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Role of Hsp-70 in Triptolide-Mediated Cell Death of Neuroblastoma

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Abstract

Background—Our recent work demonstrated that treatment of neuroblastoma with triptolide causes apoptotic cell death *in vitro* and decreased tumor size *in vivo*. Triptolide therapy has been associated with reduced expression of Hsp-70, suggesting a mechanism of cell killing involving Hsp-70 inhibition. The principal objective of this study was to investigate the role of Hsp-70 in triptolide-mediated cell death in neuroblastoma.

Materials and Methods—Neuroblastoma cells were transfected with Hsp-70-specific siRNA. Viability, caspase activity, and phosphatidylserine externalization were subsequently measured. An orthotopic, syngeneic murine tumor model was developed, and randomized mice received daily injections of triptolide or vehicle. At 21 days, mice were sacrificed. Immunohistochemistry was used to characterize Hsp-70 levels in residual tumors, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed to identify cells undergoing apoptosis.

Results—Targeted silencing of Hsp-70 with siRNA significantly decreased cellular viability, augmented caspase-3 activity, and resulted in increased Annexin-V staining. These effects parallel those findings obtained following treatment with triptolide. Residual tumors from triptolide-treated mice showed minimal staining with Hsp-70 immunohistochemistry, while control tumors stained prominently. Tumors from treated mice demonstrated marked staining with the TUNEL assay, while control tumors showed no evidence of apoptosis.

Conclusions—Use of siRNA to suppress Hsp-70 expression in neuroblastoma resulted in apoptotic cell death, similar to the effects of triptolide. Residual tumors from triptolide-treated mice expressed decreased levels of Hsp-70 and demonstrated significant apoptosis. These findings support the hypothesis that Hsp-70 inhibition plays a significant role in triptolide-mediated neuroblastoma cell death.

Keywords

triptolide; apoptosis; neuroblastoma; heat shock protein; Hsp-70

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INTRODUCTION

Advanced-stage cases of neuroblastoma, the most common pediatric extracranial tumor, are highly resistant to conventional chemotherapy. Our recent work demonstrated that treatment with triptolide, from the Chinese plant *Tripterygium wilfordii*, kills neuroblastoma cells *in vitro* and *in vivo*. We have also shown that triptolide exposure causes elevations in caspase activity and phosphatidylserine externalization, indicating that triptolide-mediated cell death occurs via an apoptotic pathway. Triptolide therapy in neuroblastoma has been correlated with decreased expression of Hsp-70 mRNA and protein, suggesting that cells treated with triptolide undergo apoptosis via inhibition of Hsp-70 expression¹. However, causative evidence is lacking.

In this study, we evaluated the hypothesis that triptolide's effects on neuroblastoma are mediated through inhibition of Hsp-70. We performed additional *in vitro* and *in vivo* experiments with triptolide, confirming our earlier results, and conducted further analysis of residual tumors from all *in vivo* experiments. To support our hypothesis that Hsp-70 inhibition plays a key role in triptolide's effects on neuroblastoma, we used siRNA to specifically suppress Hsp-70 expression and measured consequent results on cellular viability and markers of apoptosis *in vitro*. Additionally, we examined the role of Hsp-70 in the effects of triptolide therapy on neuroblastoma tumors *in vivo* by measuring markers of apoptosis and levels of Hsp-70 expression in residual tumors of treated and control mice.

MATERIALS AND METHODS

Neuroblastoma cells

N2a murine and SKNSH human neuroblastoma cells were purchased from American Type Culture Collection (Manassas, Virginia). Our culturing techniques for these cell lines have been previously described¹.

Transfection of siRNA

Hsp-70 siRNA was obtained from Qiagen (Hilden, Germany), prepared according to manufacturer's instructions, and dissolved in siRNA suspension buffer at concentration of 20 μ M. The sense strand was 5'- CCAUUGAGGAGGUAGAUUAtt-3' and the antisense strand was 5'- gtGGUAAACUCCUCCAUCUAAU-3'. A second siRNA sequence for Hsp-70 was also used, with sense strand 5'- GUUACUCAAAGUAAAUAAtt-3' and antisense strand 5'- acCAAUGAAGUUUCAUUUAUU-3'. We subsequently refer to these two sequences as sequences (a) and (b), respectively. Scrambled nonsilencing siRNA (Qiagen) was used as a control, with sense strand 5'-UUCUCCGAACGUGUCACGUDtT-3' and nonsense strand 5'- ACGUGACACGUUCGGAGAAAdTtT-3'. For transfection, SKNSH cells were plated into 96-well (2×10^3 cells/well) and 6-well (6×10^4 cells/well) plates and incubated for 72 hours. LipofectAMINE 2000 reagent (Invitrogen) was added to Opti-MEM transfection medium (Invitrogen) for a final concentration of 2 μ L/mL and incubated at room temperature for 5 minutes. Hsp-70 or nonsilencing (NS) siRNA were added at concentrations of 25 and 50 nM and incubated for another 20 minutes. The transfection reagent and siRNA were then added to the cells (100 μ L/well for 96-well plates and 1 mL/well for 6-well plates) and incubated at 37° C for 5 hours, after which time regular media in the absence of antibiotics was replaced.

Hsp-70 measurement

Seventy-two hours after transfection, cells were collected from 6-well plates for protein isolation. Cell lysates were prepared by resuspending cells in Rapid Immuno Precipitation Assay Buffer (Boston BioProducts, Worcester, Massachusetts) with protease inhibitor (20

μL per mL of lysis buffer) for two hours on ice, and cleared by centrifugation for 10 minutes at 18000 rcf. Following supernatant collection, protein concentration was determined using a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, Illinois) with bovine serum albumin standards. Western blotting was performed with mouse Hsp-70 antibody (Stressgen, Ann Arbor, Michigan) with actin expression as control (goat-polyclonal actin antibody from Santa Cruz Biotechnology, Inc, Santa Cruz, California).

Viability and caspase activity measurement

The viability and caspase-3 activity of cells treated with siRNA as above were measured at 24, 48, 72, and 96 hours. Details of these techniques have been previously described².

Annexin-V staining

Phosphatidylserine externalization was determined 48 hours after transfection with Annexin-V staining using Guava Nexin Reagent (Guava Technologies, Hayward, California). Following plating on 6-well plates and transfection as above, cells were prepared with the reagent according to manufacturer's instructions and flow cytometry used for result determination, as we have described in earlier reports¹.

Triptolide

For all *in vivo* experiments, triptolide (Calbiochem, EMD Chemicals, Inc., Gibbstown, New Jersey) was dissolved in DMSO (Sigma-Aldrich) at a concentration of 1 mg/mL. Aliquots were kept at -20 C and therapeutic doses prepared daily for mice.

In vivo tumor model

Following IACUC approval (# 0611A97266) an orthotopic, syngeneic tumor model was developed as previously described¹. Mice were randomized into treatment and control groups immediately following inoculation. Three days after tumor injection, treated mice began receiving daily intraperitoneal injections of triptolide (0.4 mg/kg) or vehicle (DMSO) delivered in 100 μL of PBS (Invitrogen). Mice were examined daily by a blinded observer for signs of morbidity and were sacrificed if they met IACUC criteria for moribund status. Remaining mice were sacrificed at Day 21, and necropsy was conducted. Tumor masses and dimensions were recorded by blinded individuals, and volume was calculated as $\frac{4}{3} \pi r^3$, where $r = 0.5 \times \text{average of length, width, and depth}$. Experiments were conducted three times.

Hsp-70 measurement in tumors

Immunohistochemistry (IHC) was used to characterize levels of Hsp-70 in residual tumors. Tumors samples were sectioned and fixed. Slides were immersed in xylene (Sigma-Aldrich) and sequentially more dilute ethanol (Pharmco Products, Inc., Brookfield, CT) for hydration and deparaffinization. IHC steps were then carried out via methodology as we have previously described³. Sections were viewed under light microscopy and photographs obtained. Western blotting was also performed in order to provide quantitative measurement of tumor Hsp-70 levels. Tumor tissues were homogenized and protein content was isolated. Western blotting was then carried out according to *in vitro* protocol above.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

TUNEL was performed on tumor tissue as a measurement of *in situ* apoptosis. Previously sectioned and fixed tumor samples were processed using the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Negative controls were obtained for each sample by omission of incubation with the TUNEL reaction mixture. Sections were viewed under light microscopy and photographs obtained.

Statistical analysis

Data are expressed as mean result $\pm$ SEM. Student's t-test was used to determine the significance of the differences between treatment and controls, with $p < 0.05$ considered statistically significant. Results from IHC and TUNEL assays were qualitative and statistical analyses were not appropriate.

RESULTS

Triptolide's effects on *in vitro* viability and *in vivo* tumor growth

Previous studies from our laboratory demonstrated that triptolide decreases neuroblastoma tumor viability *in vitro* in a dose- and time-dependent fashion¹. Results from our current studies confirm our previous findings. We have also shown that mice receiving triptolide therapy develop smaller neuroblastoma tumors than control mice. Our present experiments demonstrate an average tumor volume of $0.33 \pm 0.11 \text{ cm}^3$ in triptolide-receiving mice, compared to 1.99 ± 0.54 in controls, and average tumor mass of $0.35 \pm 0.12 \text{ g}$, compared to 1.84 ± 0.53 in controls ($p < 0.01$).

Extent of Hsp-70 silencing with siRNA treatment

To show that our siRNA treatment effectively suppressed expression of Hsp-70, Western blot analysis was conducted 72 hours after transfection. This showed dose-dependent suppression of Hsp-70 (Fig. 1), with near total suppression of Hsp-70 expression following transfection with 50 nM siRNA. Beta-actin was used as a control, demonstrating equal protein loading. At time periods less than 72 hours, both siRNA sequences had very little, if any, effect on suppressing Hsp-70 levels in this neuroblastoma cell line (data not shown).

Effect of Hsp-70-specific siRNA on cell viability and markers of apoptosis

In order to demonstrate the cytotoxic effect of Hsp-70 suppression, cellular viability was measured following transfection with Hsp-70 siRNA. Two different Hsp-70 siRNA sequences were used to rule out any off-target effects. We found that impaired expression of Hsp-70 resulted in cell killing in a dose-dependent fashion (Fig. 2). Optimal Hsp-70 silencing was achieved with 50 nM siRNA; therefore, all data shown herein are for studies performed with this concentration. Treatment with 50 nM Hsp-70-specific siRNA with both siRNA sequences [(a) and (b)] resulted in cell killing in greater than one-half of neuroblastoma cells at 96 hours (Fig. 2). We note that effects prior to this time were less significant, which we attribute to a delay in maximal Hsp-70 silencing, a feature inherent to the slow turnover of this particular cell line.

In order to show that inhibition or suppression of Hsp-70 results in cell death via apoptosis, caspase-3 activity and phosphatidylserine externalization were measured following treatment with Hsp-70 siRNA. We found that use of both Hsp-70 siRNA sequences significantly augmented caspase-3 activity at 96 hours, elevating caspase-3 activity to $>1200\%$ of control with sequence (b) and $>1500\%$ of control with sequence (a) (Fig. 3). As a marker of early apoptosis, flow cytometry for Annexin-V staining was measured, showing elevation to levels nearly 300% of control (Fig. 4). Baseline levels of apoptosis in the control cells were found to be approximately 4%. Though triptolide is known to have a number of additional effects, these data support our hypothesis that the inhibition of Hsp-70 expression serves as a fundamental step in the apoptotic neuroblastoma cell death triggered by triptolide therapy.

Hsp-70 expression in triptolide-treated tumor tissue

Immunohistochemistry for Hsp-70 resulted in considerable staining of tumor tissues from control mice. Minimal staining was evident in the tumors from mice treated with triptolide, with an obvious decrease in staining apparent in comparison to the control tumors (Fig. 5). Examination of negative controls for each sample confirmed the absence of any Hsp-70 staining. These IHC results substantiate the notion that triptolide decreases expression of Hsp-70, and specifically clarify that Hsp-70 levels are reduced in residual neuroblastoma tumor tissues from mice that have been treated with triptolide.

This effect was examined quantitatively with Western blotting. Our findings from this experiment further confirmed that Hsp-70 levels were markedly decreased in residual tumors from mice who received triptolide compared mice in the control group (Fig. 6).

In situ apoptosis in triptolide-treated tumor tissue

Tumors from mice receiving triptolide demonstrated marked staining with the TUNEL assay, indicating *in situ* apoptosis in the residual tumor tissues. Tumor samples from mice receiving vehicle failed to stain with this assay, therefore showing no evidence of apoptosis (Fig. 7). Examination of negative controls for each sample revealed absence of TUNEL staining. The results of these TUNEL assays further confirmed that neuroblastoma tumor cells in our *in vivo* model were killed by triptolide via an apoptotic pathway. Taken along with the results of our tumor model and Hsp-70 IHC, our findings from this assay clearly show that neuroblastoma tumors in mice receiving triptolide are not only significantly smaller than those of the mice receiving vehicle; in addition, these tumors demonstrate inhibited expression of Hsp-70 and consequently undergo active apoptosis.

DISCUSSION

Advanced-stage cases of neuroblastoma are highly aggressive and frequently resistant to conventional chemotherapy, with five-year survival rates in the range of 30–40%. Though much research is devoted to improving the outcomes for these patients, there has been minimal recent progress in prolonging survival for patients with advanced disease⁴. Clearly, there is an important need for development of novel therapeutic options for this cohort of pediatric patients with poor prognostic outlook.

We have previously demonstrated the efficacy of triptolide in killing neuroblastoma cells both *in vitro* and *in vivo*. However, the exact mechanism through which triptolide's effects occur has not been clearly identified. We have shown that Hsp-70 is overexpressed in neuroblastoma cells, and that triptolide exposure is associated with reduced levels of Hsp-70 *in vitro*¹. However, the potential exists that decreased expression of Hsp-70 in triptolide-treated cells is a downstream consequence, rather than the inciting mechanism through which triptolide takes effect. It is possible that triptolide-mediated cell killing occurs via alternative cellular mechanisms, which may include interference with the NF- κ B pathway or inhibition of the activities of members of the Inhibitor of Apoptosis Protein (IAP) family. In order to confirm that Hsp-70 inhibition serves as a key *causative* component of triptolide's mechanism of neuroblastoma cell killing, we conducted the experiments described herein.

Triptolide therapy has been applied to a wide range of medical indications, and has been used in both Eastern and Western medicine as an anti-immune agent. It is known to have a broad scope of cellular effects. Though we feel that Hsp-70 inhibition serves as a significant mechanism through which triptolide causes apoptotic cell death, it is possible that additional cellular activities may contribute. Prior to these studies, it was clear that triptolide-mediated cell death in neuroblastoma occurred in association with observed drops in Hsps-70 levels; however, causative evidence was lacking. We have shown that, like treatment with

triptolide, Hsp-70 suppression with siRNA results in apoptotic cell killing associated with elevation of caspase-3 activity and phosphatidylserine externalization. This proof of principle strongly supports our hypothesis that Hsp-70 inhibition is vital to triptolide's antineoplastic effects in neuroblastoma.

Residual neuroblastoma tumors from triptolide-treated mice expressed decreased levels of Hsp-70 by immunohistochemistry and demonstrated a significant extent of apoptosis with the TUNEL assay. In contrast, tumors from control mice receiving only vehicle expressed greater Hsp-70 levels by immunohistochemistry, with an insignificant extent of apoptosis revealed by TUNEL assay. These studies corroborate with our previous data, which showed that triptolide exposure *in vitro* inhibits neuroblastoma Hsp-70 expression and is associated with elevation of apoptotic markers such as caspase activity and phosphatidylserine externalization¹. These current findings establish evidence that triptolide therapy also triggers apoptosis of neuroblastoma tumors *in vivo* through inhibition of Hsp-70 expression.

The apoptotic cell death initiated by triptolide is not uniquely seen in neuroblastoma. Other authors have demonstrated triptolide's efficacy in inhibition of proliferation of a number of additional malignancies, including cholangiocarcinoma, gastric cancer, genitourinary malignancies, melanoma, breast cancer, and leukemia^{5, 6, 7}. However, the mechanisms and cellular targets for triptolide are not known. Previous work from our laboratory has shown that treatment with triptolide induces apoptotic death of pancreatic cancer cells through Hsp-70 inhibition, with performance of TUNEL assay generating similar findings to those shown here for neuroblastoma³. However, reports of triptolide therapy for neuroblastoma are limited¹; moreover, the mechanisms by which triptolide induces cell death in neuroblastoma have not been elucidated.

Our findings indicate that Hsp-70 inhibition serves as a key mechanism for triptolide-mediated cell death. Studies by Dudeja *et al* directed at clarifying the cellular pathways through which Hsp-70 inhibition causes apoptosis in cancer cells have identified two simultaneous and independent mechanisms⁸. Inhibition of Hsp-70 has been shown to increase intracellular Ca²⁺ levels, leading to apoptotic cell death. Additionally, blocking expression of Hsp-70 results in increased cytosolic cathepsin B activity, suggesting loss of lysosomal integrity. Pretreating cancer cells with an intracellular calcium chelator or an inhibitor of cathepsin B each independently provided protection from cell death in the face of Hsp-70 inhibition. These findings support the importance of both pathways; further, the authors provide evidence that these pathways have additive effects independent of each other. Finally, loss of Hsp-70's chaperone activities may represent a third possible mechanism through which Hsp-70 inhibition results in apoptotic cell death in cancer cells. Future studies delving into this possible mechanism are warranted.

Neuroblastoma is thought to result from failure of embryonal precursors to undergo programmed cell death⁹, and it is believed that altered cellular responses to apoptosis may be involved in drug resistance seen in more aggressive neuroblastoma tumors¹⁰. Poor-prognosis neuroblastomas are known to demonstrate silencing of caspase expression, contributing to their chemoresistance¹¹. Therefore, treatment with triptolide to induce apoptotic cell death via the caspase cascade may be of significant benefit to patients with tumors associated with unfavorable outcomes. Published work on neuroblastoma has shown that levels of Hsp-70 are elevated in those tumors that are more poorly differentiated as well as those associated with lymphatic metastases¹². Consequently, we further believe that induction of apoptotic pathways through inhibition of Hsp-70 may represent a very important modality of therapy for patients with aggressive neuroblastoma tumors.

Our studies strongly suggest that triptolide may have a future role in targeted therapy for neuroblastoma, and further translational studies are clearly warranted.

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Figure 1. Hsp-70 levels after siRNA treatment

a) Western blot demonstrates suppression of Hsp-70 expression in SKNSH following treatment with 50 nM Hsp-70 specific siRNA [sequence (a)]. **b)** Quantification of Western blot band densities shows that silencing of Hsp-70 with siRNA occurs in a dose-dependent fashion. N=3; * indicates $P < 0.05$ vs control.

Figure 2. Viability after siRNA treatment

Suppression of Hsp-70 expression with siRNA resulted in dose-dependent cell killing of human neuroblastoma cells at 96 hours, demonstrating the cytotoxic effects of inhibition of Hsp-70 expression. N=3; * indicates P<0.05 vs control.

SKNSH Caspase-3 Activity After siRNA

Figure 3. Caspase activity after siRNA treatment

Following treatment with Hsp-70 siRNA, caspase-3 activity was measured. Significant elevation compared to control was found at 96 hours, and a greater effect was seen with higher concentrations of siRNA. N=3; * indicates $P < 0.05$ vs control.

Annexin-V Staining in SKNSH

Figure 4. Annexin staining after siRNA treatment

Annexin-V staining was used to measure phosphatidylserine externalization as an additional marker of apoptosis. Following treatment with Hsp-70 siRNA [sequence (a)], Annexin-V staining was elevated compared to control, further showing that inhibition of Hsp-70 expression results in neuroblastoma cellular apoptosis. N=3; * indicates $P < 0.05$ vs control.

Figure 5. Immunohistochemistry

Representative results of Hsp-70 immunohistochemistry in control tumors (top row) and residual tumors from mice treated with triptolide (bottom). Note significant Hsp-70 staining in control mice, with inhibition of Hsp-70 expression in residual tumors from treated mice.

Hsp-70 Levels from Residual Tumor

Figure 6. Quantification of Hsp-70 levels from residual tumor

Western blot demonstrating Hsp-70 levels in tumors from four control mice (columns on left) and mice who received triptolide (columns on right). Actin is used as a control for protein loading. There is a marked decrease in Hsp-70 levels in tumor tissues from mice who received triptolide therapy.

Figure 7. TUNEL

Representative results from TUNEL assay in control tumors (top row) and residual tumors from mice treated with triptolide (bottom). Note minimal TUNEL staining in control mice, with obvious evidence of *in situ* apoptosis shown by marked TUNEL staining in residual tumors from treated mice.