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Soluble heat shock protein 70 members in patients undergoing allogeneic hematopoietic cell transplantation

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ABSTRACT

Background: Heat shock proteins (HSP) are highly conserved immunogenic proteins serving as potent danger signals. They are upregulated under stress conditions like fever and hypoxia. Extracellular HSP are involved in antigen presentation, cytokine release and maturation of antigen presenting cells.
Methods: The release of the inducible members of the HSP70 family, Hsp72 and Hsp70B′, into the serum of 20 patients undergoing allogeneic hematopoietic cell transplantation and 20 healthy donors was evaluated using enzyme linked immunosorbent assay (ELISA) kits.
Results: Eight patients (40%) did not receive anti-thymocyte globulin (ATG) for prophylaxis of graft versus host disease (GvHD). These patients had no detectable or low serum levels of Hsp72 ($n = 3$, 0.03 to 1.92 ng/ml) which were in line with levels detected in 20 healthy individuals ($p = 0.07$). Measurable HSP was not associated with any medication or transplantation-related procedures. In twelve patients (60%) receiving ATG, detected high levels of HSP reflected cross-reactivity of the rabbit-derived ATG with the anti-rabbit antibody used in the ELISA.
Conclusions: Assumed HSP70 expression detected such ELISA has to be regarded carefully after ATG application. Neither radiochemotherapy, nor inflammation or sepsis during aplasia induced HSP70 release into the serum. Thus, soluble HSP70 may not be involved in the pathogenesis of acute GvHD.

1. Introduction

Heat shock proteins (HSP) are highly conserved immunogenic danger signals and molecular chaperones expressed in all organisms [1–3]. Under stress conditions, several HSP (e.g. Hsp60, Hsp72, Hsp90) can be translocated to the plasma membrane and also released into the extracellular environment [4,5]. This extracellular expression is currently believed to follow two different mechanisms: First, the release of soluble HSP as a free protein from dying cells (as evaluated in this study), and second the active transport of liposomal HSP via membrane export from living cells [6].

Although HSP were originally described to be primarily stress-induced proteins (fever, sepsis, hypoxia, glucose deprivation, exposure to pro-inflammatory cytokines like TNF or IFN-γ, ozonation and oxidative stress [7–10]), each family also includes constitutively expressed members. The HSP70 family consists of 13 homologous members ranging in size from 66–78 kDa and comprises two inducible members: Hsp72 (Hsp70 or HSPA1A) [11] which can be also expressed at low levels during normal conditions by all cells [12] and the strictly stress-inducible Hsp70B′ (HSPA6) which shares a 77% gene sequence homology with Hsp72 and has no detectable basal levels in most cells [12].

HSP exert multiple functions in protein folding and degradation [13,14], assembly of subunit complexes, thermotolerance [15], cell survival [16] and antigen presentation [17,18], but play a dual role in tissue damage. They are tissue protective by inhibition of apoptosis [16,19], but they are also one of the most potent danger signals for the immune system and able to chaperone antigenic peptides derived from tumor or virus infected cells [7,20–23]. Hence, extracellular HSP can deliver antigens to antigen presenting cells (APC). These antigenic peptides can be loaded onto MHC molecules and presented to $CD4^+$ as well as to $CD8^+$ T cells via cross-priming, finally inducing antigen specific T cells [17,18].

Abbreviations: AML, acute myeloid leukemia; APC, antigen presenting cell; ATG, anti-thymocyte globulin; BSA, bovine serum albumin; CD, cluster of differentiation; DC, dendritic cell; ELISA, enzyme linked immunosorbent assay; ET, essential thrombocytemia; FC, fragment crystallizable; GvHD, graft versus host disease; HCT, hematopoietic cell transplantation; HLA, human leukocyte antigen; HSP, heat shock protein; IFN, interferon; MDS, myelodysplastic syndrome; MF, myelofibrosis; MM, multiple myeloma; NHL, non-Hogdkin lymphoma; PMF, primary myelofibrosis; TNF, tumor necrosis factor.

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Since HSP can be induced by several cytokines, the so called *cytokine storm* after hematopoietic cell transplantation (HCT) leads to massive release of multiple proinflammatory factors into the serum [24–26] and could hence also induce HSP release. Since HSP can deliver antigenic peptides to APC and induce T cell responses and also modulate the innate immune system, HSP might also have an impact on the development of graft versus host disease (GvHD) constituting the main complication of allogeneic HCT. In line with this hypothesis, elevated Hsp72 expression was observed in an in vitro generated human skin model of acute GvHD and has been found to positively correlate with the GvHD severity [27]. Since the development of GvHD in certain organs is strongly influenced by induced cell damage through external pathogens and treatment related toxicity [26,28,29] this study was focused on the evaluation of free HSP in the serum of HCT patients. This study represents the first evaluation of the systemic release of inducible free HSP70 in patients undergoing allogeneic HCT.

2. Objective

In this study, we aimed to analyze the induction of soluble HSP70 family members into the serum of patients undergoing allogeneic hematopoietic cell transplantation and to evaluate their potential role in the development of graft versus host disease.

3. Methods

3.1. Nomenclature of HSP

HSP printed in upper case annotate the HSP family. Hsp printed in lower case denotes HSP family members. For inducible proteins, we used the terms Hsp72 (also referred as Hsp70 or HSPA1A) and Hsp70B′ (also referred as HSPA6) [11,12].

3.2. Patient characteristics

This study was approved by the institutional ethics committee of the University of Tuebingen, Germany. All patients gave their written informed consent before entering the study. Hsp72 and Hsp70B′ levels were analyzed in sera obtained from 20 subsequent patients (Table 1) undergoing allogeneic HCT, as well as from 20 healthy donors. Serum samples were obtained daily between the start of conditioning regimen and hematologic regeneration (defined as neutrophils ≥ 500/μl reached on a median day 20, range day 11 to day 28). Furthermore, 40 serum samples were obtained between days 18 and 220 during follow-up visits. As controls, 80 serum samples from 20 healthy lab donors were obtained on 4 subsequent days. Control individuals were not gender or age matched. Blood samples were allowed to coagulate for 4 h at room temperature. Serum was isolated by three centrifugation steps at 818 *g* and 4 °C, aliquoted, frozen and stored at −80 °C. Upon testing, samples were gently thawed at 4 °C overnight.

Table 1
Patient characteristics.

Patients	n = 20	
Male	n = 13	65%
Female	n = 7	35%
Median age (range)	53	(29–71)
Donor		
HLA-identical	n = 20	100%
Related	n = 7	35%
Unrelated	n = 13	65%
ATG	n = 12	60%
10 mg/kg	n = 4	20%
20 mg/kg	n = 8	40%
No ATG	n = 8	40%
Disease		
AML	n = 7	35%
MDS	n = 3	15%
MM	n = 1	5%
PMF	n = 3	15%
MF	n = 2	10%
Lymphoma	n = 4	20%

Abbreviations: ATG: anti-thymocyte globulin; AML: acute myeloid leukemia; HLA: human leukocyte antigen; MDS: myelodysplastic syndrome; MF: myelofibrosis; MM: multiple myeloma; PMF: primary myelofibrosis.

3.3. Heat shock protein detection

The first ELISA used for the detection of Hsp72 (HSPA1A) was not specific for the human protein and also detected rat and mouse derived Hsp72 (HSP70 high sensitivity EIA kit, Enzo LifeSciences, Loerrach, Germany; used were two charges produced 2011 and 2015, respectively), and had a cross-reactivity of 5.4% for Hsp70B′ (HSPA6) for which the second ELISA was specific (HSP70B′ EIA kit, Enzo LifeSciences, Loerrach, Germany; used were two charges produced 2011 and 2015, respectively). Results presented in Fig. 1 were generated using ELISA kits produced 2011, results presented in Fig. 2 and in the supplementary figures were generated using ELISA kits produced in 2015.

Both ELISA were used according to the manufacturer's instructions: In brief, sera were diluted 1:4, 1:10 and 1:30 in sample diluent for detection in the Hsp72 ELISA, and 1:4, 1:8, 1:10 or 1:20 for detection in the Hsp70B′ ELISA. The respective dilutions were used as indicated. All samples were measured in duplicates. Absorbance was measured at 450 nm using a SPECTRA-MAX 340 ELISA reader (Molecular Device Corporation, Sunnyvale, CA, USA). Mean Hsp levels were calculated using a standard curve obtained by serial dilutions as provided with the ELISA ($R^2 \geq 0.985$). The sensitivity of the Hsp72 ELISA and of the Hsp70B′ ELISA was 90 pg/ml and 62 pg/ml, respectively, as denoted by the manufacturer.

3.4. Evaluation of therapeutic antibodies as controls

As control antibodies, anti-thymocyte globulin (ATG-Fresenius® S, Fresenius Biotech, Graefelfing, Germany), Bevacizumab (Avastin®, Roche Pharma, Grenzach-Wyhlen, Germany) and Rituximab (MabThera®, Roche Pharma, Grenzach-Wyhlen, Germany) were measured in the ELISA. Antibodies were diluted 1:100, 1:500 and 1:1000 and measured in duplicates.

3.5. Validation of the ELISA kits

For further validation of the ELISA kits, spiking and blocking experiments were performed as follows:

Recombinant Hsp72 and Hsp70B′ were added to patient's serum before and after ATG application, as well as to serum of healthy donors. HSP concentrations were based on the titration as provided with the ELISA kits. Furthermore, therapeutically applied lipids (ClinOleic 20%, Baxter Healthcare, Unterschleissheim, Germany) were added to assay buffer with recombinant HSP at 0.7 μg/ml soy oil. This concentration was based on the daily applied dose of 50 g soy normalized on 7 l of hypothesized blood volume. Also, serum samples were visually inspected for their lipid content and graded into three subdivisions: strong lipid content (hazy serum), limited lipid content (limited turbidity) and no lipid content (clear serum).

Hemolysis was visually determined and also graded into three subdivisions: strong hemolysis (red serum), limited hemolysis (reddish serum) and no hemolysis (yellow serum).

Furthermore, ATG-bearing serum of patients was added to the serum of healthy donors and measured in the ELISA. For blocking experiments, bovine serum albumin (BSA; 1% in PBS) and non-fat dry milk (5% in PBS) were added to the pre-blocked ELISA plate and incubated for 1 h at room temperature. Before measurement of samples, blocking agents were removed and plates were washed.