Eradication of Glioblastoma, and Breast and Colon Carcinoma Xenografts by Hsp70 Depletion[1]

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Abstract

Heat shock protein 70 (Hsp70) is an antiapoptotic chaperone protein highly expressed in human tumors. Here we demonstrate that locoregional application of adenovirus expressing antisense Hsp70 cDNA (Ad.asHsp70) eradicates orthotopic xenografts of glioblastoma and breast carcinoma, as well as s.c. xenografts of colon carcinoma in immunodeficient mice. Ad.asHsp70-treated tumors showed massive apoptosis-like cell death and recruitment of macrophages. Human monocyte-derived macrophages effectively removed the corpses of Ad.asHsp70-treated tumor cells *in vitro*. Interestingly, both tumor cell death and phagocytosis were caspase-independent. Thus, Hsp70 appears as a promising target for the treatment of cancers resistant to classic caspase-mediated apoptosis.

Introduction

The major stress-inducible Hsp70[3] (also known as Hsp72 or iHsp70) is an antiapoptotic chaperone protein expressed abundantly and preferentially in human tumors and tumor cell lines (1–3). The role of Hsp70 in tumorigenesis is supported by experimental data showing that it enhances the tumorigenic potential of rodent cells *in vivo* (4–7). Furthermore, its high expression in various human tumors correlates with therapy resistance and poor prognosis (1). The tumorigenic potential of Hsp70 has been suggested to depend on its ability to transform cells and/or its antiapoptotic function (4, 6). The latter hypothesis is supported by data showing that depletion of Hsp70 by antisense technology induces massive apoptosis-like death in tumorigenic cells but not in nontumorigenic epithelial cells or embryonic fibroblasts (8, 9). Despite the apoptosis-like morphology as judged by electron microscopy, the antisense Hsp70-induced death of breast cancer cells is independent of known caspases. Furthermore, Bcl-2 and Bcl-X_L, which protect tumor cells from most forms of programmed cell death, fail to rescue cancer cells from death induced by Hsp70 depletion. Because Bcl-2/Bcl-X_L expression as well as other alterations resulting in defective caspase activation are common in cancer cells and because many cancer cells can even survive the activation of caspases, Hsp70 depletion may provide a new option to combat tumors resistant to therapies dependent on classical mitochondrion- and caspase-mediated death pathways (10–12).

Materials and Methods

Cell Culture. All of the cells were propagated in RPMI 1640 with glutamax (Life Technologies, Inc.) supplemented with 10% FCS (Biological Industries) and antibiotics. The medium for MCF-10A cells was additionally supplemented with 100 ng/ml of choleratoxin, 20 ng/ml of epidermal growth factor, 500 ng/ml of hydrocortisone, and 100 ng/ml of insulin.

Recombinant Adenoviruses and Infections. Ad.asHsp70 and Ad.β-gal are adenoviral shuttle vectors carrying bases 475–796 of the published human Hsp70 sequence in antisense orientation or the *β-gal* gene, respectively (9). Ad.ΔE1 is an empty adenoviral vector lacking the insert. The infections were performed as described (9).

Human Tumor Xenografts. Before implantation, the tumor cells were washed twice with PBS, counted, and resuspended in PBS. MDA-MB-468 cells (1×10^7 cells in 100 μl PBS) were inoculated into a mammary fat pad of female FOX CHASE SCID mice, and LoVo-36 cells (3×10^6 cells in 100 μl PBS) were inoculated s.c. into the right flank of athymic BALB/cA *nu/nu* (nude) mice. Breast tumors were treated intratumorally on days 7, 10, and 12, and s.c. colon carcinomas on days 4 and 6 after implantation with 3×10^8 pfu and 5.4×10^9 pfu of virus in 100 μl PBS, respectively. For intracranial implantation, athymic CD1 *nu/nu* mice (Charles River, Sulzfeld, Germany) were anesthetized and placed in a stereotactic fixation device (Stoelting). A burr hole was drilled into the skull 2 mm lateral to the bregma. The needle of a Hamilton syringe was introduced to a depth of 3 mm. Five $\times 10^4$ U373MG cells in 4 μl of PBS were injected into the right striatum. At day 7 after implantation, indicated amounts of virus in 8 μl of 10% glycerol in PBS or vehicle alone were injected intralesionally. The mice of the first set of experiments were observed daily and sacrificed by an overdose of anesthetic when the first one developed adverse neurological symptoms. In the second set of experiments, Kaplan-Meier curves were generated. All of the animal work was carried out in accordance with the NIH guidelines.

Apoptosis and Survival Assays. The MTT reduction assay was used to analyze the survival of the cells (13). Apoptotic nuclear changes were detected by a TUNEL kit (Roche). zVAD-fmk was from Bachem.

Immunoblot Analysis, Immunocytochemistry, and Immunohistochemistry. The primary antibodies used included mouse monoclonal antibodies against Hsp70 (2H9), Hsc70 (N69; both kindly provided by Boris Margulis, Russian Academy of Sciences, St. Petersburg, Russia), GAPDH (Biogenesis), and ED1 (Serotec). Immunodetection of proteins (20 μg/lane) separated by 8% SDS-PAGE and transferred to nitrocellulose was performed using ECL Western blotting reagents (Amersham) indicated primary antibodies and appropriate peroxidaseconjugated secondary antibodies from Dako.

Cryostat sections (16 μm) of tumors were cut, air-dried, and stored at -20°C. For the assessment of tumor volume, sections were stained with H&E and analyzed by MCID software (Imaging Research Inc.). For immunohistochemistry slides were incubated with acetone for 10 min, air-dried, incubated with 1% H_2O_2 for 30 min, washed with PBS, and blocked with BSA (10 mg/10 ml PBS) for 10 min. Primary antibodies were incubated overnight. Bound primary antibody was labeled with biotinylated antimouse IgG antibodies, washed, incubated with avidin-biotin reagent (Vectastain Elite ABC peroxi-

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[3] The abbreviations used are: Hsp70, 70-kDa heat shock protein; Ad., adenoviral; as, antisense; β-gal, β-galactosidase; HMDM, human monocyte-derived macrophage; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling; zVAD-fmk, z-Val-Ala-D,L-Asp- CH_2F; SCID, severe combined immune deficient; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

dase; Vector, Wiesbaden, Germany), and developed in diaminobenzidine (Sigma Chemical Co., St. Louis, MO).

Phagocytosis and Macrophage Activation. Human monocytes were isolated by elutriation, differentiated to macrophages by 8–12-day culture on 48-well microtiter plates, and stained for 1 h with DiI (Molecular Probes; 2.5 mg/ml) to visualize cell bodies (14). Tumor cells infected with Ad.asHsp70 or Ad.β-gal were stained with Fast Blue (Sigma; 1 μg/ml) and added to macrophages at a ratio of 5:1 for 2 h at 37°C. The phagocytosis index was scored by counting ingested cells per phagocyte as described (14). For the analysis of macrophage activation, macrophages were incubated with or without LPS (Sigma) in the presence of cell corpses. TNF concentration in the supernatant was determined by ELISA (Pierce, Rockford, IL).

Statistical Analysis. The significance of the observed effects was evaluated by *t* test. Kaplan-Meier curves were drawn as detailed (15).

Results

Hsp70 Depletion Induces Cell Death in Cancer Cells of Various Origins. We have earlier shown that partial depletion of Hsp70 by Ad.asHsp70 induces apoptosis-like cell death in MDA-MB-468, MCF-7, BT-549, and SK-BR-3 human breast cancer cell lines (9). To test whether the cytotoxic effect of Ad.asHsp70 was specific for cancers of mammary origin, we infected human cancer cell lines originating from glioblastoma (U373MG and LN-18), colon carcinoma (LoVo-36), prostate carcinoma (PC-3 and DU145), or hepatocellular carcinoma (HUH-7) with Ad.asHsp70 at a multiplicity of infection (250–1500) required to achieve a 100% infection. Akin to breast cancer cells, tumor cells of other origins also responded to Ad.asHsp70 treatment with reduction in Hsp70 protein level, shrinkage, detachment, and a loss of viability 3–5 days after the infection (Fig. 1; data not shown). Similar infections with control viruses resulted in some cases in a slight growth inhibition (maximum 20% over 4 days compared with noninfected logarithmically growing cells) but not in any detectable morphological changes or loss of viability. Importantly, Ad.asHsp70 had no effect on the survival of nontumorigenic breast- or prostate-derived epithelial cells (Fig. 1*c*).

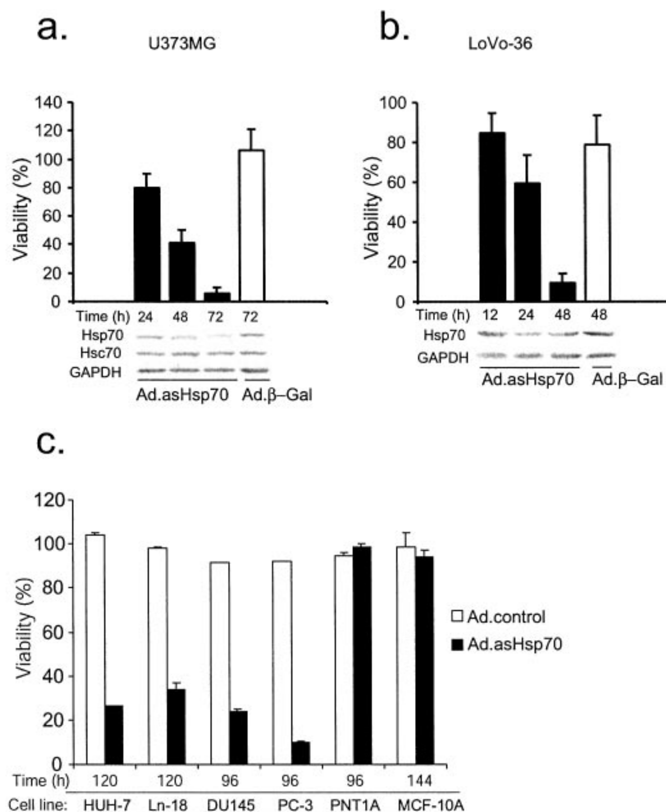

Fig. 1. Hsp70 depletion results in decreased survival of cancer cells. U373MG glioblastoma, LoVo-36 colon carcinoma, HuH-7 hepatocellular carcinoma, LN-18 glioblastoma, and Du145 and PC-3 prostate carcinomas, as well as immortalized PNT1A prostate and MCF-10A breast epithelial cells were left untreated, or infected with Ad.asHsp70 or control adenovirus at the multiplicity of infection resulting in 100% infection. The survival of cells was analyzed by MTT assay at indicated times following infection. The cell density is expressed as a percentage compared with untreated cells. The *columns* represent triplicate MTT measurements; *bars,* ± SD. *a* and *b*, proteins (20 μg/lane) from similarly infected cell lysates were analyzed for Hsp70, Hsc70, and GAPDH by immunoblotting. All experiments were repeated one to three times with essentially same results.

Ad.asHsp70 Treatment Induces Apoptosis-like Cell Death in Tumor Xenografts. To ensure that Hsp70 is not merely needed for tumor cell growth in culture conditions, we next investigated whether Hsp70 expression is also required for the *in vivo* growth of tumors. MDA-MB-468 breast carcinoma cells left untreated or infected with Ad.β-gal or Ad.asHsp70 were inoculated into the mammary fat pad of nude mice 24 h after the infection. Whereas all of the mice injected with noninfected cells or cells infected with Ad.β-gal developed aggressively growing tumors, none of the mice injected with Ad.asHsp70-infected cells showed progressive tumor growth during the observation period of 2 months (Fig. 2*a*).

The dramatic anticancer effects of Ad.asHsp70 observed *in vitro* may have great clinical relevance. Therefore, we next tested the effect of Ad.asHsp70 on the growth of progressively growing tumors *in vivo*. For this purpose we chose three tumor xenograft models in immunodeficient mice: MDA-MB-468 breast carcinoma growing in mammary fat pad, s.c. LoVo-36 colon carcinoma, and U373MG glioblastoma inoculated into the striatum. Remarkably, Ad.asHsp70 treatment induced a dramatic reduction in tumor volumes in all of the *in vivo* model systems (Fig. 2, *b* and *c*; Fig. 3*a*). Furthermore, 28 days after the inoculation of glioblastoma cells, 83% of control animals presented with neurological symptoms, whereas only 1 of 17 animals treated with Ad.asHsp70 showed mild symptoms (Fig. 3*b*). To additionally evaluate the clinical usefulness of Ad.asHsp70 treatment, we investigated the survival of glioblastoma-bearing mice. The median survival of mice treated with a single Ad.asHsp70 injection 1 week after the implantation of tumor cells was twice as long as that of mice treated with the vehicle alone or an empty control virus (Fig. 3*c*).

Next, we examined the Ad.asHsp70-induced reduction in the tumor volume in more detail. To this end, we treated the animals 5 days after the intracerebral implantation of U373MG cells, sacrificed them 2, 5, or 7 days later, and prepared cryostat sections for additional analysis. The treatment of tumors with a single injection of Ad.asHsp70 resulted in a significant reduction in the expression level of Hsp70, an increased number of cells with condensed nuclei, and massive DNA degradation (Fig. 3*d*). These changes were evident already 2 days after the treatment (data not shown) and peaked 5 days after treatment (Fig. 3*d*). One week after the treatment, an area of apoptotic/necrotic tissue surrounded the injection site (data not shown), and the tumor volumes of the Ad.asHsp70-treated mice were significantly smaller than the tumors of the Ad.ΔE1-treated mice (2 mm^3 *versus* 7 mm^3).

To test whether Ad.asHsp70-treated glioblastomas were able to attract microglia, we stained the tumor samples with an anti-ED1 antibody recognizing cells of macrophage linage. Ad.asHsp70-treated tumors ($n = 3$) contained 578 ± 81 ED1-positive cells/mm^2, whereas control tumors ($n = 3$) only had 179 ± 30 ED1 positive cells/mm^2 as analyzed on day 10 (Fig. 3*d*).

Phagocytosis of Ad.asHsp70-treated Tumor Cells by Macrophages Is Not Associated with Suppression of Inflammatory Response. Classic caspase-dependent apoptosis is associated with the triggering of an anti-inflammatory response in phagocytosing cells (Fig. 4*a*; Ref. 16) and a blunted immune attack toward tumor tissue (16). Therefore, we examined the effect of Ad.asHsp70-treated tumor cells on HMDM. Tumor cells dying after Ad.asHsp70 treatment were

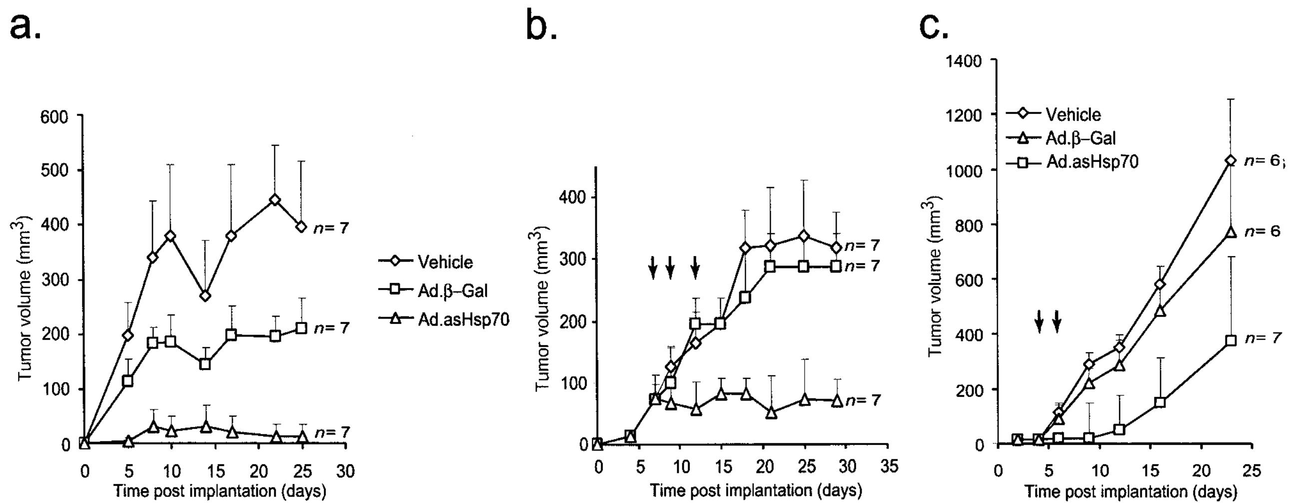

Fig. 2. Ad.asHsp70-infected cells show decreased tumor growth *in vivo*. *a*, 1×10^7 MDA-MB-468 breast carcinoma cells left untreated or infected with indicated viruses were inoculated into the mammary fat pad of female SCID mice 24 h after the infection. *b*, progressively growing MDA-MB-468 breast tumors in the mammary fat pad of SCID mice were treated with intratumoral injections of Ad.β-gal, Ad.asHsp70, or PBS (*vehicle*) on days 7, 10, and 12 after inoculation of 1×10^7 MDA-MB-468 cells. *c*, progressively growing s.c. LoVo-36 colon carcinomas in nude mice were treated with intratumoral injections of Ad.β-gal, Ad.asHsp70, or vehicle on days 4 and 6 after inoculation of 3×10^6 LoVo-36 cells. *a–c*, tumor sizes were measured twice a week. All experiments were repeated with essentially similar results. $P < 0.01$ for Ad.asHsp70 *versus* Ad.β-gal in all tumor models; *bars,* ± SD.

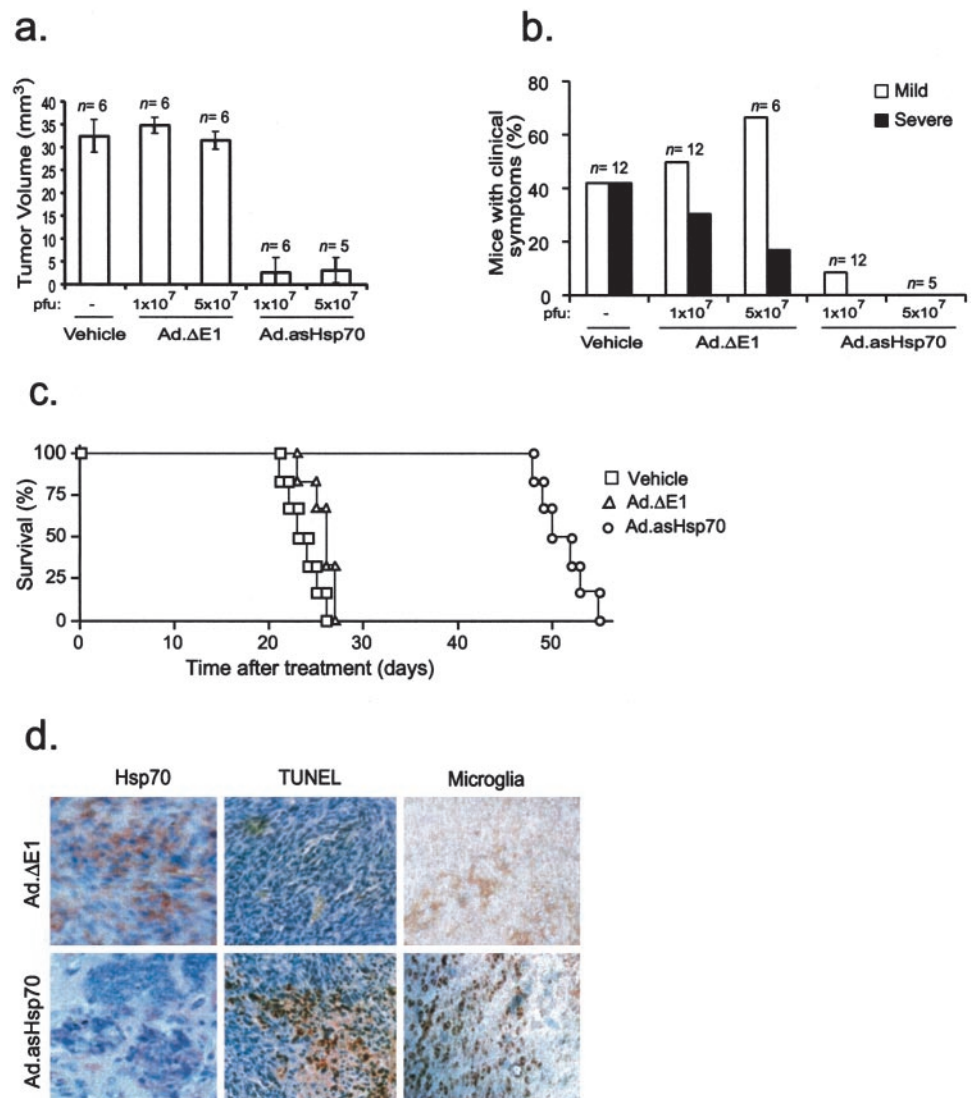

Fig. 3. Ad.asHsp70-induced apoptosis-like cell death reduces tumor volumes and clinical symptoms, and prolongs survival of glioblastoma-bearing immunodeficient mice. Five $\times 10^4$ U373MG human glioblastoma cells were implanted stereotactically into the striatum of nude mice as detailed in "Materials and Methods." *a–c*, at day 7 indicated amounts of Ad-asHsp70 or Ad.ΔE1, or 8 μl vehicle were administered intralesionally. *a*, one group of animals was sacrificed on day 30 for the evaluation of tumor volumes (*a*, $P < 0.01$ for Ad.asHsp70-treated groups *versus* all control groups), whereas in an independent set of experiments the animals were observed at regular intervals until death and Kaplan-Meier curves were drawn to demonstrate the survival in the different groups (*c*). Mice from both experiments were carefully evaluated for clinical symptoms (levels of alertness, behavior and weight, and the appearance of focal neurological deficits, *e.g.,* epileptic fits or pareses) every day. A compound score of all categories formed on day 28 is presented in *b*. *d*, in an independent set of experiments the treatment was carried out with the indicated viruses at day 5 after tumor implantation and at day 10 the animals ($n = 3$/group) were sacrificed and the brains fixed, cut, and analyzed for Hsp70 expression, DNA strand breaks (TUNEL), and presence of ED1-positive microglia. Representative slides are demonstrated; *bars,* ± SD.

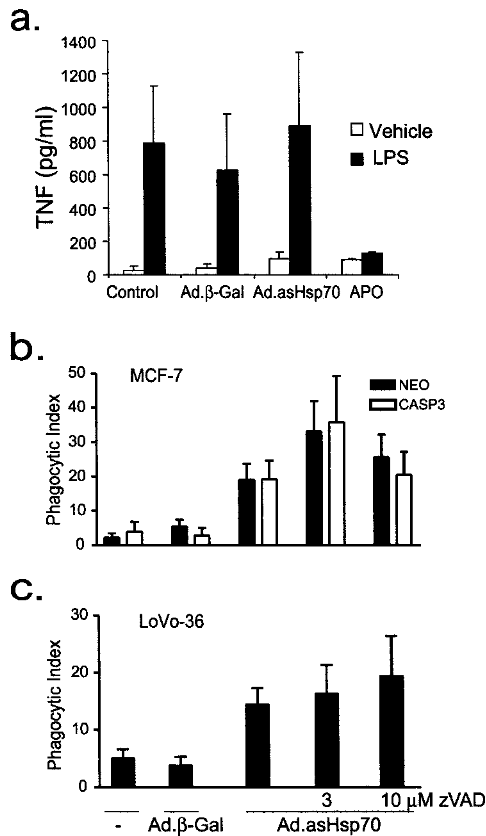

Fig. 4. Phagocytosis of Ad.asHsp70-treated tumor cells does not suppress macrophage activation. *a*, HMDM were incubated alone (*Control*), or exposed to LoVo-36 cells infected with Ad.β-gal or Ad.asHsp70 for 48 h or deprived for serum for 24 h (*Apo*). After 2 h HMDM were stimulated by either PBS (*vehicle*) or 100 ng/ml LPS. TNF concentrations in the supernatant were determined 5 h later by ELISA. *b* and *c*, MCF-7 and LoVo-36 cells were infected with Ad.asHsp70 or Ad.β-gal and incubated in the presence or absence of indicated concentrations of zVAD-fmk. After 72 h (*MCF-7*) or 48 h (*LoVo-36*), cells were added to HMDM and 2 h later the phagocytic index was scored microscopically. All data are means from three independent experiments, each based on triplicate determinations. NEO and CASP3 refer to MCF-7 cells transfected with an empty vector and caspase-3, respectively (17); *bars,* ± SD.

effectively phagocytosed by HMDM. The significant phagocytic uptake of cell corpses as well as tumor cell death appeared to be independent of caspases, because it was neither affected by zVAD-fmk nor by forced overexpression of caspase-3 in MCF-CASP3 cells (Fig. 4, *b* and *c*; data not shown). It should be noted that contrary to parental or vector-transfected MCF-7 cells, Ad.asHsp70 triggered caspase activation in MCF-CASP3 cells. However, the overexpression of caspase-3 had no significant effect on the susceptibility of MCF-7 cells to Ad.asHsp70-induced death (data not shown). Most importantly, cells killed by Ad.asHsp70 did not reduce the LPS-induced activation of macrophages as analyzed by the ability of macrophages to produce TNF (Fig. 4*a*). In fact Ad.asHsp70-treated tumor cells had some stimulatory effect on macrophages even in the absence of LPS (Fig. 4*a*). In this respect, the Ad.asHsp70-treated cells differed from classic apoptotic cells, which effectively suppressed the proinflammatory macrophage response (Fig. 4*a*).

Discussion

The data presented above suggest that the depletion of Hsp70 may offer an effective means to combat cancer. *In vitro* treatment with Ad.asHsp70 was cytotoxic to all of the tumorigenic cell lines tested, pending that they could be effectively infected by adenovirus. More importantly, Ad.asHsp70 treatment resulted in a remarkable reduction in tumor volumes in human brain, breast, and colon cancer xenografts in mice without causing any detectable side effects. Furthermore, Hsp70 depletion resulted in the recruitment of macrophages into the tumor site and caspase-independent engulfment of dying cells. Because Hsp70-depleted cells did not inhibit the activation of macrophages in a way apoptotic cells do, the infiltrating macrophages may enhance the therapeutic effect of Ad.asHsp70 by inciting an inflammatory response and secretion of cytotoxic cytokines such as TNF.

The ineffective delivery of adenoviral vectors into the tumor site appears to be the major limitation for the usage of the Ad.asHsp70 therapy in the treatment of human cancer, which is often a systemic disease. Thus, clinical applications of this approach require additional development of the delivery systems or other means to neutralize the antiapoptotic effect of Hsp70. In the case of tumors that kill their host as a result of local tissue infiltration and invasion, but not distant metastasis, *e.g.,* glioblastoma, locoregional treatment with Ad.asHsp70 may be particularly useful.

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