Heat shock protein derived from a non-autologous tumour can be used as an anti-tumour vaccine

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SUMMARY
Antigenic cross-reactivity between certain tumours has allowed the development of more widely applicable, major histocompatibility complex-disparate (allogeneic) whole-cell vaccines. This principle should also allow heat shock proteins (hsp) derived from certain tumours (and carrying cross-reactive antigens) to be used as vaccines to generate anti-tumour immunity in a range of cancer patients. Here, hsp70 derived from gp70-antigen† B16 melanoma generated cytotoxic-T-lymphocyte-mediated immune protection in BALB/c mice against challenge with gp70-antigen† CT26 colorectal tumour cells. Using ovalbumin as a model tumour antigen, it is shown that hsp70 enhances peptide re-presentation by dendritic cells via class I over equimolar whole ovalbumin antigen. However, while transfection of tumour cells with inducible hsp70 increases hsp yield from tumours, it does not enhance antigen recognition via purified hsp70 nor via whole cells or their lysate.

INTRODUCTION
The promise of utilizing the immune response in combating cancer has encouraged a great deal of laboratory and clinical research, but has come to relatively little fruition in terms of providing patient treatment options.1 This may be because tumour immunotherapy is generally a complicated, expensive, patient-specific and unknown treatment avenue. There is little doubt that the immune system can recognize and destroy cancer cells. Indeed, a large number of tumour-associated or tumour-specific antigens have been identified, often based on their recognition by the T cells of cancer patients.2 However, the ability of cytotoxic T lymphocytes (CTLs) to kill tumour cells, which is considered one of the most important events in tumour immunotherapy, is likely to be inefficient if the tumour burden is very high for logistical reasons (magnitude of immune response3) as well as because of immune suppression.4 Apart from reducing the tumour burden by surgery and other means a way of tipping the balance against tumours is to modify and increase the anti-tumour response by vaccination. An anti-tumour vaccine may take a number of forms including whole irradiated tumour cells, often with additional adjuvants such as bacillus Calmette–Guérin5 and transduced cytokines,6 tumour-derived antigens possibly pulsed onto autologous dendritic cells (DCs)7 and antigen–DNA vaccines.8 One form of subunit tumour vaccine comprises chaperone proteins [heat shock proteins (hsp)] extracted from autologous tumour.9 These hsp, the main players being hsp70, hsp90, gp96, gp110 and calreticulin, are able to bind to and transport tumour-derived antigens to host antigen-presenting cells (chiefly DC), thus promoting the priming of antigen-specific T cells.10–12 The hsp also have the ability to stimulate and activate antigen-presenting cells,13 enhancing their ability to prime T cells. Moreover, the cellular induction of hsp by various stresses in addition to heating has been shown to enhance both the immune recognition and immunogenicity of tumour cells.14–18

It has been maintained that hsp must be derived from the tumour against which vaccination is pursued, similar in principal to a need for autologous whole-cell vaccination,19 so that antigens are exactly matched. However, it is clear that the majority of tumours of a given histological origin will express a number of antigens in common which could serve as targets for an effective immune response.20 This principle has allowed the investigation and use of major histocompatibility complex (MHC)-disparate (allogeneic) tumour cells as effective

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vaccines.21–24 Thus, in principle, hsp derived from non-auto-
logous but related tumours should also be able to prime or
activate effective T cells. This has recently been shown for
hsp70 derived from human melanoma in vitro using CTL clones
specific for known melanoma antigens.25,26

The aim of this study was to evaluate the use of an hsp70
vaccine, in BALB/c mice, derived from an allogeneic tumour
cell line (B16), which expresses an antigen common to the
syngeneic tumour challenge (CT26). A model antigen, ovalbu-
min, was used to investigate mechanisms by which hsp70 may
facilitate antigen re-presentation.

METHODS

Animals and cell lines

BALB/c (H-2b) and C57BL/6 (H-2b) mice were purchased from
Harlan UK (Bicester, UK) and used at between 6 and 12 weeks
of age. All procedures were carried out in accordance with
Government guidelines. CT26 (H-2b) murine colorectal tumour,
B16-F10 (H-2b) and K1735 (H-2b) murine melanoma and
RENCA (H-2b) renal carcinoma lines have been described in
our previous studies.27–29 Ovalbumin-transfected B16 cells
(B16-OVA) were provided by P. DellaLuna.30 the
DC2.4 DC line was provided by K. Rock31 and B3Z SIIN-
FEKL-specific T-cell hybridoma was given by N. Shastri.32
Tumour cell lines were grown in Dulbecco’s modified Eagle’s
medium supplemented with 10% heat inactivated fetal calf
serum, 2 mM glutamine, penicillin (100 U/ml) and streptomyc-
in (100 μg/ml) (Sigma, Poole, UK). The adherent lines were
detached from the flasks with 0.05% trypsin/0.02% EDTA and
for in vivo inoculation were washed three times in phosphate-
buffered saline (PBS). Cell lines were all free of mycoplasma,
as determined by the Gene-Probe method (Gene-Probe, San
Diego, CA). Retroviral transfection with the inducible murine
hsp7014 was carried out as described previously.33 Puromycin,
resistance to which was encoded by the gene construct, was used
in the medium at 1.25 μg/ml.

RNA isolation and reverse transcription–polymerase chain
reaction (RT-PCR)

Total RNA was isolated from tumour cell pellets by homo-
genization with TRIZOL solution (Sigma), according to the
manufacturer’s instructions. Two micrograms of RNA, as esti-
mated by the absorbance ratio for 260 : 280 nm, were reverse
transcribed at 37°C for 1 hr using the ‘first-strand cDNA syn-
thesis kit’ (Novagen/Merck, Darmstadt, Germany), following
the manufacturer’s instructions. An equivalent of 100 ng of result-
ing cDNA was added to reaction mixtures containing 2 μl 10×
buffer (10 mM Tris HCl, 50 mM KCl, 0.01% gelatine), 160 μM
dNTPs (Amersham Pharmacia-Biotech, St Albans, UK),
2.5 mM MgCl2, 0.25 U Taq polymerase (Perkin Elmer, Nor-
walk, CT) and 0.5 μM of each primer, the sequences for gp70
have been previously published.34 Amplification was performed
on a PTC-100 DNA thermal cycler for 35–40 cycles. Products
were visualized on 1.5% (w/v) agarose gels with appropriately
sized markers (Amersham Pharmacia-Biotech). All samples
were initially subjected to PCR with primers for the house-
keeping gene GAPDH to confirm successful RNA extraction
and reverse transcription, and also to verify that the amounts of
input cDNA were constant for each reaction. RNA was shown to
be free of genomic DNA contamination by directly amplifying
the equivalent amount of RNA as was used in the subsequent
RT-PCR reactions (not shown).

Extraction of hsp70

The hsp70 was extracted from B16 tumour cells by a modified
method.35 Tumour cells that had been growing as a solid mass in
vivo were excised from killed mice. It was found that these
tumours were of an unstructured nature and so did not require
enzymatic digestion, but simply vigorous mixing by shaking in
PBS to release the cells; 4 g of tissue was processed. Washed
and pelleted cells were homogenized on ice in a glass dounce
and then sonicated on ice in 10 mM HEPES buffer, pH 7.
Following two rounds of freeze–thawing and centrifugation
at 10 000 g, the soluble fraction was made up to 15 mM 2-
ME, 3 mM MgCl2, 20 mM NaCl, and was passed over a
Sepharose–ADP column (Sigma), washed thoroughly and then
eluted with ADP without 2-ME. Excess ADP was removed at
the same time as buffer exchange to PBS, and concentration,
using a centricon filter system. Protein concentration was
determined using a Bradford assay and by reference to the
gel used for sodium dodecyl sulphate–polyacrylamide gel
electrophoresis (SDS–PAGE). For Western blotting the samples
were transferred to nitrocellulose and probed with an anti-hsp70
monoclonal antibody (BRM-22, Sigma). The same procedure
was used to extract hsp70 from K1735 cells.

In vivo experiments

Groups of four to eight female, age-matched mice were injected
subcutaneously in the shaven flank either with lethal tumori-
genic doses of tumour cells, with irradiated (100 Gy) cell
vaccine, or with 15 μg of tumour-derived hsp70. Vaccines were
routinely given on the right flank whilst challenge was given 10–
14 days later on the left flank. Tumour volume was measured
two or three times weekly with callipers in two diameters. Mice
were judged to possess a tumour when it was possible to
measure a lump (>2 mm) and were killed when the tumours
reached 15 mm in any diameter. Representative data from at
least two separate experiments are given.

In vitro experiments

Spleens from killed mice that had previously received vaccina-
tion were removed, teased apart and the red cells were lysed
with 0.87% ammonium chloride for 1 min. The resulting lym-
phocytes were washed and set up in culture (at 1 × 106/ml)
with irradiated (50 Gy) syngeneic tumour cells at a ratio of 50
lymphocytes to one tumour cell. The medium used was
RPMI-1640 supplemented with fetal calf serum, glutamine,
penicillin and streptomycin as above, plus 50 μM 2-mercap-
tetoehanol. After 5 days, lymphocytes were harvested and tested
for CTL activity by a colorimetric lactate dehydrogenase (LDH)
-release assay (Promega, Madison, WI), following the manufac-
turer’s instructions.

Statistical analysis

Statistical analysis was carried out using the log-rank test for
survival and Student’s t-test for mean values, both on PRISM
software.

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RESULTS

Antigen expression
To confirm that the tumour cell lines expressed the gp70 antigen, RT-PCR was carried out with specific oligonucleotide primers (Fig. 1). A ubiquitous housekeeping gene (GAPDH) was amplified as a control to ensure successful RT-PCR and loading. Both CT26 and B16 expressed gp70, although the level in B16 was lower. K1735, serving as a negative control, did not express this antigen.

Extraction of hsp70
The 4 g of B16 tumour tissue, calculated to comprise approximately 10^9 tumour cells, was able to yield approximately 1 µg of purified hsp70 as determined by Bradford assay and SDS–PAGE/Western blot (Fig. 2). To monitor antigen processing in conjunction with hsp70 in more detail an ovalbumin transfected of B16 was used. In addition, this transfected was further transfected with the gene encoding inducible hsp70 using a retroviral construct with puromycin selection.

Vaccination
Vaccination of BALB/c mice with irradiated syngeneic CT26 tumour cells (1 × 10^6) routinely afforded protection from challenge with CT26 of between 20% (Fig. 3a) and 50% of mice. Vaccination of BALB/c mice with an equal number of B16 cells caused increased survival in these mice, although all mice succumbed eventually to tumour (Fig. 3b). When mice were vaccinated with B16-derived hsp70, 50% of mice remained tumour-free for the duration of the experiment (>40 days) (Fig. 3c). In contrast, vaccination of mice with hsp70 extracted from K1735 elicited no protection (none or four mice) against CT26 as shown by no inhibition in tumour growth (Fig. 3d). Moreover, mice vaccinated with hsp70 from B16 were not protected against a challenge with syngeneic RENCA cells (Fig. 3d).

CTL assay
To examine possible immune mechanisms, spleens of vaccinated mice were tested for CTL activity by 5-day restimulation followed by an LDH-release assay. Similar levels (approximately 12%) of CTL activity against CT26 targets were observed in both CT26 and hsp70-vaccinated groups, double that of PBS-treated mice (Fig. 3f). No activity against YAC-1 target cells was observed (not shown).

hsp70 re-presentation mechanisms
To examine antigen re-presentation in conjunction with hsp70 in more detail, hsp70 was extracted from an OVA-transfectant of B16. In an attempt to enhance the yield and function of hsp preparations, the B16-OVA was additionally transfected with inducible hsp70 (to give B16-OVA-hsp). The hsp70 was extracted from 4 g of tumour tissue as before and the yield from the B16-OVA-hsp (2 mg) was approximately double the yield from the B16-OVA (1 mg). The system for examining antigen re-presentation was recognition of the OVA peptide SIINFEKL on the surface of DC2.4 cells by B3Z T-cell hybridoma. Initial experiments demonstrated the sensitivity of the T cells to given peptide and OVA antigen concentrations, and demonstrated the sensitivity of the system and the range of peptide/antigen that could be recognized (Fig. 4a). Re-presentation of OVA peptide via hsp70 derived from B16-OVA and B16-OVA-hsp was of a similar magnitude (Fig. 4b), at a concentration of 0.57 µm, this being 1 log lower concentration than was required for OVA antigen. As has been described previously whole B16-OVA cells were recognized by the B3Z T-cell hybridoma but transfection with hsp70 did not significantly increase this recognition (Fig. 4c). Lastly, freeze–thaw lysates of B16-OVA applied to DC2.4 were not recognized by the T cells whether or not they were transfected with hsp70 (not shown).
Figure 3. Immunity following vaccination. BALB/c mice (n = 4 per group) were vaccinated or were mock-vaccinated (PBS), and were subsequently challenged with a tumorigenic dose (2 × 10⁶) of live CT26 cells (except d); % tumour-free survival is shown. Significance of protection: with (a) CT26 cells P = 0.064, (b) B16 cells P = 0.029, (c) hsp70 derived from B16 P = 0.031, (d) hsp70 derived from B16 and challenged with RENCA P = 1. (e) CT26 tumour volume on day 17 post-challenge, following vaccination with hsp70 from B16 compared to hsp70 from K1735 P = 0.02*. (f) Vaccination with hsp70 elicited similar CTL activity (% specific lysis at 50:1 effector to CT26 target ratio) to vaccination with CT26 cells.
DISCUSSION

The search for effective cancer immunotherapies has explored numerous vaccination approaches. Consistently, it has been found that autologous whole tumour cells, administered as an irradiated vaccine alone or with adjuvants, can elicit immune responses that are effective at controlling tumour growth. It remains a matter of debate whether tumour cells can directly prime T cells, or whether tumour antigens are cross-presented by antigen-presenting cells. Clearly, both are probable, but hsp derived from tumours that chaperone tumour antigens must use the latter pathway. In an attempt to produce more practical and generic tumour vaccines, whole allogeneic tumour cell vaccines have been developed that probably possess antigens in common with the patient’s cancer (usually of the same histological type). Since this approach has shown effects in preclinical and clinical studies, a logical next step could be to produce subunit tumour vaccines, that can be used for a range of patients, in the form of tumour-cell-derived hsp. This study aimed to support and examine such a principle.

The two murine tumour cell lines were used in this study because of reports of their expression of the murine retroviral envelope protein gp70. It is known that both lines express the whole gene and translate the protein (E. Jaffee, personal communication). However, because their sublines may have lost the antigen, expression in both lines was confirmed by RT-PCR, although B16 expresses considerably less gp70 than CT26.

The ability of a vaccine comprising allogeneic B16 cells to elicit a degree of protection in BALB/c mice against CT26 challenge also confirmed their antigenic cross-reactivity, and reduced protection compared to CT26 may be the result of its lesser expression of gp70. An allogeneic K1735 vaccine (which does not possess gp70) failed to induce immunity (data not shown) nor did hsp70 extracted from K1735 (Fig. 3e). Since efficacy was seen with the allogeneic cell line vaccine, it was logical to assume that hsp derived from the tumour would also be effective. Preparations of hsp70, derived in a simple process from B16 tumour material were indeed able to elicit protective immunity against CT26 challenge. It was possible to detect low but significant levels of anti-tumour CTL activity in the spleens of hsp70-vaccinated mice, that were of a similar magnitude to those detected in whole CT26-vaccinated mice (and to results published previously), which is a moderately immunogenic
vaccine. Both the lack of cytolytic activity against natural-killer-sensitive YAC-1 cells and the lack of protection against RENCA cells suggest that the lysis of CT26 cells was the result of CTL activity.

Being able to increase the yield of hsp obtained from tumour material would be of practical use in generating vaccine material. This was attempted by transducing B16-OVA cells with the inducible hsp70 gene, previously cloned from murine tumour cells after induction by thymidine kinase/ganciclovir gene therapy and subsequently inserted into a retroviral vector. The yield was indeed increased, but only by a modest two-fold. Nevertheless, this may be useful in vaccine production.

To attempt to dissect mechanisms of hsp70-mediated representation of tumour antigen, hsp70 was extracted from B16-OVA and used in conjunction with DCs and a T-cell hybridoma. This hsp material was able to chaperone OVA antigen to DC to allow recognition by T-cell hybridomas at a molarity approximately 10-fold lower than achieved with OVA antigen. Thus hsp appeared to allow the antigens being chaperoned better access to the class I pathway than whole antigen, as has been described previously by other means. Since hsp70 is likely to chaperone a range of cell-derived proteins, the enhancement is substantial. It has been suggested that inducible hsp70 may have different chaperoning properties than constitutive hsp70 as a result of differing structural characteristics. This study, however, did not show a difference between the activity of hsp70 from hsp-transduced and non-transduced tumours. Another suggestion is that hsp70 over-expression by tumours may increase the tumours’ ability to be recognized by antigen-specific T cells by increasing MHC expression or antigen processing. Here, over-expression of hsp70 did not increase whole tumour recognition by the T cells. Lastly, preliminary data (not shown) suggest that hsp70 from OVA-transfectants does not mediate re-presentation of a class II epitope to epitope-specific transgenic T cells, and this is the subject of current investigation.

The present study demonstrates that hsp70 derived from tumour material of one mouse strain is able to generate immune-mediated protection against a tumour challenge in another mouse strain when the tumours have antigen in common. The hsp70 facilitates antigen cross-priming but increased intracellular expression does not enhance antigen presentation by the tumour cells or the cross-presentation of the tumour cell lysate. We propose that hsp70 derived from tumours of known antigenic makeup could be used as a more generic form of subunit tumour vaccine. Such material may also serve as a better source of multivalent antigen for pulsing ex-vivo DC for subsequent re-administration, than native antigen or tumour lysate.

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