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Hsp70 confines tumor progression of rat histiocytoma and impedes the cytotoxicity induced by natural killer cells and peritoneal macrophages

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ABSTRACT

Objective: To study the role of inducible form of heat shock protein 70 (Hsp70) in the host tumor regression of rat tumor model. **Methods:** We examined the role of Hsp70 in host tumorigenicity and *in vitro* cellular cytotoxicity using a rat histocytoma. The differential tumor growth and regression kinetics were studied and correlated with the expression of Hsp70, activation of macrophages and natural killer (NK) cells, and circulating or tumor infiltrating immune molecules in the host system. **Results:** The sub cuteaneous (s.c.) tumor regression was correlated with increased serum cytokines such as IL-12, TNF α , IFN γ and Hsp70. Despite of similar increase of Hsp70 in intraperitoneal (i.p.) tumor implanted animals, animals succumb to tumor growth, further, evidently, no immune molecule activation was observed. The viral promoter driven Hsp70 over expression in these tumor cells restrained solid tumor growth, however, failed to inhibit ascites growth. The NK cells from s.c. immunized animals induces cytotoxicity in the presence of anti-tumor antibody, which necessitated CD40-L expression, conversely, NK cells from i.p. immunized animals failed to induce cytotoxicity. The NK cells from s.c. or i.p. implanted animals with Hsp70 positive tumor cells failed to induce such cytotoxicity. The peritoneal macrophages isolated from s.c. tumor implanted animals when co-cultured with parental BC-8 cells lyses tumor cells, nevertheless entail macrophage specific TNF α expression. On the contrary, Hsp70 expressing BC-8 tumor cells were resistant to peritoneal macrophage induced cytolysis. **Conclusions:** This study brings out that Hsp70 possibly involved in regulating the host tumor response and cellular cytotoxicity.

1. Introduction

Tumors employ multiple mechanisms to escape from immune-mediated rejection[1,2]. Rejection of host tumor needs appropriate functioning and sufficient activation of the immune system. The multi-faceted molecular mechanisms acquired by tumor cells help in immune invasion, however, majority of spontaneous or chemically induced tumors fails to elicit such strong immune response[3,4]. There is a correlation between tumor cell immunogenicity and the expression of some heat shock proteins (Hsps) in both animal models and human cancers[5-7]. And Hsps are found to play important roles in eliciting potent anti-cancer immune responses mediated by T-cells, antigen presenting cells (APCs), and natural killer (NK) cells. It is proposed that Hsps mimic classical T-cell epitopes, therefore serve as carriers for immunogenic peptides that present antigenic peptides to the MHC-I molecules (Major Histocompatibility Complex-I) by APCs. In addition to the adaptive immunity, Hsps participate in the innate immune system acting as natural adjuvants in activating cells of the immune system for cytokine release, up-regulation of MHC-II complexes in antigen presenting cells, and in the increased dendritic cell maturation[8-10].

Although Hsp72 is involved in evasion of cell death pathways in tumor cells[11], a membrane selective expression of Hsp70 sensitize tumor cells to NK cell mediated immune lysis, and this is attributed because the membrane-bound Hsp70 is not associated with immunogenic peptides[12]. Therefore Hsp70 proteins if not involved in the cargo of antigenic peptides efficiently stimulates the immune response through its direct interaction with NK cells. On the other hand, the intraperitoneal macrophages, which serve as first line of defense during host response appears to play important role in the immunological surveillance, especially in the tumor immunity[13].

AK-5 is a spontaneous tumor developed in the laboratory that induces solid tumor with s.c. implantation which spontaneously regresses through the effector NK cells. However, these cells upon peritoneum injections develop i.p. tumor and animals succumb to the tumor growth. Since AK-5 is a heterogeneous sub-population of tumor cells, a single clone of AK-5, named BC-8 was used in the present study. The parental BC-8 and Hsp70 over expressing

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BC-8 tumor cells were tested for the host tumorigenic potential and *in vitro* cytotoxicity. We present differential sensitivity and tumorigenic potential of parental BC-8 and Hsp70 over expressing BC-8 tumor cells in the host. We also demonstrate differential *in vitro* cytotoxic effects of tumor implanted, host activated NK cells and peritoneal macrophages.

2. Materials and methods

2.1. Animal handling

All animal maintenance and handling was accomplished as per the institutional ethical committee approval at Centre for Cellular and Molecular Biology, Hyderabad, India.

2.2. Cell culture and injections

AK-5 tumor cell line is established from i.p. injections of cell-free ascites fluid of a chemically induced and established rat liver tumor, Zajdela ascetic hepatoma (ZAH). These cells possess typical characteristics of macrophages. To avoid ambiguity with the heterogenic population of *in vivo* tumors, single clone of AK-5 tumor, called BC-8, was adapted to grow in culture for several generations in Dulbecco's modified Eagle's medium (DMEM) with 10% heat inactivated fetal calf serum (FCS) in the presence of penicillin (100 U/mL) and streptomycin (50 μg/mL) is used in the present study.

2.3. Construction of hsp70-pEGFP vector and transfection

The full length coding sequence of the hsp70 gene was PCR amplified (forward primer, 5′-ATT CGA ATT CCA GAA GCA GA-3′ and reverse primer, 5′-AGC CTC TGC TGG ATC CTA T-3′ from plasmid containing the full-length cDNA (a kind gift from Prof. Lakhotia, Banaras Hindu University), the PCR conditions as follows:, denaturation -94 ℃ for 30 sec, annealing -55 ℃ for 30 sec, and extension -72 ℃ for 1 min for 30 cycles and the PCR reaction was performed in a Perkin Elmer Thermo Cycler. The PCR amplified cDNA was cloned in pEGFP vector (Clontech) with a CMV promoter and a neomycin selection marker. BC-8 cells then transfected (electroporation, BTX electroporator) with the recombinant plasmid construct and stable clones were selected by G-418 antibiotic selection. Individual clones were expanded, tested for hsp70 expression and used.

2.4. Inoculation of s.c. and i.p. tumors and measurement

BC-8 cells (8×10^6 cells) were used for injection either for s.c. or i.p. of six-week-old naïve male Wistar rats and tumor growth was monitored. The s.c. tumor size was measured using Vernier calipers, taking two diameters of the tumor at right angles to each other and calculating the volume of the tumor according to the formula, tumor size =4/3 д a^2b, where 'a', shorter diameter and 'b', longer diameter. The i.p. tumor development approximated by the mean total cell mass calculated from the percentage of packed cells and the total ascites weight. The former is determined by centrifugation, the latter by weighing the animal before and after careful peritoneal drainage. Experiments represented are from four individual sets, having minimum five animals in each set.

2.5. Collection of serum and immunoblot analysis

Blood from control and tumor implanted animals was collected at different intervals (between 2 to 4 day intervals unless otherwise indicated) by puncturing the ear lobe or tail vein and after removing the major fraction of albumin, it was used to study the expression of Hsp70 by anti Hsp70 immunoblot assay. The autoradiograms were scanned and the arbitrary densitometric values are plotted. Anti BC-8 serum was obtained from immunized animals, and the immunized represents those animals that had rejected s.c. tumor. Anti Hsp70 and actin antibodies were purchased from Stressgen (USA).

2.6. Isolation of AK-5 antibody

The anti AK-5 antiserum was collected from rats that had rejected the subcutaneous tumor after re-challenging with 5×10^6 AK-5 cells intraperitoneally. The sera were tested for the presence of anti AK-5 antibody by their ability to lyse AK-5 cells through complement fixation, and the positive samples were pooled, precipitated first with 18% sodium sulphate and re-precipitated with 12% sodium sulphate. The antibody dialyzed against phosphate-buffered saline (PBS), tested for activity and stored frozen with 0.1% NaN_3.

2.7. Induction of apoptosis by serum factor

AK-5 antiserum from s.c. immunized animals was collected, decomplemented by heat inactivation at 56 ℃ for 30 min. The serum fractions were stored in 100 μL aliquots at -70 ℃ refrigerator for further use. Since the serum factor from decomplemented antisera induces caspase-dependent apoptosis in BC-8 tumor cells, apoptosis activation was monitored in BC-8 tumor cells. For 2×10^5 cells/mL, twenty micro liters (200 μg/mL) of decomplemented serum was used.

2.8. DNA fragmentation and FACS analysis of tumor cells

Parental and hsp70-pEGFP transfected BC-8 cells (2×10^6) were incubated with serum factor for 8 h at 37 ℃ and the cells were fixed in 70% methanol, stained with propidium iodide reagent (50 μg in 0.1% sodium citrate containing 0.1% Triton X-100) are either analyzed by flow cytometry (FACS Calibur, USA) or subjected to DNA fragmentation assays.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Circulating serum cytokines were estimated by ELISA. Briefly, 50 μL of serum (diluted in 1:10 ratio with PBS) was taken in a 96-well ELISA plates (NUNC) and incubated at 4 ℃ for 4 h. Then samples were incubated with 2% bovine serum albumin (BSA) to block non-specific binding. The cytokine antibodies for IL-12, TNF-α and IFN-γ were incubated with the serum for 1 h at 37 ℃, the wells were washed with PBST (PBS with 0.1% Tween-20) and further incubated with HRPO-conjugated sheep anti rat IgG (1:5000 dilution) for 30 min at 37 ℃. After subsequent PBST washes, wells treated with OPD and H_2O_2 to develop the color; absorbance of color was measured at 490 nm using ELISA reader.

2.10. Isolation of NK cells

Spleen lymphocytes from normal and tumor-rejecting animals were isolated by Ficoll-Hypaue centrifugation. Splenocytes were subjected to two-step (50% and 60%)

discontinuous Percoll density gradient centrifugation, and the cells in the interphase were used as enriched NK cells. Contamination free sorting of NK cell population was attained by staining cells with anti rat NKR-P1 mAb 3.2.3 (Endogen Inc., Boston, MA) and anti mouse immunoglobulin fluorescence isothiocyante, FITC on FACS (Becton Dickinson, San Jose, CA). Sorted NK cells were used for subsequent experiments.

2.11. Isolation of peritoneal macrophages

Control, s.c. and i.p. bearing rats were sacrificed by CO_2 inhalation by the animals. The rats were then placed on the dissection board with its ventral side disinfected with alcohol, and the outer layer of the skin was peeled off to expose the inner skin layer of the abdominal cavity. Phosphate buffered saline (PBS, 20 mL, chilled), pH 7.4, containing 10 U/mL heparin was injected into the peritoneal cavity using sterile syringe, the abdominal area was patted to move the liquid within the abdominal cavity, and then the lavage was aspirated out and placed in a Falcon tube on ice. Peritoneal lavages were centrifuged at 800×g for 10 min in a Sorval 5B centrifuge. Pellet obtained was resuspended in Iscove's modified Dulbecco's medium (IMDM) containing 20% FCS. The cells were placed on 6-well plate (NUNC) in an incubator (5% CO_2; 37 ℃) for 2 h to adhere. The non-adherent cells were washed off with warm PBS (37 ℃) and the attached cells were characterized as macrophages. The obtained macrophages are 95% positive for macrophage specific characters.

2.12. Co-culture of NK cells with BC-8 tumor cells

NK cells from spleen lymphocytes of normal, i.p. and s.c. tumor bearing/rejecting animals were plated in 96/24-well ELISA (NUNC) plates, and co-cultured with BC-8 and Hsp70 positive BC-8 cells in the presence or absence of anti AK-5 antibody at an effector: target ratio of 10:1 for 4 h and the percent cytotoxicity was calculated.

2.13. Co-culture of peritoneal macrophages with BC-8 tumor cells

Peritoneal macrophages isolated from normal, s.c, and i.p. tumor bearing/rejecting animals isolated were plated in 96-well ELISA (NUNC) plates, overlaid with BC-8 and Hsp70 positive BC-8 at 1:5 ratio of macrophage:tumor cell. The co-culture was incubated for 8 h and the cytotoxicity was estimated.

2.14. Cytotoxicity assay

Macrophages or NK cells from normal, s.c, and i.p. tumor transplanted animals were incubated with 51Cr-labelled AK-5 cells in the presence of anti AK-5 antibody at an effector:target (E:T) ratio of 100:1 for 4 h. Chromium released in the supernatant was counted in a packerd gamma counter, and the percent cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100$$

2.15. RT-PCR (Reverse transcription-polymerase chain reaction) analysis

Total cellular RNA was isolated using Trizol, reverse transcribed using random hexamer and MMLV reverse transcriptase enzyme (Promega). The cDNA obtained was PCR amplified with primers, (1) β-actin 5′ primer: 5′-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′, and 3′ primer: 5′-CGT CAT CCT GCT TGC TGA TCG ACA TCT GC-3′; (2) TNF α 5′ primer: 5′-TTC TGT CTA CTG AAC TTC GGG GTG ATG GGT CC-3′, and 3′ primer: 5′-GTA TGA GAT AGC AAA TCG GCT GAC GGT GTG GG-3′; (3) CD40-L 5′ primer: 5′-AAG CCT TCA CGA GGA TTT TG-3′: 3′ primer: 5′-TCC CAT TTT CCA GCA CTA CC-3′, (4) Fas 5′ primer: 5′-CTG CAG ATA TGC TGT GGA TCA-3′; 3′ primer: 5′ TTT GGT GTT GCT GGT TGG T-3′: (5) Fas-L 5′ primer: 5′-AAA GAC CAC AAG GTC CAA CA-3′, 3′ primer: 5′-AGT CTC TAG CTT ATC CAT GA-3′. The PCR conditions were set as follows, hot start -94 ℃ for 1 min, for 30 cycles each cycle comprises of denaturation at 94℃- 30 sec, annealing at 55 ℃- 30 sec, and extension at 72 ℃ - 30 sec in a Perkin- Elmer Thermo Cycler.

2.16. Statistical analysis

Twenty animals were used for testing tumorigenicity experiments in each set and out of which 70% animals were tumor positive. The *in vitro* statistical data represented are from minimum of four individual experiments unless and otherwise indicated. Experimental data was analyzed by student *t*-test and error bars represent standard error, on average value. The significance (*P*) values are calculated using paired student's *t*-test. A P value< 0.05 is considered significant.

3. Results

3.1. Evaluation of tumorigenic potential of BC-8 tumor cells in the host

Considering the ambiguity in AK-5 tumor cells due to its tumor heterogeneity, in the present study, a single clone of AK-5, the BC-8 is examined for its tumorigenic potential. The BC-8 cells were able to induce s.c. and i.p. tumors when implanted subcutaneously and intraperitoneally respectively. However, a high density of tumor cells, 6×10^6 cells for s.c. tumor development, and 4×10^6 cells for the i.p. tumor development is required. The s.c. tumor regressed after 18 days whereas the i.p. tumor bearing animals succumb to tumor growth (Figure 1A and 1B).

3.2. Analysis of circulating immune molecules and Hsp70

The Hsp70 bound antigen complexes deliver antigens to MHC-I and MHC-II molecules on the APC cell surface and lead to the presentation of tumor antigens to T-lymphocytes [14,15]. Having observed tumor regression 18 days after tumor growth, we assumed that the regression may possibly correlate with increased circulatory Hsp70 levels. Therefore we estimated Hsp70 levels in the serum of tumor bearing animals. There was an increase in circulatory Hsp70 levels in s.c. tumor bearing animals, and even after tumor regression the levels are maintained. Though no tumor regression is observed in i.p. tumor bearing animals, the Hsp70 levels found increased with increase in tumor growth

(Figure 1A and 1B).

3.3. Over expression of Hsp70 in BC-8 tumor cells limits s.c. tumor growth

Abundance of circulatory Hsp70 in immunized animals imply a role for Hsp70 protein in the host immunity[16,17]. The extracellularly expressed Hsps have been shown to elicit potent anticancer effects through innate as well as adaptive immunity[5,10]. To elucidate Hsp70 role in the immune process, BC-8 tumor cells were tested for their tumor forming ability. No s.c. tumor development was observed with Hsp70 positive BC-8 cells, similarly there was no significant change in the circulating Hsp70 levels (Figure 1C). The s.c. implantation with increasing number of BC-8 tumor cells (12×10^6) also failed to induce s.c. tumor growth. Contrary to s.c. tumor development, i.p. implantation of Hsp70 positive BC-8 developed an i.p. tumor which was found to be similar to parental BC-8. Interestingly, the i.p. tumor growth with Hsp70 positive BC-8 cells showed an advancement of tumor growth where animals succumb to tumor growth, by 6 days after implantation. The i.p. tumors

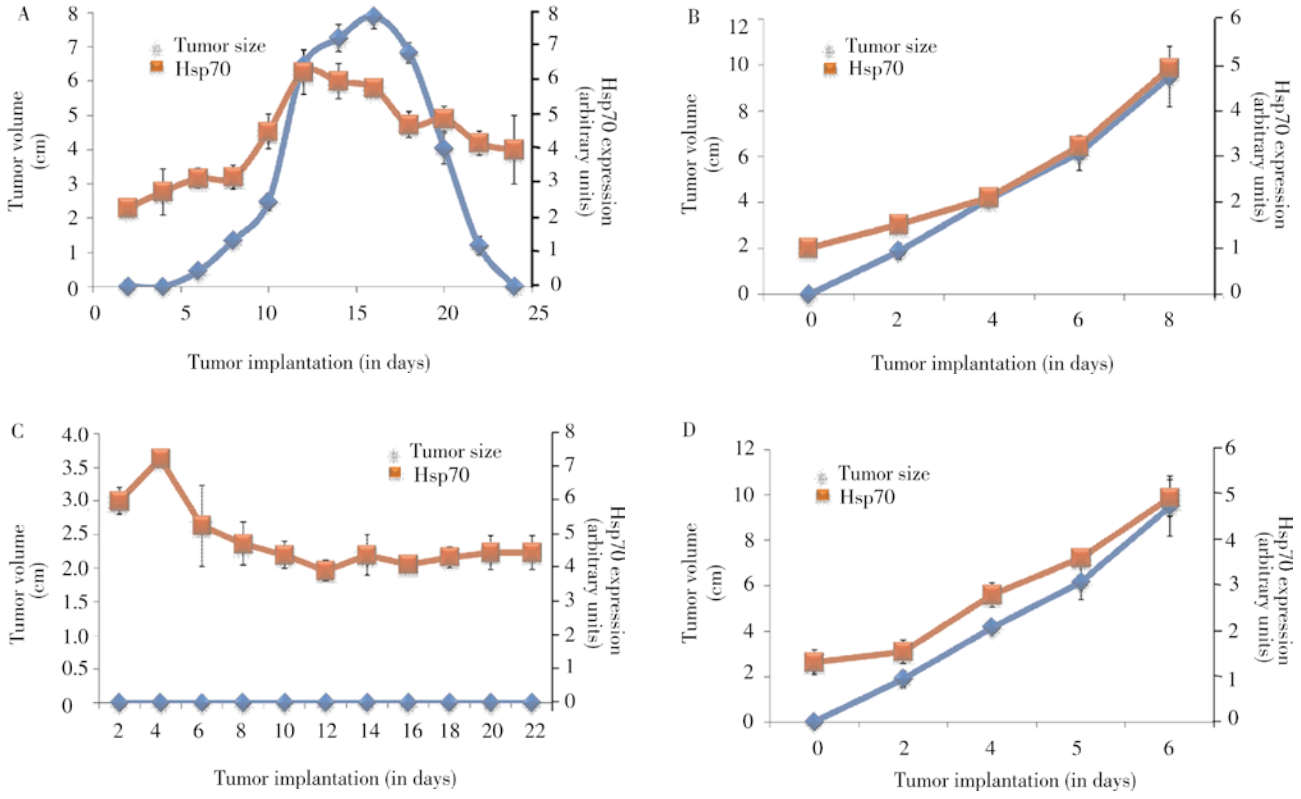

Figure 1. Tumor development in the host animal by BC-8 tumor cells implanted both by s.c. and i.p. and a correlation of tumor regression with circulating Hsp70.

also show a concomitant increase (a two-fold) of circulating Hsp70 compared to parental BC-8 induced i.p. tumors (Figure 1D).

3.4. Correlation of circulating immune molecules with Hsp70

After respective tumor implantations, circulating immune molecules was estimated from tumor bearing animals at respective time intervals. We observed a correlation between increased immune molecules with serum Hsp70 levels and enhancement of circulating immune molecules in tumor regressing animals (Figure 2A). Contrast to parental BC-8, No significant change in the circulating immune molecules was observed in Hsp70 over expressing BC-8 s.c. tumor implanted animals (Figure 2B). Though there is an initial increase in cytokine levels, a subsequent decrease is observed in i.p. tumor implanted animals (Figure 2C). And no significant change in cytokine profile was observed with Hsp70 over expressing BC-8 i.p. implanted animals (Figure 2D).

3.5. Hsp70 expression in tumor cells develops resistance to serum factor induced apoptosis

The spontaneous regression of AK-5 tumor is mediated by NK cells through antibody-dependent cellular cytotoxicity (ADCC) and the target cell death involves necrosis (perforin-mediated) and apoptosis. In the present study, the serum factor induced cell apoptosis was examined in BC-8 tumor cells. Challenging BC-8 cells with serum factor induced a significant apoptosis in the target cell (Figure 3A), however a significant decrease (seven fold, $P<0.001$) in Hsp70 positive BC-8 cells was observed compared to the parental phenotype. The DNA fragmentation assay further confirmed apoptosis resistance of these tumor cells (Figure 3B).

3.6. NK cells from immunized animals show cytotoxicity in the target cell

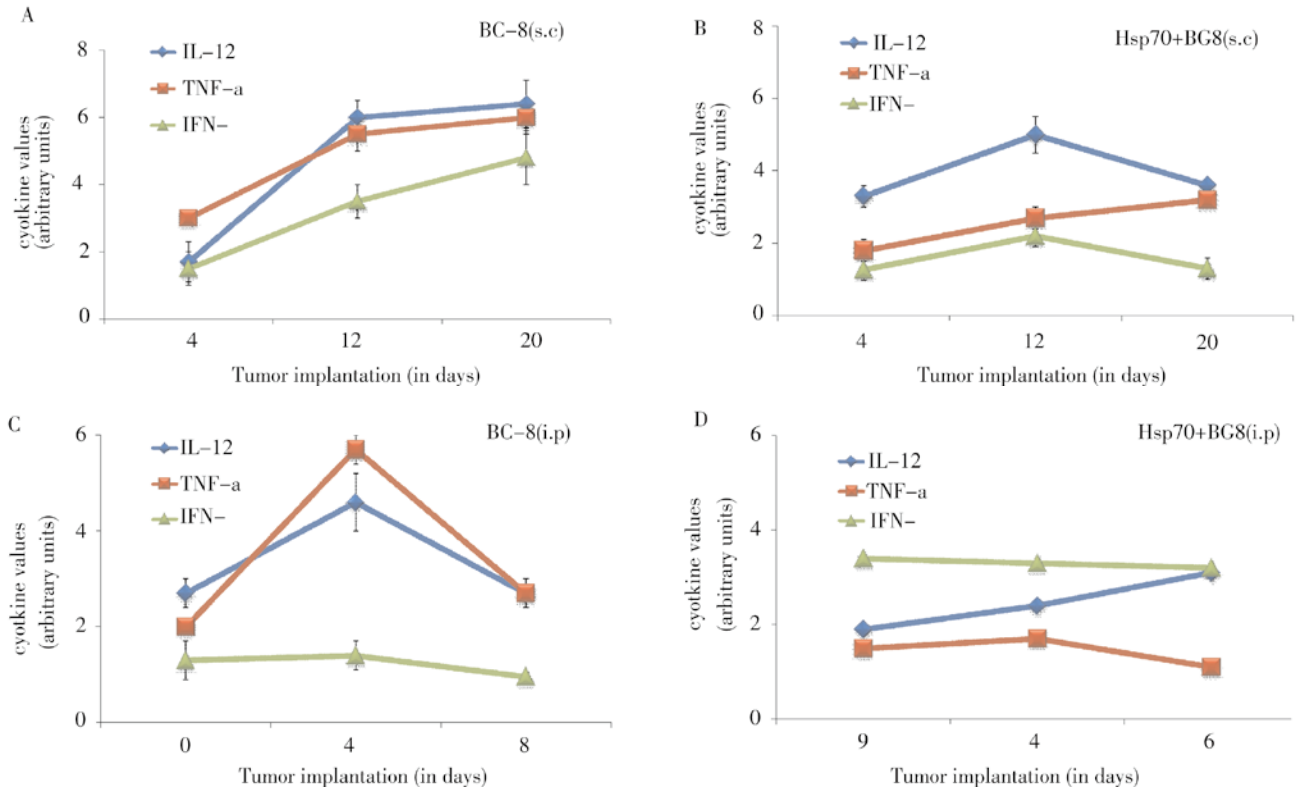

Figure 2. Comparative analysis of cytokine expression for cytokines IL-12, TNF- α and IFN- γ from the tumor implanted host animal.

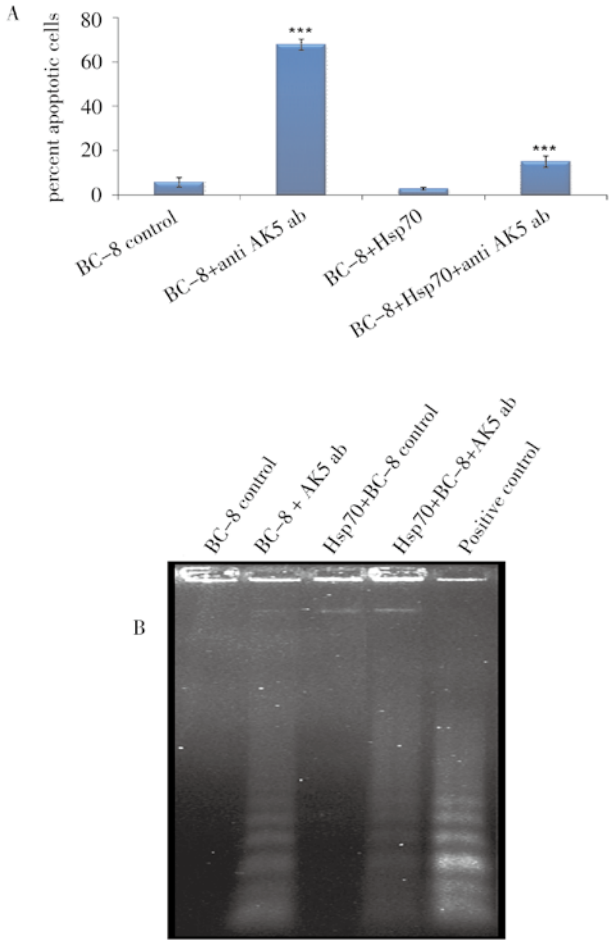

Figure 3. The antibody induced cytotoxicity of BC-8 and Hsp70 expressing BC-8 tumor cells in presence of AK-5 antibody was measured by flow cytometric analysis.

To evaluate specificity of NK cell involvement in tumor killing *in vitro*, NK cells were isolated from s.c. and i.p. implanted animals. NK cells from s.c. (parental BC-8 tumor positive) animal showed 70% cytotoxicity, whereas from Hsp70 positive BC-8 implanted animals showed negligible cytotoxicity. Interestingly NK cells from i.p. implanted animals (both parental BC-8 and Hsp70 expressing BC-8) showed only 20% of cytotoxicity (Figure 4A).

3.7. The cytotoxic ability of NK cells constrained to CD40-L expression

NK cell recognition is regulated by a delicate balance between positive signals initiating their effector functions, and inhibitory signals preventing them from proceeding to cytolysis. NK cells share several common features with T-cells, therefore express components such as CD3 and CD40-L[18]. Hence we studied the CD40-L expression and its influence on cytotoxicity. NK cells from s.c. immunized animals showed a significant two-fold increase of CD40-L over i.p. (Figure 4B). And when these cells were co-cultured with BC-8 and Hsp70 positive BC-8 cells, only parental BC-8 cells showed a two-fold increase in cytotoxicity (Figure 4C). In addition to CD40 system, an alternative mode of NK cell mediated target cell lysis suggested was through Fas and Fas-L interaction. We did not observe a significant change in the Fas expression in these NK cells, but BC-8 tumor cells are positive for Fas-L expression. Therefore we induced Fas expression in naïve NK cells with IL-2 (10 μg/mL) for 2 h followed by CD16 mAb (10 μg/mL) for 6 h and examined for tumor cell killing. The Fas expression (Figure 4D), however was found to be ineffective inducing the cytotoxicity neither of cell types, BC-8 or BC-8 cells expressing Hsp70 (Figure 4E).

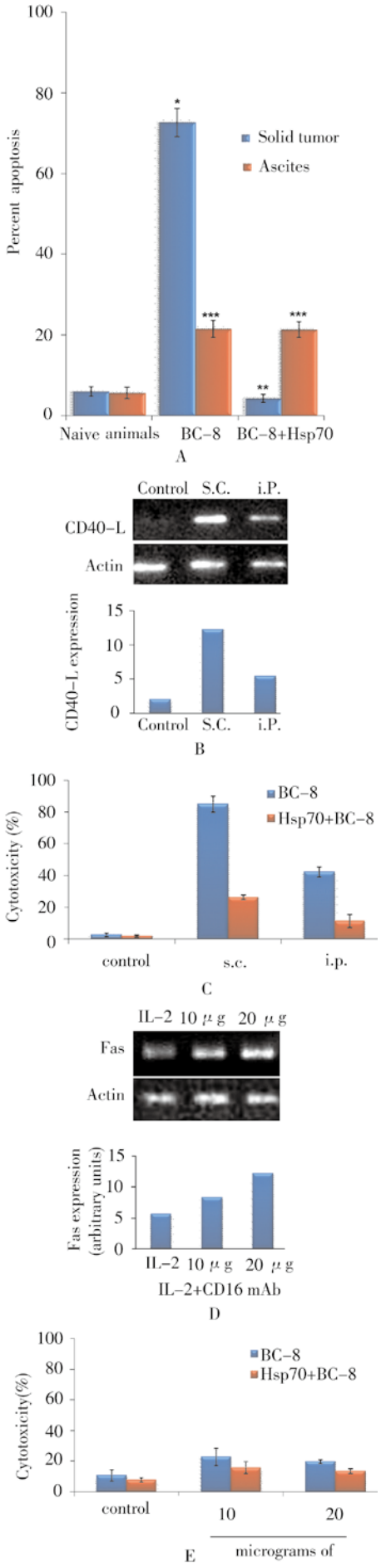

Figure 4. Natural killer cells induced cytotoxicity.

3.8. Analysis of peritoneal macrophages induced cytolysis

Macrophages can induce cytotoxicity in tumor cells, however involves multiple molecules, therefore thought to be multi-factorial. Usually macrophages migrate to the tumor site and induce regression of the tumor. Considering the importance of these peritoneal macrophages, we studied their role in inducing cytotoxicity in target cells.

First we have tested the cytolytic ability of peritoneal macrophages (AK-5 immunized s.c. tumors) with cultured BC-8 cells and found that peritoneal macrophages were effective in lysing the target cell. Macrophages from the peritoneum of parental tumor bearing animals showed differential lytic sensitivity *in vitro*. The peritoneal macrophages were collected at different time intervals, 0, 2, 4, 6 and 8 days for i.p. tumors, and 0, 6, 12, 18, 24, 30 days for s.c. tumors (in case of tumors induced by both BC-8 and Hsp70 positive BC-8 tumor cells) and tested against parental BC-8 cell lysis. Compared to macrophages isolated from s.c. tumors implanted by Hsp70 positive BC-8, macrophages from parental BC-8 implanted animals showed a significant increase in the *in vitro* cytotoxicity (Figure 5A). However, with i.p. tumors implanted by parental BC-8, there was an abrupt increase in cytotoxicity which continued till the fourth day of tumor implantation and sharply declined thereafter. However, in case of Hsp70 positive BC-8 tumor implantation, there was a negligible cytotoxicity observed (Figure 5B).

3.9. The cytolysing ability of peritoneal macrophages constrained to TNF α expression

The Macrophage induced cytolysis of tumor cells involves TNF α [19], therefore, we examined for TNF α expression in the peritoneal macrophages isolated from tumor bearing animals. Between the s.c. and i.p. of parental BC-8 tumors, peritoneal macrophages from s.c. tumors showed a significant enhancement of TNF α mRNA expression; on the other hand macrophages from i.p. tumors showed low level TNF α expression (Figure 5C). Contrary to these findings, peritoneal macrophages isolated from Hsp70 positive BC-8 tumors (both s.c. and i.p. tumors) showed a negligible expression of TNF α (data not presented). Thus interference of Hsp70 expression with the cytotoxic property of macrophages was found to be evident from these experiments (Figure 5D). To understand the significance of non-tumor TNF α mediated macrophage activation in target cell lysis, peritoneal macrophages isolated from naïve animal (Wistar rat; male) stimulated with 100 ng/mL of lipopolysaccharide (LPS from *E. coli*) for 2 h and 4 h and co-cultured with both Hsp70 negative and positive BC-8 cells for 12 h. The LPS treatment primarily induced a time dependent expression of TNF α mRNA (Figure 5E), and when these stimulated macrophages was tested for their cytotoxic property, the cytotoxicity was observed only in parental BC-8 tumor cells. The Hsp70 expression appeared to be limiting the cytotoxic ability of these activated macrophages (Figure 5F).

4. Discussion

Spontaneous regression of tumors is a rare phenomenon,

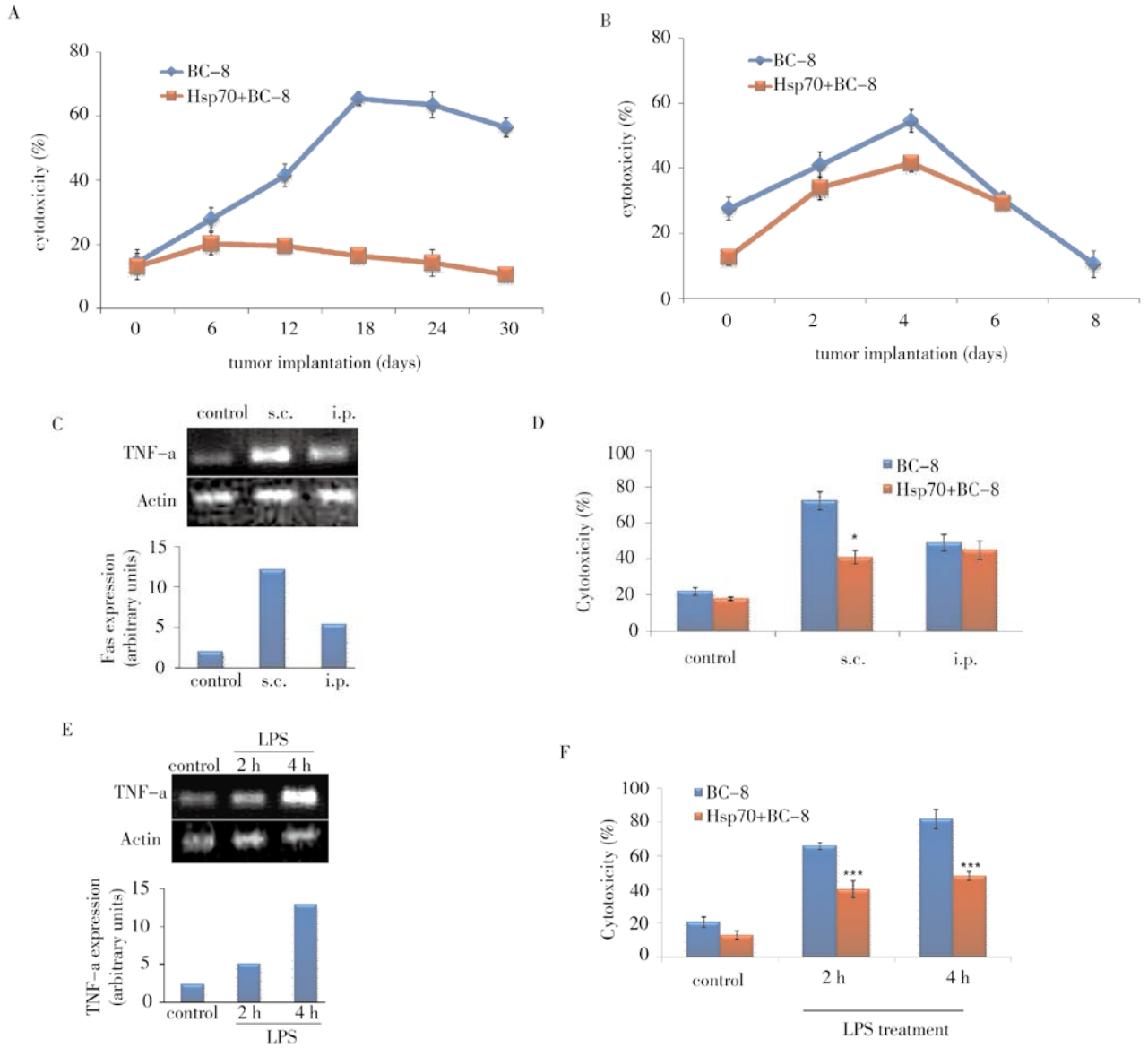

Figure 5. Effect of peritoneal macrophages isolated from immunized animals on cytotoxicity

restricted to tumors which are highly immunogenic[20], suggesting the participation of tumor-rejection antigens in stimulating the host immune system. Several studies have identified a correlation between Hsps and tumor cell immunogenicity in both animal models and human cancers. In accordance with this we also observed a gradual increase in circulating immune molecules and Hsp70 in our tumor bearing animals. In the present study, similar to s.c. tumors, i.p. tumor bearing animals also showed a gradual increase in serum Hsp70 levels, however, in the latter, absence of circulating immune molecules correlated with tumor progression.

Generally Hsp70 chaperones deliver antigens to MHC-I and MHC-II molecules on the APC cell surface thus involved in the presentation of tumor antigens to T-lymphocytes. The antigen unbound Hsps are assumed to be non-immunogenic for T-cells, however, binding of peptide-free Hsp70 to TLR2/TLR4 on APC has been found to induce the secretion of immunomodulatory cytokines[21]. Therefore un-bound Hsp70 and peptide - bound Hsp70 exhibit diverse immune-stimulatory capacities. Since BC-8 tumors showed enhanced circulatory cytokines as well as increased Hsp70 levels, we purified Hsp70-bound fractions from s.c. tumor bearing animals by ADP-sepharose column chromatography and the Hsp70 bound complexes were analyzed by 2D-gel electrophoresis and MALDI-TOF. The proteomic study showed tumor antigens such as stathmain, oncoprotein-18 etc., however, the AK-5 tumor antigen itself was not pulled down with Hsp70 (data not shown).

Another interesting finding from our study was Hsp70 positive BC-8 cells failed to develop s.c. tumor, however, they were able to induce i.p. tumor, which was similar to parental cells. Since involvement of T-lymphocyte mediated immunization is ruled out with our earlier observations, we assumed that there could be a correlation between Hsp70 expression and NK cells interaction. The Hsp70 has been shown to involved in the NK cell mediated cell lysis of tumor cells. In addition to this, Elsner *et al*[12] have shown in mouse models that Hsp70 promotes NK cell activity therefore works against tumors. Hence the cytotoxic effector mechanisms of the cellular immune system may dominate over the protective stress response. Nevertheless when NK cells from immunized animals were tested against Hsp70 positive tumor cells, the Hsp90 positive cells

showed a significant decrease in tumor cell lysis. Although our findings contradict with earlier studies that Hsp70 expression limits NK cell mediated cell lysis, these studies were done with only membrane-bound Hsp70. In our study, Hsp70 in BC-8 cells was majorly cytosolic, therefore, a decrease in NK cell mediated cytotoxicity in Hsp70 positive BC-8 may be relate to lack of NK cell activation.

Macrophages, being first line of defense, play an important role in the immunological surveillance. In BC-8 tumors, the differential expression of TNF α correlated with differential sensitivity in inducing cytolysis of tumor cells. Further, *in vitro* stimulation of naïve macrophages with bacterial lipopolysaccharide also provided information on functional significance of TNF α expression and its role in cytotoxicity. Lack of cytotoxic ability of NK cells from animals with Hsp70-positive BC-8 implantation suggested an inhibitory role of Hsp70 in the cytotoxic process. In agreement with this LPS stimulated NK cells, despite of TNF α expression failed to induce cytotoxicity.

It has been shown that extracellular expression of Hsp70 activates complement lysis, however, in the present study, the serum factor induced, caspase-dependent apoptosis appears to be independent of antibody-mediated NK cell killing. Since the upstream mechanism of serum factor induced apoptosis is not known, we could not demonstrate how Hsp70 is inhibiting antibody induced apoptosis in BC-8 cells. However, in our previous study, we demonstrated that Hsp70 by inhibiting death receptor activation (Fas or CD95) inhibits heat induced tumor cell death. The lytic pathway attributed to NK cells is through granule-mediate killing *via* perforin and an alternative pathway is through Fas and Fas-L mediated induction of cell death. Although Fas mediated cytotoxicity of tumor cells by NK cells needs expression of Fas-L on the surface, no Fas receptor expression was reported in BC-8 cells except its induction after heat stress. This could be the reason the Fas induced BC-8 tumor cells showed a significant apoptosis by Fas-L positive NK cells.

In conclusion, the cytotoxicity of BC-8 tumor cells is examined by (1) TNF α mediated peritoneal macrophages, (2) activated NK cells, and (3) serum factor specific to AK-5. We presented a differential cell-mediated humoral response from s.c. and i.p. tumors induced by these tumor cells. We also investigated Hsp70 involvement in the host immunity as well as in the cytotoxic assays. The Hsp70 expression in tumor cells not only limited the s.c. tumor growth but inhibited the activation of host peritoneal macrophages and NK cells. Our study gains importance in the perspective of Hsp-based anticancer treatments.

Conflict of interest statement

The authors declare no competing interest with the present work.

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