ORIGINAL RESEARCH

Pilot study of intratumoral injection of recombinant heat shock protein 70 in the treatment of malignant brain tumors in children

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Abstract: Intratumoral injections of recombinant heat shock protein (Hsp)70 were explored for feasibility in patients with brain tumors. Patients aged 4.5–14 years with untreated newly diagnosed tumors (n=12) were enrolled. After tumor resection, five injections of recombinant Hsp70 (total 2.5 mg) were administered into the resection cavity through a catheter. Before administration of Hsp70 and after the last injection, specific immune responses to the autologous tumor lysate were evaluated using the delayed-type hypersensitivity test. Further, peripheral blood was monitored to identify possible changes in lymphocyte subpopulations, cytokine levels, and the cytolytic activity of natural killer cells. The follow-up period in this trial was 12 months. Intratumoral injections of Hsp70 were well tolerated by patients. One patient had a complete clinical response documented by radiologic findings and one patient had a partial response. A positive delayed-type hypersensitivity test was observed in three patients. In peripheral blood, there was a shift from cytokines provided by Th_2 cells toward cytokines of a Th_1-cell-mediated response. These data corresponded to changes in lymphocyte subpopulations. Immunosuppressive T-regulatory cell levels were also reduced after injection of Hsp70, as well as production of interleukin-10. The cytolytic activity of natural killer cells was unchanged. The present study demonstrates the feasibility of intratumoral delivery of recombinant Hsp70 in patients with cancer. Further randomized clinical trials are recommended to assess the optimum dose of the chaperone, the treatment schedule, and clinical efficacy.

Keywords: heat shock protein 70, malignant brain tumors, immunotherapy, glioblastoma

Introduction

Primary malignant brain tumors are the second most common pediatric malignancies after leukemia, representing 20% of all cancers in children. In the majority of cases, treatment consists of surgery followed by radiotherapy and concomitant chemotherapy.[1] Despite this aggressive multimodal approach, the prognosis for patients is poor. Among the new treatments currently being investigated, immunotherapy is theoretically very attractive because it offers the potential for highly tumor-specific effects.[2] In recent years, evidence has accumulated for the potential immunotherapeutic anticancer activity of the molecular chaperone, heat shock protein (Hsp)70.[3] The immunologic function of Hsps was first discovered because of their ability to induce tumor rejection when they were purified from the tumor.[4] Later it was shown that tumor-derived Hsps can carry cancer-associated peptides as cargo into antigen-presenting cells for subsequent presentation with major histocompatibility complex class I to CD8+ (cluster of differentiation 8) T-lymphocytes, triggering formation of subpopulations of tumor-specific effector cells.[5] This

hypothesis was investigated in several clinical trials where autologous tumor-derived Hsps were used as vaccines in patients with cancers at various locations.[6–8]

Recently, much attention has focused on the peptide-independent immunomodulatory activity of chaperones. It has been shown that Hsps can move into the extracellular space encased in exosomes or in soluble form, where they function as natural adjuvants, enhancing innate and adaptive immune responses.[9,10] By binding specific receptors, ie, Toll-like receptor (TLR)-2 and TLR-4, they induce expression of costimulatory molecules, maturation of dendritic cells, and secretion of proinflammatory cytokines, eg, tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-12, IL-6, and granulocyte-macrophage colony-stimulating factor.[11,12] Moreover, Hsp70 can activate the cytolytic and migratory activity of natural killer cells.[13] Since Hsps modulate the immune system toward a cell-mediated response, they could be useful in approaches to the treatment of cancer.

Previously we have demonstrated the immunomodulatory antitumor activity of Hsp70 in models of B16 melanoma, RA-2 rhabdomyosarcoma, and C6 glioblastoma,[14–16] and intratumoral injection of recombinant Hsp70 was demonstrated to be therapeutically relevant in the treatment of C6 rat glioblastoma.[15] Infusion of Hsp70 delayed the rate of tumor growth and prolonged survival in tumor-bearing rats. Therapeutic efficacy was accompanied by enhanced antitumor activity of natural killer cells (production of granzyme B) as well as CD8+ cytotoxic T-lymphocytes, as shown by interferon-gamma (IFN-γ) ELISPOT (enzyme-linked immunospot) assay.[15] In a recent in vitro study, we further explored the possible antitumor immunomodulatory

mechanism of Hsp70 activity,[17] and demonstrated that exogenously delivered Hsp70 penetrated cancer cells and induced translocation of its endogenous analog to the cell surface. This process significantly increased the sensitivity of tumor cells toward the cytolytic activity of lymphocytes.[17]

Following in vitro and in vivo assessments of Hsp70 activity, we investigated the feasibility, safety, side effects, and immunologic responses associated with Hsp70 therapy in pediatric patients with malignant brain tumors.

Materials and methods
Clinical study design

Patients of both sexes with newly diagnosed malignant brain tumors without any previous treatment were enrolled in the study (Table 1). The study protocol stipulated that tumor resections be as complete as believed safely feasible by the surgeons. All resections were done by two designated study surgeons in a neurosurgical institution. Surgery was done using a neurosurgical microscope (Carl Zeiss Surgical GmbH, Oberkochen, Germany). Other intraoperative diagnostic procedures (eg, sonography, neuronavigation) were performed for planning of the surgical approach and for tumor localization. During tumor resection, a silicone catheter was implanted into the resection cavity. Following surgery, a course of five injections of Hsp70 were delivered into the resection cavity through the catheter, ie, 500 µg of Hsp70 in saline solution was infused per single injection on days 1, 3, 5, 7, and 9 following surgery, for a total of 2.5 mg of protein per course. Immunologic changes (lymphocyte subpopulations, cytokine levels, delayed-type hypersensitivity test for antitumor immune reaction) were monitored before and after the course of Hsp70 infusions. The patients

Table 1 Patient characteristics

Case	Age	Gender	Histopathology	KPS	Previous treatment	Surgery
1	13	M	Glioblastoma multiforme	60	–	IR
2	13	M	Anaplastic astrocytoma (G III)	80	–	IR
3	7	M	Anaplastic ependymoma (G III)	80	–	IR
4	13	M	B-cell non-Hodgkin's lymphoma	80	–	IR
5	4	M	Choroid plexus carcinoma (G IV)	70	–	IR
6*	12	F	Primitive neuroectodermal tumor	60	S	IR
7	10	F	Anaplastic astrocytoma (G III)	80	–	IR
8	14	F	Anaplastic ependymoma (G III)	80	–	IR
9*	12	M	Astrocytoma (G II)	60	S	IR
10	4,5	F	Astrocytoma (G II)	70	–	IR
11	6	F	Anaplastic astrocytoma (G III)	60	–	IR
12*	10	M	Glioblastoma multiforme	60	S	IR

Notes: *Patients 6, 9, and 12 had undergone ventriculoperitoneal shunting surgery. Patients did not receive any conventional treatment before the course of heat shock protein 70. Within 24 hours after surgery, the residual tumor was assessed by postoperative magnetic resonance imaging.
Abbreviations: KPS, Karnofsky performance status; S, surgery; IR, incomplete resection; G, grade.

received no other treatment except for corticosteroid therapy during the Hsp70 infusions. Pretreatment with dexamethasone 4 mg (from various manufacturers) twice daily was given for at least 2 days before surgery and continued for 5 days thereafter. For evaluation of the short-term response of the tumor to chaperone treatment with Hsp70, magnetic resonance imaging (MRI) was performed in the 24 hours following surgery and again 4 weeks after the final Hsp70 infusion. All patients were monitored for adverse events. The follow-up period for the study was 12 months. Following the course of chaperone treatment, patients received conventional radiotherapy or chemotherapy.

Eligibility

Patients of both sexes with newly diagnosed malignant brain tumors and whose parents provided written informed consent according to a resolution of the local ethics committee of the AL Polenov Russian Research Scientific Institute of Neurosurgery (St Petersburg, Russian Federation) were eligible for this study. Inclusion criteria were: intraoperative histologically proven diagnosis of malignant brain tumor on review by two independent pathologists; 3–18 years of age; Karnofsky performance status >60; absolute neutrophil count (neutrophils and bands) $\geq 1.5 \times 10^9/L$; platelets $\geq 100 \times 10^9/L$; aspartate aminotransferase or alanine aminotransferase ≤ 5 times the upper limit of normal, total bilirubin ≤ 1.5 times the upper limit of normal, serum creatinine ≤ 1.5 times the upper limit of normal, or estimated creatinine clearance ≥ 60 mL per minute; no known immunosuppression; negative human immunodeficiency virus serology; no evidence of acute or chronic hepatitis on standard hepatitis C and B screening tests; no second malignancies; absence of active infection; no history or clinical evidence of active autoimmune disease; informed consent by parents. Exclusion criteria were: not proven diagnosis of malignant brain tumor by the biopsy during the operation of malignant brain tumor after surgical resection; concomitant life-threatening disease; presence of acute (including respiratory) infection; known immunosuppressive disease; positive serology for human immunodeficiency virus, hepatitis B or C; history of an autoimmune disease; history of other malignancies; psychologic, family, sociologic, or geographic conditions not permitting adequate medical follow-up; and compliance with the study protocol.

Preparation of recombinant human Hsp70

Recombinant Hsp70 was produced in the Research Institute of Highly Pure Biopreparations (St Petersburg, Russian Federation) from *Escherichia coli* transformed with a pMSHsp70 (plasmid maintenance sequence heat shock protein 70) plasmid according to Good Manufacturing Practice standards.[18,19] Briefly, Hsp70 was purified by anion exchange chromatography using DEAE-Sepharose (GE Healthcare Life Sciences, Cleveland, OH, USA) followed by adenosine triphosphate (ATP)-affinity chromatography on ATP-agarose (Sigma, St Louis, MO, USA). Endotoxin was depleted by polymyxin B-sepharose endotoxin-removing gel (Sigma). Quantitation of endotoxin was performed using the *Limulus amoebocyte* lysate assay (QCL-1000; Cambrex Bio Science, Walkersville, MD, USA). The resulting endotoxin content was below 0.1 EU/mg Hsp70. To confirm that the immunomodulatory activity of Hsp70 was not due to endotoxin contamination, we boiled Hsp70 (at 95°C) and assessed its activity in a series of in vitro tests as described previously (ie, activation of lymphocytes in the cytotoxic T-lymphocyte test).[15] The results demonstrated that the heated preparation of Hsp70 had no immunostimulatory activity. Protein concentration was measured using the Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) and purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Evaluation of clinical status

The toxicity of Hsp70 was monitored and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0.[20] Immediate and delayed toxicities were assessed following each delivery of Hsp70 solution. Evaluation of autoimmune symptoms and neurologic examinations were done before and 30 minutes after vaccination, and at all 4-weekly follow-up visits. MRI was done to monitor intracranial lesions after treatment with Hsp70. MRI scans were performed preoperatively and within 24 hours after surgery (before the course of Hsp70). The presence of residual tumor was assessed by postoperative MRI. Residual tumor was defined as contrast enhancement with a volume >0.175 cm³. The cutoff volume represented the size of one voxel in the T1 image and corresponded to the minimal resolution obtained on MRI. The cutoff volume was defined to prevent interpretation problems when distinguishing between tumor and nonspecific enhancement. The volumes of residual tumors were segmented on individual scans, and the individual volumes were summed for the final volume. All patients were divided into groups of complete or incomplete resection whether or not they had a contrast-enhancing lesion. Four weeks after the last intratumoral injection of Hsp70, we performed MRI for assessment of tumor responses to

chaperone therapy. Clinical responses were classified based on the criteria devised by Macdonald et al:[21] complete response, defined as complete disappearance of the tumor for at least 4 weeks; partial response, defined as a reduction of 50% or more in tumor size for at least 4 weeks; minor response, defined as a 25%–50% decrease in size of the lesion lasting at least 4 weeks or a more than 50% decrease in lesion size lasting less than 4 weeks; stable disease, defined as either a decrease of less than 25% or an increase of less than 25% in tumor size for at least 4 weeks; and progressive disease, defined as an increase of 25% or more in tumor size.

Immunofluorescence analyses of Hsp70 expression in tumor samples

Hsp70 was analyzed in tumor biopsies obtained from all patients during surgery. Serial frozen sections of biopsy specimens were cut at a thickness of 7 µm and stained with monoclonal anti-Hsp70 antibodies (clone BRM-22; Sigma) and secondary goat anti-mouse Alex568 conjugated antibodies (Thermo Fisher Scientific, Waltham, MA, USA). The nucleus was detected using 4′,6-diamidino-2-phenylindole. Monoclonal anti-Hsp70 antibody reacts specifically against inducible (Hsp72) and constitutive (Hsp73) forms of Hsp70. Mouse immunoglobulin G1 isotype antibodies were used as the control. Sections were mounted using DAKO fluorescent mounting medium (Dako North America Inc., Carpinteria, CA, USA). Fluorescence images were captured with a Leica TCS SP5 confocal system, equipped with lasers exciting at 543 nm (He/Ne) and 405 nm (diode laser) on a Leica DM IRBE microscope stand with an HCX PL APO 100×1.40 NA, HP 40×1.25 oil immersion objective lens (Leica Microsystems, Heidelberg, Germany).

Evaluation of immunologic status
Delayed-type hypersensitivity test

A mixture of 0.05 µg of purified tuberculin and 10 µg of autologous tumor lysate was administered intradermally into the forearm before and after 1 month of treatment[22] to evaluate the specific cell-mediated immune response in the treated patients. A positive delayed-type hypersensitivity skin test reaction was defined as >2 mm diameter induration after 48 hours.

Cytotoxic T-lymphocyte test

For the cytotoxic T-lymphocyte test, peripheral blood lymphocytes were obtained after tumor resection and again 2 weeks after the last intratumoral infusion of Hsp70. Peripheral blood lymphocytes were isolated by Histopaque® 1.077 (Sigma)

gradient centrifugation. Cell viability was determined by 0.4% trypan blue exclusion before the experiments. Lymphocytes were challenged with K562 (human erythroleukemia) cells as target cells for analysis of the cytolytic activity of the natural killer cells obtained. Prior to the cytotoxic assay, the K562 cells were labeled with 5 µM CellTracker Orange CMTMR (Thermo Fisher Scientific). They were then added to the effector cells (peripheral blood lymphocytes) at different effector to target cell ratios of 12.5:1, 25:1, and 50:1 and incubated for 4 hours. A fluorescent compound 7-amino-actinomycin D (BD Biosciences, San Jose, CA, USA) was added to each tube at a final concentration of 1 µg/mL, as recommended by the manufacturer. The stained samples were analyzed by multicolor flow cytometry immediately after the end of the incubation period. Cytotoxicity was calculated as the percentage of CellTracker Orange CMTMR+, 7-amino-actinomycin D+ cells of the total CellTracker Orange CMTMR+ cells.

Cell surface analyses

To assess possible changes in lymphocytes and their subpopulations before and after administration of Hsp70, peripheral blood lymphocytes were collected from the patients, resuspended in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide (Sigma), and stained with monoclonal antibodies against human CD3, CD4, CD8, CD19, CD25, CD56, or HLA (human leukocyte antigen) DR for 30 minutes at 4°C. T-regulatory cells were detected as CD4+CD25+FoxP3+ cells. For intracellular staining for FoxP3+, we applied anti-FoxP3 antibodies (R&D Systems Inc., Minneapolis, MN, USA). The stained cells were then washed and analyzed using FACScan (Becton Dickinson, San Jose, CA, USA). Species-matched and isotype-matched monoclonal antibodies were used as controls.

Detection and quantification of cytokines

Quantification of IL-1β, IL-1Ra, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α in serum (or cerebrospinal fluid) and culture supernatants of stimulated lymphocytes was performed using commercially available enzyme-linked immunosorbent assay kits (eBiosciences, San Diego, CA, USA), in accordance with the manufacturer's instructions. For stimulation, peripheral blood lymphocytes were treated with phytohemagglutinin (Biological Industries, Beit HaEmek, Israel) at a concentration of 20 mg/mL for 48 hours.

Statistical analysis

Continuous variables were compared using the paired Student's *t*-test. Statistical significance was determined at the

$P<0.05$ level. The two-tailed Mann–Whitney log-rank test was used to compare the study and control groups.

Results

Patient characteristics

Twelve patients were enrolled in the pilot study (Table 1) and comprised five girls and seven boys of mean age 9.9 (4.5–14) years. A histologic diagnosis of malignant brain tumor on intraoperative biopsy was the key criterion for inclusion in the study. On subsequent evaluation of paraffin block sections (when administration of Hsp70 was performed), different histologic types of malignant tumors were identified. All biopsy material was checked for the presence of Hsp70 by immunofluorescence analysis. As expected, we observed marked expression of cytoplasmic Hsp70 in all 12 patients. Three patients (cases 6, 9, and 12) had previously undergone surgery (ventriculoperitoneal shunting) at the AL Polenov Russian Research Scientific Institute of Neurosurgery before participating in the study. All patients were treated before and after surgery with corticosteroids in standard doses. Starting on the day following tumor debulking, all patients received a series of injections of purified chaperone Hsp70 through a silicone catheter implanted in the resection cavity. The total amount of infused protein was 2.5 mg (500 µg per injection). Evaluations for toxicity and treatment response were conducted from the first day of administration of Hsp70. All patients had baseline MRIs within 24 hours of tumor resection before starting infusion of Hsp70. According to their postoperative MRIs, all 12 patients had a residual contrast-enhancing lesion related to incomplete resection.

Assessment of safety and clinical responses

Intratumoral infusions of purified chaperone Hsp70 were well tolerated, except in three patients (cases 5, 6, and 10) in whom major (grade 3) adverse effects were observed (Table 2). Five patients had grade 2 fever, three patients had grade 3 headaches, three further patients had grade 2 headaches, and one patient each had grade 1 and grade 2 vomiting. In seven patients, clinical symptoms started to appear 1.5–2 hours after each chaperone injection and abated in 5–6 hours to a normal level. There were no treatment-related hematologic, hepatic, or renal toxicities, nor any clinical or radiologic signs of autoimmune reactions in any patient.

The radiologic criteria devised by Macdonald et al[21] were used to evaluate the clinical responses to intratumoral infusion of Hsp70.[21] Four weeks after the last injection of Hsp70, nine patients showed no change on MRI scan (stable disease); one patient (case 7) showed a complete response; one patient (case 5) showed a partial response (Figure 1); and one patient (case 12) showed disease progression (Table 2). The MRI of one patient (case 7) showed no signs of the contrast enhancement lesion 4 weeks after the final administration of chaperone Hsp70.

Antitumor cell-mediated responses were analyzed in all patients following assessment of the MRI scans. For this purpose, purified tuberculin with autologous tumor lysate was administered into the forearm before and after Hsp70 injection.[22] The test was positive in only three patients, including the patient with a clinical complete response (case 7), and was negative in nine patients. Eleven patients

Table 2 Results of administration of heat shock protein 70

Case	Total amount of injected protein (mg)	Radiologic response	DTH	Adverse effects	Outcome
I	2.5	SD	Positive	Fever (grade 2)	Alive
2	2.5	SD	Negative	No	Alive
3	2.5	SD	Negative	No	Alive
4	2.5	SD	Negative	No	Alive
5	2.5	PR	Positive	Fever (grade 2), headache (grade 3), vomiting (grade 2)	Alive
6	2.5	SD	Negative	Fever (grade 2), headache (grade 3)	Alive
7	2.5	CR	Positive	Fever (grade 2), headache (grade 2)	Alive
8	2.5	SD	Negative	Fever (grade 2), headache (grade 2)	Alive
9	2.5	SD	Negative	Headache (grade 2)	Alive
10	2.5	SD	Negative	Fever (grade 2), headache (grade 3), vomiting (grade 1)	Alive
11	2.5	SD	Negative	No	Alive
12	2.5	PD	Negative	No	Dead

Notes: Before the course of heat shock protein 70, all patients had baseline MRI. Four weeks after the last intratumoral infusion of heat shock protein, the tumor was assessed on MRI and clinical responses were classified based on the criteria of Macdonald et al.[21] The follow-up period was 12 months.
Abbreviations: DTH, delayed-type hypersensitivity test; SD, stable disease; CR, complete response; PR, partial response; PD, progressive disease; MRI, magnetic resonance imaging.
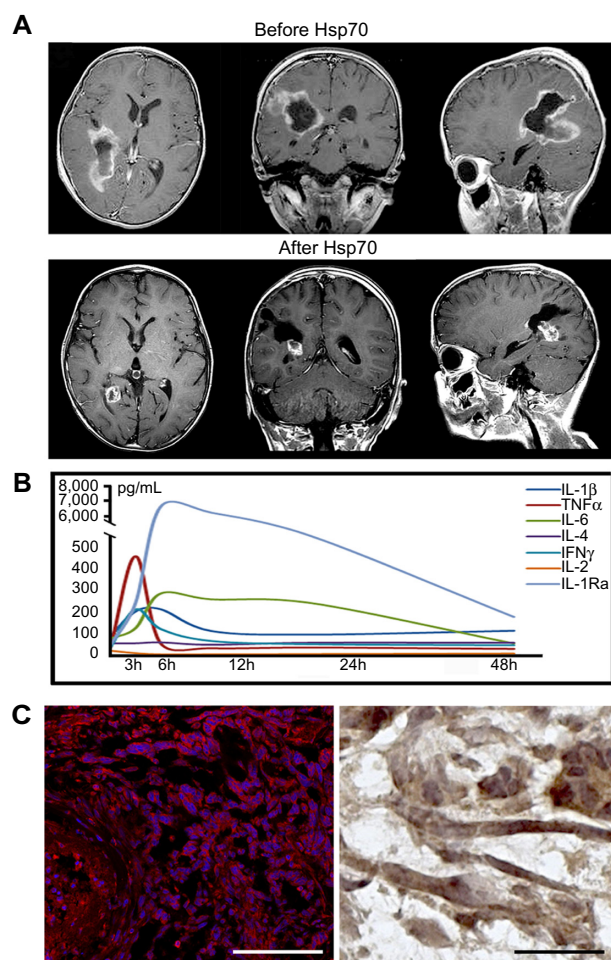
Figure 1 Case report of intratumoral Hsp70 therapy in patient 5.

Notes: (**A**) MRI brain images in a 4-year-old boy with a choroid plexus carcinoma before intratumoral injections of Hsp70 (upper panels) with MRI 4 weeks after the last intralesional infusion of Hsp70. (**B**) Analysis of cytokine (IL-1β, IL-1Ra, IL-2, IL-4, IL-6, interferon-gamma, tumor necrosis factor-alpha) levels in cerebrospinal fluid after a single Hsp70 infusion. Cerebrospinal fluid was collected 3, 6, 12, 24 and 48 hours after injection of chaperone and cytokines were detected by enzyme-linked immunosorbent assay. (**C**) Immunofluoresence image of choroid plexus carcinoma section, with Hsp70 detected by monoclonal antibodies BRM-22a (with Alexa568 conjugated secondary antibodies, red color). Nuclei were stained by DAPI (blue color). Scale bar, 75 μm. On the right is a magnified image of tumor cells on the immunohistochemistry assay with BRM-22a antibodies to Hsp70 revealed by standard biotin-peroxidase reaction (nuclei stained by hematoxylin). Scale bar, 10 μm.

Abbreviations: h, hours; Hsp70, heat shock protein 70; TNFα, tumor necrosis factor alpha; IL-1Ra, interleukin-1 receptor antagonist; IFNγ, interferon gamma; DAPI, 4',6-diamidino-2-phenylindole; IL, interleukin; MRI, magnetic resonance imaging.

were alive and the patient who progressed had died by the end of the follow-up period.

Local administration of Hsp70 leads to a T helper (Th)$_1$-polarizing immune response

It has been demonstrated previously that the peptide-independent chaperone Hsp70 has important cytokine activity, the main function of which could be summarized as polarizing the immune system toward a T-cell mediated

response.[10] To assess this hypothesis, the numbers of immune cells in peripheral blood were evaluated before and after administration of Hsp70. Using monoclonal antibodies, the numbers of distinct lymphocyte subpopulations in peripheral blood were analyzed on the first day after tumor resection and again 1 week after the last Hsp70 injection (Table 3). The relative level of each lymphocyte subpopulation was assessed in comparison with that of total lymphocytes. The levels of each individual cell type immediately after resection were used as controls, so that the immune condition before Hsp70 administration was a reference point for evaluation of immunologic changes during treatment. An increase in relative content of T-lymphocytes after Hsp70 infusion (including CD4+, CD8+) was observed in all 12 patients. Intriguingly, an increase in the relative proportion of the activated CD3+HLA DR+ cell population (from 2.3%±0.5% to 4.8%±0.6%, $P<0.001$) was observed. Parallel to the increase in T-cell level, there was a statistically significant decrease in the relative proportion of B-lymphocytes (CD19+) from 27.3%±2.3% to 16.8%±2.0% ($P<0.001$). The observed change in the relative proportions of T-cell and B-cell populations could be due to the influence of Hsp70. Cytokine (IL-4, IL-6, IFN-γ, TNF-α) levels were measured in order to evaluate the immune system function better. It is already well known that IL-4 and IL-6 are predominantly produced by Th$_2$ cells and B-cells and are responsible for humoral immunity, whereas IFN-γ and TNF-α are secreted by Th$_1$-helper cells and CD8+ T-cells and are responsible for cell-mediated immunity. These cytokines have opposing functions. The above-mentioned cytokine levels were measured in serum, along with their spontaneous and induced (by phytohemagglutinin) levels of production by peripheral blood lymphocytes in medium (Table 4). As expected, a dramatic decrease in IL-4 and IL-6 cytokines was observed in serum, whereas IFN-γ and TNF-α levels were elevated two-fold. These changes in serum cytokine levels were reflected in their spontaneous production in vitro. IL-4 and IL-6 levels in medium abated, while spontaneous production of IFN-γ and TNF-α was elevated. Analysis of induced cytokine production also revealed the same tendency, ie, decreases in IL-4 and IL-6 along with elevations in IFN-γ (from 344.2±111.9 pg/mL to 862.9±283.0 pg/mL) and TNF-α (from 66.4±17.5 pg/mL to 225.3±47.9 pg/mL). These data provide evidence that there was a shift toward T-cell-mediated responses maintained by Th$_1$-helper cells (production of IFN-γ and TNF-α) after administration of Hsp70.

An elevation in temperature was observed in six patients immediately after intratumoral injection of Hsp70 (1.5–2 hours). The peak of the temperature curve (up to 38.5°C) in all patients

Table 3 Main parameters in peripheral blood lymphocyte subpopulations

Parameter	Before			After		
	Mean ± SD	Minimum–maximum	Me (LQ; UQ)	Mean ± SD	Minimum–maximum	Me (LQ; UQ)
CD3*	63.3±2.1	53–79	62 (58; 67.5)	71.9±1.5	64–82	72 (67.5; 75)
CD4*	30.3±2.3	20–44	33.5 (22; 35.5)	36.8±1.8	27–46	36.5 (31; 42.5)
CD8*	24.8±2.5	19–50	23 (19.5; 23.5)	32.3±1.6	26–45	31 (28.5; 35.5)
CD3 HLA DR*	2.3±0.5	0.7–5.8	1.6 (1.1; 3.4)	4.8±0.6	1.2–9.7	4.6 (3.9; 5.6)
CD19*	27.3±2.3	13–35	30.5 (20; 33.5)	16.8±2.0	7–30	14.5 (13; 21.5)
Tregs*	8.1±0.6	4.8–10.9	8.0 (6.7; 10.1)	5.7±0.5	2.4–8.9	5.3 (4.9; 6.8)
NK cells	5.4±0.8	0–11	5 (4; 6)	8.2±1.0	3–16	7.5 (5.5; 9.5)
Cytotoxicity	35.2±2.0	24–45	35 (29; 40.5)	31.3±2.0	17–40	32.5 (27.5; 36.5)

Notes: Immunomonitoring was performed for all 12 patients. Blood samples were taken before the course of heat shock protein 70 and after the last heat shock protein 70 injection. The numbers represent the percentages of the gated lymphocyte populations. No statistical significance was observed for the change in natural killer cell populations and its cytotoxicity ($P>0.05$). For other lymphocyte subpopulations (marked with *) statistical significance was $P<0.001$.
Abbreviations: SD, standard deviation; Me, median; LQ, lower quartile; UQ, upper quartile; Tregs, T-regulatory cells; NK, natural killer; CD, cluster of differentiation; HLA, human leukocyte antigen; HLA, human leukocyte antigen.

was 2.5 hours after chaperone injection. At this time, other symptoms appeared (such as nausea, vomiting, and headache). Patients were treated with nonsteroidal anti-inflammatory drugs and all symptoms subsided after 5–6 hours. These symptoms could be due to stimulation of local cytokine production by Hsp70, so although not planned in the pilot study, cytokine levels in cerebrospinal fluid were evaluated 3, 6, 12, 24, and 48 hours after protein injection in one patient (case 5, Figure 1B). After injection of Hsp70, as expected, there was a transient increase in production of IL-1β, IL-1Ra, IL-6, TNF-α, and

IFN-γ (reaching a peak 3 hours after Hsp70 injection) with a subsequent decrease from 6 hours onwards. Cytokine levels subsided to control levels (before injection of chaperone) within 6 hours.

Administration of Hsp70 decreases immunosuppressive regulatory T-cells

The role of various immunosuppressive factors has been widely discussed in the literature. Previously, many studies have demonstrated the immunosuppressive role of

Table 4 Main parameters in cytokine levels in peripheral blood

Parameter	Before			After		
	Mean ± SD	Minimum–maximum	Me (LQ; UQ)	Mean ± SD	Minimum–maximum	Me (LQ; UQ)
IL-4						
Serum	20.5±7.6	6–103	13 (10; 18)	6.8±2.4	1–24	2.5 (2; 9.5)
Spontaneous	24.3±7.3	2–67	13 (6; 47)	9.25±3.0	1–34	4.5 (2; 13.5)
Induced	236.6±29.7	89–421	223.5 (160.5; 308.5)	110.9±17.6	23–210	99.5 (67.5; 159)
IL-6						
Serum	15.8±3.3	2–39	13 (8.5; 17)	8±2.7	2–27	2.5 (2; 10)
Spontaneous	62.2±18.8	2–248	47 (24.5; 81)	20.1±5.5	2–54	15.5 (2; 34)
Induced	1,362.8±273.8	378–3,257	1,226 (586.5; 2,011.5)	601.6±102.7	226–1,504	510.5 (353.5; 734.5)
IL-10						
Serum	24.3±3.7	8–46	21 (16; 33.5)	9.3±2.7	2–34	7 (2.5; 10)
Spontaneous	40.3±16.4	2–199	13 (8; 53.5)	5.6±1.7	2–23	3 (2; 7)
Induced	241.8±32.3	125–510	239 (135.5; 299.5)	110.9±22.9	29–290	89.5 (51.5; 146)
IFN-γ						
Serum	27.8±5.6	2–70	23 (17; 35)	63.1±13.7	20–181	46 (30.5; 76)
Spontaneous	35.8±10.1	3–133	27 (13; 47)	158.5±63.4	36–730	73 (54; 97.5)
Induced	344.2±111.9	64–1,367	183 (141; 347.5)	862.9±283.0	165–3,641	458.5 (227; 1,180)
TNF-α						
Serum	8.6±2.3	2–32	5.5 (4.5; 10)	23.4±6.1	6–75	13.5 (7.5; 33)
Spontaneous	8.0±2.2	2–27	4.5 (3; 11.5)	37.4±9.4	4–99	31.5 (9; 59.5)
Induced	66.4±17.5	2–189	38 (23; 114)	225.3±47.9	56–564	160.5 (77; 346.5)

Note: For all cytokines, the statistical significance of serum level (pg/mL) change, spontaneous and induced production (pg/mL) was $P<0.001$.
Abbreviations: IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; IL, interleukin; SD, standard deviation; Me, median; LQ, lower quartile; UQ, upper quartile.

regulatory T-cells on tumor-reactive effector T-lymphocytes. In all 12 patients in the present study, a decrease in the relative level of regulatory T-cells (CD4+CD25+Foxp3+) in peripheral blood was observed (from 8.1%±0.6% to 5.7%±0.5%, $P < 0.001$, Table 3). Further, we assessed for a possible correlation between the decrease in regulatory T-cells and levels of IL-10, and found that serum IL-10 levels abated from 24.3±3.7 pg/mL to 9.3±2.8 pg/mL. Spontaneous IL-10 production also decreased from 40.3±16.4 pg/mL to 5.6±1.7 pg/mL; the same phenomenon was observed for induced production by peripheral blood lymphocytes (from 241.8±32.3 pg/mL to 110.9±22.9 pg/mL). The decrease in regulatory T-cell content in blood after treatment with Hsp70 also corresponded to a decrease in IL-10 production (Table 4).

Hsp70 infusion does not alter the cytolytic activity of natural killer cells

Relative levels of natural killer cells (CD3–CD16+CD56+) in peripheral blood before and after chaperone administration were analyzed first (Table 3). A slight increase in natural killer cells from 5.4%±0.8% to 8.2%±1.0% was observed, although this was not statistically significant ($P > 0.05$). The cytolytic activity of natural killer cells was then assessed in a cytotoxicity assay (cytotoxic T-lymphocyte test) where effector cells were challenged with K562 cells; no statistically significant increase was found in the cytolytic activity of the natural killer cells (35.2%±2.0% before and 31.3%±2.0% after Hsp70 administration, $P > 0.05$).

Discussion

The molecular chaperone Hsp70 was recently shown to efficiently stimulate an innate and adaptive antitumor immune response.[3,5] Direct intratumoral delivery of Hsp70 has been investigated in in vivo experiments,[14,15] and it was found that the locally delivered chaperone essentially reduces tumor growth via activation of the immune response.[15] The basic mechanisms of the antitumor immunomodulatory activity of molecular chaperones have been well established since the original discovery of the enhancement of chaperone-mediated representation of tumor antigens and initiation of specific anticancer immunity, as well as the recruitment of Hsp70 to the surface of cancer cells, inducing a cytotoxic response by natural killer cells.[6,13] According to our research, both parts of the immune system are involved in the response induced by Hsp70.[15]

Local injections of Hsp70 into the resection cavity were well tolerated by patients, with an acceptable toxicity profile.

Previously it was shown that cytokine therapy might have very significant toxicity. High doses of recombinant IL-2 used in patients with renal cancer led to severe complications that significantly limited application of this treatment in the clinic.[23,24] In the case of local administration of Hsp70, no major side effects beyond grade 3 were observed and the safety profile was comparable with that of other cytokines such as IFN-α-2b or granulocyte-macrophage colony-stimulating factor, which are now used successfully in the treatment of patients with melanoma.[23–26]

Untreated patients with newly diagnosed tumors were enrolled in the present study. In the late stages of cancer progression, there is a certain rate of immunosuppression (via the immunosuppressive activity of tumor-associated macrophages and microglia, tumor-infiltrating lymphocytes, and regulatory T-cells) and exhaustion of tumor-specific CD8+ T-cells, causing immunotherapy to be ineffective.[27–31] Functional impairment of tumor-associated macrophages and microglia and exhaustion of T-lymphocytes occurs relatively late in the course of tumor growth, potentially providing a window of opportunity for therapeutic strategies directed toward preventing their functional impairment.[32] Thus, in a Phase III clinical trial of autologous tumor-derived heat shock Hsp gp96 peptide complexes for treatment of patients with stage IV melanoma, the survival rates in patients with the earlier M1a and M1b substages were longer than for patients with substage M1c.[8]

Based on the in vivo data thus far, it was decided to infuse Hsp70 locally in order to provide a high concentration of chaperone inside the resection tumor cavity. It was also proposed that Hsp70 therapy should be performed immediately after tumor debulking. As demonstrated previously, the extent of tumor burden frequently overcomes the magnitude of any immune response induced by vaccination.[33] In advanced disease, the tumor mass is a harsh environment for immune effectors to enter into and function effectively. It is well known that tumor cells secrete inhibitory cytokines, such as IL-10, transforming growth factor-beta-β, and prostaglandin E2, that suppress T-cells.[34,35] After tumor debulking, which may be regarded as a cytoreductive measure, the counterbalance of cytokines could be shifted toward an antitumor response by local application of Hsp70. It was also suggested Hsp70 immunotherapy be performed before conventional treatment, ie, radiotherapy or chemotherapy. Studies have shown that treatment with a tumor vaccine can increase the response to subsequent chemotherapy.[36–38] The enhanced clinical response to chemotherapy and radiotherapy seen in

vaccine responders may be due to triggering of pre-existing immunity via treatment-related apoptosis.[39–41]

To assess short-term changes in tumor growth and the response to Hsp70, postoperative MRI was performed within 24 hours of surgery and again 4 weeks after the final Hsp70 injection. Progression of tumor growth was not observed, except in case 12. Moreover, in one case each, a complete response (case 7) and a partial response (case 5) were observed (Figure 1). It is difficult to evaluate the therapeutic potency of Hsp70 given the heterogeneity of this group of patients, but conclusions can be drawn regarding its safety and absence of stimulation of tumor growth. Following their course of Hsp70, the patients received further conventional treatment.

Several explanations have been proposed for the possible anticancer effects of purified Hsp70, and recently, a further explanation for the role of Hsp70 has been suggested.[17] In previous in vitro experiments using different cell lines, it was shown that exogenous Hsp70 could penetrate cancer cells and induce translocation of intracellular Hsp70 to the cell surface.[17,42] In our study, elevated expression of membrane-bound Hsp70 increased the immunorecognition of cancer cells by cytolytic lymphocytes, as confirmed by the cyto-toxic T-lymphocyte test. In all 12 patients, elevated levels of Hsp70 were observed in tumor cells. Thus, exogenous Hsp70 introduced intratumorally could penetrate tumor cells and induce translocation of intracellular Hsp70 toward the plasma membrane, enhancing immunorecognition of the tumor cells. Uptake of Hsp70 by tumor cells has been reported previously in models of C6 glioblastoma and B16 melanoma.[14,15,43] The preferential accumulation of Hsp70 protein in C6 glioblas-toma cells could be explained by CD40 receptor-mediated uptake of chaperone.[44] However, the true importance of this mechanism in overall antitumor immunomodulation by Hsp70 in patients remains debatable.

In the present trial, a shift from cytokines provided by Th_2-helper cells (IL-4, IL-6) to cytokines of the Th_1-helper-mediated response (TNF-α, IFN-γ) was observed. These data corresponded to an increase in the overall T-cell subpopula-tion (including CD4+, CD8+, and activated CD3+HLA DR+) and a concomitant decrease in numbers of B-lymphocytes. Gene expression studies in multiple solid tumor types indicate that upregulation of IFN-γ may be associated with improved survival or prognosis.[45] Investigators have identified a cluster of genes (T-box transcription factor 21, IFN regulatory factor 1, IFN-γ, CD3, CD8, granulysin, and granzyme B) associated with Th_1 immunity and generation of CD8+ cytolytic T-cells. A tumor environment rich in type I cytokines (especially IFN-γ and TNF-α) would result in increased antigen presen-tation and generation of cytotoxic T-cells, and would favor tissue destruction.[39] Moreover, in a study by Donson et al, it was shown that upregulation of immune function-related genes in pediatric patients with ependymoma was associated with a good prognosis and a beneficial immune response.[46] The present study demonstrates a reversion of the immune system toward a Th_1-guided response due to treatment with Hsp70. This may have an impact on control of tumor growth, but further randomized studies are needed. This phenomenon of a Th_1-polarizing function of Hsp70 is also consistent with results demonstrated by other researchers using various cell lines.[9] Analysis of cytokine levels in cerebrospinal fluid after injection of Hsp70 showed short-term increases in IFN-γ and TNF-α for several hours. Long-term administration of Hsp70 may be needed to maintain elevated levels of these cytokines in the tumor microenvironment, as demonstrated in our recent in vivo study.[15]

Another objective of Hsp70 application is to activate the cytolytic and migratory activities of natural killer cells.[13] However, while evaluation of natural killer cell levels in peripheral blood showed a slight increase in their numbers, their cytotoxic activity was not altered. In part, this could be explained by the concomitant corticosteroid therapy that the patients received in the preoperative and postoperative periods. On the other hand, activated tumor-reactive natural killer cells could migrate to the tumor site from peripheral blood, and thus could not be detected by routine cytotoxic T-lymphocyte testing. Previously it was demonstrated that although Hsp-based vaccines might not induce generation of cytotoxic natural killer cells, activated natural killer cells are contributing to the antitumor response.[47–50] Thus, in the study by Zeng et al, it was shown that chaperone vaccination stimulated production of proinflammatory cytokines and chemokines by natural killer cells.[50]

Apart from stimulation of the cell-mediated immune response, a decrease in immunosuppressive regulatory T-cells was observed after administration of Hsp70. Regulatory T-cells function to maintain immune homeostasis and limit acute inflammation.[51] These cells interfere with T-cell priming and can affect the antitumor function of CD8+ cells via secretion of transforming growth factor-beta and IL-10.[52] The clinical importance of this phenomenon was demonstrated in the case of ovarian cancer, where it was shown that high levels of regulatory T-cells were associated with decreased survival.[53] Clinical trials of regulatory T-cell depletion along with antigen-specific vaccination suggest that increases in the induced tumor-specific immune response occurred with reductions in

the level of regulatory T-cells.[54–56] Whether this phenomenon of diminished regulatory T-cells as observed in our trial is due to the direct influence of Hsp70 on regulatory T-cells or via cooperation with other cells remains to be explored.

Previously it was reported that positive results on the delayed-type hypersensitivity test might correlate with T-cell antitumor responses.[57] However, this test was only positive in three patients, including the one patient with a complete response. For precise analysis of specific antitumor-generated responses, other immune assays (ie, ELISPOT) may be needed. Also, an insufficient specific antitumor immune response observed in patients could be an indication to escalate the dose of Hsp70.

Local application of purified recombinant human Hsp70 is feasible and safe, and has an acceptable toxicity profile. We assume that the observed changes in immunologic parameters may correspond to the influence of Hsp70 on the immune system, but might not be relevant to control of tumor growth or total eradication. The present study aimed to determine the feasibility and safety of administration of purified recombinant Hsp70 into the resection cavity in pediatric patients with tumors of the central nervous system. Further randomized clinical trials will determine further the antitumor effect of Hsp70.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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