not directly inhibit caspase 9 activity. Percent of inhibition of caspase 9 by equal molar amounts of HSP 27 or caspase 9 inhibitor. Error bars represent standard error of the means. Data was averaged from one experiment run in triplicate. There are no statistically significant differences in values between the control and the HSP 27 containing caspase assay.
Figure 7  HSP 27 inhibits caspase 9 activation. Time course of Caspase 9 activity in DB46 (+HSP27) and DC4 (-HSP 27) cells at 0, 15, 30, 45 minutes after treatment with 10mM sodium butyrate. Error bars represent standard error of the means. (*: p=0.05)
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Figure 8  
HSP 27 can bind to cytochrome c. Western blot showing direct interaction between HSP 27 and cytochrome c. Lane 3 shows that there is a low level of cross reactivity between the HSP 27 antibody and cytochrome c. When both HSP 27 and cytochrome c are added together and immunoprecipitated with an antibody specific for HSP 27, a strong signal for cytochrome c is observed in lane 8. This indicates that HSP 27 and cytochrome c form a complex in vitro.
Figure 9 Addition of cytochrome c results in an increase in caspase 3 activity in cell free extracts. Relative caspase 3 activity in the presence of various amounts of cytochrome c in DC4 cell lysate. Error bars represents standard error of the means.
Figure 10  Effects of caspase 9 inhibitor on caspase 3 activation in cell free lysate. Relative caspase 3 activity in the presence of various amounts of caspase 9 inhibitor. Error bars represent standard error of the means. (* : p=0.05 compared to control)
Figure 11  HSP 27 inhibits cytochrome c activation of caspase 3 in cell free extracts. Relative caspase 3 activity in the presence of various concentrations of cytochrome c (in micrograms) with and without the presence of 5 μg of HSP 27 in DC 4 cell lysate.
Figure 12  Granzyme B can overcome HSP 27 inhibition of caspase activation. Relative caspase 3 activity in the presence of 2\(\mu\)g cytochrome c or 1\(\mu\)g granzyme B with and without the addition of equal molar amounts of HSP 27 in DC 4 cell lysate. Error bars represent standard error of the mean. This was one experiment done in triplicate. (*: p = 0.05 compared to control)
CHAPTER 4

DISCUSSION

Many research groups have shown that HSP 27 inhibits apoptosis but the mechanism for this has not been determined. Garrido et. al (12) have indirect evidence that HSP 27 binds to cytochrome c and thus inhibits apoptosis. In this thesis, HSP 27 was shown to bind directly to cytochrome c. This was demonstrated in estrogen receptor negative cell lines derived from MDA-MB-231 cells. The cell line DB 46 had a constitutively expressing HSP 27 vector while the DC 4 cell line had a control vector, which did not express HSP 27. With these cell lines, the caspase 3, then caspase 9 and cytochrome c components of the caspase cascade were evaluated.

*In vivo* studies revealed that HSP 27 blocks the activation of caspase 3 in the presence of an apoptotic stimulus (NaB). Figure 4 (page 23) demonstrates a complete time course. Caspase 3 activation was observed at 6 hours following exposure to NaB in the control transfected cell lines. These results are similar to what others have reported for caspase 3 activation (23). In the HSP 27 over-expressing cells, caspase 3 was not activated between 0 and 24 hours. This lead to the conclusion that HSP 27 might be directly inhibiting caspase 3 or acting at a point prior to the caspase cascade. Apoptosis appears not to occur at a later time because of the cell survival data (figure 3, page 22). The cell survival data indicate that the cells that over-express HSP 27 are close to control values which indicate the HSP 27 expressing cells have not undergone apoptosis within
values which indicate the HSP 27 expressing cells have not undergone apoptosis within fourteen days of exposure to NaB. A direct interaction between caspase 3 and HSP 27 was assessed. Since it had been reported that HSP 27 could inhibit serine proteases, it was best to investigate any inhibition of cysteine proteases (37). When recombinant HSP 27 and recombinant caspase 3 were added together and incubated, there was no inhibition in the amount of substrate that caspase 3 could cleave (figure 5, page 24). This suggested that HSP 27 does not directly inhibit caspase 3.

Since HSP 27 did not inhibit caspase 3, the caspase cascade was evaluated at the level of caspase 9. Once again, time courses were conducted and it was determined that caspase 9 was activated at fifteen minutes following addition of NaB in the control transfected cell line. While in the HSP 27 expressing cells, caspase 9 was not active up to and past 24 hours. Once again this result was consistent with published findings. Sun et. al. (23) reported that caspase 9 is activated at 45 minutes and peaks at about four hours. This thesis utilized breast cancer cells while Sun et. al. worked with Jurkat T cells. This may be the reason that a sharp increase and decrease was observed in breast cancer cells while Sun et. al. reported a steady increase in caspase 9. HSP 27 does not directly inhibit caspase 9 (figure 6, page 25). It was also shown that caspase 9 does play a role in the activation of caspase 3 (figure 10, page 29). Because there is a reduction in caspase 3 activity if there is a caspase 9 inhibitor present, this suggests that caspase 9 activates most of the caspase 3 in the cell. The proposed caspase cascade suggests that caspase 9 is activated before caspase 3. The time course data support this mechanism in breast cancer cells since caspase 9 was activated at 15 minutes while caspase 3 was activated at 6 hours.
following exposure to NaB. Since the presence of HSP 27 is inhibiting caspase 9 activation the caspase cascade was further evaluated at the level of cytochrome c.

The data shows that HSP 27 binds directly to cytochrome c. Garrido et. al. (12) reported that HSP 27 inhibits at the level of cytochrome c but a direct inhibition was not demonstrated. Data in this thesis demonstrates that HSP 27 directly binds to cytochrome c (page 27, figure 8). HSP 27 may be binding to cytochrome c when it is released from the mitochondria or after it has become part of the apoptosome. Since HSP 27 is a molecular chaperone and binds to denatured proteins, it can be assumed that cytochrome c is denatured when it leaks out of the mitochondria. Knowing that a direct interaction with cytochrome c is occurring, an attempt to overcome this inhibition was performed by adding more cytochrome c to cell free extracts. Using a dose response design, this inhibition was overcome with the addition of more cytochrome c. These results indicate that HSP 27 can only bind a finite amount of cytochrome c. The excess cytochrome c, which is not bound to HSP 27, can activate caspase 3. At the cellular level it would be possible that exogenous cytochrome c may be able to activate the apoptotic pathway in cells that over express HSP 27.

In order to initiate apoptosis via another mechanism and also to show that HSP 27 is working at the level of cytochrome c, granzyme B was evaluated since it is known to activate all known caspases. We determined the ability of granzyme B to activate caspase 3 in the presence of HSP 27. It was shown that HSP 27 does not inhibit the activation of caspase 3 by granzyme B (figure 12, page 31). These results help to confirm that HSP 27 does not inhibit caspase 3 activation via another pathway of apoptosis. The use of granzyme B might be a way to kill breast cancer cells that over
express HSP 27. Since granzyme B is produced in killer T cells, researchers could develop a T cell that can detect a cancer cell that over expresses HSP 27 and have the T cell distribute granzyme B to kill the cancer.

As described in the introduction, there are many known inhibitors of apoptosis. All inhibitors of apoptosis bind to caspases to inhibit their activation or stop their activation. HSP 27 has no sequence homology to any of these other inhibitors suggesting that it works at another point in the caspase cascade. This thesis has demonstrated that HSP 27 is a novel inhibitor of apoptosis because it binds directly to cytochrome c and nowhere else in the cascade (figure 8, page 27). Also with the addition of excess cytochrome c we can restart the caspase cascade which also suggests that HSP 27 only binds at the level of cytochrome c.

With the over-expression of HSP 27, cancer cells will be able to receive death signals (cytotoxic drugs and heat shocks) but will not undergo apoptosis. This is because HSP 27 is halting the caspase cascade at the level of cytochrome c as seen in figure 13 (page 38). When the apoptotic stress is applied, it causes a release of cytochrome c from the mitochondria. This released cytochrome c may or may not be denatured when released. Since HSP 27 is known as a molecular chaperone and binds to denatured proteins, we can speculate that cytochrome c is denatured. We know from work in our laboratory that HSP 27 can help to maintain protein function (such as citrate synthase and α-glucosidase) from thermal denaturation. Similar studies have reported that HSP 27 binds to citrate synthase where the enzyme retains its function (39). HSP 27 might be binding cytochrome c and inhibiting the formation of the apoptosome or HSP 27 might be binding to cytochrome c on the apoptosome and inhibiting the activation of caspase 9.
HSP 27 will bind to denatured proteins independent of its phosphorylation states (40). With the over-expression of HSP 27, the cell is more likely to survive an apoptotic stimulus.

In conclusion, the hypothesis of this study was proven to be false. It was thought that HSP 27 inhibited caspase 3 activity, but it was demonstrated that HSP 27 inhibits the activation of caspase 3 and caspase 9. Therefore, our current hypothesis is that HSP 27 is a novel inhibitor of apoptosis because it directly binds to cytochrome c released from the mitochondria. When HSP 27 binds to cytochrome c the caspase cascade is inhibited which leads to an inhibition of apoptosis. We have also shown that the addition of excess cytochrome c will reactivate and start the caspase cascade. Granzyme B will also bypass the inhibitory actions of HSP 27 and activate the caspases, hopefully, causing apoptosis in cancerous cells that over-express HSP 27 (figure 13, page 38).

Future Work

Future work to complete the mechanistic outline of HSP 27 binding to cytochrome c would be to determine if cytochrome c is in the native form or a denatured form when released from the mitochondria. Another experiment would be to determine if HSP 27 can refold cytochrome c to its native forms and releases it. Both of these experiments could reveal if cytochrome c is in a native or denatured form. Apoptosis could be induced in cells that over-express HSP 27 by adding varying concentrations of granzyme B. Since granzyme B does not easily cross the cell membrane, perforin would be used to facilitate granzyme B into the cells. When perforin is in high concentrations it will make holes in the cell membrane and granzyme B can enter the cell.
unknown mechanism, **perforin** makes a hole in the vesicle and granzyme B is released (38). With these experiments, they will show if granzyme B can enter a cell and activate the caspase cascade even in the presence of an over-expression of HSP 27. This piece of information might be useful in determining if granzyme B could be used as a therapeutic drug for breast cancer patients.
Apoptotic stimuli

Mitochondria

cytochrome c

procaspase 9, dATP and Apaf-1

HSP 27

apoptosome

caspase 9

procaspase 3

caspase 3

DNA repair (NuMa, DNA-Pk)

Other caspases Structural proteins Kinases ICAD Cell cycle

(caspases 6,7 lamin, G-actin, (PKC d, fodrin, gelsolin) (PARP, pRb,Mdm-2)

3, 9) FAK, PAK2)

Figure 13 Caspase Cascade Pathway

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