Molecular Immunology 46 (2008) 181–191

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Heat shock protein–antigen fusions lose their enhanced immunostimulatory capacity after endotoxin depletion

Boris-Christian Marincek[a], Marie-Cristine Kühnle[a], Cathy Srokowski[b], Hansjörg Schild[b], Günter Hämmerling[a], Frank Momburg[a,c,*]

[a] Division of Molecular Immunology (D050), German Cancer Research Center (DKFZ), Heidelberg, Germany
[b] Institute of Immunology, University of Mainz, Mainz, Germany
[c] Translational Immunology Unit (D015), German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

ARTICLE INFO

Article history:
Received 30 June 2008
Received in revised form 29 July 2008
Accepted 29 July 2008
Available online 18 September 2008

Keywords:
Heat shock protein 70
Fusion protein
Endotoxin
Tumor vaccine
TLR ligands

ABSTRACT

Heat shock proteins (HSPs) induce cross-presentation of antigens by dendritic cells (DC) as well as DC maturation. These properties make HSP antigen complexes good candidates to prime CD8 T cell responses against tumor-associated antigens. In this study, we analyzed four different members of the HSP70 family fused to a fragment of ovalbumin (OVA) as a model tumor antigen. *E. coli*-derived recombinant HSP70-OVA fusion proteins efficiently primed antigen-specific cytotoxic T cells in short-term *in vivo* immunization assays. Because of concerns that the adjuvant effect of HSPs may be due to endotoxin contamination, we studied this issue in detail. Induction of OVA-specific cytotoxicity was significantly decreased in mice deficient for the LPS receptor, TLR4. After careful removal of endotoxins, immunization with HSP70-OVA failed to prime cytotoxic T cell responses. However, we obtained strong *in vivo* kill responses when endotoxin-depleted HSP70-OVA was used in combination with the TLR9 ligand CpG oligodeoxynucleotide 1668. Importantly, prophylactic and therapeutic treatment with endotoxin-depleted HSP70-OVA together with CpG significantly delayed the outgrowth of OVA-expressing B16 melanoma cells. However, we were unable to detect significant differences in the magnitudes of immune responses against endotoxin-depleted recombinant OVA *vs.* endotoxin-depleted HSP70-OVA fusion protein. Thus, immunization with recombinant HSP70-antigen fusion protein does not provide an advantage over recombinant antigen alone when combined with a suitable adjuvant. Altogether, our data suggest that the adjuvant effect of the HSP70 part of the fusion protein is completely lost after endotoxin removal.

1. Introduction

Heat shock proteins (HSP) serve as molecular chaperones in various intracellular compartments. Their function is to prevent aggregation of polypeptide chains and support correct protein folding (Bukau and Horwich, 1998). The ability to bind polypeptide chains via hydrophobic regions and to unfold them is important for HSP function. HSPs are expressed by prokaryotes and eukaryotes and can be classified into different families, based on their molecular weight: small HSPs, HSP40, HSP60, HSP70, HSP90, and HSP110 (Lindquist and Craig, 1988; Fink, 1999; Hartl and Hayer-Hartl, 2002). In addition to their cellular functions, it is recognized that they possess immunological properties. These include strong immunostimulatory capacities that can lead to the maturation and activation of antigen-presenting cells (Asea et al., 2000; Harmala et al., 2002; Massa et al., 2005). Furthermore, HSPs bind antigenic oligopeptides and induce adaptive immune responses against these peptides (Srivastava, 2002). This unique feature of immune stimulation and cross-presentation of bound antigen has been further exploited and HSP molecules covalently linked to a protein antigen have been produced (Suzue and Young, 1996; Suzue et al., 1997). Such HSP-antigen fusion proteins were shown to enter the MHC class I processing pathway of antigen-presenting cells and to stimulate antigen-specific $CD8^+$ T cells (Suzue et al., 1997).

HSP receptors on antigen-presenting cells (APCs) enable HSP proteins to propagate signaling pathways. Among these are CD91 (Binder et al., 2000; Basu et al., 2001), several scavenger receptors such as CD36 (Panjwani et al., 2002) and LOX-1 (Delneste et al., 2002), CD40 (Wang et al., 2001; Becker et al., 2002), and the

Abbreviations: APC, antigen-presenting cell; DC, dendritic cells; BMDC, bone marrow-derived dendritic cells; IFN-γ, interferon-γ; HSP, heat shock protein; HSP70, member of the heat shock protein 70 kD family; LPS, lipopolysaccharide; mHsp70, mouse inducible Hsp70; mHsc70, mouse heat shock cognate protein 70; mHsp70L1, mouse Hsp70-like protein 1; mtubHsp70, *M. tuberculosis* Hsp70; ODN, oligodeoxynucleotide; OVA, ovalbumin; rOVA, recombinant OVA fragment.

* Corresponding author. Tel.: +49 6221 423729; fax: +49 6221 401629.
E-mail address: f.momburg@dkfz.de (F. Momburg).

0161-5890/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2008.07.039

Toll-like receptors TLR-2 and 4 (Asea et al., 2002; Vabulas et al., 2002a,b). The finding that TLR-2 and 4 can bind HSPs has raised the question if the strong immunostimulatory capacities of the HSPs are actually due to inherent structural features or to a contamination with bacterial compounds, such as the TLR-4 ligand LPS. As many of the HSPs have been produced recombinantly in a bacterial expression system, one has to anticipate endotoxin contamination of the recombinant proteins (Dayan, 1995; Magalhaes et al., 2007). Recent reports have raised doubts about the inherent immunostimulatory capacities of HSPs, not only of HSPs produced in *E. coli* (Bausinger et al., 2002; Wallin et al., 2002; Bendz et al., 2007), but also of HSP purified from tissue (Warger et al., 2006).

Different mycobacterial and murine Hsp70 preparations have been described as potent in eliciting adaptive immune responses *in vivo*. *Mycobacterium tuberculosis* Hsp70 (Suzue et al., 1997; MacAry et al., 2004), murine Hsp70 (Udono and Srivastava, 1993; Faure et al., 2004), murine Hsp70-like protein 1 (Wan et al., 2004; Wu et al., 2005) and murine heat shock cognate 70 protein (Udono et al., 2001) have been analyzed individually, but have never been compared side by side for their immunogenicity. In this study we compared these four HSP70 fused to a large C-terminal fragment of OVA for their capacity to cross-prime OVA-specific $CD8^+$ T cells and their use as tumor vaccines in an OVA-transgenic melanoma model. To address the issue of endotoxin contamination of recombinant HSP70-OVA fusion proteins, the effect of endotoxin depletion on their immunostimulatory capacities was examined.

Out of the four HSP70-OVA fusions studied, the Hsp70 from *M. tuberculosis* most efficiently activated OVA-specific $CD8^+$ T cells. Notably, careful endotoxin removal led to a complete loss of the immunostimulatory effect of the HSP70-OVA fusion proteins. Endotoxin-depleted HSP70-OVA fusion proteins alone were not effective as tumor vaccines, but required addition of the adjuvant CpG-ODN 1668. Unexpectedly, the anti-tumoral effect of HSP70-OVA in combination with CpG was not stronger than the recombinant OVA fragment administered together with CpG. Thus, the potential co-stimulatory effect of HSP70 appears to be abolished after endotoxin removal.

2. Materials and methods

2.1. Recombinant proteins

cDNA constructs coding for the HSP70-OVA fusion protein variants, OVA fragment (rOVA), or unconjugated HSP70 molecules were cloned into a pET-21d(+) (Novagen, Darmstadt, Germany) modified by an extended multicloning site and a C-terminal streptavidin-binding peptide (Keefe et al., 2001) in frame with the vector-provided hexa-histidine (His_6) tag sequence. To generate the OVA fragment in HSP70-OVA fusion proteins and rOVA, a portion of chicken ovalbumin cDNA (kindly provided by Dr. Y. Reiss, Tel Aviv, Israel) was PCR-amplified using primers with suitable restriction sites. This sequence (encompassing a.a. 146–359 of OVA; GenBank accession no. V00383) harbors the H-$2K^b$ epitope SIINFEKL (a.a. 257–264) and the I-A^b epitope ISQAVHAAHAEINEAGR (a.a. 323–339). cDNAs coding for murine (m) Hsp70 (murine heat shock protein 1A; GenBank accession no. BC054782), mHsc70 (murine heat shock protein 8; GenBank accession no. BC006722), and mHsp70L1 (murine heat shock protein 14; GenBank accession no. BC002056) were obtained from the German Resource Center for Genome Research (Berlin, Germany). The HSP70 cDNAs were PCR-amplified without stop codons using primers containing suitable restriction sites for the extended multicloning site of pET21d(+) and cloned at the 5′ end of the OVA fragment. Likewise, a mycobacterial Hsp70 fragment (*M. tuberculosis* DnaK; GenBank accession no. NC_00962; kindly provided by Dr. P.J. Lehner, Cambridge, UK) containing the C-terminal peptide-binding domain (a.a. 359–610) was cloned in frame with OVA. This truncated mycobacterial Hsp70 (mtubHsp70) is able to stimulate chemokine and cytokine release (Wang et al., 2002) and to induce CTL responses (MacAry et al., 2004). To obtain antigen-free recombinant HSPs, SBP/His_6-tagged HSP70 variants were also cloned without the OVA cDNA. As irrelevant control antigen, a fragment of the human HER-2/neu protein (a.a. 299–579; GenBank accession no. BC080193) was PCR-amplified and inserted at the 5′ end of the SBP/His_6 tag in the modified pET21d(+) vector. All constructs were confirmed by DNA sequencing.

Recombinant protein was purified as described previously (Udono et al., 2004). Recombinant proteins were expressed in *Escherichia coli* strain Rosetta 2(DE3) (Invitrogen, Eggstein, Germany). His_6-tagged unconjugated HSP70 variants, His_6-tagged HSP70-OVA variants, and His_6-tagged rOVA fragment were purified under denaturing conditions (8 M urea) by affinity chromatography on Ni^{2+}-NTA agarose (Qiagen, Hilden, Germany). Recombinant proteins were refolded by a quick removal of urea with phosphate-buffered saline (PBS) and eluted with PBS containing 200 mM imidazole (Sigma–Aldrich, Taufkirchen, Germany). The purified recombinant proteins were dialyzed against PBS to remove imidazole and analyzed by Western Blot using the anti-His_6-tag mAb 13/45/31a (kindly provided by Dr. G. Moldenhauer, DKFZ, Heidelberg, Germany) or by staining with streptavidin-peroxidase (Vector Laboratories/Linaris, Wertheim-Bettingen, Germany). Protein purity was determined by SDS-PAGE and densitometric analysis with a Lumi-Imager (Roche, Mannheim, Germany). Murine gp96 was purified according to a protocol described previously (Warger et al., 2006).

2.2. Endotoxin removal

Quantitative measurement of the endotoxin contamination was performed by the limulus amoebocyte lysate assay using an Endosafe-PTS device (Charles River Laboratories, L'Arbresle, France) by diluting the recombinant proteins 1:40 in endotoxin-free water. In order to efficiently remove contaminating endotoxins, we adapted a method by Nicchita and colleagues (Reed et al., 2003). Briefly, Ni^{2+}-NTA agarose-bound recombinant protein was extensively washed in TTT buffer (25 mM Tris–Cl, pH 7.8, 150 mM NaCl, 0.4% (v/v) Tween 20, 0.4% (v/v) Triton X-114). Refolding of recombinant protein and removal of remaining detergent was conducted by extensive washing with endotoxin-free phosphate-buffered saline (PBS; Invitrogen). Recombinant proteins were eluted as described above and imidazole was subsequently removed by dialysis against endotoxin-free PBS.

2.3. CpG, peptides

Phosphorothioate-stabilized CpG-ODN 1668 (5′-TCCATGACGTTCCTGATGCT-3′) was synthesized by the oligonucleotide core facility of the German Cancer Research Center (Heidelberg, Germany). The peptide SIINFEKL was synthesized by the peptide synthesis core facility of the German Cancer Research Center (Heidelberg, Germany) and purified to >95% purity by HPLC.

2.4. Mice

C57BL/6J wild-type mice (B6; Thy 1.2^+) were purchased from Charles River Wiga (Sulzfeld, Germany) and bred in our animal facility. C57BL/6 OT-I mice express a transgenic TCR (Va2/Vb5.1) specific for the H2-K^b-restricted peptide OVA(257–264) (SIINFEKL) derived from chicken OVA and were kindly provided by M. Zenke (Max Delbrück Center for Molecular Medicine, Berlin, Germany).