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Abstract

Multidrug resistance is a reoccurring dilemma faced in the field of chemotherapy. In a number of studies carried out signs of reverse transformation has occurred in multidrug resistant tumor lines, where the cells begin to adopt a more normal phenotype and become much less tumorigenic. In reverse transformed neuroblastoma cell sublines this reversal in phenotype has been correlated with increased protein amounts of p53, P-glycoprotein, and Hsp70. This study examines mRNA levels of Hsp70 and an isoform of Hsp90, another heat shock protein involved in response to stress. In this study we report that mRNA levels of Hsp70 but not Hsp90 were slightly elevated in the reverse transformed sublines as compared to parental lines. These findings support previous studies carried out and suggests that the increase in Hsp70 protein observed in reverse transformed lines may be due to increased expression levels of steady state Hsp70 mRNA.

Introduction

Multidrug resistance is a reoccurring obstacle faced in the chemotherapeutic management of cancer. Multidrug resistance (MDR) is characterized by high levels of resistance to a number of chemically unrelated drugs (Biedler and Riehm 1970, de Bruijn et al. 1986). MDR is established in cell lines by expressing high levels of plasma membrane efflux pumps such as P-glycoprotein and MRP-1, both members of the ABC superfamily of transporters (Chen et al. 1990, Obzen 2006). These transporters decrease

cellular levels of a particular drug, and through a mechanism not yet identified, also create cellular resistance for other drugs (de Bruijn et al. 1986, Inaba et al. 1981).

Interestingly, in a number of cases associated with MDR, cell lines have shown signs of reverse transformation. Reverse transformation is a phenomenon that occurs in transformed cell lines, in which the malignant cells revert back towards a more normal phenotype and become significantly less tumorigenic (Biedler and Spengler 1994, Nielson and Puck 1980). Chinese hamster lung and ovary cells, mouse tumor cells, and human neuroblastoma cell lines selected for drug resistance have all shown reversion from a tumorigenic cell line to that of a normal cell phenotype (As cited in Meyers and Biedler 1988, Nielson and Puck 1980). Although the exact molecular mechanism of reverse transformation is still unknown, in transfected Chinese hamster ovary cells reverse transformation has been linked to treatment and restoration of cAMP levels (Nielson and Puck 1980). In human neuroblastoma cell lines, overexpression of *MDR*1 gene, which codes for the P-glycoprotein transporter, has been correlated with reverse transformation (Spengler et al. 2006 unpublished).

Hsp70 and Hsp90, Heat Shock Protein of about 70 and 90 kilodaltons, respectively, are chaperone proteins that have both constitutive and inducible forms, and have a number of vital cellular functions (Hartl 1996, Morimoto 1993). For example, Hsp70 proteins have an essential role in cells to aid in proper protein folding and degradation, membrane translocation, and regulatory processes (Hartl 1996). Similarly, Hsp90 aids in the proper folding of transcription factors and protein kinases and preventing protein aggregation. Also, Hsp70 and Hsp90 work together to produce mature, properly folded proteins through a chaperone complex that binds immature proteins

(Caplan 1999, Lee et al. 2004). To accomplish this Hsp70 binds the immature protein and interacts with the Hop protein, which ultimately allows for Hsp90 to dock onto the complex and complete proper protein folding though the use of energy (Lee et al. 2004, Wegele 2006). Originally, it was thought that heat shock proteins were only involved in response to thermal stress, however, it has been found that expression levels of these proteins are elevated under all kinds of stressful conditions such as: oxidant injury, toxic metals, inflammation, trauma to tissues and cancer (Morimoto 1993, Somji 1999).

This study examines levels of Hsp70 and an isoform of Hsp90 mRNA in reverse transformed, multidrug-resistant neuroblastoma cell lines using RT-PCR. These reverse transformed neuroblastoma lines are under stress from high level exposure to chemotherapeutic drugs. In a previous study Spengler et al. (2006) demonstrated an increase of Hsp70 protein correlated with reverse transformed MDR neuroblastoma cell lines using Western Blotting. Thus altered expression of chaperone proteins may play a role in the reverse transformation phenomenon. This prompted an examination of the mRNA levels of Hsp70 as well as other heat shock proteins to determine if the elevated amounts of protein were a result of increase levels of mRNA expression or protein stabilization.

Materials and Methods

Cell Lines

Cell Lines BE(2)-C and multidrug resistant sublines BE(2)-C/ACT(0.2) (ACT0.2), and BE(2)C/VCR(20) (VCR20) were supplied by Dr. Robert Ross and Barbara Spengler. These cells were all cultured in a 1:1 mixture of MEM and F12 supplement with 10% fetal bovine serum at 37°C with 5% CO_2. Resistant sublines were

routinely grown in presence of drug (0.2µg/mL for ACT0.2 and 20µg/mL of vincristine for VCR20) and then removed from drug 4-7 days prior to analysis.

RNA Purification

Total RNA was isolated from cells in exponential growth phase using a Guanidine Thiocynate procedure (Chomcynski and Sacchi 1987, Farrell 1989). Briefly, cells were harvested from each sample, rapidly frozen using liquid nitrogen and stored at -80°C. 4mL of Guanidine Thiocynate and 668µl of β-mercaptoethanol were used to resuspend the pellets. Sodium Acetate and phenol were then added before centrifuging at 4°C for 30 minutes at 10,000rpm. The upper phase of the sample was then transferred to 8mL of pre-chilled 100% ethanol and left overnight at -20°C. After the precipitation period, the tubes were centrifuged again at 4°C for 30 minutes at 10,000rpm. The pellet was resuspended in 400µl of Guanidine Thiocynate buffer and mixed with 800µl of pre-chilled ethanol to precipitate overnight again. Finally the sample was centrifuged again as above and resuspended in 400µl of 70% ethanol and again centrifuged at 4°C at 7,000rpm for 15minutes. After air-drying the pellet was resuspended in 20µl of DEPC water and frozen at -80°C.

Primers

Primers were designed to specifically amplify Hsp70 and Hsp90 isoform 1 (Hsp90) mRNA by spanning large introns to prevent genomic amplification during RT-PCR. In the case of Hsp70, primers were designed to span introns 7 and 8, which are approximately 1000bp and 2000bp, respectively, to generate a cDNA product of 341 base pairs from the mRNA. The forward primer (Hsp7078-F) located in exon 7 was 5'-

GTGCATTATTACGACTCTG-3' (nucleotides 1063-1081 from Accession number NM_002154.3) and the reverse (Hsp7078-R) in exon 9 was 5'-TCAGAGTGACAGCTTCATCAG-3' (nucleotides 1403-1384 from Accession number NM_002154.3). Primers for Hsp90 were selected to span intron 1, which is nearly 37000bp in length. The cDNA product generated using these primers is 380 base pairs. The forward Hsp90 primer (Hsp901-F), designed to anneal in exon 1, was 5'-TGTCATGAGCCTGAGGTGAAC-3' (nucleotides 130-150 of Accession number NM_001017963.1) and the reverse primer (Hsp901-R), located in exon 2, was 5'-GTGGATCCAGACACCAACAG-3' (nucleotides 509-490 from Accession number NM_001017963.1) Primers for GAPDH were a gift from Dr. Berish Rubin. All primers were diluted to a 10pmol/µl concentration.

RT-PCR

RT-PCR was performed on mRNA from cell lines BE(2)-C, ACT0.2, and VCR20 using the Qiagen One-Step RT-PCR kit. Twenty nanograms of RNA were used in amplification along with 3µl of 5x RT Buffer, 0.6µl of dNTPs, 0.6µl of RT Enzyme, 0.75µl of forward primer, and 0.75µl of reverse primer for each set of primers in a final reaction volume of 15µl. The One-Step reaction for Hsp70 and Hsp90 samples was carried out as follows: 50°C x 30 mins, 95°C x 15 mins, followed by 34 and 35cycles respectively for Hsp70 and Hsp90 of 94°C for 20 sec for denaturation, a 30 sec annealing period at 56°C, and an extension period of 72°C for 30 sec. A final extension period of 7 mins at 72°C was carried out for all samples. The same RT-PCR protocol was carried out for GAPDH but only for 22 cycles in the same machine at the same time as the other

samples. All samples were analyzed on a 1% agarose gel and then quantified using SigmaGel software to determine relative amount of Hsp70, Hsp90 and GAPDH, respectively.

PCR Purification

RT-PCR products were purified using a Rapid PCR Purification kit (Marligen Bioscience, Inc). Following the manufacturer's protocol, products were purified by mixing 400µl of Binding Solution (H1) on a membrane cartridge and centrifuged at 12,000 x g for 1 minute. Centrifuge flow-through was discarded and 700µl of Wash Buffer (H2) was applied to the membrane and centrifuged at 12,000 x g for 1min. Finally, 35µl of warm ddH_2O was used to wash the column and collect the purified product after 2 rounds of centrifugation at 12,000 x g. The resulting purified product's OD was determined using a spectrophotometer to determine the product concentration.

Sequencing and Alignment

Purified RT-PCR products were sequenced using the Ampicycle Sequencing kit (Perkin Elmer), using a modification of the Sanger dideoxy method for DNA sequencing. Briefly 50 fmol from Hsp70 or Hsp90 RT-PCR product were mixed with 4µl of 10x Cycling Buffer, 2µl of the respective forward primer, 0.3µl of ^{33}P-dATP and water to a final volume of 30µl for a master mix. From this master mix, 6µl was added to each of 4 tubes containing 2µl of either ddGTP, ddATP, ddTTP or ddCTP to a final volume of 8µl, and overlaid with 12µl of mineral oil. The reaction were then exposed to the following

PCR conditions: 94°C x 3min followed by 35 cycles of 94°C x 30sec, 58°C x 30sec and 72°C x 1min. Upon completion of the final cycle, 4µl of Stop Solution were added to each tube and then the mixture was denatured at 94°C for 3 minutes. 3µl of the resulting reactions were then run on a denaturing polyacrylamide gel and visualized by autoradiography. Sequences were then manually read and aligned on GenBank.

Results

mRNA was isolated from the ACT0.2 and VCR20 cell lines are what representative MDR reverse transformed lines, as well as BE(2)-C, the I-type neuroblastoma cell line from which the MDR lines were derived. RT-PCR was carried out on these mRNA samples using primers specific for Hsp70 and Hsp90 and these reactions generated the predicted cDNA products of 341bp and 380bp, respectively.

Figure 1: RT-PCR product results from primers located in exon 7 (nucleotides 1063-1081 of Accession number NM_002154.3) and exon 9 (nucleotides 1403-1384 of Accession number NM_002154.3) using 20ng of RNA from cell lines BE(2)-C, VCR20, and ACT0.2, respectively. Hsp70 samples were subjected to 33 cycles of amplification. The PCR for the GAPDH primers was performed for 22 cycles (nucleotides 100-119 and 327-308 of accession number NM_002046). Samples were then run on a 1% agarose gel with ethidium bromide and visualized using UV light.

Cell Line	Hsp70/GAPDH	Fold Difference
BE(2)-C	0.5956	1
VCR20	0.8144	1.36
ACT0.2	0.9238	1.55

Table 1: Quantitative data of mRNA amount for Hsp70 in the cell lines BE(2)-C, VCR20, and ACT0.2 with all data being normalized to the parental line BE(2)-C. The program SigmaGel was used to determine these values. Fold values were calculated by dividing by values for Hsp70 by values for GAPDH.

As seen in Figure 1, an increase of Hsp70 mRNA was seen in the two reverse transformed MDR neuroblastoma lines as compared to BE(2)-C. The level of GAPDH amplification was used in the three cell lines as a loading control for RNA amounts. These results were quantified using SigmaGel. Table 1 shows quantitative results from the RT-PCR results. The reverse transformed lines, VCR20 and ACT0.2 respectively showed a 1.36 and 1.55 fold increase over the parental BE(2)-C cell line. Sequencing of the RT-PCR product generated using the Hsp70 specific primers to determine whether the amplified product was in fact Hsp70. The sequence generated from the RT-PCR product was aligned with the NCBI database and it was determined that the amplified product was in fact Hsp70 (Figure 2).

Formatted Alignments

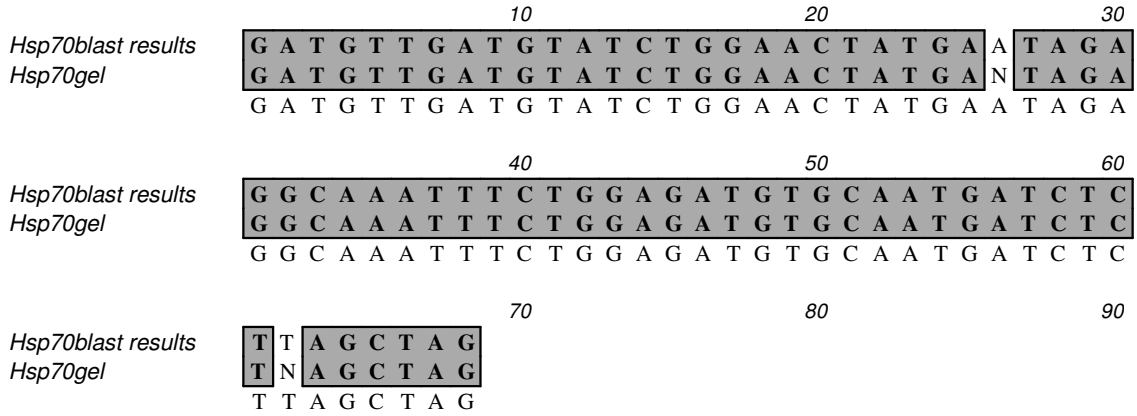

Figure 2: Sequence analysis of RT-PCR product for primers targeting Hsp70. 68 nucleotides of the RT-PCR product was compared to the NCBI database. 66 of the 68 nulceotides matched exactly. Values of "N" on the Hsp70 gel were the result of unclear sequence. Alignment was done using MacVector.

RT-PCR was performed on Hsp90 using primers specific for Hsp90 isoform 1 (accession number NM_001017963.1). Amplification of GAPDH again served as a loading control. Levels of mRNA were quantified using SigmaGel program. As seen in Figure 3, the reverse transformed lines show no change in mRNA levels compared to the BE(2)-C control line. When these products were quantified there was no difference in mRNA levels (Table 2). The amplified cDNA product generated using the Hsp90 specific primers. The sequence was determined to match the Hsp90 product (Figure 4).

Figure 3: RT-PCR product from primers specific for Hsp90 and GAPDH. Primers for Hsp90 were located in exon 1 (nucleotides 130-150 of accession number NM_001017963.1) and the reverse in exon 2 (nucleotides 509-490 of accession number NM_001017963.1). Primers for GAPDH were the same as in Figure 1. Samples from the cell lines BE(2)-C, VCR20, and ACT0.2 were amplified for 35 cycles for Hsp90, and for 22 cycles for GAPDH.

Formatted Alignments

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                                10                  20                  30
Hsp90blastresults   C G C T T C G G A G C C A G C A C G C G G G G T A C C C T A
Hsp90gel            C G C T T C G G A G C C A G C A C G C G G G G T A C C C T A
                    C G C T T C G G A G C C A G C A C G C G G G G T A C C C T A

                                40                  50                  60
Hsp90blastresults   C G G G G A G C G C G G A T G C C C C C G T G T T C G G G C
Hsp90gel            C N G G G A G C G C G G A T G C C C C C G T G T T C G G G C
                    C G G G G A G C G C G G A T G C C C C C G T G T T C G G G C

                                70                  80                  90
Hsp90blastresults   G G G G A C G G C T C C A C C C
Hsp90gel            N N G G A C G G C T C C A C C C
                    G G G G A C G G C T C C A C C C
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Figure 4: Sequence analysis of RT-PCR product for primers targeting Hsp90. 76 nucleotides of the RT-PCR product was compared to the NCBI database. 73 of the 76 nucleotides matched exactly. Values of "N" on the Hsp90 gel were the result of unclear sequence reaction result. Alignment was done using Mac Vector.

Cell line	Hsp90/GAPDH	Fold Difference
Be(2)-C	0.4258	1
VCR20	0.3736	0.88
ACT0.2	0.5674	1.33

Table 2: Quantitative results of Hsp90 mRNA levels in the three cell lines BE(2)-C, VCR20, and ACT0.2. SigmaGel was used to quantify the RT-PCR results and normalized to the parental cell line BE(2)-C. Fold values were calculated by diving SigmaGel values for Hsp90 by values for GAPDH in each cell line.

Finally, the results found in this study were compared to results found in a previous study, Spengler et al. (2006). A comparison of the data is seen in Table 3. In the previous study, protein levels for the reverse transformed lines above as much as a 4.0 fold increase in Hsp70 protein levels.

Cell Line	**Hsp70 mRNA amounts**	**Hsp70 protein amounts**
BE(2)-C	1	**1**
VCR20	1.36	**ND**
ACT0.2	1.55	**4.0**

Table 3: Table 3 shows expression of steady state mRNA and protein amounts for Hsp70. mRNA levels were determined in this study using RT-PCR. Protein amounts were determined by Spengler et al. (2006) using Immunoblotting. Data from Spengler et al. is a result of a single immunoblot. Both mRNA and protein amounts of the reverse transformed MDR lines VCR20 and ACT0.2 were set relative to the parental line BE(2)-C.

Discussion

RT-PCR for Hsp70 and Hsp90 reveals that there is in slight increase in levels of mRNA for Hsp70 in the reverse transformed multidrug-resistant neuroblastoma sublines as compared to the parental line. This result supports a previous study by Spengler et al. (2006), which showed an increase in Hsp70 proteins in the same reverse transformed

lines. However, when compared with the data from Spengler et al. (2006) there is a large discrepancy between increased amounts of Hsp70 protein and the amount of Hsp70 mRNA detected in the present study. Although there is a slight increase in the mRNA levels for Hsp70 it does not account for the reported 4 fold increases in the ACT0.2 subline compared to the parental BE(2)-C (Spengler et al. 2006).

By contrast, no consistent increase in Hsp90 amount was seen in the MDR reverse transformed lines. It was hypothesized that an increase in Hsp70 mRNA levels would be accompanied by an increase in Hsp90 mRNA levels as well, since Hsp70 sequesters Hsp90 in protein folding. In previous studies involving cancer and cancer-related stressors, a number of the heat shock proteins like Hsp70 and Hsp90 have shown variable levels and degrees of expression (Jolly and Morimoto 2000).

The difference in mRNA levels between Hsp70 and Hsp90 may be due to the fact that both play multiple roles in the cell. Hsp70 is involved in cytosolic protein folding and in preventing protein aggregate formation. Hsp70 also: 1) functions in the nucleus to regulate and respond to stress and growth-induced gene expression, 2) translocates proteins destined for specific cellular compartments, and 3) suppresses the apoptosis pathway (as cited in Jolly and Morimoto 2000). Hsp90 aids in the degradation of certain receptors, promotes proper protein folding (in conjunction with Hsp70), and plays an integral role in proper cell signaling (Siriani et al. 2005). Since these heat shock proteins have a variety of functions, their expression levels within a cell may be different depending upon the particular stresses experienced (as seen in Martinez et al. 1999).

In this study the mRNA levels for isoform 1 of Hsp90 (not isoforms 2) mRNA levels was analyzed in this study. Previous studies examining Hsp70 levels in the reverse

transformed MDR cell lines used immunoblotting with an antibody that likely recognizes more isoforms than the primers used in this study. Both Hsp70 and Hsp90 have constitutive and inducible forms, examining mRNA levels for all of these isoforms is necessary to determine what accounts for the increase found by Western Blotting performed by Spengler et al. (2006).

To better determine Hsp70 and Hsp90 a number of future studies may be carried out. It may prove fruitful to determine the half lives of both proteins in reverse transformed MDR cell lines compared to drug-sensitive neuroblastoma lines to see if there is an increase or decrease in protein half life that might explain the observed increase in Hsp70 protein levels. Further studies involving Hsp90 isoform 2 expression levels can also give insight into overall expression on Hsp90 in the reverse transformed MDR lines. Isolating and characterizing the promoter sequences of these chaperones can help to determine if the different Heat Shock Proteins respond to different stress signals.

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