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Dissertation thesis

THE ROLE OF HUMAN HEAT SHOCK PROTEIN HSP70, HSP60 AND *MYCOBACTERIUM BOVIS* HSP65 IN PATHOGENESIS OF GRAFT *VERSUS* HOST DISEASE AND RHEUMATOID/JUVENILE IDIOPATHIC ARTHRITIS

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Table of contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

Å	angstrom
AA	adjuvant arthritis
17-AAG	17-N-Allylamino-17-demethoxygeldanamycin
ADCC	antibody-dependent cellular cytotoxicity
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
Akt/PKB	protein kinase B or Akt , a serine/threonine kinase
AML	acute myeloid leukemia
ANA	antinuclear antibodies
Apaf-1	apoptosis protease - activating factor - 1
APCs	antigen-presenting cells
APG	ATP-binding-domain; P: peptide-binding-domain; G: germ cell derived
Ask1	apoptosis signal-regulating kinase 1
ATP	adenosine triphosphate
BAG	nucleotide exchange factor
Bax	an apoptosis regulating protein
BCG	Bacille Calmette-Guérin
Bid	pro-apoptotic member of the Bcl-2 protein family
βME	β-Mercaptoethanol
BMMC	bone marrow mononuclear cell
CAD	caspase-activated DNAse
CCR5	chemokine (C–C motif) receptor 5
CCT	chaperonins containing TCP-1
CD	cluster of differentiation
CHIP	carboxyl-terminus of HSP70 Interacting Protein
CIA	collagen-induced arthritis

CML	chronic myeloid leukemia
CMML	chronic myelomonocytic leukemia
CMV	cytomegalovirus
CNBr	cyanogen bromide
CTL	cytotoxic T lymphocyte
Daxx	a mediator of Fas-induced apoptosis
DCs	dendritic cells
DNA	deoxyribonucleic acid
ELISA	enzyme -linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
FAB	The French-American-British classification
FACS	fluorescence activated cell sorting
Fas	a member of the tumor necrosis factor (TNF) receptor superfamily
GAD65	glutamic acid decarboxylase
GM-CSF	granulocyte-macrophage colony-stimulating factor
Grp	glucose regulated protein
GvHD	Graft-*versus*-Host Disease
Hif1	a hypoxia-inducible factor 1
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HOP	the HSP70/HSP90 Organizing Protein
HPV	human papillomavirus
HS	heat shock
HSCT	hematopoietic stem cell transplantation
HSE	heat shock element
HSF	heat shock factor

HSPs	heat shock proteins
HspBP1	nucleotide exchange factor
HSV	herpes simplex virus
IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILAR	International League of Associations for Rheumatology
JCA	juvenile chronic arthritis
IDDM	insulin-dependent diabetes mellitus
JIA	juvenile idiopathic arthritis
JNK	c-Jun N-terminal kinase
JRA	juvenile rheumatoid arthritis
kDa	kilodalton
LDL	low density lipoprotein
LPS	lipopolysaccharide
LRP1	lipoprotein receptor-related protein 1
MB, *M. bovis*	*Mycobacterium bovis*
MCP-1	monocyte chemotactic protein-1
MDS	myelodysplastic syndrome
MHC	major histocompatibility complex
MKBP	myosin dystrophy binding protein
MIP-1	macrophage inflammatory protein 1
MMP	matrix metalloproteinase
MRP	multidrug resistance associated protein
MS	multiple sclerosis
M. tuberculosis	*Mycobacterium tuberculosis*

MyoD	a protein with a key role in regulating muscle differentiation
NF-kB	nuclear factor-kB
NHLs	non-Hodgkin's lymphomas
NK cells	natural killer cells
NO	nitric oxide
NOD	non-obese diabetic
OD	optical density
OxLDL	oxidized low density lipoprotein
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PBSCT	peripheral blood stem cell transplantation
Pgp	the drug-resistance related protein
PIA	pristane-induced arthritis
RA	refractory anemia
RA	rheumatoid arthritis
RAEB	refractory anemia with excess blasts
RAEB-T	refractory anemia with excess blasts in transformation
Raf-1	a serine/threonine-specific kinase
RANTES	member of the interleukin-8 superfamily of cytokines
(RANTES: Regulated upon Activation, Normal T-cell Expressed, and Secreted)	
RARS	refractory anemia with ringed sideroblasts
RF	rheumatoid factor
rh	recombinant human
RIP	receptor interacting protein
SC	synovial cell
SDS	sodiumdodecyl sulphate

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	shared epitope
SF	skin fibroblast
sHSPs	small heat shock proteins
SIV	simian immunodeficiency virus
SLE	systemic lupus erythematosus
SR-A	scavenger receptor class A
TCP-1	t-complex polypeptide 1
TGF	transforming growth factor
T_H	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR-1	tumor necrosis factor receptor 1
UV	ultraviolet
VEGF	vascular endothelial growth factor
WB	Western blotting

Chapter 1: Introduction

Heat shock proteins are extremely versatile and potent molecules, the importance of which to biological processes is highlighted by the high degree to which their structure and function are phylogenetically conserved. Our knowledge of the physiological role of heat shock proteins is currently limited; however, a better understanding of their function and thereby the acquisition of the capacity to harness their power might lead to their use as therapeutic agents and revolutionize clinical practice in a number of areas.

Chapter 2: Basic characteristics of heat shock proteins

2.1 History of heat shock proteins

In 1962 Ritossa and co-workers first discovered the heat shock (HS) response while observing the salivary cells of Drosophila melanogaster (Ritossa et al., 1962). It was noted that heating of these cells induced puffs forming at various regions of the polytene chromosomes. Further analysis revealed that these puffs were actually areas of localized transcription that correlated with the increase of several families of proteins. This response was termed "heat shock response" because heat shock was the most common inducer. However, until 1974 the first products of these genes were identified and the term "heat shock protein" was adopted (Tissieres et al., 1974).
Heat shock proteins are present, and can be induced in all species. They are among the most phylogenetically conserved proteins (Pockley, 2001).

2.2 Classification of heat shock proteins

The heat shock proteins (HSPs) are classified into different families based on their molecular mass in kilodaltons (kDa): small HSPs, HSP60 or chaperonins, HSP70, HSP90 and HSP110. No uniform system of naming stress proteins and genes has been adopted but several helpful conventions are in broad use. The fully capitalized names denote an entire protein family, eg HSP70. The gene encoding HSP70 is designated *hsp70*. Where possible, the inducible members of a stress protein family such as Hsp70 should be distinguished from constitutively expressed or cognate members such as Hsc70. (http://web.uconn.edu/cscedoffice/authors.html)

2.3 Expression of heat shock proteins

Each family of HSPs is composed of members expressed either constitutively or regulated inductively and are targeted to different subcellular compartments. For instance, while Hsp90 is constitutively abundantly expressed in the cells, Hsp70 and Hsp27 are highly induced by different stresses such as heat, oxidative stress, or anticancer drugs (Schmitt et al., 2007).

A marked increase in HSP synthesis, known as the stress response, is induced by a variety of stressful situations including heat shock, environmental (ultraviolet radiation or heavy metals), pathological (infection or malignancies) or physiological (growth factors or cell differentiation) stimuli (Jaattela, 1999; Lindquist and Craig, 1988).

Regulation of heat shock protein gene transcription is mediated by the interaction of the transcription factor (heat shock factor, HSF) with heat shock elements (HSEs). The heat shock response is one of the most potent inducers of gene expression. This gene iduction is achieved at different levels. The primary mechanism is the activation of HSFs, which bind to HSEs present upstream to all heat shock genes, to initiate transcription. All conditions capable of inducing HSPs do so by activating HSFs. Two HSFs exist in humans, HSF1 and HSF2. HSF1 is activated by heat shock and other stresses. HSF2 is to be activated during embryonic development and cell differentiation. In unstressed cells, HSF1 is present as a non-active form, which has DNA-binding activity. Stress-induced activation of HSF1 involves phosphorylation, oligomerization and redistribution within the nucleus. In contrast to HSF1, HSF2 is not changed in cells under stressful conditions. In vivo, HSF2 is known to be active as a trimer during embryonic development and spermatogenesis. In addition, HSF2 has a role in the differentiation of multipotential hematopoietic cells into erythroid lineage precursors (Thomas et al., 2005-1).

2.4 General functions of heat shock proteins

2.4.1 Heat shock proteins as molecular chaperones

Molecular chaperones are defined as "proteins that assist the correct non-covalent assembly of other proteins-containing structures in vivo but are not permanent components of these structures when they are performing their normal biological functions "(Ellis et al., 1996).

It has been shown that most HSPs have strong cytoprotective effects and behave as molecular chaperones for other cellular proteins. Small heat shock protein Hsp27 functions as chaperone in protection against protein aggregation (Schmitt et al., 2007). HSP60 competes in the pathway of protein misfolding and aggregation (Fenton and Horwich, 2003). HSP70 transports proteins between cellular compartments, degradates unstable and misfolded proteins, prevents and dissolves protein complexes, folds and re-folds proteins and controls regulatory proteins (Daugaard et al., 2007). HSP90 acts as molecular chaperone essential for activating many signaling proteins in the eukaryotic cell (Terasawa et al., 2005). HSP110 binds to unfolded proteins and prevents their aggregation (Oh et al., 1999).

Inappropriate activation of signaling pathways could occur during acute or chronic stress as a result of protein misfolding, protein aggregation, or disruption of regulatory complexes. The action of chaperones, through their properties in protein homeostasis, is thought to restore balance (Schmitt et al., 2007).

2.4.2 Heat shock proteins and extracellular functions

HSPs can also have an extracellular location. HSP70 has been found externally expressed, bound to the plasma membrane. HSPs like HSP70, HSP90 have been found in the extracellular medium. Several mechanisms may account for the cellular release of HSPs, including the necrosis of the cells. The function of extracellular HSPs is immunogenic through the chaperoning of antigenic peptides (Schmitt et al., 2007) or

extracellular HSPs (regardless of chaperoned peptides) can induce the secretion of inflammatory cytokines by antigen-presenting cells (APCs) (Binder et al., 2004). More details about extracellular functions of HSPs will be in chapter 3.

2.4.3 Heat shock proteins are involved in antigen presentation

2.4.3.1 HSPs and antigen presentation by major histocompatibility complex (MHC) class I molecules

HSPs chaperone antigenic peptides that are generated within cells. Such chaperoning is a part of the endogenous pathway of antigen presentation by major histocompatibility complex (MHC) class I molecules. HSPs such as HSP70, HSP90 and HSP110 associate with a broad array of peptides generated within cells. These peptides include normal self-peptides as well as antigenic peptides derived from antigens expressed by the cells. The antigenic peptides may be tumor antigens, bacterial antigens, viral antigens or minor histocompatibility antigens. There are two suggestions with respect to the role of HSP-peptide complexes either within the cell in which they are generated or outside it: **first** ("the inside story"), the chaperoning of peptides by the HSPs of the cytosol (HSP70, HSP90 and HSP110) and of the endoplasmic reticulum (ER) (gp96, a mem ber of HSP90 family) is a mechanism for their journey to the MHC class I molecules of the cells in which the HSP-peptide complexes are formed; **second** ("the outside story"), HSP-chaperoned peptides exposed on the cell surface or released from the cells, due to stress or cell death, are taken up by the surrounding APCs, resulting in re-presentation (cross-presentation) of the peptides by MHC class I molecules on the APCs (Li et al., 2002).

2.4.3.2 *HSPs and antigen presentation by major histocompatibility complex (MHC) class II molecules*

In contrast to MHC class I, the role for HSP: peptide complexes in the MHC class II pathway is less clear, although several findings indicate that HSP: peptide complexes also facilitate the presentation of MHC class II-restricted epitopes. A role for an involvement of members of the HSP70 family in the MHC class II processing and presentation pathway was first implicated by DeNagel and Pierce (1992). It was shown that overexpression of the constitutively expressed Hsc73 in a macrophage cell line led to an increased presentation of exogenous antigen via MHC class II (Panjwani et al., 1999). Moreover, DnaK (*Escherichia coli* HSP70) was found to enhance the processing and presentation of exogenous antigen to a human CD4+ T cell clone, whereas the effect was dependent on the allelic variants of human leukocyte antigen HLA-DR (Roth et al., 2002). A direct interaction between HLA-DR-derived peptide fragments and HSP70 molecules was demonstrated by Maier et al. (2002), showing that only peptide fragments derived from HLA-DR molecules associated with protection from rheumatoid arthritis (RA) did not bind to HSP70. In a murine model, gp96: peptide complexes facilitated the in vivo presentation of an MHC class II-restricted epitope, but the secretion of effector cytokines by the CD4+ T cells could not be detected (Doody et al., 2004). Furthermore, an involvement of self-inducible Hsp70 in MHC class II-dependent autoantigen processing was reported by Mycko et al. (2004), and recent data indicate that bacterial HSP70 facilitates the processing and presentation of antigenic peptides by MHC class II (Tobian et al., 2004). However, most of these studies have been done in murine systems or with human T cell clones.

Recently, Haug et al. (2005) investigated the potential of antigenic peptides from tetanus toxin and influenza hemagglutinin complexed to the human stress-inducible Hsp70 to enhance activation and proliferation of human memory CD4+ cells. Hsp70:

peptide complexes were found to amplify the proliferation of antigen-specific CD4+ T cells.

2.4.4 Heat shock proteins act as inhibitors of apoptosis

Cell death can occur in two main forms, apoptosis and necrosis. Apoptosis is formally defined by the apprearance of distinct morphologic changes, including membrane blebbing, chromatin condensation, and fragmentation of the nucleus. Considered as a "physiologic cell suicide" program, apoptosis is involved in embryonic development, tissue homeostasis, and immune system function. Disturbances in apoptosis contribute to numerous diseases, including cancer (Aghdassi et al., 2007).
Mediated by a family of cysteine proteases known as caspases, two distinct pathways characterize apoptotic processes. The extrinsic pathway is triggered by death receptor, such as Fas or the tumor necrosis factor (TNF) receptor with their respective ligands, resulting in caspase-8 activation. In response to growth factor withdrawal, hypoxia, or DNA damage, the intrinsic pathway is initiated, resulting in cytochrome c release, loss of mitochondrial membrane potential, and the apoptosome formation, a complex consisting of cytochrome c, apoptotic protease-activating factor-1 (Apaf-1), and procaspase-9. Both pathways converge at the level of caspase-3, an effector caspase that leads to the typical morphologic and biochemical changes of the apoptotic cell. Tumor cells express several proteins that suppress apoptosis. Among them, the anti-apoptotic members of the Bcl-2 ([1]) protein family, members of the inhibitor of apoptosis protein family, and heat shock proteins play a major role (Aghdassi et al., 2007).

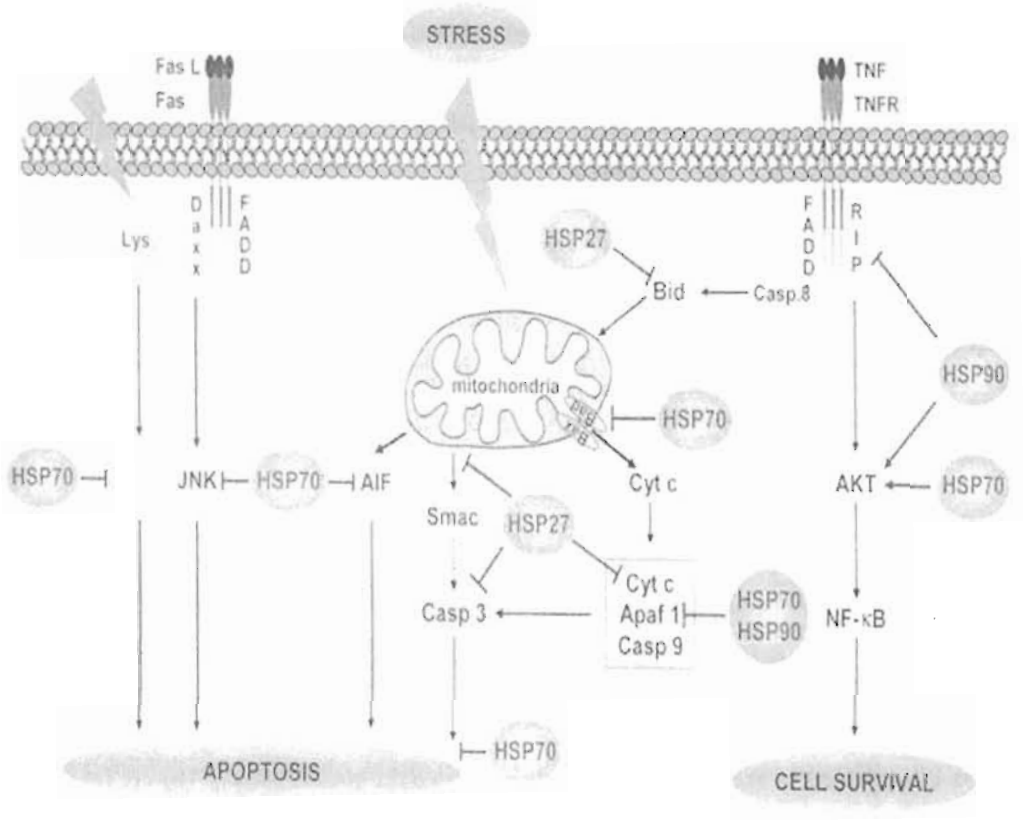
([1]) Bcl-2 is the prototype for a family of mammalian genes and the proteins they produce. The sub-families and their members are: Bcl-2 subfamily (pro-survival): Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1; Bax subfamily (pro-apoptotic): Bax, Bak and Bok; BH3 subfamily (pro-apoptotic): Bad, Bid, Bik, Blk, Hrk, BNIP3 and Bim. In humans, BCL-2 is a proto-oncogene located on chromosome 18. The gene was discovered as the translocated locus in a B-cell leukemia (hence the name). This translocation is also found in some B-cell lymphomas. Its product is an integral membrane protein (called Bcl-2) located in the membranes of the endoplasmic reticulum (ER), nuclear envelope, and in the

outer membranes of the mitochondria. The Bcl-2 protein suppresses apoptosis by preventing the activation of the caspases that carry out the process.

Heat shock proteins contribute to the tight regulation of the apoptotic process. They are involved in regulation of both extrinsic and intrinsic pathways. The small heat shock protein Hsp27, stress-inducible Hsp70 and Hsp90 have been shown to act as inhibitors of apoptosis (Schmitt et al., 2007). Figure 1 shows how HSPs are contributed in apoptotic pathways. Concerning the intrinsic (mitochondrial) pathway, at the mitochondrial level, Hsp27, through Bid, and Hsp70, by inhibiting Bax, inhibit the mitochondrial release of proapoptotic proteins. At the postmitochondrial level, Hsp27 binds to cytochrome c, Hsp70 and Hsp90 bind to Apaf-1 in all cases resulting in the inhibition of apoptosome formation and thereby prevention of caspase activation and apoptosis. Regarding extrinsic pathway, Hsp27 can interact with and inhibit Daxx apoptotic pathway whereas Hsp70 bind to JNK 1, resulting in inhibition of JNK activation. Hsp90 interacts with RIP1 kinase and Akt, resulting in both cases in promotion of NF-kB-mediated inhibition of apoptosis.

Figure 1: Modulation of apoptotic pathways by HSPs

(Schmitt et al., 2007)



Explanatory notes: ***AIF**: apoptosis-inducing factor; **Akt**: protein-kinase B; **Apaf-1**: apoptotic protease-activating factor-1; **Bax**: an apoptosis regulating protein; **Bid**: pro-apoptotic member of the Bcl-2 protein family; **Casp**: caspase; **Daxx**: a mediator of Fas-induced apoptosis; **Cyt c**: cytochrome c; **NF-kB**: Nuclear factor-kB, a ubiquitous transcription factor that controls the expression of genes involved in immune responses, apoptosis, and cell cycle. **JNK**: c-Jun N-terminal kinase; **RIP**: receptor interacting protein.*

The involvement of each HSP in apoptosis will be described in details in next section.

2.5 Heat shock protein families

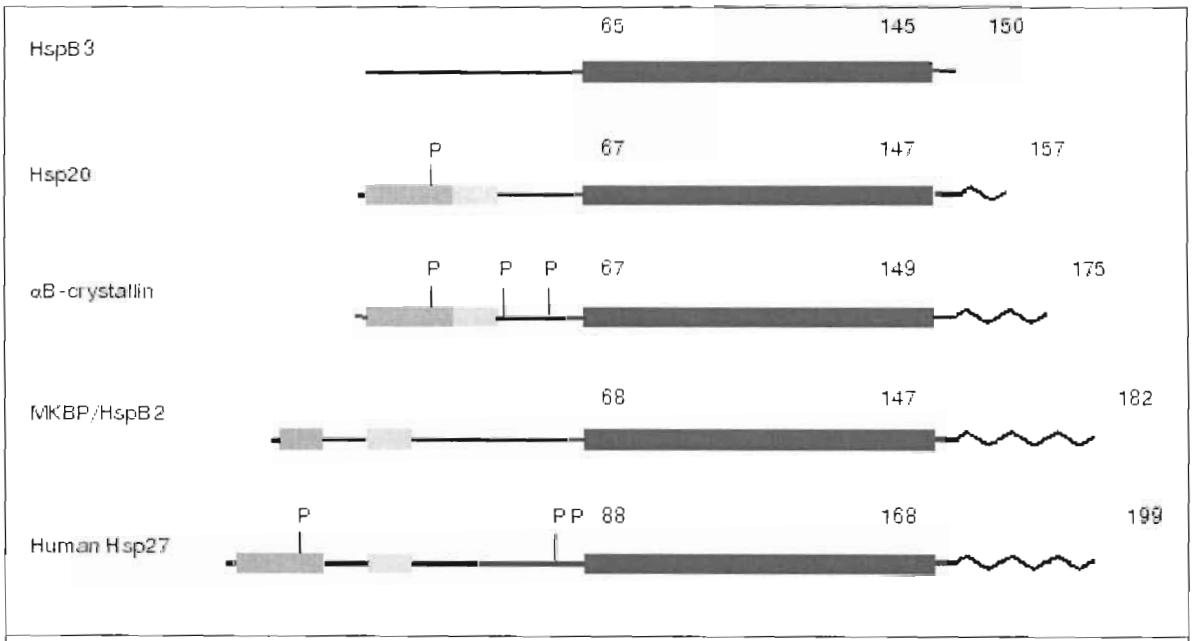
2.5.1 Small HSPs

The group of small heat shock proteins (sHSPs) combines proteins with molecular weight in the range of 12 up to 43 kDa that were isolated from archaea, bacteria, plants, and animals. It is supposed that vertebrates synthesize five major classes of sHSPs. These are α-A-crystallin and α-B-crystallin; sHSPs with molecular weight 25-27 kD (Hsp25/27); sHSPs with molecular weight 20 kD (Hsp20); sHSPs with molecular weight 17 kD (HspB3); and so-called activator of myotonic dystrophy protein kinase (MKBP, myosin dystrophy binding protein) that sometimes is designated as HspB2. Recently a new class of sHSPs with molecular weight of 22 kD was described in the literature (Gusev et al., 2002).

Small HSPs have some structural features in common: very characteristic is a homologous and highly conserved amino acid sequence, the so-called α-crystallin domain at the C-terminus. These sequences consist of 80 to 100 residues with a homology ranging from 20% (between remote members of sHSPs isolated from bacteria and mammals) to 60% (between closely related mammalian sHSPs) and form β-sheets, which are important for the formation of stable dimmers. The N-terminus consists of a less conserved region, the so-called WDPF domain, followed by a short variable sequence with a rather conservative site near the C-terminus of this domain. The C-terminal part of the sHSPs consists of the above mentioned α-crystallin domain, followed by a variable sequence with high mobility and flexibility (Figure 2) (Gusev et al., 2002).

Small HSPs tend to form large oligomeric complexes. Oligomers of mammalian sHSPs are very flexible, and this is probably the reason for the lack of X-ray crystallographic data for mammalian sHSP (Gusev et al., 2002).

Figure 2: Scheme of the structure of several representatives of the family of small heat shock proteins (Gusev et al., 2002)



The dark shaded area marks the so-called WDPF-domain, the light shaded area marks conservative region in the N-terminal part of sHSP and the black area denotes conservative α-crystallin domain. P, the sites of phosphorylation; zigzag, flexible C-terminal region.

Small HSPs display chaperone function in vitro, and in addition it has been suggested that they are involved in the inhibition of apoptosis, organization of the cytoskeleton and establishing the refractive properties of the eye lens in the case of α-crystallin (Haslbeck, 2002).

Currently, human Hsp27 is between the most well studied sHSPs. Human Hsp27 is encoded by a gene family of four members mapped at chromosome 7q; three genes have promoter elements and are heat-inducible, while one other is a pseudogene lacking promoter elements (Sarto et al., 2000). Hsp27 appears in many cell types, especially all types of muscle cells. It is located mainly in cytosol, but also in the perinuclear region,

ER and nucleus. It is overexpressed during different stages of cell differentiation and development.

Hsp27 has been shown to interact and inhibit components of both apoptotic pathways. Overexpressed Hsp27 protects against apoptotic cell death trigerred by various stimuli, including hyperthermia, oxidative stress, staurosporine (an inhibitor of kinase proteins), ligation of the Fas death receptor, and cytotoxic drugs (Schmitt et al., 2007). The phosphorylated form of Hsp27 inhibits Daxx (a mediator of Fas-induced apoptosis) and prevents the association of Daxx with Fas and Ask1 (apoptosis signal-regulating kinase 1) (Charette et al., 2000). Hsp27 interacts with the outer mitochondrial membranes and interferes with the activation of cytochrome c/Apaf-1/dATP complex (the apoptosome) and therefore inhibits the activation of procaspase-9 (Sarto et al., 2000).

2.5.2 HSP60 or chaperonins

The HSP60 family has been found in all bacteria, chloroplasts, mitochondria and cytoplasm of eukaryotes and the amino acid sequence among its members has been conserved at approximately 50% (Becker and Craig, 1994). This family can be divided into 2 groups: **group 1** includes mitochondrial Hsp60 of eukaryotes, Hsp65 of mycobacteria (eg *Mycobacterium tuberculosis* and *Mycobacterium bovis*), GroEL (HSP60 of *Escherichia coli*), and ribulose-1, 5-bisphosphate carboxylase/ oxygenase (Rubisco) subunit binding protein of plastid (major organelle found in plants and algae) ; **group 2** involves cytosolic CCT (chaperonins containing TCP-1 - t-complex polypeptide 1) of eukaryotes and chaperonins of archaea (Yokota et al., 2000).

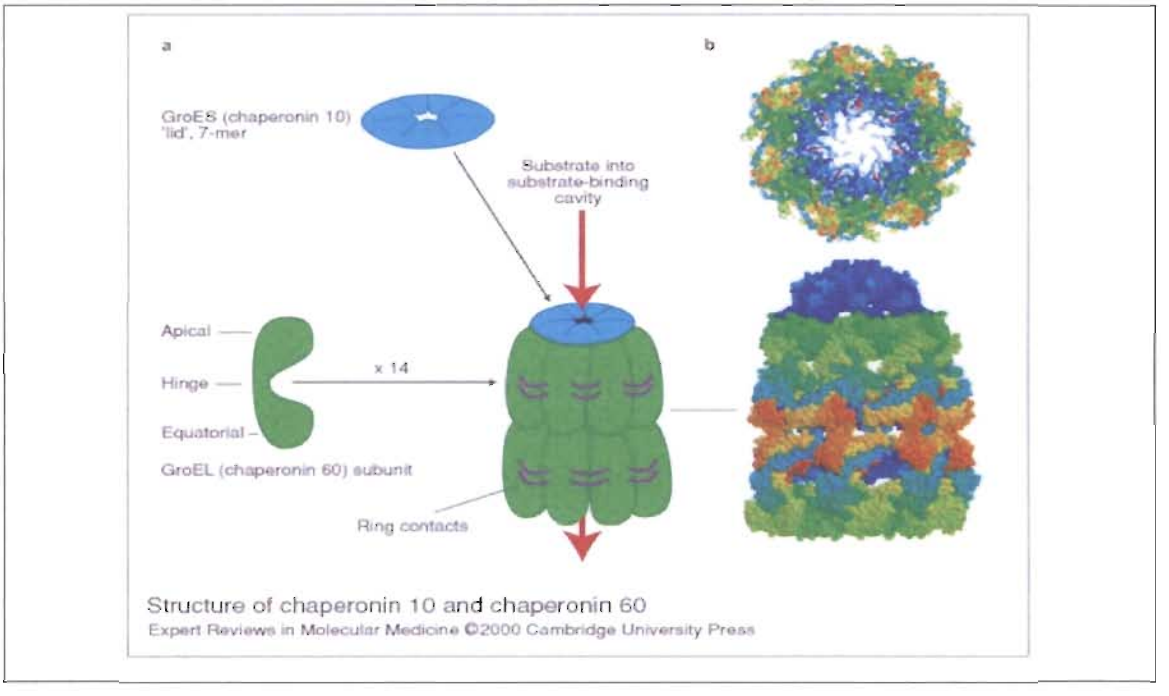
HSP60 is slightly taller than it is wide with a hydrophobic central cavity or channel that may be used to link polypeptides (Braig et al., 1994). HSP60 structure consists of a porous cylinder of 14 subunits made of two nearly 7-fold rotationally symmetrical rings stacked back-to-back with dyad symmetry (Figure 3).

Each HSP60 subunit consists of three domains:

- A large equatorial domain that form the foundation of the assembly and the waists of the domains hold the rings together
- A large loosely structured apical domain that forms the end of the cylinder
- A small slender intermediate domain that connect the two

Figure 3: 3-Dimensional image of HSP60 protein structure

(The picture source: http://www-ermm.cbcu.cam.ac.uk/00002015h.htm)



The intermediate domain induces a conformational change when ATP is bound allowing for an alternation between the hydrophilic and hydrophobic substrate binding sites. In its inactive state, the protein is in a hydrophobic state. When activated by ATP, the intermediate domain undergoes a conformational change that exposes the hydrophilic region. This insures fidelity in protein binding. Chaperonin 10 (Hsp10 in human and GroES in *Escherichia coli*) aids HSP60 in folding by acting as a dome-like

cover on the ATP active form of HSP60. This causes the central cavity to enlarge and aids in protein folding (Ranford et al., 2000).

In humans, it was demonstrated that two genes *hsp60* and *hsp10* are localised head to head on chromosome 2 separated by a bidirectional promoter. The transcriptional activity of the promoter fragment in the *hsp60* direction is increased approximately 12 fold upon heat-shock (Hansen et al., 2003).

HSP60 constitutes approximately 15-30% of all cellular proteins (Ranford et al., 2000). In additional to HSP60's typical role as HSPs, studies have shown that HSP60 plays an important role in the transport and maintenance of mitochondrial proteins as well as the transmission and replication of mitochondrial DNA.

HSP60 possesses two main responsibilities with respect to mitochondrial protein transport. It functions to catalyze the folding of proteins destined for the matrix and maintain protein in an unfolded state for transport across the inner membrane of the mitochondria. Many proteins are targeted for processing in the matrix of the mitochondria but then are quickly exported to other parts of the cells. The hydrophobic portion of HSP60 is responsible for maintaining the unfolded conformation of the protein for transmembrane transport. Studies have shown how HSP60 binds to incoming proteins and induces conformational and structural changes. Subsequent changes in ATP concentrations hydrolyze the bonds between the protein and HSP60 which signals the protein to exit the mitochondria. HSP60 is also capable of distinguishing between proteins designated for export and proteins destined to remain in the mitochondrial matrix by looking for an amphilic alpha-helix of 15-20 residues (Koll et al., 1992). The existence of this sequence signals that the protein is to be exported while the absence signals that the protein is to remain in the mitochondria. The precise mechanism is not yet entirely understood.

In addition to its critical role in protein folding, HSP60 is involved in the replication and transmission of mitochondrial DNA. It has been proposed that HSP60 binds

preferentially to the single stranded template DNA strand in a tetradecamer like complex. This tetradecamer complex interacts with other transcriptional elements to serve as a regulatory mechanism for the replication and transmission of mitochondrial DNA (Kaufman et al., 2003). Mutations in HSP60 increase the levels of mitochondrial DNA and results in subsequent transmission defects.

2.5.3 HSP70

The HSP70s are found in most, if not all, cellular compartments of eukaryotes, including nuclei, mitochondria, chloroplasts, endoplasmic reticulum and the cytosol, as well as in all bacteria examined so far (Becker and Craig, 1994). This class of proteins has been universally conserved with at least 50% identity among its members. It consists of at least 8 chaperone proteins that differ from each other by their pattern of expression and intracellular localization: **Bip** (in ER), **mtHsp70** (in mitochondrial matrix), **Hsc70** (the only cytosolic HSP70 that is abundantly and ubiquitously expressed in all cells), **Hsp70t** (expressed at high levels in testis), **Hsp70-2** (expressed at lower or non-detectable levels in ovary, small intestine, colon, brain, placenta, kidney), **Hsp70-1A** and **Hsp70-1B** (collectively reffered to as Hsp70-1, that is expressed at low or non-detectable levels in most unstressed normal cells, but its expression is rapidly induced upon a variety of physical and chemical stresses; furthermore, its expression is high in several cancers, where it is often found in the plasma membrane and the inner leaflet of the membranes of the endo-lysosomal compartment), finally, **Hsp70-6** represents a poorly studied intronless member of the family that shows almost no basal expression and is induced only by extreme stresses (Daugaard et al., 2005).

Table 1 shows human *hsp70* genes and their encoded proteins.

Table 1: Human heat shock protein 70 genes (Daugaard et al., 2005)

Locus	Position	Protein name(s)	Homology (*)	Cellular localization	Notes
HSPA 1A	6p21. 3	Hsp70-1(A), Hsp70	100%	Cytosol, nucleus, membranes	Intronless; MHC-linked; stress-inducible
HSPA 1B	6p21. 3	Hsp70-1(B) Hsp70	99%	Cytosol, nucleus, membranes	Intronless; MHC-linked; stress-inducible
HSPA 1L	6p21. 3	Hsp70-t, Hsp70-Hom	91%	Cytosol	Intronless; MHC-linked; constitutively expressed at high levels in testis, at very low levels in other tissues
HSPA8	11q23.3-q25	Hsc70, Hsp73	86%	Cytosol, nucleus, lysosomes	House-keeping protein; constitutively expressed in most tissues
HSPA2	14q24.1	Hsp70-2	84%	Cytosol, nucleus	Constitutively expressed at high levels in testis and brain, at very low levels in other tissues
HSPA6	1cen-qter	Hsp70-6	84%	Cytosol, nucleus	Intronless; strictly stress-inducible

Table 1: Human heat shock protein 70 genes (Daugaard et al., 2005)

(Continued)

Locus	Position	Protein names	Homology (*)	Cellular localization	Notes
HSPA5	9q33-q34.1	Bip, Grp78	64%	Endoplasmic reticulum	House-keeping protein; constitutively expressed; compartment-specific protein
HSPA9	5q31.1 PBP74, Mot-2	mtHsp70, Grp75	52%	Mitochondria	House-keeping protein; constitutively expressed

* Amino acid homology to the protein encoded by HSPA 1A

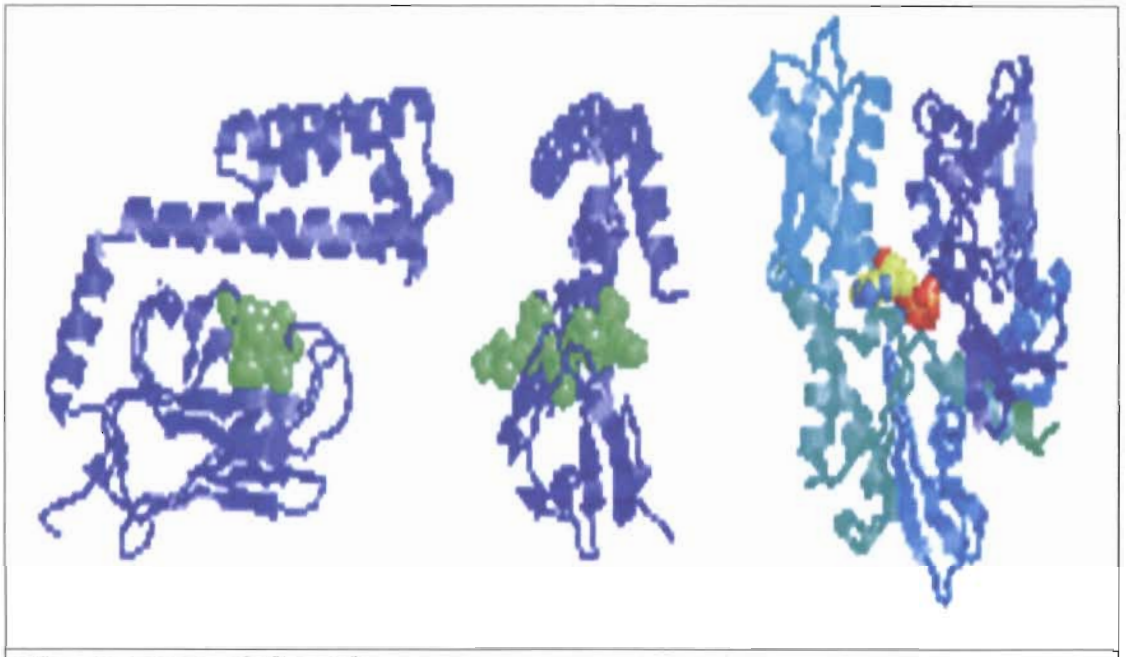
All of the HSP70 proteins have three major functional domains.

- An N-terminal ATPase domain binds ATP (adenosine triphosphate) and hydrolyzes it to ADP (adenosine diphosphate). The exchange of ATP drives conformational changes in the other two domains
- A substrate binding domain contains a groove with an affinity for neutral, hydrophobic amino acids residues. The groove is long enough to interact with peptides up to seven residues in length
- A C-terminal domain rich in alpha helical structure acts as a "lid" for the substrate binding domain. When HSP70 is ATP bound, the lid is open and peptides bind and release relatively rapidly. When HSP70 proteins are ADP bound the lid is closed, and peptides are tightly bound to the substrate binding domain.

(Wegele et al., 2004)

Figure 4: The substrate-binding domain of HSP70

(The picture source: http://people.cryst.bbk.ac.uk/~ubcg16z/hsplec.html)



The structure of the substrate binding domain of the *Escherichia coli* HSP70 protein DnaK (front and side views, left and center), with a bound peptide (green) in a channel penetrating right through the DnaK domain. On the right is the ATPase domain of another member of the HSP70 family, Hsc70. The ATP (space filling) binding site is in a cleft. The ATPase domain structure is homologous to those of actin and hexokinase.

When not interacting with a substrate peptide, HSP70 is usually in an ATP bound state. HSP70 by itself is characterized by a very weak ATPase activity, such that spontaneous hydrolysis will not occur for many minutes. As newly synthesized proteins emerge from the ribosomes, the substrate binding domain of HSP70 recognizes sequences of hydrophobic amino acids residues, and interacts with them. This spontaneous interaction is reversible, and in the ATP bound state HSP70 may relatively freely bind and release peptides.

However, the presence of a peptide in the binding domain stimulates the ATPase activity of HSP70, increasing its normally-slow rate of ATP hydrolysis. When ATP is hydrolyzed to ADP, the binding pocket of HSP70 closes, tightly binding the now-trapped peptide chain. Further speeding ATP hydrolysis enable cochaperones: primarily Hsp40 in eukaryotes, and DnaJ in prokaryotes. These so-called J-domain cochaperones dramatically increase the ATPase activity of HSP70 in the presence of interacting peptides.

By binding tightly to partially-synthesized peptide sequences (incomplete proteins) HSP70 prevents them from aggregating and being rendered nonfunctional. Once the entire protein is synthesized, a nucleotide exchange factor (BAG-1 and HspBP1 are among those which have been identified) stimulates the release of ADP and binding of fresh ATP, opening the binding pocket. The protein is then free to fold on its own, or to be transferred to other chaperones for further processing. HOP (the HSP70/HSP90 Organizing Protein) can bind to both HSP70 and HSP90 at the same time, and mediates the transfer of peptides from HSP70 to HSP90.

HSP70 also aids in transmembrane transport of proteins, by stabilizing them in a partially-folded state.

HSP70 proteins can act to protect cells from thermal or oxidative stress. These stresses normally act to damage proteins, causing partial unfolding and possible aggregation. By temporarily binding to hydrophobic residues exposed by stress, HSP70 prevents these partially-denatured proteins from aggregating, and allows them to refold.

HSP70 seems to be able to participate in disposal of damaged or defective proteins. Interaction with CHIP (Carboxyl-terminus of HSP70 Interacting Protein) - an E3 ubiquitin ligase - allows HSP70 to pass proteins to the cell's ubiquitination and proteolysis pathways (Wegele et al., 2004).

Panoply of stimuli, including mild hyperthermia, anoxia, UV irradiation, or anticancer agents, induces the synthesis of stress-inducible Hsp70 (HSP70-1), which enhances the ability of the cell to survive those otherwise lethal conditions. Induced Hsp70 expression block the apoptotic pathway at different levels. At a pre-mitochondrial stage Hsp70 inhibits p53 (2), it also binds to and functions as inhibitor of stress activated kinases, eg c-Jun N-terminal Kinase (JNK-1); at the mitochondrial stage Hsp70 prevents mitochondrial membrane permeabilization through the blockage of Bax (an apoptosis regulating protein) translocation, and finally, at the post-mitochondrial level it interacts with AIF (apoptosis-inducing factor) and Apaf-1 (apoptosis protease – activating factor-1), thereby preventing the recruitment of procaspase-9 to the apoptosome. The binding of Hsp70 to AIF leads to AIF-induced chromatin condensation. In TNF-induced apoptosis, Hsp70 does not preclude the activation of caspase-3 but prevents downstream morphologic changes that are characteristic for dying cell. During the final phases of apoptosis, chromosomal DNA is digested by the DNAse CAD (caspase-activated DNAse), its enzymatic activity and proper folding has been reported to be regulated by Hsp70. At the death receptor level, Hsp70 can mediate Bcr-Abl (3)-mediated resistance to apoptosis in human leukemia cells. Hsp70 binds to the death receptors DR4 and DR5, thereby inhibiting TRAIL (a death ligand)-induced assembly and activity of death-inducing signaling complex (DISC) (Schmitt et al., 2007).

(2) p53, also known as tumor protein 53 is a transcription factor that regulates the cell cycle and hence functions as a tumor suppressor. It is important in multicellular organisms as it helps to suppress cancer.

(3)Bcr-Abl or the Philadelphia Chromosome: In most cases of CML, the leukemic cells share a chromosome abnormality not found in any nonleukemic white blood cells, nor in any other cells of the patient's body. This abnormality is a reciprocal translocation between one chromosome 9 and one chromosome 22. This translocation is designated t(9;22). It results in one chromosome 9 longer than normal and one chromosome 22 shorter than normal. The latter is called the Philadelphia chromosome and designated Ph[1]. The DNA removed from chromosome 9 contains most of the proto-oncogene designated c-ABL. The break in chromosome 22 occurs in the middle of a gene designated BCR. The resulting Philadelphia chromosome has the 5' section of BCR fused with most of c-ABL.

2.5.4 HSP90

The HSP90 family is highly conserved and expressed in a variety of different organisms from bacteria to mammals with sequence homology from 40% to 55%.

The human cell contains three isoforms: cytosolic inducible **Hsp90α,** cytosolic **Hsp90β** that is constitutively expressed, and the constitutive **GRP94/gp96** in the ER. The α- and the β-form are showing 85% sequence identity and functional differences between these isoforms are poorly understood (Sarto et al., 2000). Recently, a membrane associated variant of cytosolic Hsp90, lacking an ATP-binding site, has been identified and was named **Hsp90N** (Grammatikakis et al., 2002).

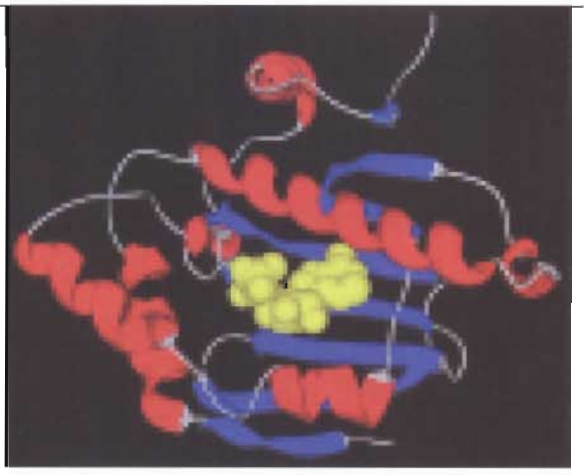
The human gene encoding the inducible α-Hsp90 is mapped to chromosome 14q32.3, and pseudogenes to chromosome segments 1q21.2-q22, 4q3.5, and 11p14.1-14.2. The constitutively expressed *hsp90β* gene is located at chromosome 6p21 and two pseudogenes are at chromosomes 4q21-q25 and 15pter-q21 (Sarto et al., 2000).

HSP90 consists of three structural domains:

- a highly conserved N-terminal domain of ~25 kDa contains the binding pocket for ATP (Stebbins et al., 1997; Prodromou et al., 1997)
- middle domain of ~40 kDa is likely to be involved in client binding (Sato et al., 2000; Fontana et al., 2002)
- a C-terminal domain of ~12 kDa is supposed to possess an alternative ATP-binding site, which becomes accessible when the N-terminal pocket is occupied (Marcu et al., 2000; Soti et al., 2002) and

a "charged linker" region , that connects the N-terminus with the middle domain (Pearl et al., 2000; Prodromou et al., 2003)

Figure 5: Structure of the N-terminal ATP-binding domain of yeast HSP90 complexed with ADP (Prodromou et al., 2003)



The domain consists of a twisted eight-stranded β-sheet with a cluster of α-helices arranged on top of it. The nucleotide (yellow) is bound in an unusual kinked conformation in a deep pocket formed by the surrounding helices and loops. This pocket also constitutes the binding site of the antitumor and antibacterial drug geldanamycin (Stebbins et al., 1997).

The ATPase binding region of HSP90 is currently under a great degree of study, because of the interest of its role in cancer and protein maintenance. This area of the protein is near the N-terminus and has a high affinity site to bind ATP at an uncharacteristically bent manner compared to other proteins, thus, tumor related experiments involving this section of HSP90 are commonly conducted with an antitumor and antibacterial drug geldanamycin (Goetz et al., 2003). More information about geldanamycin will be in chapter 6.

HSP90 associates with a number of signaling proteins including ligand-dependent transcription factors such as steroid receptor, ligand-independent transcription factors such as MyoD (a protein with a key role in regulating muscle differentiation), tyrosine

kinases such as v-Src ([4]), and serine/threonine kinases such as Raf-1, which functions in the MAPK/ERK signal transduction pathway as part of a protein kinase cascade. The main chaperone role of HSP90 is to promote the conformational maturation of these receptors and signal-transducing kinases (Schmitt et al., 2007).

([4]) Some tumor-inducing retroviruses eg Rous sarcoma virus - RSV contain a gene called v-src; it was found that the v-src gene in RSV is required for the formation of cancer.

HSP90 seems to have different molecular partners involved in apoptosis. Concerning the intrinsic pathway, it was reported that HSP90 inhibits apoptosis as a result of a negative effect on apoptosis protease-activating factor 1 (Apaf-1) function. The anti-apoptotic action of HSP90 is also reflected by its capacity to interact with phosphorylated serine/threonine kinase Akt/PKB, a protein that generates a survival signal in response to growth factor stimulation. Phosphorylated Akt can phosphorylate the Bcl-2 family protein Bad and caspase-9, leading to their inactivation and to cell survival.

In extrinsic apoptotic pathway, HSP90 has been shown to interact with and stabilize the receptor interacting protein (RIP). Upon ligation to TNFR-1 (tumor necrosis factor receptor 1), RIP-1 is recruited to the receptor and promotes the activation of NF-kB ([5]) and JNK (c-Jun N-terminal kinase). Degradation of RIP-1 in the absence of HSP90 precludes activation of NF-kB mediated by TNF-α and sensitizes cells to apoptosis.

([5]) NF-κB (nuclear factor-kappa B) is a protein complex which is a transcription factor. NF-κB is found in all cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens. NF-κB plays a key role in regulating the immune response to infection.

Another pathway of cell survival in which HSP90 can be involved implied p53. Other client proteins of HSP90 through which this chaperone could participate in cell survival are the transcription factors Her2 ([6]) and Hif1a (hypoxia-inducible factor 1) (Schmitt et al., 2007).

([6]) Her2/neu (also known as ErbB-2) is a member of the ErbB protein family, more commonly known as the epidermal growth factor receptor family. Her2/neu is notable for its role in the pathogenesis of breast cancer and as a target of treatment. It is a cell membrane surface-bound receptor tyrosine kinase and is normally involved in the signal transduction pathways leading to cell growth and differentiation.

HSP90 is inevitable for induction of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase eNOS ([7]) (Fontana et al., 2002). Both are important for *de novo* angiogenesis that is required for tumor growth beyond the limit of diffusion distance of oxygen in tissues (Calderwood et al., 2006). It also promotes the invasion step of metastasis by assisting the matrix metalloproteinase MMP2 (Eustace et al., 2004).

([7]) eNOS or NOS3 generates NO in blood vessels and is involved with regulating vascular function. That constitutive Ca^{2+} dependent NOS provides a basal release of nitric oxid NO. eNOS is associated with plasma membranes surrounding cells and the membranes of Golgi bodies within cell.

Finally one can say that HSP90 participates in many key processes in oncogenesis such as self-sufficiency in growth signals, stabilization of mutant proteins, angiogenesis, and metastasis. Targeting HSP90 with drugs like the geldanamycin derivative 17-N-Allylamino-17-demethoxygeldanamycin (17-AAG) has shown promising effects in clinical trials.

2.5.5 HSP110

Several reports described large members of the HSP70 family that were far more diverged in sequence than any of the previously described HSP70 proteins. Cloning mammalian HSP110 showed the evidence that HSP110 and the other above sequences comprised a statistically significant, distict HSP70 subfamily (Lee-Yoon et al., 1995).

While other HSP families have been studied extensively, the HSP110s have been virtually ignored.

HSP110 is a family of large stress proteins referred to as the HSP110/SSE family (Lee-Yoon et al., 1995). The mammalian protein has recently been cloned and found to share 30-33% amino acid identity with members of the HSP70 family, most of which occurs in the conserved ATP-binding domain of these molecules.

The mammalian members of the HSP110/SSE family include: **Hsp110** (or Hsp105α and Hsp105β), **APG-1** (or Osp94), and **APG-2** (or irp94) (A: ATP-binding-domain; P: peptide-binding-domain; G: germ cell derived) (Easton et al., 2000).

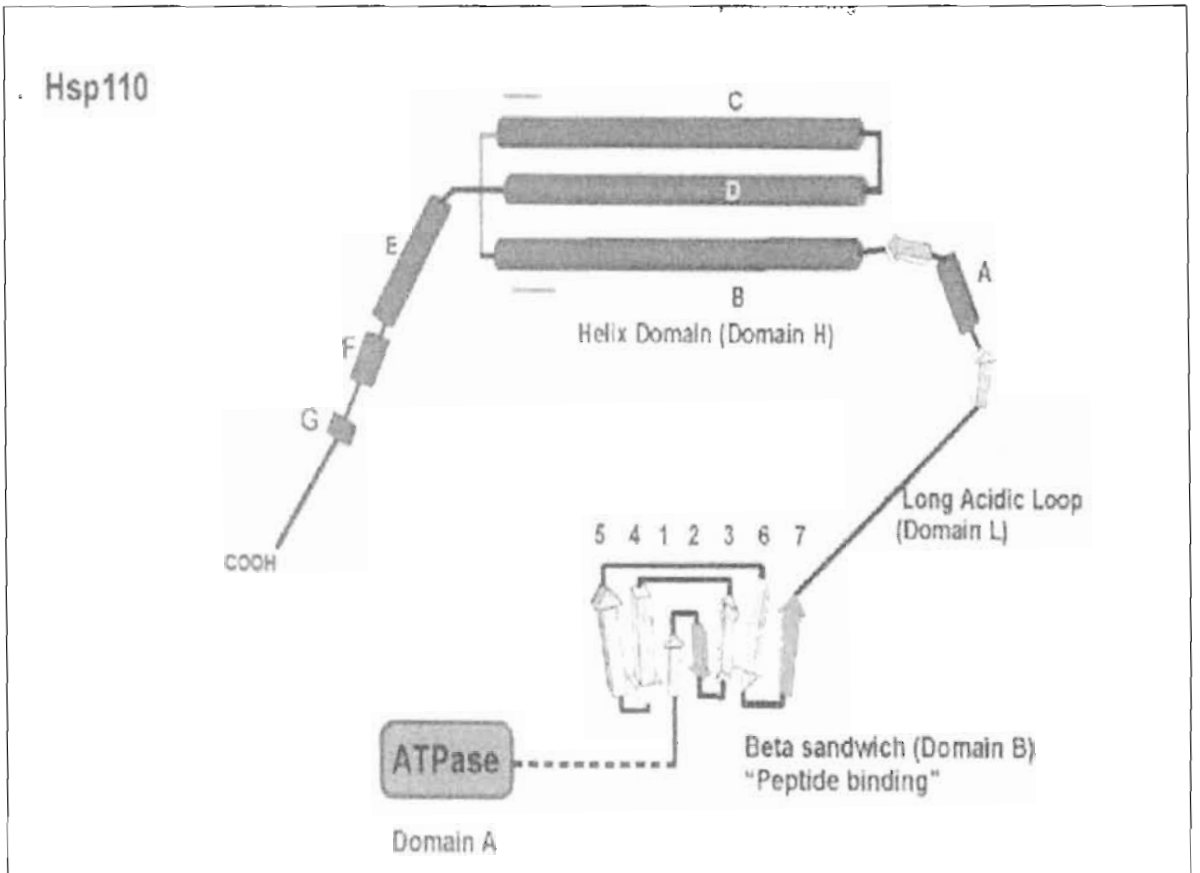
The Genbank database now includes HSP110 protein from hamster, mouse, humans, Drosophila etc, however no prokaryotic member has yet been identified (Easton et al., 2000). In humans, the gene for Hsp110 is located on chromosome 13q12.2-13.3, while *Apg-1* and *Apg-2* genes were maped to chromosomal loci 4q28 and 5q23.3-q31.1, respectively (Nonoguchi et al., 1999).

After HSP70 and HSP90, HSP110 is the third or fourth most abundant HSP in most mammalian cell lines and tissues. Studies suggested that HSP110 is primarily cytoplasmic and becomes nuclear after heating (Easton et al., 2000).

A model of Hsp110 was built based on its sequence similarity with the DnaK protein (*E. coli* HSP70) and is shown in figure 6.

Figure 6: Model for the fold of Hsp110

(Easton et al., 2000)



Residues 1-394 of Hsp110 (designated as ATPase) show 34% identity in aa sequence to the same region of DnaK. From aa 394 to aa 509, Hsp110 is predicted to exhibit 7 β strands (the β-sheet domain or B). The following 98 aa of Hsp110 are composed of a number of negatively charged residues and is reffered to as the loop domain. Finally, distal to the loop domain the C-terminal residues of Hsp110 are predicted to form a series of α-helices (residues 608-858 or domain H).

HSP110 has been found in all vertebrates examined but has been characterized best in mouse, hamster, and humans. APG-1 and APG-2 were found from both mouse and humans. Presumably, similar proteins will be found in other mammals. Although these proteins are primarily expressed in the gonads, they are also expressed at lower levels in all tissues of the mouse. APG-1 is found in highest concentration in testis, particularly in germ cells. The expression level of APG-1 increases with the maturation of germ cells, although it is not heat inducible in germ cells, suggesting the involvement of APG-1 in normal germ cell development. Conversely, APG-1 is heat inducible in somatic cells; however, the optimal heat condition for the induction is different from that of Hsp70 (stronger induction is observed by a shift from 32°C to 39°C rather than by a shift from 32°C to 42°C or the traditional shift from 37°C to 42°C). This preference of heat induction is also observed in the induction of Hsp110. Although the biological meaning of a shift from 32°C to 39°C remains unknown, the difference in the optimal heat conditions may reflect certain differential roles between Hsp110/APG-1 and Hsp70 during heat response (Easton et al., 2000).

APG-2 is highly expressed in both ovarian and testicular tissue of the mouse. It is also expressed to a lesser extent in somatic tissues. APG-2 is not heat inducible by either traditional temperature shifts or shifts that induce APG-1 (Easton et al., 2000).

Hsp110 is also denoted as Hsp105. In mouse FM3A cells, two alternative forms, 105α and 105β are observed, with 105β having 43 fewer amino acids than 105α. It has been proposed that the smaller version is a result of alternative splicing. The smaller version is only observed by a continuous heat shock at 42°C, whereas the larger form is preferentially induced after recovery from heat shock at 42°C or higher. Interestingly, the amino acids deleted in the smaller version are in the long acidic domain of full-length Hsp110. The differential roles played by these two versions of Hsp110 remain unknown (Easton et al., 2000).

Of significant interest is the fact that Hsp110 can also be induced by the human papilloma virus oncoprotein E7, which is a viral transcription factor. The significance of this observation is not yet known. That this induction requires the presence of the E7 conserved region 2, which is essential for the binding of E7 to retinoblastoma family proteins, suggests that Hsp110 induction may be coordinated with the cell cycle (Easton et al., 2000).

Chapter 3: Extracellular functions of heat shock proteins

3.1 Immune and inflammatory effects of extracellular HSPs

In addition to the intracellular response, stress also triggers the release of proteins into the extracellular space. Indeed stress proteins such as Hsp27, 60, 70, 90 and 110 and glucose regulated proteins (Grp) 78, 94, 170 and calreticulin are released from cells in a variety of circumstances and interact with adjacent cells or in some cases enter the bloodstream (Calderwood et al., 2007).

A variety of cell types secrete stress proteins, including neuronal cells, monocytes, macrophages, B cells and tumor cells of epithelial origin (Clayton et al., 2005; Davies et al., 2006; Robinson et al., 2005). This suggests that stress protein release is a fairly widespread phenomenon and may be implicated in a number of physiological or pathological events. Furthermore, it appears that some cell types may be adapted for specialized secretion of stress proteins into the bloodstream (Campisi and Fleshner, 2003). In addition, HSPs are released from cells undergoing necrosis after extremes of heat stress or other toxic treatments (Mambula and Calderwood, 2006; Todryk et al., 1999). Extracellular HSP60 and HSP70 may indeed be physiological alarm signals for cell trauma. Just as HSP70 release is common to multiple cell types, the ability to bind stress proteins is also shared by many cell types including many cells of the hemopoietic lineage, neuronal cells, vascular and other epithelial cells (Singh-Jasuja et al., 2000-1; Srivastava, 2002; Theriault et al., 2005).

Extracellular stress proteins of the HSP and Grp families have powerful effects on the immune response. During exposure to many pathogens, prokaryotic HSPs are released at high levels and are dominant antigens in the immunological responses to such pathogens (Burnie et al., 2006; Young and Elliott, 1989). Mammalian cells express endogenous stress proteins to high levels after trauma or exposure to bacteria or

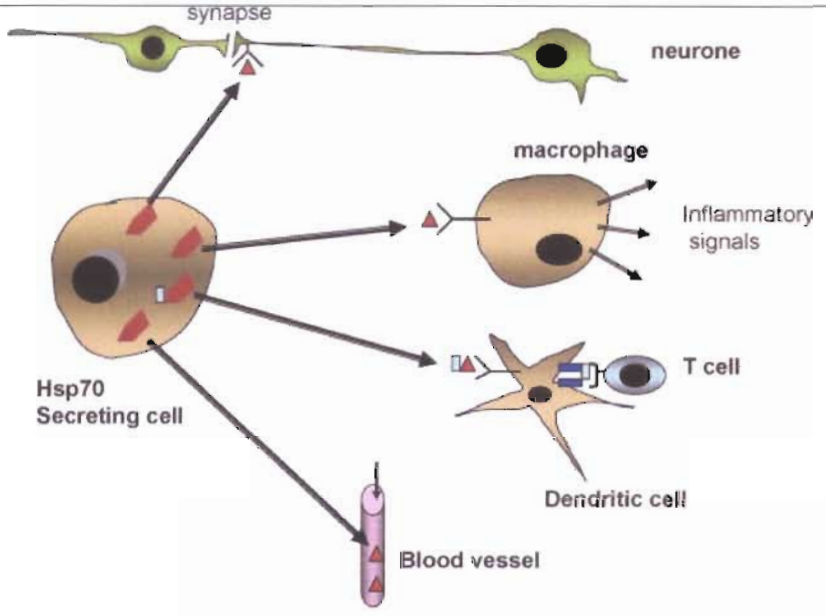
bacterial proteins (Hunter-Lavin et al., 2004). Such stress proteins can be pro-inflammatory and lead to cytokine transcription and release (Asea et al., 2000, 2002). In addition, stress proteins can act as stimulants of the adaptive immune response through their ability to bind antigenic peptides during antigen processing (Noessner et al., 2002). When such stress protein-peptide complexes are released from dead and dying cells, they bind to receptors on antigen processing cells (APCs) and antigens can be delivered to MHC class I molecules on the surfaces of such cells through a process known as **antigen cross-presentation** (Figure 7) (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000-1). Such interactions form the basis for molecular chaperone based anti-cancer vaccines. HSP–peptide complexes extracted from tumors can stimulate a specific CD8+ T cell mediated immune response in the tumor bearing host (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000-2). The potency of such vaccines has been ascribed to the ability of stress proteins to stimulate both the innate and adaptive arms of the anti-tumor immune response (Srivastava and Amato, 2001).

It has been suggested that HSP-chaperoned antigenic peptides may be presented also via MHC class II molecules and thus enhanced activation of antigen-specific CD4+ T cells (Haug et al., 2007). Extracellular Hsp70: peptide complexes can be detected in MHC class II-enriched compartments after receptor-mediated endocytosis (Lipsker et al., 2002); complexes from the cytosol may reach the MHC class II presentation pathway via autophagic processes (Dengjel et al., 2005).

Diseases such as rheumatoid arthritis (RA) can be triggered by cross reactive T cells which recognize common epitopes in mammalian and highly immunogenic prokaryotic HSP (Van Eden et al., 2005; Hauet-Broere et al., 2006). It was speculated that the close degree of conservation in the sequences of these stress proteins might trigger an autoimmune response to mammalian stress proteins. Interestingly however, application of the corresponding mammalian HSPs suppresses the pro-inflammatory responses to bacterial HSP epitopes and leads to remission of inflammatory diseases (Kingston et al.,

1996). HSPs can thus be both profoundly immunostimulatory or immunosuppressive, depending on context (Van Eden et al., 2005; Daniels et al., 2004).

Figure 7: HSP70 is released from cells and may interact with a wide range of target cells (Calderwood et al., 2007)



HSP70 may be released by active secretion mechanisms or from cells undergoing necrosis. The resulting extracellular HSP70 (red triangles) may then interact with neuronal cells, monocytes or macrophages or enter the circulation. HSP70 may also be released conjugated to antigenic peptides (blue rectangles) and HSP70–peptide complexes are taken up by APCs such as dendritic cells. Such peptides may then be transferred to MHC class I molecules (dark blue rectangles) through process known as **cross-presentation**, and such MHC I–peptide complexes can be recognized by CD8+ T lymphocytes leading to T cell activation (similar properties have been shown for other HSPs).

3.2 Heat shock proteins (HSP) receptors

3.2.1 An overview of HSP receptors

The existence of HSP receptors on antigen presenting cells (APCs) was hypothesized in 1994. The first such receptor, CD91 (identified previously as a α-2-macroglobulin/low density lipoprotein receptor), was identified and characterized in 2000. Futhermore, seven putative HSP receptors have been identified. These include Lox-1, scavenger receptor class A (SR-A), Toll-like receptor (TLR) 2 and 4, CD14 (a receptor for endotoxin - lipopolysaccharide), CD40 (a receptor molecule on the cell surface of B cells, endothelial and epithelial cells) and CD36 (collagen type I and thrombospondin receptor) (Binder et al., 2004). Table 2 reviews the sugessted HSP receptors.

Table 2: Molecules suggested as HSP receptors

(Modified from Binder et al., 2004)

Proposed receptor	Characteristics and common ligand(s)	HSP Ligand
CD91	Also known as α 2 Macroglobulin (α 2M) receptor/low density lipoprotein (LDL) receptor. CD91 has a broad cellular distribution, but in the hematopoietic system it is expressed on monocyte lineage cells. The α 2M/LDL receptor mediates endocytosis of a variety of ligands including α 2M-protenase complexes, plasminogen activators in complex with plasminogen activator inhibitor, *Pseudomonas* Exotoxin A.	gp 96 HSP70 HSP90
Lox-1	LOX-1 is a recently identified oxidized low density lipoprotein (OxLDL) receptor that is abundantly expressed in vascular endothelial cells. It binds, internalizes and degrades OxLDL but not native LDL or acetylated LDL. OxLDL is implicated in atherosclerosis and glomerulosclerosis.	HSP70
SR-A	Scavenger receptors (SR) are cell surface proteins that can bind and internalize modified lipoproteins, such as acetylated LDL and oxidized LDL. They are categorized into classes A, B, and C according to their structural characteristics. In addition to the ligand binding activity for modified lipoproteins, SR-A (mainly expressed on macrophages) is involved in the binding and clearance of lipopolysaccharide in vivo and the binding of lipoteichoic acid of Gram-positive bacteria such as *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Listeria monocytogenes*. In addition to its role in the uptake of modified lipoproteins and other polyanions, as well as in cell-cell interaction, SR-A has been shown to be relevant in cell adhesion, cell activation, and inflammatory response. SR-A was proposed to play a critical role in modified lipoprotein metabolism and atherosclerosis.	gp96

Table 2: continued

Proposed receptor	Characteristics and common ligand(s)	HSP Ligand
TLR-2 and **TLR-4**	Members of the Toll-like receptor (TLR) family probably play a fundamental role in pathogen recognition and activation of innate immunity. TLR2 and TLR4 are expressed in myelomonocytic cells. TLR2 recognizes peptidoglycan from Gram-positive bacteria, lipoproteins and lipopeptides from several bacteria, glycophosphatidylinositol, lipoarabinomannan, porins, and zymosan from yeast. TLR4 is a transmembrane lipopolysaccharide receptor. Activation of it causes the release of antimicrobial peptides, inflammatory cytokines and chemokines, and costimulatory molecules that initiate the innate immune response to common Gram-negative bacteria. TLR4 also interacts with respiratory syncytial virus, fibronectin, fibrinogen, and hyaluronic acid.	HSP70 gp96 HSP60
CD14	CD14 was the first pattern recognition receptor to be identified. It is expressed on, and secreted by myeloid cells. CD14-negative cells, such as epithelial and endothelial cells, become responsive to bacterial pathogens in the presence of soluble CD14 (sCD14), a protein present in the serum in microgram amounts and secreted by monocytes and hepatocytes. Membrane-bound and sCD14 bind a variety of bacterial products, eg lipopolysaccharide (LPS) from Gram-negative bacteria, lipoteichoic acids from Gram-positive bacteria, mycobacterial glycolipids, and mannans from yeast. At the molecular level, CD14 acts by transferring LPS and other bacterial ligands from circulating LPS-binding protein to the Toll-like receptor 4/MD-2 signaling complex. Engagement of this complex result in the activation of innate host defense mechanisms, such as release of inflammatory cytokines, and in upregulation of costimulatory molecules, thus providing cues that are essential to directing adaptive immune responses.	HSP70

Table 2: continued

Proposed receptor	Characteristics and common ligand(s)	HSP Ligand
CD40	A receptor molecule on the cell surface of all mature B lymphocytes, most B-cell malignancies, monocytes, dendritic cells (in the nervous system), endothelial cells (within blood vessels), and epithelial cells. CD40 is a member of the tumor necrosis factor (TNF) superfamily. Together with CD40 ligand (CD154), CD40 is an important contributor to B cell differentiation/costimulation, isotype switching and rescuing B cells from apoptosis. It is also involved in inflammatory processes that lead to atherosclerosis and thrombosis (clotting).	HSP70
CD36	The receptor for extracellular matrix proteins such as collagen and thrombospondin. CD36 is known to mediate the adhesion of *Plasmodium falciparum*. CD36 is expressed on monocytes, platelets, endothelial cells, and some human tumor cell lines, but not on lymphocytes and granulocytes. It is a very early marker for erythroid differentiation.	gp96

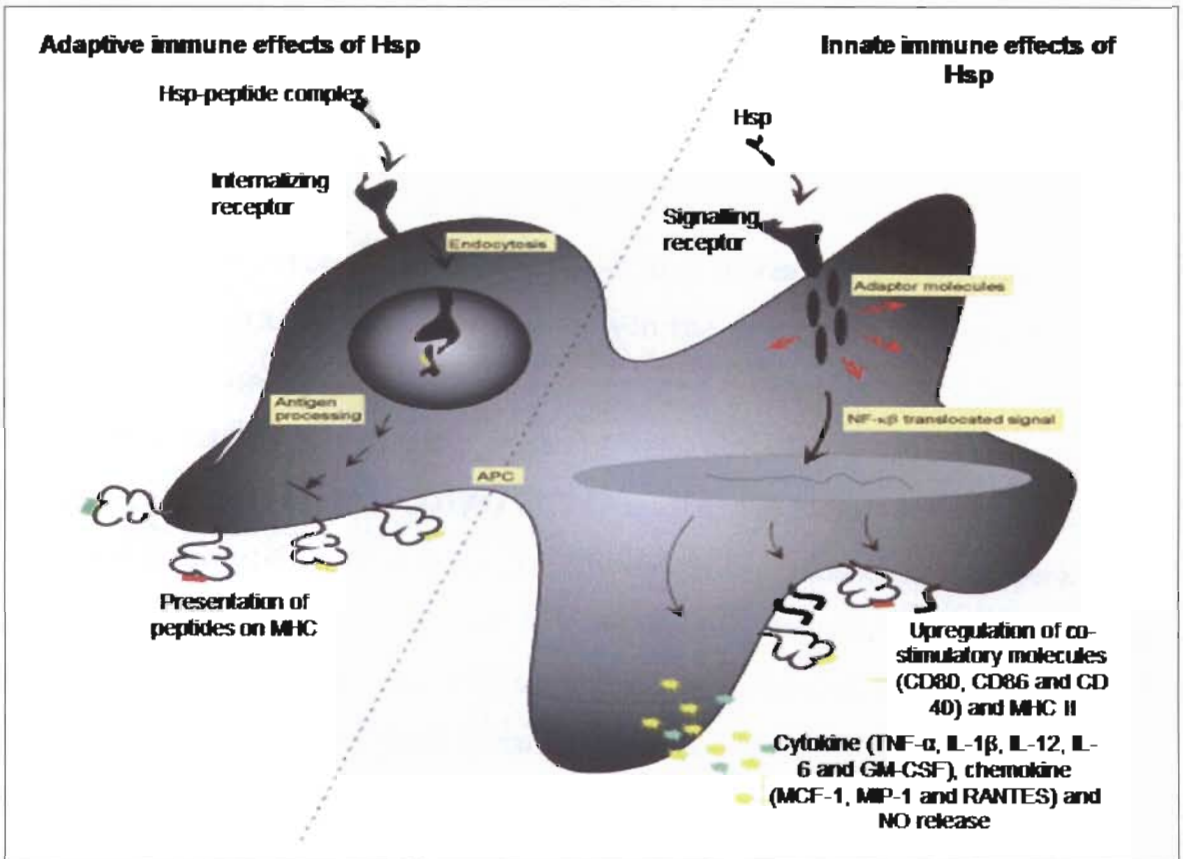
3.2.2 HSP-APC interaction

HSP–APC interaction has two distinct consequences (Figure 8): **firstly**, the HSP-peptide complexes are taken up by the APCs and the peptides re-presented on MHC class I molecules of the APC (Suto et al., 1995; Doody et al., 2004); **secondly**, HSPs (regardless of chaperoned peptides) induce the secretion of inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-12, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) - by macrophages and dendritic cells (DCs) (Chen et al., 1999; Kol et al., 1999; Basu et al., 2000; Singh-Jasuja et al., 2000-2; Asea et al., 2000; Moroi et al., 2000), chemokines - such as MCP-1, MIP-1 and RANTES - by macrophages (Lehner et al., 2000; Panjwani et al., 2002), nitric oxide (NO) by macrophages and DCs (Chen et al., 1999; Panjwani et al., 2002), upregulation of

maturation markers - such as MHC class II, CD86, CD83 and CD40 - on DCs (Basu et al., 2000; Singh-Jasuja et al., 2000-2) and migration of DCs to draining lymph nodes (Binder et al., 2000). These effects involve receptor engagement, signalling and translocation of NF-κB to the nucleus of macrophages and DCs (Kol et al., 1999; Basu et al., 2000).

Figure 8: Two outcomes of the interaction of heat-shock proteins (HSPs) with receptors on antigen-presenting cells (APCs)

(Binder et al., 2004)



In the specific or adaptive outcome, HSP-chaperoned peptides are internalized by endocytosing receptors and the peptides are processed and presented on MHC molecules of the APC. In the innate or peptide-non-specific outcomes, HSPs engage signalling receptors that trigger NF-κB activation. The APCs release cytokines, chemokines and nitric oxide (NO) and upregulate the expression of co-stimulatory molecules and MHC-II.

Chapter 4: Heat shock proteins in normal aging

Age-related deterioration in immune function has been recognized in many species. In humans the clinical manifestation of such immune dysfunction is age-related. It increases the susceptibility to certain infections and the incidence of some autoimmune diseases and certain cancers. Laboratory investigations reveal age-related changes in the peripheral T cell pool (eg increase of CD4+ T cells, a switching from T_H1 to T_H2 response, decrease of CD8+ T cells) and cytokine production profile [a decreased production of interferon-γ (IFN-γ) and IL-2 and increased production of IL-4, IL-6] (Krejsek and Kopecky, 2004). These changes in the properties of peripheral T cells are thought to be causally linked to an age-associated involution in the thymus (Aspinall and Andrew, 2000). Concerning humoral response, there is a proliferation of B lymphocytes of subset B1 (CD19+/CD5+) and a production of autoantibodies (eg antinuclear factor, rheumatoid factor, antithyroglobulin) (Krejsek and Kopecky, 2004).

As it was mentioned in chapter 3, stress protein release may be implicated in a number of physiological events. HSPs are present in circulation of normal individuals (Pockley et al., 1998), and their circulating levels are decreased in aging. In study involving 60 normal individuals aged between 20 and 96 years, extracellular Hsp60, anti-Hsp60, anti-Hsp70 and anti-mycobacterial Hsp65 antibodies were detected in all sera, whereas extracellular Hsp70 was detectable in only 77% of the samples analysed. Regression analysis revealed a progressive decline in extracellular Hsp60 (759 ng/ml < 40 years; 294 ng/ml > or = 90 years) and extracellular Hsp70 (400 ng/ml < 40 years; 20 ng/ml > or = 90 years) levels with age whereas no relationship was apparent for anti-Hsp60 and Hsp65 antibody levels. Hsp70 antibody levels tended to increase with age (115 U/ml < 40 years; 191 U/ml > or = 90 years) (Rea et al., 2001).

In vitro studies have shown that Hsp70 expression in heat-stressed lung cells (Fargnoli et al., 1990), hepatocytes and liver (Heydari et al., 1995; Hall et al., 2000), splenocytes

(Pahlavani et al., 1995), myocardium (Gray et al., 2000) and mononuclear cells is reduced with increasing age (Richardson and Holbrook, 1996), as is the induction of Hsp70 expression in response to ischemia (Nitta et al., 1994) and mitogenic stimulation (Faassen et al., 1989). Heat shock cognate-70 gene expression declines during normal aging in human retina (Bernstein et al., 2000), and heat shock-induced Hsp70 expression is decreased in senescent and late-passage cells, both of which suggest that the process of aging itself might be associated with reduced Hsp70 production (Liu et al., 1989; Luce and Cristofalo, 1992; Effros et al., 1994).

Although currently uncertain, possible mechanism underlying an attenuated stress response during aging might include a reduced availability of heat shock factor (HSF) (Richardson and Holbrook, 1996) or age-associated increase in abnormal or denatured proteins that could interfere with HSF binding to heat shock elements (HSEs) (Munro and Pelham, 1985). Alternatively, age-related decrease in the capacity of HSF to undergo the oligomerization that is essential for binding to HSEs might be involved.

Increasing age is associated with a reduced capacity to maintain homeostasis in all physiological systems and it might be that this results, in part at least, from a parallel and progressive decline in the ability to produce heat shock proteins. If this is so, an attenuated heat shock protein response could contribute to the increased susceptibility to environmental challenges and the more prevalent morbidity and mortality seen in aged individuals (Richardson and Holbrook, 1996; Shelton et al., 1999).

Chapter 5: Heat shock proteins and different pathological conditions

Heat shock proteins, particularly those of the HSP60 and HSP70 families, are immunodominant molecules, and a significant element of the immune response to pathogenic microorganisms is directed towards peptides derived from HSPs (Kaufmann, 1990; Young, 1990). This phenomenon, together with the ubiquitous nature of human HSPs and the high degree of sequence homology between mammalian and microbial HSPs (~50–60% identical residues in the case of the HSP60 family) has led to debate whether the immune system recognizes HSPs as dominant microbial antigens or potentially harmful self-antigens (Kaufmann, 1990). It has also been suggested that HSPs might provide a link between infection and autoimmunity, either through recognition of conserved epitopes or via cross-reactivity/molecular mimicry (Lamb et al., 1989). Evidence for a link between HSP reactivity and pathogenesis of infection, autoimmune diseases and malignancies has arisen from several studies.

5.1 Heat shock proteins and infectious diseases

Strong humoral and cellular immune reactivity against different members of HSP families accompanies many infectious diseases since these proteins are abundant in different pathogens and immunologic memory for cross-reactive determinants of conserved HSP is generated during life due to frequent re-stimulation with pathogens with different degrees of virulence. Under these conditions, infection of an individual with a virulent pathogen would enable the already prepared immune system to react quickly before the immune system response to more pathogen-specific antigens develops. Table 3 shows the immune responses to HSPs in different infections, in that HSPs represent major antigens.

Table 3: HSPs, immunodominant antigens in several infectious diseases (Zugel and Kaufmann, 1999)

Pathogen	Disease	HSP family
Helminths		
Schistostoma Mansoni	Schistosomiasis	HSP70, HSP90
Onchocerca volvulus	Onchocercosis	HSP70
Brugia malayi	Filiariasis	HSP70
Protozoa		
Plasmodium falciparum	Malaria	HSP70, HSP90
Trypanosoma cruzi	Chagas' disease	HSP70, HSP90
Leishmania major	Leishmaniasis	HSP70
Leishmania donovani	Leishmaniasis	HSP70
Leishmania braziliensis	Leishmaniasis	HSP70
Toxoplasma gondi	Toxoplasmosis	HSP60
Fungi		
Candida albicans	Candidiasis	HSP90
Histoplasma capsulatum	Histoplasmosis	HSP60, HSP70
Bacteria		
Mycobacterium tuberculosis	Tuberculosis	HSP60, HSP70
Mycobacterium leprae	Leprosy	HSP10, HSP60, HSP70
Chlamydia trachomatis	Trachoma	HSP60, HSP70
Borrelia burgdorferi	Lyme disease	HSP60, HSP70
Helicobacter pylori	Gastritis	HSP60
Yersinia enterocolitica	Yersiniosis	HSP60
Legionella pneumophila	Legionnaires' disease	HSP60
Treponema pallidum	Syphilis	HSP60
Bordetella pertussis	Pertussis	HSP60
Listteria monocytogenes	Listeriosis	HSP60, HSP70

5.2 Heat shock proteins and autoimmune diseases

Experimental and clinical observations have confirmed that HSPs are involved in regulation of some autoimmune diseases such as autoimmune arthritis including rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA), insulin-dependent diabetes mellitus (IDDM), multiple sclerosis (MS), systemic lupus erythematosus (SLE) and other autoimmune reactions (Raska et al., 2005). Recently, various studies have shown that HSP-reactive T cells have an immunoregulatory phenotype, indicating that HSPs - in particular HSP60 and HSP70 - constitute a group of "autoantigens" with the potential to trigger immunoregulatory pathways, which can suppress immune responses that occur in human inflammatory diseases. RA/JIA and IDDM are the autoimmune diseases, for which immune responses to HSPs have been most extensively studied (Van Eden et al., 2005).

5.2.1 Heat shock proteins and rheumatoid arthritis/juvenile idiopathic arthritis

5.2.1.1 Rheumatoid arthritis (RA):

RA is an autoimmune disease that causes chronic inflammation of the joints, the tissue around the joints as well as other organs in the body such as eyes, lung, heart, blood vessels etc. Therefore, RA is referred to as a systemic illness. While RA is a chronic illness, meaning it can last for years, patients may experience long periods without symptoms. Typically, however, RA is a progressive illness that has the potential to cause joint destruction and functional disability. RA is a worldwide condition with variable incidence. It is estimated that around the world, 1 in every 100 people suffer from RA (Hartzheim and Goss, 1998). In Western countries, it affects 1-3% of the population with a 3:1 female preponderance (Abdel-Nasser et al., 1997; Hartzheim and Goss, 1998). While patients most frequently present in the fourth to sixth decades of life, RA can manifest at any age (Ahern and Smith, 1997). In some families, multiple members can be affected, suggesting a genetic basis for the disorder.

The etiology of RA is not clear. It is postulated that a genetically susceptible host is exposed to an unknown pathogen (antigen) and this interaction gives rise to a persistent immunological response. There are several factors that are considered to be involved in etiology of RA such as **genetic predisposition, infectious agents, gender and HSPs**.

The vast majority of patients with RA carry human leukocyte antigen-DRB1 (HLA-DRB1) alleles involving HLA DRB1*0401, DRB1*0404 and DRB1*0101 encoding QKRAA, QRRAA, or RRRAA amino acid sequences in positions 70 to 74 of the DR-β chain, a motif commonly known as the RA 'shared epitope' (SE). Immunogenetic analyses have identified the SE as the main genetic risk factor in diverse ethnic groups and have documented a particularly strong association with a severe form of the disease. It has also been suggested that a gene-dose effect may exist in which patients carrying two SE-positive alleles are afflicted with more severe disease than patients with one SE allele or none (Ling et al., 2007).

The greater prevalence of RA among women suggests that sex hormones are implicated in the development of the disease. Many viruses and bacteria have been implicated as causative agents, eg Epstein-Barr virus, parvovirus, lentiviruses, Mycoplasma, Mycobacteria and Yersinia (Buch and Emery, 2002).

5.2.1.2 *Juvenile idiopathic arthritis (JIA)*:

JIA, a compromise for the American term juvenile rheumatoid arthritis (JRA) and the European classification juvenile chronic arthritis (JCA) is not a single disease, but a term that encompasses all forms of arthritis that begin before the age of 16 years, persist for more than 6 weeks, and are of unknown cause. Studies in developed countries have reported a prevalence that varies between 16 and 150 per 100,000. International League of Associations for Rheumatology (ILAR) categorized JIA to these forms: systemic arthritis (frequency 4-17%), oligoarthritis (27-56%), rheumatoid factor (RF)-positive polyarthritis (2-7%), rheumatoid factor-negative polyarthritis (11-28%), enthesitis-

related arthritis (3-11%), psoriatic arthritis (2-11%) and undifferentiated arthritis (11-21%)(Ravelli and Martini, 2007).

The cause and pathogenesis of JIA are still poorly understood but seem to include both genetic and environmental components. Moreover, the heterogeneity of this disease implies that different factors probably contribute to the pathogenesis and cause. The notion that an infection triggers chronic arthritis in genetically susceptible individuals is still unproven. The results of the first genome-wide scan of children with the disease lend support to the assumption that several genes, including at least one in the HLA region, affect susceptibility to JIA. **Oligoarticular disease** has been consistently associated with HLA antigens. Positive associations include HLA-A2, HLA-DRB1*11 (a subtype of HLA-DR5), and HLA-DRB1*08. **RF-positive polyarthritis** in children has been reported to be associated, as in adults, with HLA-DR4. In **enthesitis-related arthritis**, 76% of patients were reported to be HLA-B27 positive compared with a population frequency of around 10%. A single nucleotide polymorphism (-174) in the regulatory region of the interleukin-6 gene, has been associated with **systemic disease**. A polymorphism in the 5′ flanking region (-173) of macrophage inhibitory factor has been shown to be associated with JIA, and in systemic disease, to be correlated with high serum and synovial fluid macrophage inhibitory factor concentrations, and worse long-term functional outcome (Ravelli and Martini, 2007).

5.2.1.3 *HSPs and rheumatoid arthritis/juvenile idiopathic arthritis*

The first evidence of a role for HSPs as antigens in inflammatory responses was obtained in the 1980s in the rat model of heat-killed-mycobacteria-induced adjuvant arthritis (ADJUVANT-INDUCED ARTHRITIS, AA). After *Mycobacterium tuberculosis* immunization, both antibody and T cell responses to mycobacterial Hsp65 (a member of HSP60 family) were detected in Lewis rats. It has been found that arthritogenic and protective T cell clones obtained from arthritic rats recognized the 180-188 sequence of

Hsp65. The arthritogenic T cell clone also reacted to an epitope of cartilage proteoglycan, suggesting that targeting of inflammation to the joints might be due to cross-reactivity between aa 180-188 of Hsp65 and a self component in the cartilage (Van Eden et al., 1985). Furthermore, it was evident that successful transfer of arthritis resistance to naive recipients depends on the transfer of Hsp65 specific T cells. It seems that Hsp65 plays a crucial role in the T cell regulatory events involved in both the induction of AA and protection against AA (Hogervorst et al., 1992). Following these findings, several studies reported immune reactivity to HSPs in human arthritis. Elevated levels of IgG antibodies to human HSP60 were detected in sera of children with JIA (De Graeff-Meeder et al., 1993). Arthritic synovial tissue samples from patients with RA or JIA expressed increased levels of HSP60 in mitochondria. T cells from the synovial fluid and blood of patients with RA or JIA proliferated in response to *Mycobacterial bovis* HSP60 that has 100% homology with the HSP60 of *M. tuberculosis* (Van Eden et al., 2005).

In contrast to healthy controls, synovial fluid and peripheral blood mononuclear cells from patients with JIA had substantial proliferative responses to self-HSP60. Cellular responses to *Escherichia coli* DnaJ (also known as HSP40) were also reported in JIA patients. Further studies showed that CD4+ T-cell responses to human HSP60 at the onset of JIA are correlated with responses to mycobacterial HSP60 and with a favourable prognosis. These findings indicated that microbial-HSP-crossreactive responses of T cells to endogenous human HSP60 might have a regulatory role in the course of self-remitting JIA. T-cells recognizing self-HSP60 that are isolated from patients with JIA mainly have a regulatory phenotype: after activation with human HSP60, T cells from patients with remitting JIA express CD25 (interleukin 2 receptor α chain) and CD30 [a member of the tumor necrosis factor (TNF) - nerve growth factor (NGF) superfamily] and can produce regulatory cytokines, such as interleukin IL-4, IL-10 and transforming growth factor β (TGF- β) (Van Eden et al., 2005).

Studies of adult RA have shown a more diverse situation with regard to T-cell proliferation in response to HSPs and have shown a role for different HSPs, including the HSP70-family member Bip (HSP70 in endoplasmic reticulum). Corrigall et al. (2001) reported that 30% of RA patients possess serum autoantibodies to BiP and 60% show synovial T cell proliferation. In the same study, DBA/1 mice with collagen-induced arthritis (CIA) and pristane-induced arthritis (PIA) produce anti-BiP antibodies. Finally, BiP, given before immunization, inhibits the development of CIA in mice and adjuvant arthritis (AA) in Lewis rats. These results collectively suggest that BiP may indeed be an autoantigen in RA with important immunoregulatory properties for arthritis induction (Corrigall et al., 2001). However, similar to the findings for patients with JIA, human HSP60 seems to be driving the production of regulatory cytokines in patients with RA. When stimulated with human HSP60, HSP60-reactive synovial fluid T-cells from patients with RA produced less IFN-γ and more IL-4 than when stimulated with bacterial HSP60. These HSP60-specific T cells supressed TNF-α production by autologous peripheral blood mononuclear cells in co-cultures (Van Eden et al., 2005).

During periods of active arthritis, inflammation in the joints leads to local cellular stress and therefore the up-regulation of self-HSP expression in the synovial tissue. It has been proposed that T cells recognizing these self HSPs can be activated in the inflamed joint, thereby inducing the activation of several regulatory T-cell subsets, including the following: T_H3 cells, which produce TGF-β; T regulatory 1 (T_R1) cells, which produce IL-10; and CD4+CD25+ regulatory T (T_{Reg}) cells, which are characterized by expression of forkhead box P3 - FOXP3 ([8]) (Van Eden et al., 2005).

[8] FOXP1, FOXP2, and FOXP3 all bind the FOX-binding site within the IL-2 promoter, and all but FOXP2 suppress IL-2 promoter activity.

5.2.2 HSPs and insulin-dependent diabetes mellitus (IDDM)

5.2.2.1 Insulin-dependent diabetes mellitus or Type 1 diabetes

IDDM, a form of diabetes mellitus, is an autoimmune disease that results in the permanent destruction of insulin producing β-cells of the pancreas. Type 1 diabetes is not primarily a childhood problem. The adult incidence of Type 1 is similar to that for children. Many adults who contract Type 1 diabetes are mis-diagnosed with Type 2, due to the mis-conception of Type 1 as a disease of children. The most useful laboratory test to distinguish Type 1 from Type 2 diabetes is the C-peptide ([9]) assay, which is a measure of endogenous insulin production since external insulin has included no C-peptide. Lack of insulin resistance, determined by a glucose tolerance test, would also be suggestive of Type 1. Testing for GAD 65 ([10]) antibodies has been proposed as an improved test for differentiating between Type 1 and Type 2 diabetes. Autoantibodies against islet cell cytoplasmic autoantigen (ICA), insulin (IAA), GAD 65, and IA2/ICA512 autoantigen (IA2A, a protein tyrosine phosphatase) are extremly effective for predicting eventual development of Type 1 diabetes in otherwise healthy individuals (Wasserfall and Atkinson, 2006).

([9]) C-peptide is a peptide which is made when proinsulin is split into insulin and C-peptide. They split when proinsulin is released from the pancreas into the blood in response to a rise in serum glucose – one C-peptide for each insulin molecule.

([10]) Glutamic acid decarboxylase (GAD65) is an enzyme that is produced primarily by pancreatic islet cells. Patients with insulin-dependent diabetes mellitus (IDDM) often have antibodies to GAD65 and several other islet cell antigens.This is consistent with the hypothesis that IDDM is an autoimmune disease and that autoantibody production is an early step in the development of IDDM. Autoantibodies can be detected in many cases prior to the onset of glucose intolerance. The presence of GAD65 autoantibodies has been shown to be a strong predictive marker for the eventual onset of IDDM.

Type 1 diabetes exhibits dramatic geographic and ethnic variation. The highest incidence rates in the world (> 35/100,000 per year) have been reported for Finland and Sardinia, Italy. The lowest incidence rates are observed in the Asian countries (< 3/100,000 per year) including Japan, China and Korea. The Native American (American

Indians), Cuban, Chilean and Mexican populations also have extremely low rates of Type 1 diabetes. In most other Caucasian populations in Europe and the Americans, incidence rates are moderate (~ 10-20/100,000 per year) (Dorman and Bunker, 2000). Several factors play a role in pathogenesis of Type 1 diabetes such as genetic predisposion, infection and diatery habits. The autoimmune attack may be triggered by reaction to an infection, eg Coxsackie virus type B, probably based on molecular mimicry. In terms of genetic markers, Type 1 diabetes is primarily determined by genes in the HLA region. It has been shown that the alleles HLA DRB1*0401, HLA DRB1*0402, HLA DRB1*0405, HLA DQA1*0301, HLA DQB1*0302 increase Type 1 diabetes risk. In contrast, the alleles HLA DRB1*1501, HLA DQA1*0102 and HLA DGB1*0602 are protective for the disease (Krejsek and Kopecky, 2004).

In a number of studies, breast-feeding has been proposed to have a protective effect: a high frequency of breast-feeding has been reported to be associated with a low incidence of Type 1 diabetes. Duration of breast-feeding of less than 3–4 months has been shown to be associated with development of Type 1 diabetes in a meta-analysis, which is a statistical analysis of a large collection of analysis results from individual studies. The possible protective effect of breast-feeding may be due to a delayed introduction of cow's milk, and several studies have reported an earlier exposure to cow's milk or solid foods in children with Type 1 diabetes compared with healthy children. Most studies on the association between breast-feeding and Type 1 diabetes are retrospective, and the results may be compromised by recall bias. Thus, prospective studies are needed to investigate the possible association between β-cell autoimmunity, infant feeding in general and breast-feeding in particular. In a Finnish study, short-term exclusive breast-feeding and an early introduction of a cow's milk-based formula were associated with an increased risk of β-cell autoimmunity in genetically predisposed children, but the duration of breast-feeding has not been associated with an increased

risk of β-cell autoimmunity in children with a first-degree relative with Type 1 diabetes in Germany, Australia or the USA (Holmberg et al., 2007).

5.2.2.2 HSPs and insulin-dependent diabetes mellitus (IDDM)

Nonobese diabetic (NOD) mice exhibit a susceptibility to spontaneous development of Type 1 diabetes. The mice were first reported in 1980. Since that time the strain has been widely established around the world and used by investigators to test compounds or devices to either prevent development of diabetes or to provide therapy. Susceptibility to IDDM in NOD mice is polygenic and environment exerts a strong effect on gene penetrances. The major component of susceptibility, however, is the unique MHC haplotype (H-2g7 Chr 17). Environmental factors including housing conditions, health status, and diet all effect development of diabetes in the mice. Diabetes development in NOD mice is characterized by insulitis, a leukocytic infiltrate of the pancreatic islets. Marked decreases in pancreatic insulin content occur in females at about 12 weeks of age and several weeks later in males (Makino et al., 1980).

In the NOD mouse model of Type 1 diabetes, immunization with *M. tuberculosis* HSP60 has been shown to protect against disease. However, this protection depends on the form of HSP administration, because mycobacterial HSP60 causes disease earlier when administered in IFA (incomplete Freund's adjuvant) but protects against disease when administered in PBS (phosphate-buffered saline). Other studies have led to the identification of a mouse HSP60 - derived peptide, known as p277 (aa position 437-460), which is effective at protecting NOD mice against insulitis. Although the protective effect of this peptide was confirmed in another rodent model of Type 1 diabetes, a separate study did not replicate these results. It has also been shown that it is possible to predict resistance to future insulitis in NOD mice that have spontaneously developed natural IgG specific for epitopes within HSP60: that is, NOD mice that have "vaccinated" themselves against HSP60 were protected from developing disease.

Recently, it has been documented that gp96 (a member of HSP90 family) has disease-suppressive immunomodulatory activity in NOD mice, although the mechanisms involved remain to be elucidated (Van Eden et al., 2005).

Similar to the findings for animal models, the presence of IgG autoantibodies and T cell specific for human as well as *M. bovis* HSP60 has been documented following several studies of patients with Type 1 diabetes (Ozawa et al., 1996; Horvath et al., 2002; Weitgasser et al., 2003; Abulafia-Lapid et al., 1999). Recently, one study has shown that children with newly diagnosed Type 1 diabetes manifest heightened T-cell autoimmunity to human HSP70 and HSP60, but not to HSP90 (Abulafia-Lapid et al., 2003). Cohen et al. (2002) showed that the HSP60-derived peptide p277 could bind human MHC class II molecules and induce proliferative T-cell responses in patients with Type 1 diabetes. However, further studies are required to reveal the role of these responses in the disease.

5.3 Heat shock protein reactivity and atherosclerosis

Atherosclerosis is thought to develop as a result of inflammatory responses to an initial injury of an arterial wall, with macrofages and T cells being important in atherosclerotic plaques at all stages of development, mainly through their production of pro-inflammatory mediators. Infection has been proposed as a possible trigger for the initial arterial-wall injury and is thought to lead to further damage based on an autoimmune process (Wick et al., 2004). In humans, several microorganisms have been implicated as triggering factor for atherosclerosis, most importantly *Chlamydie pneumoniae* and cytomegalovirus (CMV) (Boman and Hammerschlag, 2002; Bason et al., 2003).

Similarly as oxidized low-density lipoprotein (LDL), HSP60 might be an important autoantigen in atherosclerosis. Animal models of atherosclerosis have shown a role for HSP60 in development of disease and, to a lesser extent, in protection against disease.

For rabbits and mice, immunization with mycobacterial HSP60 increased disease in the presence of atherosclerosis-promoting genetic and dietary conditions (Wick et al., 2004). However, more recent studies have shown that mycobacterial HSP60, when administered orally or intranasally, has the potential to suppress induced atherosclerosis (Maron et al., 2002; Harats et al., 2002). High levels of autoantibodies specific for human Hsp60 have been reported to be associated with cardiovascular disease and coronary heart disease (Wick et al., 2004). Recently, a B-cell epitope of human Hsp60 was identified that has homology with two CMV-encoded proteins. Patients with coronary heart disease were found to have antibodies specific for this human Hsp60 epitope, as well for the homologous viral peptides, whereas healthy control individuals did not. So, infection with CMV might induce a cascade of immune responses that lead to damage of the endothelium through molecular mimicry involving Hsp60 (Bason et al., 2003).

By contrast, the presence of high levels of inducible Hsp70 in human sera seems to be associated with protection against cardiovascular disease (Pockley et al., 2003; Zhu et al., 2003). So, although there is some evidence that HSP60 might be involved in development of disease (possibly through a molecular mimicry mechanism), in certain circumstances, such as after oral administration of HSP60 to animals or when serum level of Hsp70 are high, HSPs might have disease-suppressive capacities (Van Eden et al., 2005).

5.4 Heat shock protein reactivity in malignancies

5.4.1 Heat shock proteins and solid tumors

Heat shock proteins (HSPs) are overexpressed in a wide range of human cancers and are implicated in tumor cell proliferation, differentiation, invasion, metastasis, death, and recognition by the immune system. Although HSP levels are not informative at the diagnostic level, they are useful biomarkers for carcinogenesis in some tissues and

signal the degree of differentiation and the aggressiveness of some cancers. In addition, the circulating levels of HSP and anti-HSP antibodies in cancer patients may be useful in tumor diagnosis. Furthermore, several HSPs are implicated in the prognosis of specific cancers. Increased HSP expression may also predict the response to some anticancer treatments (Ciocca and Calderwood, 2005).

Elevated HSP expression in malignant cells plays a key role in protection from spontaneous apoptosis associated with malignancy as well as the apoptosis generated by therapy, mechanisms which may underlie the role of HSP in tumor progression and resistance to treatment. HSP transcription requires activated HSF1 (heat shock transcription factor 1), which is itself overexpressed in cancer and plays a role in invasion and metastasis. However, the molecular mechanisms linking increased HSP and HSF1 expression to tumor progression are not currently understood (Ciocca and Calderwood, 2005).

HSP expression is tailored for induction by the stress response, and the proximal signal for HSP induction is apparently the accumulation of denatured proteins. One hypothesis explaning the overexpression of HSPs in cancer is that the physiopathological features of the tumor microenvironment (low glucose, pH, and oxygen) tend toward HSP induction (Ciocca and Calderwood, 2005).

5.4.1.1 Implications of HSPs in tumor diagnosis

In these cases, HSP expression has been analyzed in the relation to the histopathological characteristics of the tumor tissue (eg tumor type, grade of differentiation), with the expression of other molecules (eg estrogen receptors, mutated p53), and with patient parameters like sex and age. The main following conclusions can be made concerning diagnostic immunopathology, carcinogenesis, differentiation and associations with other molecules.

Diagnostic immunopathology: since HSPs are overexpressed in a wide range of malignant cells and tissues, HSP detection is not useful in diagnostic immunopathology. However, it may be useful to apply in a panel of immunopathology in some cases: the detection of αβ crystanlin (small HSPs) for identification of renal cell carcinomas (Pinder et al., 1994) and the detection of Hsp27 or Hsp90 for identification of Reed-Sternberg cells ([11]) (Hsu and Hsu, 1998).

([11]) Reed-Sternberg cells (Lacunar Histiocytes) are distinctive giant cells found on light microscopy in biopsies from individuals with Hodgkin's lymphoma (Hodgkin's disease; a type of lymphoma), and certain other disorders. They are derived from B lymphocytes.

Carcinogenesis: HSP expression levels can help indicate the presence of abnormal changes during the process of carcinogenesis in certain tissues. For example, Hsp27 is overexpressed in hyperplastic endometrium, and this protein appears as a marker of squamous metaplasia in the uterine cervix; Hsp10 and Hsp60 are related with the process of carcinogenesis of the uterine cervix and colon; and inducible Hsp70 is associated with carcinogenesis of the oral epithelium and as a marker for early hepatocelullar carcinoma (Ciocca and Calderwood, 2005).

Differentiation: HSPs associated with higher differentiation are: Hsp27 and Hsp90 in endometrial carcinomas, Hsp27 in squamous carcinomas (uterine cervix, oral epithelium), and Hsp27 as a marker of keratinocyte differentiation in the skin. In contrast, HSPs associated with poor differentiation are inducible Hsp70 in cancers of the breast, ovary, and oral epithelium; Grp78 (member of HSP70 falimy) in lung carcinoma; and Hsp27 in astrocytomas (Ciocca and Calderwood, 2005).

Associations with other molecules: In general, several HSPs are coexpressed in cancer tissues; in addition, certain HSPs can be significantly associated with other molecules. For example, Hsp27 has been associated with estrogen receptor (ERα) in female breast carcinomas and endometrial carcinomas, but this protein did not appear associated with ERα in male breast carcinomas, cervical uterine carcinomas,

hepatocellular carcinomas, and meningiomas (tissues that may express ERα). It is interesting that Hsp27, which was first described as an estrogen-regulated protein, is significantly associated with ERα in the female breast and endometrium. These two organs are under strong estrogen and progesterone regulation (Ciocca and Calderwood, 2005).

5.4.1.2 Implications of HSPs in tumor prognosis

The prognosis of a particular cancer patient is very important in the clinic to individualize cancer treatments, to plan the patient's follow-up, and to answer questions from the patient or relatives. Overtherapy with cytotoxic drugs can be avoided in cancer patients if they are correctly identified as having good prognosis and vice versa. The following conclusions can be made about the HSPs that have been studied most.

Hsp27: elevated Hsp27 expression has been associated with poor prognosis in ovarian, gastric, liver and prostate cancer, and osteosarcomas. In contrast, Hsp27 over-expression has been associated with good prognosis in endometrial adenocarcinomas, oesophageal cancer, and in malignant fibrous histiocytomas. Although there are fewer studies in other cancers, the data suggest that Hsp27 has no prognostic value in head and neck squamous cancer, bladder and renal cancer (except when associated with other markers). There are contradictory data in oral cancer and ovarian cancer (Ciocca and Calderwood, 2005).

Hsp70: Overexpression of inducible Hsp70 is correlated with poor prognosis in breast cancer, endometrial cancer, uterine cervical cancer, and transitional cell carcinoma of the bladder. This is consistent with the Hsp70 associations with poor differentiation, lymph node metastasis, increased cell proliferation, block of apoptosis, and higher clinical stage, which are markers of poor clinical outcome. In contrast, high Hsp70 expression was correlated with good prognosis in oesophageal cancer, pancreatic cancer, renal cancer, and melanoma. Hsp70 expression showed no correlation with prognosis in

ovarian cancer, oral cancer, head and neck squamous cancer, gastric and prostate cancer (Ciocca and Calderwood, 2005).

Hsp90: High Hsp90 expressions in cancer tissues and presence of autoantibodies to Hsp90 have been correlated with poor prognosis in breast cancer. In contrast, Hsp90 strong expression is associated with good prognosis in endometrial cancer. Loss of Hsp90 (and Hsp60) expression has been associated in bladder carcinoma with invasive recurrence risk. Hsp90 expression was of no prognostic value in ovarian and oral cancer (Ciocca and Calderwood, 2005).

5.4.1.3 Implications of HSPs in prediction the response to anti-tumor treatment

There are studies exploring the use of the HSPs to predict the response (or lack of response) of a set of cancer patients to a specific treatment. The following conclusions can be deduced:

Hsp27: Although the expression of Hsp27 correlated with that of ERα in breast cancer, detection of Hsp27 does not predict the response to tamoxifen (an orally active selective estrogen receptor modulator which is used in the treatment of breast cancer). Regarding chemotherapy, Hsp27 overexpression has been correlated with a shorter disease-free survival in advanced breast cancer patients who received neoadjuvant chemotherapy. This clinical implication of Hsp27 expression with resistance to chemotherapy is in agreement with studies performed in ovarian cancer, in head and neck cancer, and esophageal squamous cell carcinoma (in association with other molecular markers). Hsp27 has shown no predictive value to chemotherapy in rectal cancer, malignant fibrous histiocytoma, and central nervous system tumors (induction radiochemotherapy). Regarding the brain tumors, glioblastoma multiforme is rather resistant to radiochemotherapy and that these tumors already show a high expression of Hsp27 (as well as other HSPs) and that a further elevation in HSP content may not correlate with a more resistant phenotype (Ciocca and Calderwood, 2005).

Hsp70: Although Hsp70 expression is associated with ERα expression in breast cancer, Hsp70 (like Hsp27) did not show predictive value for tamoxifen administration. In contrast, Hsp70 is emerging as a predictor of resistance to chemotherapy in breast cancer. Moreover, high Hsp70 levels predicted lower response of breast cancers to radiation and hyperthermia (Ciocca and Calderwood, 2005).

The molecular mechanisms involving Hsp27 and other HSPs in resistance to cancer therapies can be explained in several ways: (1) as molecular chaperones they can confer cytoprotection by repairing more efficiently the damaged proteins resulting from cytotoxic drug administration, (2) protecting cancer cells from apoptosis (Arrigo et al., 2002), (3) protecting the microvasculature inside tumors, because Hsp27 is found in endothelial cells (Ciocca et al., 2003), and (4) enhancing DNA repair (Mendez et al., 2003; Nadin et al., 2003).

5.4.2 Heat shock proteins and hematooncological diseases

Actually, most of studies regarding HSPs have been performed on malignant cells from solid tumor. Little is known on the role and the expression of HSPs in hematological malignancies, among that acute myeloid leukemia (AML), non-Hodgkin's lymphomas (NHLs), and myelodysplastic syndrome (MDS) are the most extensively studied.

5.4.2.1 HSPs and acute myeloid leukemia (AML)

Acute myeloid leukemia (AML), also known as acute myelogenous leukemia, is a cancer of the myeloid line of white blood cells, characterized by the rapid proliferation of abnormal cells which accumulate in the bone marrow and interfere with the production of normal blood cells. AML is the most common acute leukemia affecting adults, and its incidence increases with age. The French-American-British (FAB) classification system divided AML into 8 subtypes, M0 through to M7, based on the type

of cell from which the leukemia developed and its degree of maturity: M0 (undifferentiated AML); M1 (myeloblastic, without maturation); M2 (myeloblastic, with maturation); M3 (promyelocytic), or acute promyelocytic leukemia (APL); M4 (myelomonocytic); M4eo (myelomonocytic together with bone marrow eosinophilia); M5 monoblastic leukemia (M5a) or monocytic leukemia (M5b); M6 (erythrocytic), or erythroleukemia; M7 (megakaryoblastic) (Bennett et al., 1976).

Some few studies examined the impact of the intracellular expression of HSPs as prognostic factors alternative or additional to drug-resistance and apoptosis proteins in patients with newly diagnosed AML. HSP intracellular expression was determined on leukemic blasts derived from bone marrow as well as from peripheral blood of AML patients. HSP27 was expressed by 39%, HSP60 by 26%, HSP70 by 58%, HSP90 by 41%, and HSP110 by 30% of cases. Leukemic cells showed a heterogeneous pattern of HSPs expression among patients, among cells from individual patients, and among the different HSPs examined (Thomas et al., 2005-2). Unfortunately, in this study the authors did not characterize the concrete form of each HSP family examined.

In AML, HSP expression seems to correlate with a poor prognosis. A correlation between HSP expression and the expression of CD34 on leukemic cell surface that is known as a factor of poor outcome was observed. Indeed, higher expression of all HSPs was associated with lower complete remission (CR) rate and shorter survival duration in AML (Thomas et al., 2005-2).

The poor outcome of patients expressing HSPs could be related to a concomitant expression of drug resistance. A strong correlation between the expression of HSPs and that of MRP (multidrug resistance associated protein) was reported. This confirmed previous results reported in breast cancer, in which elevated HSP levels have been shown to correlate with drug resistance (Thomas et al., 2005-2).

In AML, high expression of Bcl-2 has been associated with CD34+ phenotype and poor response to chemotherapy. The expression of HSPs also correlated with that of Bcl-2 in AML patients (Thomas et al., 2005-2).

Other studies have focused on membrane expression of Hsp70, the major heat inducible member of the HSP70 family, on leukemic blasts derived from bone marrow of AML patients. A positive membrane expression of Hsp70 was shown to be correlated with percentage of leukemic blasts, while bone marrow of healthy human individuals or that of patients with AML in remission did not express Hsp70 on the plasma membrane. These findings suggested that Hsp70 acts as a tumor-selective recognition structure in vivo (Hantschel et al., 2000). Another study showed that high Hsp70 - membrane expression on AML leukemic cells is associated with a worse prognosis (Steiner et al., 2006). It has been reported that Hsp70 is a target for natural killer cells (NK cells) and expressed on the surface of tumor cells and mediate an increased sensitivity to allogeneic NK cells (Multhoff et al., 1997, 1999). Most interestingly, similar to full-length Hsp70 protein, the N-terminal-extended 14-mer peptide TKDNNLLGRFELSG (TKD, aa 450-463) was able to stimulate the cytolytic and proliferative activity of NK cells at concentrations equivalent to full-length Hsp70 protein (Multhoff et al., 2001). Hsp70-positive leukemic blasts can be killed by NK cells stimulated with low doses of interleukin-2 plus peptide TKD (Gehrmann et al., 2003). This Hsp70-targeted therapy could be of value, especially in patients who express Hsp70 in a high degree.

5.4.2.2 *HSPs and non-Hodgkin's lymphomas*

Non-Hodgkin's lymphoma (NHLs) is a cancer that starts in the lymphoid tissue. Such tissue makes up the lymph nodes, spleen, and other organs of the immune system. NHLs can occur at any age and are often marked by enlarged lymph nodes, fever, and weight loss. There are many different types of NHLs, which can be divided into aggressive (fast-growing) and indolent (slow-growing) types and can be classified as

either B-cell or T-cell NHLs. Lymphomas related to lymphoproliferative disorders following bone marrow or stem cell transplantation are usually B-cell NHLs. Prognosis and treatment depend on the stage and type of disease (Harris et al., 2000).

Increasing evidence has shown that specific inhibition of Hsp90 in neoplastic cells can lead to cell cycle arrest and apoptosis. Therefore, a recent study (Valbuena et al., 2005) aimed to assess for Hsp90 intracelullar expression in various types of B- and T-NHLs, in light of the potential utility of Hsp90 inhibitior (s) in the treatment of patients with NHLs. The authors found that Hsp90 is frequently expressed in many types of NHLs, including both low and high-grade tumors, but more commonly and strongly in high-grade tumors and suggested that selective inhibition of Hsp90 may be a novel target for investigational therapy in subsets of patients with NHLs.

5.4.2.3 HSPs and myelodysplastic syndromes (MDS)

The myelodysplastic syndromes (MDS, formerly known as "preleukemia") are a diverse collection of hematological conditions united by ineffective production of blood cells and varying risks of transformation to AML. Anemia requiring chronic blood transfusion is frequently present. Although not truly malignant, MDS is nevertheless classified within the hematological neoplasms. The FAB classification divided MDS into 5 categories: Refractory anemia (RA); Refractory anemia with ringed sideroblasts (RARS); Refractory anemia with excess blasts (RAEB); Refractory anemia with excess blasts in transformation (RAEB-T); and Chronic myelomonocytic leukemia (CMML).

Intracellular expression of inducible Hsp70, constitutive Hsc70 and Hsp27 was determined in bone marrow mononuclear cells (BMMC) derived from MDS patients at diagnosis, before treatment and not receiving red cell transfusions. All HSPs studied were over-expressed in MDS BMMC compared to healthy controls. An expression of inducible Hsp70 was shown to correlate with disease progression (Michalopoulou et al., 2006). Another study examined an expression of Hsp27, Hsp60, Hsp70, Hsp90 and

Hsp110 in bone marrow of patients with newly diagnosed MDS together with that of membrane differentiation antigen CD34 and the drug-resistance related protein (Pgp). An over-expression of all tested HSPs was observed in patients with poor prognosis MDS. The authors concluded that types of MDS with a good prognosis (RA, RARS) have lower HSP expression while RAEB has higher HSP expression, associated with the increased blast percentage. Expression of all HSPs, except Hsp70, correlated with CD34 and Pgp. There is a suggestion to study whether HSP antagonists can prevent or at least delay blastic evolution of MDS (Duval et al., 2006).

5.5 Heat shock proteins and Graft *versus* Host Disease (GvHD), a complication after allogeneic hematopoietic stem cell transplantation (HSCT)

Graft-*versus*-Host Disease (GvHD) is a major complication after allogeneic hematopoietic stem cell transplantation (HSCT). Clinically, GvHD is divided into acute and chronic forms: The **acute or fulminant form** of the disease is observed within the first 100 days post-transplant and the **chronic form** of GvHD is defined as that which occurs after 100 days. This distinction is not arbitrary: acute and chronic GvHD appear to involve different immune cell subsets, different cytokine profiles, and different types of target organ damage.

Acute graft-*versus*-host disease (aGvHD) is a T cell-mediated disease affecting multiple target organs including skin, liver, stomach, and/or intestines. The mechanism of the disease is complex and largely depends upon a histocompatibility antigenic difference between donor and recipient, involving both immunocompetent cells and cytokines, which can initiate apoptosis and necrosis of host tissue (Jarvis et al., 2003). Reviewing the pathophysiology of aGvHD, donor T cells activated by host alloantigens release T_H1 cytokines, IL-2 and IFN-γ, which activates macrophages and NK cells. Activated macrophages and NK cells can then be triggered by gut bacteria and by

infections to release large quantities of inflammatory cytokines and active nitrogen intermediates, a „cytokine storm", mediating tissue injury. Cell damage from preparative regimens cause as well as transient release of inflammatory cytokines, such as IL-1 and TNF-α, that increases the immunogenicity of host APCs (Hakim et al., 1997).

As it was mentioned in chapter 2, HSPs chaperone antigenic peptides that are generated within cells. Such chaperoning is a part of the endogenous pathway of antigen presentation by MHC class I molecules. In addition, peptides that are chaperoned by HSPs, or are released by cell stress or death, are taken up by antigen-presenting cells (APCs) and re-presented by their MHC molecules. It was suggested that peptide transport from the proteasome to the endoplasmatic reticulum (ER) and subsequent peptide loading of MHC class I molecules in the ER depend on battery of HSPs including cytosolic and endoplasmic members of the HSP70 and HSP90 families (Li et al., 2002). Their proposed role in peptide binding and antigen presentation could suggest a potential role in the alloreactive process that leads to GvHD after HSCT.

Goral et al. showed in a (DA x LEW) F1 rat model that both inducible and constitutive HSP70 are involved in acute GvHD pathology (Goral et al., 1995, 1998), and acute and/or chronic GvHD in humans is accompanied by an increase in anti-Hsp70 and anti-Hsp90 antibodies (Goral et al., 2002). The authors determined the levels of plasma antibodies to inducible Hsp70 and Hsp90 in human recipients after allogeneic peripheral blood stem cell transplantation (PBSCT). Plasma levels of these antibodies were correlated with GvHD status in the recipients. Patients with acute GvHD had a significant increase in IgM anti-Hsp70 and/or anti-Hsp90 early (30-90 days) after transplantation. In addition, an increase in IgM Hsp70 and/or anti-Hsp90 antibodies preceded or accompanied chronic GvHD. Antibody levels returned to normal within the next 400 days in the majority of patients (Goral et al., 2002).

Jarvis et al. found increased expression of inducible Hsp70 in the skin explant biopsy specimens significantly associated with clinical aGvHD grades II to IV

irrespective of GvHD prophylaxis. They suggested that this dramatic induction of Hsp70 in graft-*versus*-host reaction may be a result of a protective response in an attempt to refold damaged polypeptides which are correlated with HSPs' known function of protein folding and repair (Jarvis et al., 2003). However, further studies are required to clarify complex involvement of HSPs in GvHD pathogenesis.

Chapter 6: The potential therapeutic value of heat shock proteins

There are a number of indications in which heat shock proteins might be of therapeutic value. To date, the majority of studies have focused on either their capacity to regulate inflammatory responses in autoimmune disease or their ability to induce peptide-specific immune responses against tumors and pathogenic organisms.

6.1 Heat shock proteins in vaccine development against infectious diseases

HSPs could have a dual role in vaccine development against infectious diseases. Because HSPs can be early targets in the immune response against pathogens, they are being exploited as antigens for vaccine development (Zugel and Kaufmann, 1999). In addition, because HSPs potently stimulate innate and antigen-specific (adaptive) pathways, they are promising as vaccine adjuvants for a broad spectrum of pathogens.

Pathogens with prolonged intracellular persistence (eg mycobacteria and some viruses) are logical targets for vaccines aimed at augmenting cellular immunity. However, the view that cellular immunity is restricted to intracellular pathogens and that humoral immunity is restricted to extracellular pathogens is overly simplistic (Casadevall, 2003; Casadevall and Pirofski, 2003). HSP-based vaccines target multiple innate and antigen-driven pathways, making this approach attractive for intracellular and extracellular pathogens.

6.1.1 HSP-based vaccine and tuberculosis

Host defense against mycobacteria depends on cellular immunity. Tuberculosis is responsible for 2 million deaths a year, with a substantial proportion occurring in HIV-positive people. Bacille Calmette-Guérin (BCG) is a vaccine against tuberculosis that is

prepared from a strain of the attenuated live bovine tuberculosis bacillus, *Mycobacterium bovis* that has lost its virulence in humans by being specially cultured in an artificial medium for years. At best, the BCG vaccine is 80% effective in preventing tuberculosis for duration of 15 years; however, its protective effect appears to vary according to geography. The protection conferred by BCG vaccination has produced widely discrepant results in clinical trials, emphasizing the need for more-effective vaccines and more-reliable immunologic surrogates of protection (Bloom et al., 1994). Strategies to induce long-term immunity more effectively include the use of novel adjuvants and DNA vaccination.

HSP-based vaccines have been effective in several models of experimental tuberculosis. Vaccination with BCG peptides complexed to HSPs induces T_H1 responses and protect against live challenge in a murine model of pulmonary tuberculosis (Colaco et al., 2004). A DNA vaccine based on the Hsp65 *Mycobacterium leprae* gene conferred protection both prophylaxis and therapy in a mouse model of tuberculosis (Bonato et al., 2004). In another study, DNA vectors containing *M. tuberculosis* alanine–proline-rich antigen (Apa), and Hsp65 and Hsp70 mycobacterial antigens combined with BCG induced more robust immunity and conferred greater protection than BCG alone in tuberculosis in C57BL/6 and BALB/c mice (Ferraz et al., 2004). A DNA vaccine combination expressing *M. tuberculosis* Hsp65 and IL-12 was protective in a cynomolgus monkey model of tuberculosis (Kita et al., 2005).

6.1.2 HSP-based vaccine and viral infections

6.1.2.1 HSP-based vaccine and human immunodeficiency virus (HIV) infection

Binding of HSP to viral complexes can enhance antiviral immunity, including NK activity, antibody-dependent cellular cytotoxicity (ADCC), and CTL activities (Brenner and Wainberg, 2001). HSPs stimulated by stress can stimulate antiviral host defense (Oglesbee et al., 2002). Robust CD4+ and CD8+ T-cell responses are considered

important immune components for controlling HIV infection; priming and expanding these responses are likely to be crucial for developing an effective HIV vaccine.

In one recent study, gp96 complexed to HIV-1 Gag-p24 ([12]) peptide contained multiple MHC class I- and II-restricted epitopes are suitable to induce effective CTL memory by simultaneously providing CD4+ T-cell help (SenGupta et al., 2004). Other studies revealed a novel vaccine strategy in which Hsp70 was covalently linked to the CCR5 ([13]) peptides, HIV gpl20 (a human HIV-1 coat glycoprotein) and SIV (simian immunodeficiency virus) p27 capsid protein. Immunization of macaques with this complex led to induction of C-C chemokines and antibodies that block and downmodulate CCR5, as well as immune responses to the subunit SIV antigens that correlated with protection from SIV challenge. This vaccine was also effective in controlling mucosal SIV infection (Bogers et al., 2004 1-2).

([12]) Gap-p24 is a capsid protein that constitutes the core of AIDS virus HIV-1

([13]) Chemokine (C-C motif) receptor (CCR) 5, a receptor for chemokines CCL3, CCL4 and CCL5 is a major co-receptor with the CD4 glycoprotein, mediating cellular entry of CCR5 strains of HIV-1 or simian immunodeficiency virus (SIV) that may cause an AIDS-like immune deficiency known as simian AIDS. The CCR5 gene has been mapped to the short arm of chromosome 3 amongst a group of genes that encode multiple chemokine receptors. Soon after CCR5 was shown to behave as a co-receptor along with CD4 for HIV-1, the mutant allele CCR5-[Delta]32, which is characterized by a 32 bp deletion in the single coding exon of the gene, was identified in Caucasians. CCR5-[Delta]32 does not produce a functional protein, explaining the near-complete protection against HIV-1 infection in individuals homozygous for the allele.

6.1.2.2 *Other HSP-based vaccines against viral infections*

Two chaperone-based vaccines targeting chronic viral infections are currently in clinical development. Both use recombinant chaperone as carrier of defined pathogen-specific antigens, and have been shown to be safe and well-tolerated (Hoos and Levey, 2003). The chaperone E7 is used for treatment of anogenital warts caused by human papillomavirus HPV 6, 11 and cervical dysplasia caused by HPV types 16, 18, 31 and 45

(Stress Biotechnologies). The AG-702 is implicated in treatment of genital herpes caused by the *herpes simplex virus* (HSV) type 1 and type 2 (Antigenics).

6.2 Heat shock proteins in vaccine development against autoimmune diseases

Despite evidence from animal models that autoimmune conditions can also be prevented and treated with peptide therapy, the approach has been slower to translate to the clinic (Larche and Wraith, 2005). Two recent studies evaluating peptides derived from heat shock proteins for the treatment of diabetes and rheumatoid arthritis have provided encouraging results.

6.2.1 Diapep277 and Type 1 diabetes

Individuals with newly diagnosed Type 1 diabetes were treated by subcutaneous injection at three times points (at study entry, 1 month and 6 months) with 1 mg of a peptide Diapep277 whose sequence involves residues 437-460 of the human Hsp60 molecule. After 10 months, islet cell function had been maitained in the treated group but had declined in placebo controls (mannitol in the vehicle). In the treated group, peripheral blood T cells produced more IL-10 and IL-13, indicating modulation toward a T_H2-regulatory phenotype (Raz et al., 2001).

6.2.2 A dnaP1 and rheumatoid arthritis

In a phase I study of individuals with rheumatoid arthritis, a peptide derived from *Escherichia coli* cochaperone dnaJ (HSP40), a dnaJP1 (QKRAAYDQYGHAAFE) sharing sequence homology with the shared epitope among RA-associated HLA alleles was administered by mucosal (oral) delivery. Fifteen patients with active RA as defined by the American College of Rheumatology of disease duration <5 years divided in three different dose groups (0.25, 2.5, and 25 mg daily) were included in the trial. Analysis of

peripheral blood response to antigen showed that although there was no overall change in the numbers of antigen-specific cells after treatment, there were significant increases in the percentage of T cells producing IL-4 and IL-10 and concomitant reductions in IFN-γ and TNF-α (Prakken et al., 2004). These results may reflect the induction of a regulatory T cell population after therapy. But the study was not placebo controlled and did not report clinical outcomes. As a result, no conclusions can be drawn in relation to clinical efficacy.

6.3 Heat shock protein-based anti-cancer therapy

There is an exciting new door for the field of HSPs in cancer. HSP and the HSF families could provide a true Achilles heel for cancer therapy because they seem to be required for cell survival during tumor progression and metastasis (Volloch and Sherman, 1999; Hoang et al., 2000; Nylandsted et al., 2000 1-2; Jones et al., 2004). Currently, there are two aspects in use of HSPs against malignances.

Firstly, HSPs or HSFs may be targeted by drugs, and new classes of drugs targeting HSPs are beginning to be deployed, most notably at this time targeting Hsp90 (Neckers and Ivy, 2003). **Secondly**, the elevated HSPs may also provide a tempting target for immunotherapy protocols because they are able to chaperone tumor antigens and act as biological adjuvants to break tolerance to tumor antigens and cause immune killing by cytotoxic CTL and tumor regression (Arnold-Schild et al., 1999; Belli et al., 2002; Manjili et al., 2002; Noessner et at., 2002; Srivastava, 2002; Todryk et al., 2003; Castelli et al., 2004; Daniels et al., 2004). Dependence on the selective advantages for growth offered by the protective effects of HSPs may thus render tumor cells vulnerable to detection through immunosurveillance and killing by chaperone-based immunotherapy.

6.3.1 Agents that modify the molecular levels or molecular capabilities of the HSPs

This is achieved by the inhibition of Hsp90 by **geldanamycin** (benzoquinone ansamycin antibiotic), a naturally-occurring drug produced by microorganisms to protect themselves from disease-causing substances, or the geldanamycin analog **17AAG** [17-(Allylamino)-17-demethoxygeldanamycin]. The benzoquinone ansamycin antibiotics represent a class of drugs capable of affecting multiple targets in the signal transduction pathway involved in tumor cell proliferation and survival. When Hsp90 becomes activated, it forms a large complex with various cochaperones in malignant cells. In normal cells, it is found in a latent and uncomplexed state. 17-AAG binds to the complexed form of Hsp90 with a 100 fold higher affinity than the latent form in non-transformed cells. Hsp90 inhibition leads to the dissociation of various Hsp90 client proteins from the chaperone complex and to their constitutive degradation by the proteasome. Inhibition of Hsp90 induces apoptosis of various malignant cells (Thomas et al., 2005-1). These drugs target the nucleotide-binding site in the N-terminal domain of Hsp90, the same as the ATP-binding site (inhibition of the adenosine triphosphatase activity) causing inhibition of the binding of Hsp90 to the client proteins (Workman, 2002). In fact, these drugs are toxic, but the symptoms of toxicity of 17AAG such as fatigue, anorexia, diarrhea, nausea, vomiting and reversible elevations of liver enzymes are manageable (Ciocca and Calderwood, 2005).

Finally, HSF-1, Hsp27, Hsp70, and grp78 are also targets of antisense oligonucleotide therapies ([14]) or other manipulations with possibilities for anticancer therapies. These interesting approaches are still at the preclinical level.

([14]) Antisense therapy is a form of treatment for genetic disorders or infections. When the genetic sequence of a particular gene is known to be causative of a particular disease, it is possible to synthesize a strand of DNA, RNA or a chemical analogue that will bind to the mRNA produced by that gene and inactivate it, effectively turning that gene "off". This synthesized nucleic acid is termed an "anti-sense" oligonucleotide because its base sequence is complementary to the gene's mRNA, which is called the "sense" sequence.

6.3.1.1 Clinical trial with HSP90 inhibitors

In the phase I clinical trial on cancer patients, thirty patients with advanced malignancies refractory to conventional treatment were treated with 17-AAG (Banerji et al., 2005). The diagnosis were different, eg melanoma, sarcoma, breast carcinoma, colon carcinoma, mesothelioma, ovary carcinoma, and renal carcinoma. The authors demostrated that 17-AAG exhibits a tolerable toxicity profile with therapeutic plasma concentrations and target inhibition for 24 hours after treatment and some indications of clinical activity at the dose level 450 mg/m^2/week. 17-AAG produced in some patients stable disease, higher apoptosis, and less proliferation of the tumors but with lower potency than radiotherapy or chemotherapy. The dose level 450 mg/m^2/week is recommended for phase II clinical trials (Banerji et al., 2005).

6.3.2 Use of HSPs as carriers/adjuvant to present tumor molecules to the immune system

The objective is to elicit in a cancer patient a specific and active immune response against its own tumor using the HSPs as natural adjuvants that present to the immune system the molecules that have shielded the potential epitopes from immune recognition. The immunization is carried out with tumor-derived HSPs (gp96, Hsp70, and others), which bring attached the specific tumor peptides. When injected as a therapeutic vaccine, the HSPs interact with receptors on the professional antigen presenting cells (dendritic cells, macrophages). These cells introduce the antigen(s) into the MHC class I and II pathways, inducing a specific cytotoxic and helper T lymphocyte response and the production of proinflammatory cytokines. Another approach is to use recombinant HSPs with oncoproteins such as Her-2/neu or proteins from oncogenic viruses such as E7 of human papillomavirus. The tumor-derived auto-vaccines based on HSP or the recombinant HSP fusion proteins induce cytokine and costimulatory molecules with activation of CD4+ and CD8+ T cells and increase in NK cells that kill

tumor cells (Rivoltini et al., 2003). So far the most promising effects are being obtained in renal cancer and melanoma patients, but several other cancer patients are being treated with the vaccines based on HSPs including patients with colorectal, gastric and pancreatic cancers, leukemia, and lymphoma. These HSP-based vaccines exhibit minimal toxicity, and if they continue to show good results, they may ultimately be incorporated into the armamentarium against patients with limited or minimal cancer disease.

6.3.2.1 HSP-peptide complexes investigated in clinical trials

6.3.2.1.1 Oncophage

Based on proprietary HSP technology, the Oncophage vaccine is designed to capture the particular cancer's 'fingerprint.' This fingerprint contains unique antigens that are present only on that particular patient's specific cancer cells. Injection of the vaccine is intended to stimulate the patient's immune system to recognize and attack any cells bearing the specific cancer fingerprint. Oncophage is made from individual patients' tumors. Patients have surgery to remove part or all of the cancerous tissue. Using a proprietary manufacturing process, the heat shock protein gp96 and its associated peptides are isolated from the tumor. The complexes are extracted and purified from each sample, then sterilely filtered and placed into vials. The vaccine is shipped frozen back to the hospital pharmacy for use when the patient has recovered from surgery.

Currently it has been used in phase III clinical trials for **metastatic melanoma and kidney cancer.** Other clinical research evaluating Oncophage includes studies in several other cancers such as **lymphoma, pancreatic and gastric cancers**. Oncophage has been granted fast track and orphan drug designation from the Food and Drug Administration of the USA in both metastatic melanoma and kidney cancer.

Metastatic melanoma:

Investigators presented findings from a phase II trial of Oncophage as part of a combination treatment for metastatic melanoma. The study involved 18 of the evaluated patients with stage IV melanoma, all of whom underwent surgery, and then received Oncophage in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon-α (IFN- α). The researchers reported that one of two patients in this series was rendered disease-free after surgery and remained free of disease for 419 days. Of the remaining 16 patients with residual melanoma post-surgery, 10 experienced disease stabilization, lasting from 97 to 372 days. In addition, of the 16 patients evaluated for immunological response, 6 exhibited a stabilized disease process and had a demonstrable increase in anti-melanoma immune response (Belli et al., 2002).

Metastatic kidney cancer:

As reported, of the 61 metastatic kidney cancer patients who received at least one dose of Oncophage vaccine, 21 responded clinically, including one patient who remains completely free of disease for more than 2.5 years after treatment. Median progression-free survival time was 18 weeks for all patients who received oncophage. Two years after initiation of the vaccine, 30% of the patients remain alive. No significant toxicity was associated with Oncophage treatment (Assikis et al., 2003).

Metastatic colorectal cancer:

A study evaluated 29 patients with stage IV colorectal cancer metastatic to the liver, who had undergone complete resection (surgical removal) of their metastasized disease. In the beginning, the patients who responded immunologically to the vaccine (52% of study subjects) had a statistically significant survival compared with those who did not respond immunologically. The responders demonstrated an overall 2-year survival rate of 100%, compared with 50% for the nonresponders, and a disease-free survival rate of

50%, compared with 8% for the nonresponders. The patients who demonstrated immune response to Oncophage treatment also experienced a lower rate of recurrence (41%) as compared with nonresponders (92%)(Mazzaferro et al., 2003).

6.3.2.1.2 AG-858

AG-858, a kind of the autologous tumor-derived HSP70-peptide complex is a vaccine made from individual patients' cancerous cells. HSP70 associates with antigenic peptides, transporting them into antigen presenting cells for processing. Tumor-derived HSP70-peptide complexes used in vaccine preparations have been shown to prime tumor immunity and tumor-specific T cells in animal models. Patients undergo a blood-filtering process called leukapheresis, during which leukocytes are collected. The leukocytes are sent to company so that the personalized vaccines can be made. Injection of the AG-858 vaccine may cause the body to attack any cells bearing this cancer fingerprint.

The use of AG-858 in combination with Gleevec (imatinib mesylate, an inhibitor of tyrosine kinase Bcr/Abl) demonstrated responses in 7 of the 8 patients with chronic phase chronic myeloid leukemia (CML). AG-858 is composed of HSP extracted from patients' own leukemic cells. Seven patients showed cytogenetic improvement, and two tested negative for leukemic DNA in a PCR assay, indicating that the Bcr/Abl-positive cells had been eliminated from their blood. Immunological analysis indicated that vaccination was associated with an increased production of IFN-γ (indicator of T-cell activation). Vaccines were successfully prepared for and were well tolerated by all patients, with no serious toxicity (Li et al., 2003).

6.3.2.1.3 TKD

TKD has been investigated by company Multimmune GmBH, Germany to develope innovative products to treat Hsp70 membrane-positive tumors. As is was mentioned in

chapter 5, membrane-bound Hsp70 serves as a tumor-selective target structure, since Hsp70 is frequently presented on the plasma membrane of tumors and metastases but not in normal tissues. It was reported that Hsp70 N-terminal-extended 14-mer peptide (TKD, aa 450-463) was able to stimulate the cytolytic and proliferative activity of NK cells similar as full-length Hsp70 protein (Multhoff et al., 2001). Incubation of peripheral blood lymphocytes with TKD peptide plus a low dose of interleukin 2 (IL-2) iniciates the cytolytic and migratory capacity of NK cells towards Hsp70 membrane-positive tumor cells in vitro and in a xenograft tumor mouse model (Schmitt et al., 2007). Encouraged by these results, a phase I clinical trial was performed in patients with therapy-refractory, metastasized colorectal and non-small lung cell carcinoma to study the tolerability and feasibility of TKD-activated NK cells. After ex vivo stimulation of the leukapheresis product with IL-2/TKD, the peptide was removed and the activated autologous cells were reinfused intravenously. Re-infusion of TKD-activated NK cells was well tolerated, feasible, and safe. Ten of twelve patients showed significant immunological responses including an up-regulated cytolytic activity against Hsp70 membrane-positive tumors and an increase in the cell surface density of activatory NK cell receptors, including the C-type lectin receptor CD94, which serves as a surrogate marker for an Hsp70 activity. Moreover, two of five patients receiving more than four treatment cycles showed clinical responses. This result was not expected since all patients had progressive tumor disease during their last standard chemoradiotherapy (Krause et al., 2004).

In conclussion, HSP-based cancer vaccines constitute a biological way in tumor therapy, which can kill tumor cells with the help of the immune system in vivo. Their role is specific and potent in preclinical tests and their results in clinical trials have been promising. Cancer vaccines have brought hope for hundreds of cancer patients. It can be speculated that cancer vaccines may replace chemotherapy for some tumors within several years (Wang et al., 2006).

Chapter 7: The aims of the study

As it is reviewed above, heat shock proteins may be involved in pathogenesis of several pathological processes involving cancer, infection or autoimmunity. Immunological responses against HSPs can be either humoral or cellular. Further more, extracellular HSPs and the mebrane-bound HSPs on plasma membrane of cells affected by infection or tumor cells may have a role in immunoregulation. However, an involvement of HSPs in pathogenesis and prognosis of several pathological conditions such as rheumatoid arthritis/juvenile idiopathic arthritis as well as complications after hematopoietic stem cell transplantation, eg Graft *versus* Host Disease (GvHD) and infection is not completely clear. Based on these observations, our study dealed with two major aims:

7.1 Aim 1: An examination of humoral response against recombinant human (rh) Hsp60, *Mycobacterium bovis* (MB) Hsp65 and recombinant inducible (rh) Hsp70 in pediatric patients who underwent allogeneic hematopoietic stem cell transplantation (HSCT) for various malignant and non-malignant diseases and in patients with juvenile idiopathic arthritis (JIA)

By using simultaneously two methods Western blotting (WB) and Enzyme -linked Immunosorbent Assay (ELISA) we screened anti-HSP antibodies pre- and post-transplant in a cohort of pediatric patients who underwent HSCT to discover an association between the levels of anti-HSP antibodies and complications of HSCT such as infection and acute GvHD. Further we screened anti-HSP antibodies in patients with juvenile idiopathic arthritis (JIA) and determined an association between anti-HSP antibody levels and rheumatoid factor (RF), antinuclear antibodies (ANA), HLA B27 and disease duration.

In the following we examined a humoral reactivity of pediatric and young patients with various malignant and non-malignant diseases before HSCT and patients with JIA against MB Hsp65 derived fragments generated by cyanogen bromide (CNBr) digestion. Thus, we determined whether there would be qualitative and quantitative differences in the humoral response against various epitopes in different patients´ cohorts when compared to healthy controls.

7.2 Aim 2: Cell surface expression of inducible Hsp70 on synovial cells derived from patients with rheumatoid arthritis and juvenile idiopathic arthritis

By using fluorescence activated cell sorting (FACS) we screened fibroblast-like synovial cells derived from synovial tissue of rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) patients for the membrane expression of inducible Hsp70. Further we estimated the expression of the most common HSP receptors such as Toll-like receptor (TLR) 2 and 4, CD14, CD40, CD36, and CD91 on RA fibroblast-like synovial cells derived from synovial tissue. Autologous skin fibroblasts derived from the operation wound were used as controls.

Chapter 8: Results and discussion

The results of the study are reported in four papers that have been accepted for publication in international scientific journals with impact factor. A part of results are presented in two manuscripts that were submitted for publication.

8.1 An examination of humoral response against recombinant human (rh) Hsp60, *Mycobacterium bovis* (MB) Hsp65 and recombinant inducible (rh) Hsp70 in pediatric patients who underwent allogeneic hematopoietic stem cell transplantation (HSCT) for various malignant and non-malignant diseases and in patients with juvenile idiopathic arthritis (JIA)

8.1.1 The sensitivity and specificity of Western blotting and ELISA in detection of anti-HSP antibodies

(*Published in:* ***Nguyen TT****, Zlacka D, Vavrincova P, Sedlacek P, Hromadnikova I. 2006. Detection of antibodies against 60-, 65- and 70-kDa heat shock proteins in paediatric patients with various disorders using Western blotting and ELISA. Clin Chem Lab Med 44: 442-9*)

We used simultaneously WB and ELISA to detect IgG antibodies against rh-Hsp60, MB-Hsp65 and rh-Hsp70 in sera of 7 pediatric patients indicated to HSCT for different malignant and non-malignant diseases before, during and after conditioning and all the time post-transplant. Sera of 8 patients with JIA and 4 healthy controls were also tested. When using Western blotting with classical SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) with HSP amount 0.5 μg per lane we detected anti-rh Hsp60 and anti-MB Hsp65 antibodies in all tested sera, but we failed to detect antibodies against rh-Hsp70 in all samples, although they were selected on the basis of high optical density (OD) values in ELISA assay. Commercial mouse monoclonal

antibody of isotype IgG1 raised against linear epitope in the region of amino acid residues 436-503 of human Hsp70 (Stressgen, Victoria, Canada) always gave a positive reaction. Some investigators have also observed negative results when performed WB with purified recombinant human Hsp70. Munari et al. was able to detect antibodies against purified human Hsp70 by using SDS-PAGE and WB in immune-mediated hearing loss patients and healthy controls only when high amount of rh-Hsp70 was loaded on the gel (Munari et al., 2004). However our experiments carried out in the similar manner showed the loss of the specificity when 5 μg of rh-Hsp70 per lane was used. Non-specific binding occurred when the incubation with primary antibody was left out. The effect of reagents such as β-Mercaptoethanol (βME) and sodiumdodecyl sulphate (SDS) used in SDS-PAGE on the recognition of rh-Hsp70 by patients' sera antibodies was also reported (Munari et al., 2004). Under NATIVE-PAGE conditions proteins retain their higher-order structure and often retain their biological activity (Kurien et al., 2003). This method of gel electrophoresis allows one to separate native proteins according to difference in their charge density. The buffer in the gel is suitable for maintaining the protein in its native state. Concerning these facts we performed WB with NATIVE-PAGE to detect antibodies raised against native HSPs. Both anti-Hsp60 and anti-Hsp65 antibodies were detectable by using NATIVE-PAGE and WB. However, we proved repeatedly antibodies against rh-Hsp70 only in one out of fifteen tested serum samples, namely in case of a patient with active JIA systemic disease.

We concluded that SDS-PAGE/NATIVE-PAGE and WB is convenient to detect the presence of anti-rh Hsp60 and anti-MB Hsp65 antibodies, but it was not sensitive enough for the detection of antibodies against stress-inducible rh-Hsp70 in our studied cohort. ELISA, which is more sensitive, might be preferentially used to screen and quantify anti-Hsp60, anti-Hsp65 and anti-Hsp70 antibodies in sera of pediatric patients with various disorders.

8.1.2 IgG antibodies against rh-Hsp60, MB-Hsp65 and inducible rh-Hsp70 are present in sera of patients before, during and after conditioning and all the time after hematopoietic stem cell transplantation

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And: Zlacka D, Sedlacek P, Prucha M, Hromadnikova I. 2006. Antibodies to 60, 65 and 70 kDa heat shock proteins in pediatric allogeneic stem cell transplant recipients. Pediatr Transplant 10: 794-804).

By using ELISA we detected anti-Hsp60, anti-Hsp65 and anti-Hsp70 IgG antibodies in sera of 7 pediatric patients before HSCT, during conditioning and shortly after HSCT (when the patients were immunocompromised) and all the time post-transplant. Based on this study we have quantified anti-HSP antibodies and examined an association with transplant related complications such as acute GvHD, graft failure, hemorrhagic cystitis, infection, mucous inflammation of gastrointestinal tract, venooclusive disease and capillary leakage syndrome.

We found no correlation between anti-HSP antibodies and the occurrence and severity of acute GvHD and/or other transplant-related complications like graft failure, hemorrhagic cystitis and capillary leakage syndrome. However, elevated anti-HSP antibodies involving IgM and IgG isotypes were found to be associated with bacterial and fungal infection depending on etiological agents. We demonstrated de novo humoral response to HSPs in a cohort of patients with actual infection caused by *Klebsiella pneumoniae* (anti-Hsp60, anti-Hsp65 and anti-Hsp70), *Pseudomonas aeruginosa* (anti-Hsp60, anti-Hsp70) and *Aspergillus fumigatus* (anti-Hsp65).

8.1.3 The prevalence of anti-Hsp70 antibodies (IgG, IgM, Ig total) is much higher in JIA patients when compared with healthy controls and the levels of anti-Hsp70 antibodies correlate with the severity of the disease

(*Published in: Zlacka D, Vavrincova P,* ***Nguyen TT****, Hromadnikova I. 2006. Frequency of anti-hsp60, -65 and -70 antibodies in sera of patients with juvenile idiopathic arthritis. J Autoimmun 27:81-8*).

Sera of 209 juvenile idiopathic arthritis (JIA) patients and 50 healthy controls were examined for antibodies against rh-Hsp60, MB-Hsp65 and inducible rh-Hsp70 using ELISA. Anti-Hsp60 Ig total antibodies did not exceed the control level in any JIA patient. The numbers of JIA patients (16/209, 7.6%) who raised anti-Hsp65 Ig total antibodies was equal to healthy controls (4/50, 8%). Elevated levels of Ig total antibodies against Hsp70 were found in a cohort of patients with JIA (36.8%) when compared with age-matched healthy individuals (2%). These antibodies were predominantly of IgG isotype in systemic disease and IgM isotype in oligoarthritis. In polyarthritis both IgG and IgM antibodies frequently occurred. Significantly higher anti-Hsp70 antibody levels were found in RF-positive JIA patients. The levels of anti-Hsp70 antibodies correlated with the severity of disease evaluated on the basis of Steinbrocker's functional classification and rtg staging system. No association between anti-Hsp70 antibody levels and ANA, HLA B27 and disease duration (less than 2 years × more than 2 years) was observed except IgM anti-Hsp70 antibody where significantly higher levels were detected in HLA B27-positive patients. The prevalence of anti-Hsp70 antibodies is much higher in JIA patients when compared with healthy controls, suggesting their possible role in pathological mechanism of the disease.

8.1.4 Pediatric and young patients with various malignant and non-malignant diseases and patients with JIA showed similar IgG antibody levels against Mycobacterium bovis (MB) Hsp65; however, increased antibody levels against MB Hsp65 derived fragments were found in patients when compared with healthy controls

(Manuscript submitted for publication: ***Nguyen TT****, Bezouska K, Vavrincova P, Sedlacek P, Hromadnikova I. 2007. Humoral response against Mycobacterium bovis Hsp65 derived fragments in children and young people under pathologic and physiologic state).*

We used cyanogen bromide (CNBr) to cleave MB Hsp65 to three larger fragments: P1-163, molecular weight (Mw) 17.1 kDa; P191-285, Mw 10.4 kDa and P290-534, Mw 25.3 kDa. For the separation of fragments we chose Tricine SDS-PAGE because it is commonly used to separate proteins in the mass range of 1-100 kDa. Western blotting was used to detect humoral response against MB Hsp65 and its derived fragments. We measured the reflectance density (DR1, after subtraction of background value) of bands with AlphaEaseFC Stand Alone software. To increase the reproducibility of measurements we calculated the ratio of antibody concentrations (DR2) in each experiment: DR2 = DR1 of antibodies against MB Hsp65 epitope/ DR1 of antibody against MB Hsp65. Humoral reactivity against MB Hsp65 derived fragments differed on a case-by-case basis. The sera either of JIA patients (n=11) or those indicated to HSCT (n=10) reacted with individual MB Hsp65 fragments more frequently when compared with healthy controls (n=10). While IgG anti-Hsp65 antibody levels showed no significant differences between the small studied cohorts, significantly higher levels of antibodies against MB Hsp65 epitopes were observed in patients before HSCT and JIA patients when compared with healthy controls.

Comparing WB reflectance densities (DR1 and DR2), significantly elevated antibodies against P1-163 (DR1: $p=0.014$; DR2: $p=0.022$) and P290-534 (DR1: $p=0.009$; DR2: $p=0.003$) epitopes were found in patients before HSCT. Similarly, significantly

increased DR1 and DR2 values of antibodies against P1-163 (DR1: p=0.018; DR2: p=0.006) and P290-534 (DR1: p=0.05; DR2: p= 0.04) epitopes were detected in JIA patients.

The immune system of each individual would react to different epitopes of MB BCG Hsp65 immunodominant antigen. It is known that HSPs are highly conserved proteins and there is a high sequence homology between mammal and microbial HSPs. In healthy individuals, a humoral response against MB Hsp65 and its fragments may be explained by history of BCG vaccination and common infections.

An increased humoral response against individual Hsp65 derived fragments in a cohort of patients before HSCT might be explained by frequent infection caused by various pathogens in immunocompromised patients suffering from different malignant and non-malignant diseases. Members of HSP60 family were reported to be immunodominant antigens in several microbes like Mycobacteria species, *Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa*.

Enhanced levels of IgG antibodies to MB Hsp65 derived fragments detected in sera of patients with JIA might reflect anamnestic infection responsible for the onset of the autoimmunity as well as the response against highly expressed Hsp60 in synovial tissue due to a sequential homology between human Hsp60 and MB Hsp65.

8.2 Cell surface expression of inducible Hsp70 on synovial cells derived from patients with rheumatoid arthritis and juvenile idiopathic arthritis

8.2.1 Fibroblast-like synovial cells derived from patients with severe course of RA and JIA are strongly positive for membrane expressed inducible Hsp70

(*Published in:* ***Nguyen TT****, Gehrmann M, Zlacka D, Sosna A, Vavrincova P, Multhoff G, Hromadnikova I. 2006. Heat shock protein 70 membrane expression on fibroblast-like synovial cells derived from synovial tissue of patients with rheumatoid and juvenile idiopathic arthritis. Scand J Rheumatol 35: 447-53*).

We performed FACS analysis on fibroblast-like synovial cells of 15 RA patients and 3 JIA patients to investigate Hsp70 membrane expression. Skin fibroblasts derived from the operation wound (n=4) and peripheral blood mononuclear cells (PBMC) of 7 RA and 3 JIA patients were also tested. Peripheral blood lymphocytes (PBL) and skin fibroblasts of 10 healthy individuals were used as negative controls. Significantly higher percentage of Hsp70 membrane expression was found on fibroblast-like synovial cells derived from arthritis-affected joints in RA patients (mean 47.7%) when compared either with autologous skin fibroblasts (mean 9.5%, $p< 0.001$), control skin fibroblasts (mean 5.6%, $p< 0.001$) or autologous PBL (mean CD45/Hsp70-positive 10.4%, $p< 0.001$) and control PBL (mean CD45/Hsp70-positive 7.7%, $p< 0.001$).

High percentage of Hsp70 membrane expression was also observed on fibroblast-like synovial cells derived from 3 patients with JIA (mean 35.2%) when compared with autologous PBL (mean CD45/Hsp70-positive 10.4 %).

This study is the first demonstration of Hsp70 membrane expression on fibroblast-like synovial cells derived from synovial tissues of RA and JIA patients. Alike Martin et al., in the case of myeloid dendritic cells (DCs) in RA synovial fluid we speculate that Hsp70 might be translocated to the cell surface from the cytosol in response to sustained

stress and/or that Hsp70 might be captured onto the cell surface from the extracellular space via HSP receptors (Martin et al., 2003).

Inducible Hsp70 might bind autoantigens released from chronically affected synovial tissue and contribute to the autoantigen processing in RA and JIA. Hsp70-autoantigen peptide complexes released from stressed and dead cells may be endocytosed by professional antigen presenting cells (synovial fluid dendritic cells) through binding to HSP receptors (eg CD91, CD14) and represented by MHC molecules. Fibroblast-like synovial cells in rheumatoid synovial membrane show an activated phenotype with increased expression of MHC class II and adhesion molecules. It has been suggested that they also act as antigen presenting cells involved in the activation of T cells (Corrigall et al., 2000).

8.2.2 Hsp70 may be captured onto the cell surface of synovial cells derived from inflammed synovial tissue from the extracellular space via CD91 receptor

(*Manuscript submitted for publication: Hromadnikova I, **Nguyen TT**, Zlacka D, Sedlackova L, Popelka S, Veigl D, Pech J, Vavrincova P, Sosna A. 2007. Expression of HSP receptors on fibroblast-like syovial cells derived from rheumatoid arthritis - affected joints*).

We examined the cell surface expression of inducible Hsp70 and HSP receptors like TLR2, TLR4, CD14, CD36, CD40 and CD91 on fibroblast-like synovial cells (SC) derived from synovial tissue in 23 patients with rheumatoid arthritis (RA) who underwent synovectomy. For the comparison, autologous skin fibroblasts (SF) derived from the operation wound were also tested.

Similarly as in our previous study, significantly higher percentage of Hsp70 membrane expression was found on fibroblast-like synovial cells derived from arthritis-affected joints than on autologous skin fibroblasts (median SC: 21.4 % x SF: 5.0 %, $p<0.001$).

We found that both synovial cells and skin fibroblasts expressed relatively high levels of cell surface CD91 (median SC: 80.2 % x SF: 79.2 %), however no or low levels of CD14, CD40, TLR2, TLR4 and CD36. Further, we observed high membrane co-expression of CD91 and Hsp70 on RA synoviocytes (median 18.6 %), while autologous skin fibroblasts showed only background Hsp70 expression (median 3.9 %, $p<0.001$). Simultaneously by using ELISA we investigated the levels of inducible Hsp70 in synovial fluids and serum samples of patients with RA. Sera of 24 age-matched healthy controls were included in the ELISA assays as a control.

Hsp70 positivity was detected in 100 % RA synovial fluids (range 474.5 - 1078.9, mean 713.0, median 550.1 ng/ml) in contrast to control sera (range 8.0 - 53.4, mean 18.2, median 15.8 ng/ml; $p<0.001$) and RA sera (range 12.0 - 44.6, mean 24.5, median 27.7 ng/ml; $p<0.001$). The samples were considered to be positive if the OD values exceeded the mean plus 2 SD (standard deviation) of healthy control sera.

In our studies we repeatedly observed high membrane expression of Hsp70 on cell surface of fibroblast-like synovial cells derived from RA patients. It was reported that Hsp70 protects cells against a variety of toxic conditions such as oxidative stress, TNF-α, heat shock, heavy metals and cellular damage after ischaemia. In addition, Hsp70 overexpression has been shown to be protective against apoptotic death; synovial cells expressing elevated Hsp70 levels might therefore develop a certain resistance to apoptosis (Mosser et al., 1997; Schett et al., 1999). This would be in line with the observation of the low frequency of apoptosis in rheumatoid synovium despite the abundance of apoptosis-inducing factors (Sugiyama et al., 1996; Asahara et al., 1997). Extracellular stress proteins including HSPs and glucose regulated proteins (Grp) are emerging as important mediators of intercellular signaling and transport. Release of such proteins from cells is triggered by physical trauma and behavioral stress as well as exposure to immunological "danger signals". Stress protein release occurs both through physiological secretion mechanisms and during cell death by necrosis. After release into

the extracellular fluid, HSP or Grp may then bind to the surfaces of adjacent cells and initiate signal transduction cascades as well as the transport of cargo molecules such as antigenic peptides (Calderwood et al., 2007). Many of the effects of extracellular stress proteins are mediated through cell surface receptors.

Previous studies suggest that extracellular Hsp70 can initiate a potent innate and adaptive immune response (Asea et al., 2000, 2002). HSPs interacts with antigen presenting cells (APCs) through surface receptors such as scavenger receptors LOX-1 (Delneste et al., 2002; Binder et al., 2004); CD94 (Gross et al., 2003) and SR-A (Berwin et al., 2003); the LDL-receptor-related protein/α2-macroglobulin CD91 receptor (Basu et al., 2001; Binder et al., 2004); the Toll-like receptor (TLR) 2 and 4 (Asea et al., 2002; Binder et al., 2004); CD14 (Asea et al., 2000); CD36 (Binder et al., 2004) and CD40 (Wang et al., 2001; Becker et al., 2002). Formation of Hsp70-HSP receptor complex is associated with the induction of the pro-inflammatory response including a cytokine production (IL-1β, TNF-α, IL-6, etc.), expression of MHC class II (Tobian et al., 2004) and nitric oxide (NO) release (Panjwani et al., 2002).

In addition to APCs, Hsp70 can avidly bind to non-APC cell lines, especially those from epithelial or endothelial background (Theriault et al., 2005).

Since we observed simultaneously a high membrane expression Hsp70 on RA synovial cells and a high prevalence of soluble Hsp70 in RA synovial fluids, we examined whether extracellular Hsp70 is bound to the surface of RA synovial cells via any HSP receptors mentioned aboved.

We report the high membrane expression of CD91 on the cells of mesenchymal origin derived from RA affected and non-affected tissues. Further, we observed high simultaneous membrane co-expression of CD91 and Hsp70 in cell cultures derived from RA synovial tissues, while autologous skin fibroblasts showed only background Hsp70 expression.

It was proposed that the oxidized LDL binding protein CD91/LRP found on antigen presenting cells and other cell types (Basu et al., 2001) could be the common receptor for all immunogenic HSPs, including Hsp60, 70, gp96 and calreticulin (Basu et al., 2001). However, its role as a direct high/medium affinity HSP binder is still not clear. Theriault et al. examined the ability of Hsp70 in free solution to bind cells with, or without CD91 expression and observe minimal differences (Theriault et al., 2005).

The large group of HSP receptors may reflect the large and heterogeneous group of proteins often with radically different cellular effects. In addition, stress proteins likely recognize different receptors on different cell types (Theriault et al., 2005). The multiplicity of receptors may also indicate specialization for individual functions: receptors such as the TLR, CD40 and CCR5 may be adapted for transmembrane signaling while CD91 and scavenger receptor (SR) may play more important roles in internalization of HSP.

Indeed, it has been found that even between quite closely related members of the HSP70 family there were differences in interactions with individual receptors (Calderwood et al., 2007).

Since we simultaneously demonstrated the high prevalence of inducible Hsp70 in RA synovial fluids and a high membrane co-expression of Hsp70-CD91 on RA synovial cells, we speculate that Hsp70 released from inflammed synovial tissue might be captured onto the cell surface of synovial cells from the extracellular space via CD91 receptor. The significance of the Hsp70 interaction with synovial cells via CD91 remains undefined but may mediate other non-immune purposes like development of a higher resistance to stress-induced apoptosis as was described eg in adjacent neuronal cells taking up extracellular Hsp70 released from glial cells in normal conditions or during stress (Guzhova et al., 2001).

Chapter 9: Conclusion

Several studies have convincingly established the involvement of heat shock proteins in several pathological conditions. Heat shock proteins are implicated in prediction, diagnosis, prognosis and treatment of the diseases. Currently, number of heat shock protein-based drugs has been investigated in clinical trials in treatment of infections, autoimmune diseases and cancer.

Our studies dealt with the role of human inducible Hsp70, human Hsp60 and *Mycobacterium bovis* Hsp65 in pathogenesis of acute Graft *versus* Host Disease (aGvHD), a major complication after hematopoietic stem cell transplantation (HSCT) as well as in pathogenesis of rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA).

The studies with my participation as first author revealed that:

- SDS-PAGE/NATIVE-PAGE and Western blotting is not sensitive enough for the detection of IgG antibodies against human inducible Hsp70. Therefore in our further studies we used ELISA that is more sensitive to screen antibodies against all tested HSPs in sera of patients undergoing HSCT and patients with RA/JIA.
- Although the presence of inducible Hsp70 on plasma membrane was observed mostly on cancer cells, our studies detected high membrane expression of Hsp70 on fibroblast-like synovial cells derived from synovia affected by autoimmune diseases like RA and JIA. We speculated that inducible Hsp70 present on plasma membrane of RA/JIA synovial cells could be translocated from the cytosol in response to stress and/or that Hsp70 might be captured onto the cell surface from the extracellular space via HSP receptors. In further study we screened the presence of HSP receptors like CD91, CD40, CD36, CD14, TLR2, TLR4 as well as their association with inducible Hsp70 on RA derived synovial cells. We detected high co-expression of Hsp70 and CD91 on plasma membrane of RA synovial cells, while other receptors were expressed at low

levels or were not expressed at all. Simultaneously, we detected high levels of soluble inducible Hsp70 in RA synovial fluids. We speculated that Hsp70 released from inflammed synovial tissue might be captured onto the cell surface of synovial cells from the extracellular space via CD91 receptor. These findings strengthen the hypothesis that inducible Hsp70 might be involved in pathogenesis of autoimmune disorders like RA and JIA.

The studies in that I took part as a co-author have concluded that:

- Study of humoral responses against HSPs in pediatric patients undergoing HSCT for malignant and non-malignant disorders has shown that antibodies against human inducible Hsp70, human Hsp60 and *M. bovis* Hsp65 including total immunoglobulins (Ig), IgG and IgM were detected in patients' sera before conditioning, over the course of conditioning and all the time post-transplant. However, no correlation between anti-HSP antibody levels and the occurence and severity of acute Graft *versus* Host Disease was observed.
- Significanly increased levels of antibodies against human inducible Hsp70 were found in a cohort of patients with JIA when compared with age-matched healthy individuals. These antibodies were predominantly of IgG isotype in systemic disease and IgM isotype in oligoarthritis. In polyarthritis both IgG and IgM antibodies frequently occurred. The levels of anti-Hsp70 antibodies correlated with the severity of disease evaluated on the basis of Steinbrocker's functional classification and rtg staging system.

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Chapter 11: A list of author's publications

1. **Nguyen TT**, Zlacka D, Vavrincova P, Sedlacek P, Hromadnikova I. 2006. Detection of antibodies against 60-, 65- and 70-kDa heat shock proteins in paediatric patients with various disorders using Western blotting and ELISA. *Clin Chem Lab Med* 44:442-9. **IP=1.72**
2. Zlacka D, Vavrincova P, **Nguyen TT**, Hromadnikova I. 2006 Frequency of anti-hsp60, -65 and -70 antibodies in sera of patients with juvenile idiopathic arthritis. *J Autoimmun* 27:81-8. **IP= 2.15**
3. **Nguyen TT**, Gehrmann M, Zlacka D, Sosna A, Vavrincova P, Multhoff G, Hromadnikova I. 2006. Heat shock protein 70 membrane expression on fibroblast-like synovial cells derived from synovial tissue of patients with rheumatoid and juvenile idiopathic arthritis. *Scand J Rheumatol* 35:447-53. **IP=2.27**
4. **Nguyen TT**, Bezouska K, Vavrincova P, Sedlacek P, Hromadnikova I. 2007. Humoral response against Mycobacterium bovis Hsp65 derived fragments in children and young people under pathologic and physiologic state. **Article submitted for publication**
5. Hromadnikova I, **Nguyen TT**, Zlacka D, Sedlackova L, Popelka S, Veigl D, Pech J, Vavrincova P, Sosna A. 2007. Expression of HSP receptors on fibroblast-like synovial cells derived from rheumatoid arthritis - affected joints. **Article submitted for publication**

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Detection of antibodies against 60-, 65- and 70-kDa heat shock proteins in paediatric patients with various disorders using Western blotting and ELISA

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Abstract

Background: We examined antibodies against 60-, 65- and 70-kDa heat shock proteins (HSPs) in paediatric healthy individuals, patients with juvenile idiopathic arthritis (JIA) and those undergoing allogeneic stem-cell transplantation for various malignant and non-malignant diseases.

Methods: Western blotting and ELISA were used to examine HSP-directed humoral immune responses.

Results: Using ELISA we detected anti-Hsp60, -Hsp65 and -Hsp70 IgG antibodies in patient sera before, during and after conditioning and at all post-transplant times, as well as in JIA patients and controls. Western blotting showed positivity for anti-Hsp60 and anti-Hsp65 antibodies in all samples with a HSP concentration of 0.5 μg/lane. However, anti-Hsp70 antibodies were not detected at all when both sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE were used, except for one JIA patient, for whom a positive signal was only achieved in native PAGE when Hsp70 was increased to 2 μg/lane and serum dilution decreased to 1:10.

Conclusion: Western blotting is convenient for the detection of anti-Hsp60 and anti-Hsp65 antibodies, but it is not sensitive enough for the detection of anti-Hsp70 antibodies. ELISA, which is more sensitive, might be preferentially used to screen anti-Hsp60, -Hsp65 and -Hsp70 antibodies in sera of children with various disorders.

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Keywords: antibody; ELISA; heat shock protein; juvenile idiopathic arthritis; stem cell transplantation; Western blotting.

Introduction

Current evidence suggests that heat shock proteins (HSPs) might play an important role in the pathogenesis of several pathological processes, for example, autoimmune diseases and graft vs. host disease (GvHD). HSPs are among the most phylogenetically conserved proteins and can be classified into several families based on molecular weight: HSP110, HSP90, HSP70, HSP60, HSP47 and the small HSPs (HSP10–30) (1). Numerous reports have suggested that a connection between infection and the onset of autoimmune disease exists due to the cross-reactivity between some epitopes of microbial HSPs and host HSPs produced at sites of inflammation (2, 3). Therefore, the great degree of amino acid sequence conservation of host and pathogen HSPs may lead to the breakdown of immunotolerance and development of autoimmune disease (4). Several groups have detected humoral immune reactivity against HSPs in autoimmune disorders such as lupus erythematosus (5), rheumatoid arthritis (6–8), juvenile chronic arthritis (5, 9), Crohn's disease and active ulcerative colitis (10), immune thrombocytopenic purpura (1), autoimmune inner ear disease (11, 12), thyroid autoimmune processes (13), and autoimmune liver disease (14).

Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease of childhood (15–17) and is characterised by chronic inflammation of one or more joints, resulting in joint destruction and severe functional limitation in nearly 49% of affected children (18, 19). JIA consists of various subtypes, with three principal types of onset: a) oligoarthritis; b) polyarthritis; and c) systemic disease (20). This heterogeneous disease was one of the first human autoimmune diseases for which extensive immune reactivity directed toward self-HSPs has been described (21). However, few studies have examined the HSP-directed humoral immune response in a cohort of children with JIA (5, 9).

Acute GvHD is a very common complication after stem cell transplantation (SCT), which is used to treat patients with various lympho-haemopoietic malignancies, as well as non-malignant disorders (22). In this process, donor T-cells recognise the major histocompatibility complex (MHC) and their associated peptides on host-derived antigen-presenting cells (APCs), which results in tissue damage (23). Numer-

ous reports have suggested that HSPs are over-expressed at sites of inflammation. Recently, Jarvis et al. demonstrated increased expression of stress-inducible Hsp70 in a human tissue model of acute GvHD, suggesting a possible involvement in the pathogenesis of the disease (24). Goral et al. showed in a rat model that Hsp70 is involved in acute GvHD pathology (25, 26), and acute and/or chronic GvHD in humans is accompanied by an increase in anti-Hsp70 and -Hsp90 antibodies (27).

In most studies, the detection of anti-HSP antibodies was based either on an ELISA or on Western blotting (WB).

In the present study we tested the sensitivity and specificity of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE and WB, as well as ELISA, for the detection of antibodies against recombinant human Hsp60 (rh-Hsp60), recombinant *Mycobacterium bovis* Hsp65 (MB-Hsp65) and stress-inducible recombinant human Hsp70 (rh-Hsp70) in paediatric patients with various disorders, including JIA patients and those receiving allogeneic stem-cell grafts for various malignant and non-malignant diseases.

Materials and methods

Patients and sera

The first cohort tested involved seven paediatric patients (six males, one female, age range 7–19 years) treated with allogeneic SCT for various malignant and non-malignant diseases in the Bone Marrow Transplant Unit in the Department of Paediatric Haematology and Oncology at University Hospital Motol in Prague. The underlying diseases in transplanted patients were acute lymphoblastic leukaemia (ALL, n=3), severe aplastic anaemia (SAA, n=1), Wiskott-Aldrich syndrome (WAS, n=2) and myelofibrosis (MF, n=1). Patient sera were collected before and during conditioning and after SCT (range day 20–day 132). Patients received peripheral blood stem-cell (PBSC, n=2) or bone marrow (BM, n=5) grafts from unrelated donors (n=5) and/or human leukocyte antigen (HLA)-identical siblings (n=2). Patients were conditioned with total body irradiation and etoposide (TBI/VP16, n=2), busulphan and cyclophosphamide (Bu/Cy, n=2), busulphan, cyclophosphamide and etoposide (Bu/Cy/VP16, n=1), busulphan, cyclophosphamide and melphalan (Bu/Cy/Mel, n=1) or cyclophosphamide with anti-lymphocyte globulin (Cy/ALG, n=1).

Patients were given GvHD prophylaxis in the form of cyclosporine alone (CsA, n=1), cyclosporine and methotrexate (CsA/Mtx, n=1), or cyclosporine, methotrexate and anti-thymocyte globulin (CsA/Mtx/ATG, n=5).

Five patients developed acute GvHD of grade II and four out of five were good responders to corticosteroids. In two patients, GvHD subsequently reactivated. Two patients had no sign of acute GvHD. One patient died on day 37 post-transplant from pneumonitis and suspect diffuse alveolar haemorrhage.

The second cohort consisted of eight patients (four males, four females, age range 5–18 years) from the Outpatient Department of Rheumatology at University Hospital Motol in Prague with definite JIA lasting more than 2 years (range 2–13 years). According to the Idiopathic Arthritides of Childhood Classification, all patients had systemic arthritis. Patients met the standard American College of Rheumatology (ACR) criteria for disease activity measures and were divided into two groups, depending on disease activity: 1) complete or near remission, with or without ongoing treatment (n=3); and 2) active disease (n=5).

Sera of four age-matched, healthy individuals (three males, one female, age range 5–16 years) were also tested. Interestingly, one healthy donor (no. 1) was *Toxoplasma gondii* IgM-seropositive at the time of testing, without symptoms of the disease. The serum of this healthy control subject was used as a positive control for detection of anti-Hsp60 antibodies in a series of experiments.

Sera were aliquoted and stored at −20°C until used.

Local Ethics Committee approval and informed consent were obtained for all patients and healthy individuals involved in the study.

ELISA

ELISA was performed as described by Goral et al. (27). Briefly, 96-well ELISA plates (Corning-Fisher Scientific, Itasca, IL, USA) were coated with rh-Hsp60 (Lionex, Braunschweig, Germany), MB-Hsp65 (Lionex) and rh-Hsp70 (Stressgen, Victoria, Canada) at a concentration of 0.05 μg/well in 0.05 M bicarbonate buffer, pH 9.6 (Sigma Biosciences, St Louis, MO, USA). After overnight incubation at 4°C, plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST), pH 7.4, (Sigma Biosciences). PBST was used throughout the assay to wash the plates. We blocked non-specific binding using 1% bovine serum albumin (BSA) (Sigma Biosciences) in PBST for 2 h at room temperature (RT). After washing, 100 μL of serum samples diluted 1:100 in blocking buffer (PBST/1% BSA) was added to the wells in duplicate and incubated overnight at 4°C. After washing, plates were incubated with peroxidase-conjugated goat-anti-human IgG (Fab-specific) antibody (Sigma Biosciences) diluted 1:40,000 for 5 h at RT. The plates were then washed and developed with 0.4 mg/mL o-phenylenediamine dihydrochloride (OPD), 0.4 mg/mL urea hydrogen peroxide in 0.05 M phosphate citrate buffer, pH 5.0 (Sigma FAST OPD, Sigma Biosciences). The optical density (OD) was evaluated at 450 nm using an MRX II ELISA plate reader (Dynex Technologies, Chantilly, VA, USA).

SDS-PAGE and WB

SDS-PAGE was performed using a modification of the method described by Laemmli (28). HSPs were diluted in sample buffer containing SDS and β-mercaptoethanol (βME) (Bio-Rad, Hercules, CA, USA), heated at 95°C for 4 min and were then run on a 10% SDS-PAGE gel at a HSP concentration of 0.5 μg/lane using a Mini-PROTEAN 3 Cell (Bio-Rad). Separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 100 V and 350 mA for 1 h at RT. The efficiency of the transfer was confirmed by staining the membranes with Coomassie brilliant blue solution (Bio-Rad). The membranes containing HSP were then cut into pieces of 2 cm×6 cm, rinsed with PBST, and saturated with blocking buffer using gentle agitation. To prevent non-specific binding of antibodies, an optimal blocking protocol was used for each HSP. In the case of rh-Hsp60, the membrane was blocked with 5% casein (Bio-Rad) in PBST overnight at RT. For MB-Hsp65, blocking was in 3% casein in PBST for 2 h at RT. For rh-Hsp70, blocking was in 5% non-fat dry milk (Bio-Rad) in PBS for 2 h at RT. Patient sera were diluted 1:50 in appropriate blocking buffer and incubated with the membranes for 2 h at RT with gentle agitation. After

washing, horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody (172-1050, Bio-Rad) diluted 1:3000 in appropriate blocking buffer was incubated with the membranes for 1 h at RT.

Anti-Hsp60 monoclonal antibody (Clone LK2, Sigma Biosciences), anti-mycobacterial Hsp65 monoclonal antibody (SPA-882, Stressgen) and anti-Hsp70 monoclonal antibody (SPA-810, Stressgen), together with goat anti-mouse IgG HRP-conjugated antibody (170-6516, Bio-Rad) were used as relevant controls for HSP detection.

Controls without sera or primary antibody were always treated in parallel to confirm the specificity of all HSP assays.

After washing, bound anti-HSP antibodies were detected using an Amplified Opti-4CN Substrate Kit (Bio-Rad) according to the manufacturer's instructions.

Analysis of the results involved capturing strip images, locating bands a using pre-stained molecular weight marker (Precision Plus Protein standards 10–250 kDa, Bio-Rad), and measuring the reflectance density (DR1, after subtraction of background value) of anti-Hsp60, -Hsp65 and -Hsp70 bands with AlphaEaseFC Stand Alone software (Alpha Innotech, San Francisco, CA, USA). To increase the reproducibility of measurements, we calculated the ratio of antibody concentrations (DR2) by including positive and negative control serum samples in each experiment: DR1 of testing sample–DR1 of negative control/DR1 of positive control–DR1 of negative control (29).

Native PAGE and WB

Discontinuous native PAGE (30) was chosen for separation of HSPs depending on their size and charge. We used a running buffer (pH 8.3) with a pH greater than pI of the HSPs (rh-Hsp60, pI 5.7; MB-Hsp 65, pI 4.85; rh-Hsp70, pI 5.42) to impart a negative charge to proteins and allow their migration toward the positive electrode.

HSPs were diluted in sample buffer at a concentration of 2 μg/lane and loaded into the wells.

Native PAGE was carried out under the same conditions as for SDS-PAGE. In consecutive Western blotting, HSP transfer onto PVDF membranes was performed without methanol and a serum dilution of 1:10 was used.

Results

ELISA

Using ELISA, we detected anti-Hsp60, -Hsp65 and -Hsp70 IgG antibodies in patient sera before, during and after conditioning, and at all times post-transplant. Based on this study, we have quantified anti-HSP antibodies and examined an association with transplant-related complications, especially GvHD (data not shown).

Antibodies against rh-Hsp60, MB-Hsp65 and rh-Hsp70 were also detected in sera of paediatric patients with definite JIA and in healthy controls. We compared anti-HSP antibody levels between JIA subgroups involving oligoarthritis, polyarthritis and systemic disease and analysed an association of anti-HSP antibody levels with the presence of rheumatoid factor (RF), antinuclear antibodies, HLA B27 and disease duration (data not shown).

Furthermore, we used semi-quantitative WB to confirm the ELISA results.

SDS-PAGE and WB

Anti-Hsp60 and anti-Hsp65 antibodies Using SDS-PAGE and WB, we confirmed our ELISA results. All tested sera reacted with rh-Hsp60 (Figure 1) and MB-Hsp65 (Figure 2). In most cases, there was a correlation between the ratio of reflectance densities (DR) for test and control serum sample bands included in each experiment (DR2, WB) and the OD values (ELISA) (Figures 1 and 2).

Anti-Hsp70 antibodies Despite the successful detection of anti-Hsp70 antibodies in all sera tested using ELISA, we were not able to detect anti-Hsp70 at all, although mouse anti-Hsp70 monoclonal antibody always gave a positive result, and when left out of the incubation with primary antibody, verified the specificity of the assay (Figure 3). In our cohort of seven patients undergoing SCT, eight patients with JIA and four healthy individuals, no serum showed a positive WB result, although these sera were selected on the basis of high anti-Hsp70 antibody OD values detected in ELISA. The results remained negative when the amount of rh-Hsp70 was increased to 2 μg/lane and serum dilution was decreased to 1:10 (Figure 3). When 5 μg of rh-Hsp70/lane was used, non-specific binding occurred when incubation with the primary antibody was left out.

We suspected that the failure to detect anti-Hsp70 antibodies might be caused by the loss of key B-cell epitopes of Hsp70, which might be conformational and located on the surface of the protein, due to the denaturation during SDS-PAGE. Based on these observations, we performed native PAGE and WB to detect the reactivity between native HSPs and anti-HSP antibodies present in sera.

Native PAGE and WB

Both anti-Hsp60 and -Hsp65 antibodies were detectable using native PAGE and WB (data not shown).

Mouse anti-Hsp70 monoclonal antibody used as a positive control identified the 70-kDa HSP, as well as its aggregates (Figure 4).

When 15 samples of sera were screened together, we were able to identify anti-Hsp70 antibodies in just one case, JIA patient no. 5, when the Hsp70 amount was increased to 2 μg/lane and the serum dilution was decreased to 1:10, while the specificity of the assay was preserved (Figure 4). Interestingly, when this patient was tested again under the same conditions using SDS-PAGE and WB, no signal was detected.

Discussion

Many investigators have shown that antibodies against HSPs may play a role in the pathogenesis of various diseases, including JIA and GvHD.

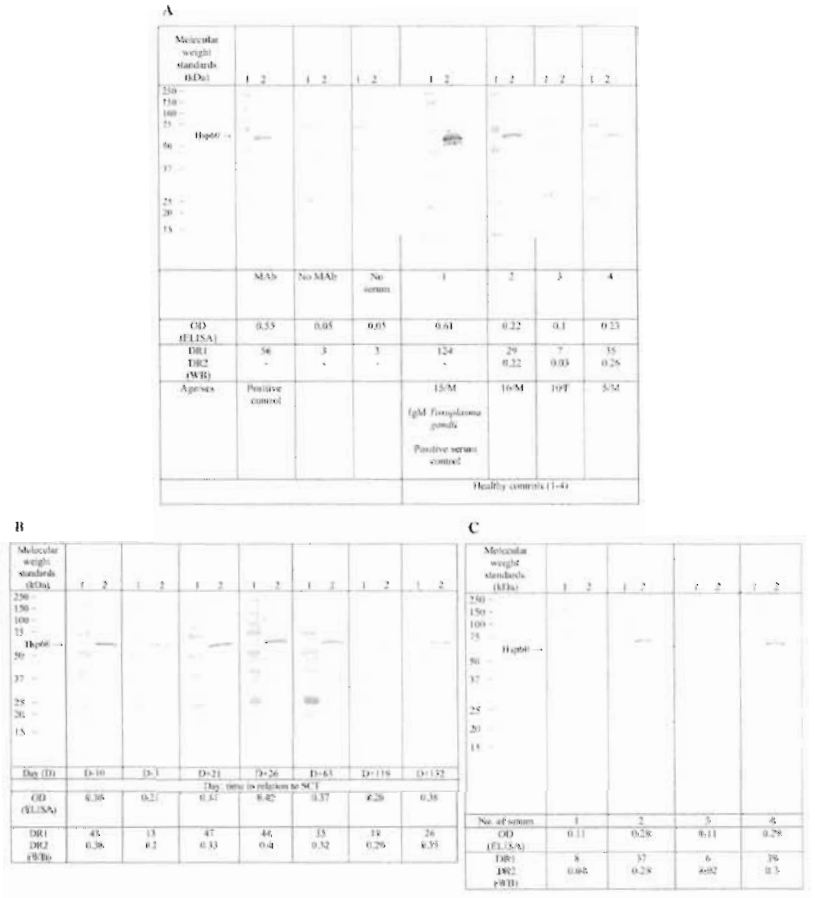

Figure 1 Comparison of SDS-PAGE/WB and ELISA in the detection of anti-Hsp60 IgG antibodies in sera of children. (A) Anti-Hsp60 monoclonal antibody and healthy control sera. (B) Patient (1-year-old female) with pre-T ALL in complete remission (CR1) before and after HLA-matched unrelated donor SCT. Conditioning: Bu/Cy/VP16. Transplant-related complications: day 23–day 44, acute GvHD grade II (skin stage 3); day 104–day 121, GvHD progression (skin); day 126, *Klebsiella pneumoniae* sepsis. (C) JIA patients with systemic active disease (1, 2, 4) or in remission (3). rh-Hsp60 (0.5 μg/lane) was run on 10% SDS-PAGE, blotted against anti-Hsp60 monoclonal antibody clone LK2 (Sigma Biosciences, St Louis, MO, USA) (MAb) diluted 1:1000 or sera diluted 1:50 in blocking buffer (5% casein in PBST). Lane 1, Precision Plus Protein standards (250, 150, 100, 75, 50, 37, 25, 20 and 15 kDa). Lane 2, anti-Hsp60 antibodies were detected in all tested sera. Healthy donor no. 1 was *Toxoplasma gondii* IgM-seropositive at the time of testing, without symptoms of the disease. The serum of this healthy control was used as a positive control for the detection of anti-Hsp60 antibodies in a series of experiments. ELISA results are expressed as the optical density (OD) and WB results as the reflectance density (DR1) of each band, and the ratio of DRs produced by the tested sera and positive and negative control sera included in each experiment (DR2).

Most authors used ELISA to detect anti-HSP antibodies. De Graeff-Meeder et al. showed that serum IgG anti-human Hsp60 antibodies in JIA patients were significantly higher when compared with control children (9). Conroy et al. proved that serum antibodies to Hsp70 were infrequent in children with JIA, although IgG antibodies to Hsp70 were detected in 50% of the synovial fluid of JIA patients (5).

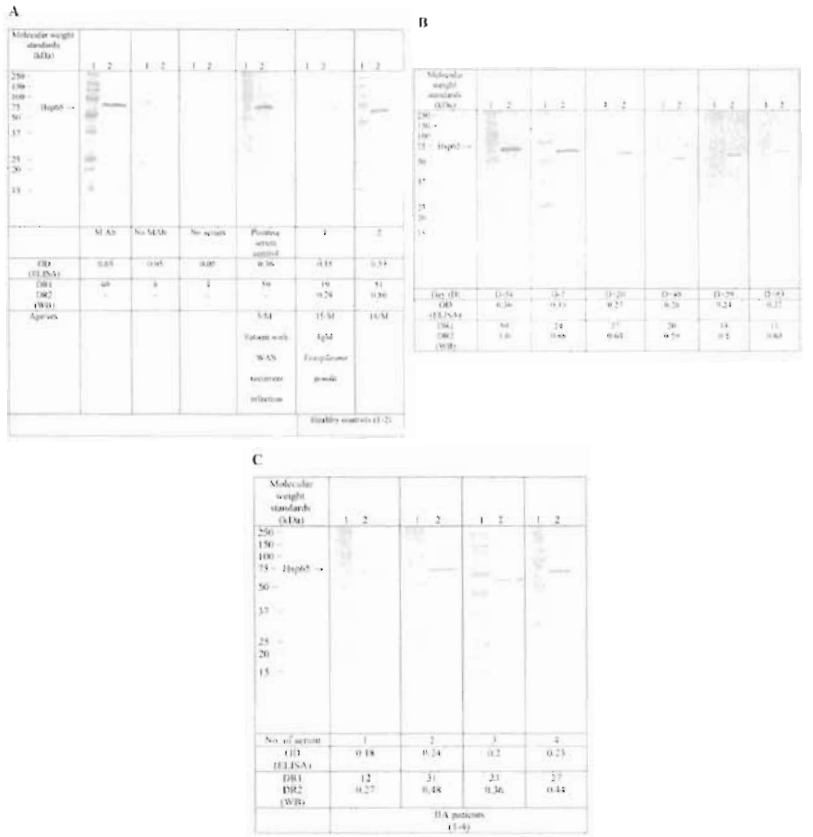

Figure 2 Comparison of SDS-PAGE/WB and ELISA for the detection of anti-Hsp65 IgG antibodies in sera of children. (A) Anti-Hsp65 monoclonal antibody and healthy control sera; (B) patient (5-year-old male) with WAS before and after HLA-matched unrelated donor SCT. Conditioning: Bu/Cy. Transplant-related complications: day 46, acute GvHD grade II (intestine); day 56, anti-CD20 monoclonal antibody for Epstein-Barr virus (EBV) re-activation; day 83, no GvHD, no EBV DNA detected. (C) JIA patients with systemic active disease (1, 2, 4) or in remission (3). MB-Hsp65 (0.5 µg/lane) was run on 10% SDS-PAGE, blotted against anti-Hsp65 monoclonal antibody (MAb) diluted 1:1000 or sera diluted 1:50 in blocking buffer (3% casein in PBST). Lane 1, Precision Plus Protein standards (250, 150, 100, 75, 50, 37, 25, 20 and 15 kDa). Lane 2, anti-Hsp65 antibodies were detected in all tested sera. The serum of a patient with WAS who suffered from recurrent infection was used as a positive control for detection of anti-Hsp65 antibodies in a series of experiments. The ELISA results are expressed as the optical density (OD) and WB results as the reflectance density (DR1) of each band and the ratio of DRs produced by the tested sera and positive and negative control sera included in each experiment (DR2).

In the case of patients undergoing SCT, Goral et al. reported that the development of acute and/or chronic GvHD in humans is accompanied by an increase in anti-Hsp70 and -Hsp90 antibodies (27).

Anti-HSP antibodies were also detected in sera of healthy adults. Rea et al. detected antibodies against rh-Hsp60, rh-Hsp70 and recombinant MB-Hsp65 in sera of normal individuals between the ages of 20 and 96 years (31).

In the present study, we screened antibodies against rh-Hsp60, MB-Hsp65 and rh-Hsp70 in paediatric patients with JIA and/or various malignant and non-malignant diseases treated with allogeneic SCT. We tested the sensitivity and specificity of ELISA and

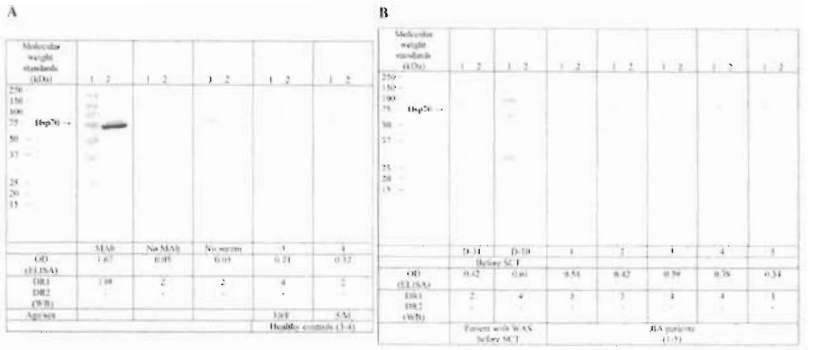

Figure 3 Comparison of SDS-PAGE/WB and ELISA for the detection of anti-Hsp70 IgG antibodies in sera of children. (A) Anti-Hsp70 monoclonal antibody and healthy control sera. (B) Patient (4-year-old male) with WAS before HLA-matched unrelated donor SCT, and JIA patients with systemic active disease (1, 2, 4, 5) or in remission (3). rh-Hsp70 (2 μg/lane) was run on 10% SDS-PAGE, blotted against anti-Hsp70 monoclonal antibody (MAb) diluted 1:1000 or sera diluted 1:10 in blocking buffer (5% non-fat dry milk in PBS). Lane 1, Precision Plus Protein standards (250, 150, 100, 75, 50, 37, 25, 20 and 15 kDa). Lane 2, anti-Hsp70 IgG antibodies were not detected in any serum sample using SDS-PAGE and WB. The ELISA results are expressed as the optical density (OD) and WB results as the reflectance density (DR1) of each band.

WB with native (native PAGE) and denatured HSPs (SDS-PAGE).

Our ELISA data are consistent with those previously reported. We detected anti-Hsp60, -Hsp65 and -Hsp70 antibodies in all subjects tested, even in immunocompromised patients during conditioning and shortly after SCT.

WB is one of the most common immunoassays frequently used for antibody detection (32). In SDS-PAGE systems, proteins are denatured by heating in buffer containing SDS and a thiol reducing agent such as βME. In some proteins this can lead to the loss of discontinuous key B-cell epitopes, which are located on the surface of the protein and are likely to be composed of amino acids from different parts of the polypeptide chain that have been brought together by protein folding. It is generally believed that most antibodies raised against intact, fully folded proteins recognise discontinuous epitopes (33). Using SDS-PAGE and WB, we detected anti-rh-Hsp60 and anti-

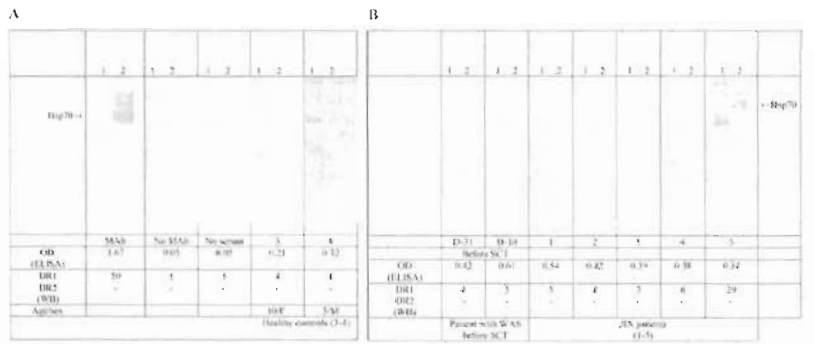

Figure 4 Comparison of native PAGE/WB and ELISA for the detection of anti-Hsp70 IgG antibodies in sera of children. (A) Anti-Hsp70 monoclonal antibody and healthy control sera. (B) Patient (4-year-old male) with WAS before HLA-matched unrelated donor SCT, and JIA patients with systemic active disease (1, 2, 4, 5) or in remission (3). rh-Hsp70 (2 μg/lane) was run on 10% native PAGE, blotted against anti-Hsp70 monoclonal antibody (MAb) diluted 1:1000 or sera diluted 1:10 in blocking buffer. Lane 1, Precision Plus Protein standards (250, 150, 100, 75, 50, 37, 25, 20 and 15 kDa). Lane 2, anti-Hsp70 IgG antibodies were detected in just one case, JIA patient no. 5. The ELISA results are expressed as the optical density (OD) and WB results as the reflectance density (DR1) of each band.

Table 1 Comparison of the sensitivity of Western blotting (SDS-PAGE and NATIVE-PAGE) and ELISA for the detection of antibodies against rh-Hsp60, MB-Hsp65 and rh-Hsp70.

Anti-HSP antibodies	Number of sera tested, n	ELISA, number of positive samples/n	Western blotting (SDS-PAGE), number of positive samples/n	Western blotting (native PAGE), number of positive samples/n
rh-Hsp60	23	23/23	23/23	23/23
MB-Hsp65	18	18/18	18/18	18/18
rh-Hsp70	15	15/15	0/15	1/15

MB-Hsp65 antibodies in all sera tested, but we failed to detect antibodies against rh-Hsp70 in all samples, although they were selected on the basis of high OD values in the ELISA assay. Commercial monoclonal antibody raised against the linear epitope in the region of amino acid residues 436–503 of human Hsp70 (Stressgen) always gave a positive reaction. Some investigators have also observed negative results when performing WB with purified human Hsp70. Munari et al. were able to detect antibodies against purified human Hsp70 using SDS-PAGE and WB in immune-mediated hearing loss patients and healthy controls only when a high amount of rh-Hsp70 was loaded onto the gel (34). However, our experiments carried out in a similar manner showed a loss of specificity when 5 μg of rh-Hsp70 per lane was used. Non-specific binding occurred when incubation with the primary antibody was left out.

The effect of reagents such as βME and SDS used in SDS-PAGE on the recognition of rh-Hsp70 by antibodies in patient sera has also been reported (34). Under native PAGE conditions, proteins retain their higher-order structure and often retain their biological activity (35). In this context, we performed WB with native rh-Hsp70 (NATIVE-PAGE). However, we repeatedly found antibodies against rh-Hsp70 in only one out of 15 serum samples tested, namely in the case of one patient with active JIA systemic disease.

SDS-PAGE/native PAGE combined with WB is convenient for detection of the presence of anti-rh-Hsp60 and anti-MB-Hsp65 antibodies, but it is not sensitive enough for the detection of antibodies against stress-inducible rh-Hsp70 in our cohort (Table 1). ELISA, which is more sensitive, might be preferentially used to screen and quantify anti-Hsp60, -Hsp65 and -Hsp70 antibodies in sera of paediatric patients with various disorders.

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Review

Frequency of anti-hsp60, -65 and -70 antibodies in sera of patients with juvenile idiopathic arthritis

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Abstract

Cross-reactivity between microbial and human heat shock proteins (hsps) led to the concept that hsp might be involved in the etiopathogenesis of autoimmune diseases. We investigated antibodies to recombinant human hsp60, recombinant *Mycobacterium bovis* hsp65 and to stress-inducible recombinant human hsp70 using enzyme-linked immunosorbent assay (ELISA) in sera of 209 juvenile idiopathic arthritis (JIA) patients and 50 healthy controls. Anti-hsp60 antibodies did not exceed the control level in any JIA patient. The numbers of JIA patients (16/209, 7.6%) who raised anti-hsp65 antibodies was equal to healthy controls (4/50, 8%). Elevated levels of antibodies against hsp70 were found in a cohort of patients with JIA (36.8%) when compared with age-matched healthy individuals (2%). These antibodies were predominantly of IgG isotype in systemic disease and IgM isotype in oligoarthritis. In polyarthritis both IgG and IgM antibodies frequently occurred. Significantly higher anti-hsp70 antibody levels were found in RF-positive JIA patients. The levels of anti-hsp70 antibodies correlated with the severity of disease evaluated on the basis of Steinbrocker's functional classification and rig staging system. No association between anti-hsp70 antibody levels and ANA, HLA B27 and disease duration (less than 2 years × more than 2 years) was observed except IgM anti-hsp70 antibody where significantly higher levels were also detected in HLA B27-positive patients. The prevalence of anti-hsp70 antibodies is much higher in JIA patients when compared with healthy controls, suggesting their possible role in pathological mechanism of the disease.

Keywords: Antibodies; Autoimmune disease; Heat shock protein; Juvenile idiopathic arthritis

1. Introduction

The heat shock proteins (hsps) are a group of highly conserved proteins classified according to their molecular weights, produced by cells of prokaryotic and eukaryotic origin under normal as well as stressful conditions [1–4]. The heat shock proteins are present in low concentrations in normal unstressed cells [5,6], where they play important physiological roles including that of chaperones to assist in proper folding and assembly of polypeptides [7–15], intracellular transport of other proteins between different intracellular compartments and MHC–peptide complex processing [16–20]. Current evidence suggests that stress proteins may be important elements in the infectious aetiology and pathogenesis of various autoimmune diseases involving rheumatoid and juvenile idiopathic arthritis [21–26].

These proteins are considered one of the superantigens and are the immunodominant antigens of various microbial pathogens inducing strong humoral and cellular immune responses in numerous infections caused by bacteria, protozoa, fungi and nematodes [27–32]. Numerous reports have suggested that a connection between an infection and the onset of autoimmune disease exists due to the cross-reactivity between some

Abbreviations: ELISA, enzyme-linked immunosorbent assay; Hsp, heat shock protein; JIA, juvenile idiopathic arthritis.

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epitopes of microbial hsps and host's hsps produced at sites of inflammation [4,33]. Therefore, the great degree of amino acid sequence conservation of host and pathogen hsps may lead to the breakdown of immunotolerance and development of autoimmune disease [34].

Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease of childhood [35–38] characterised by chronic inflammation of one or more joints resulting in joint destruction and severe functional limitation in nearly 49% of affected children [39,40]. JIA is not a homogenous disease but consists of various subtypes with three principal types of onset: (a) oligoarthritis; (b) polyarthritis; (c) systemic disease [41]. This heterogeneous disease is among the first human autoimmune diseases in which extensive immune reactivity directed toward increased expression of endogenously produced hsp60 at synovial lining cells has been described [42,43].

The goal of our study was to screen antibodies to recombinant human hsp60, recombinant *Mycobacterium bovis* BCG hsp65 and stress-inducible recombinant human hsp70 by using ELISA in a cohort of patients with JIA and compared with healthy controls. We analysed also an association between anti-hsp antibody levels and rheumatoid factor (RF), antinuclear antibodies (ANA), HLA B27 and disease duration (less than 2 years × more than 2 years).

2. Methods

2.1. Patients

Two hundred and nine patients (100 males, 109 females) with JIA aged 2–56 years (mean 15.3, median 14) from the Outpatient Department of Rheumatology, University Hospital Motol in Prague and 50 age-matched healthy controls (aged 2–47 years, mean 16, median 15) were included in the study. Four patients at the age of 41, 48, 51 and 59 years were included in the study. All of them fulfilled diagnostic criteria for JIA since the disease onset occurred before their 16th birthday.

The underlying diseases, using the Idiopathic Arthritides of Childhood Classification criteria, were 83 oligoarthritis, 109 polyarthritis (12 RF-positive and 97 RF-negative) and 17 systemic arthritis patients [41]. A total cohort involved 14 RF-positive and 193 RF-negative; 53 ANA-positive and 154 ANA-negative; 56 HLA B27-positive and 133 HLA B27-negative patients.

Steinbrocker's functional classification was used to rate the extent of physical disability on a four-level scale, ranging from Class I to Class IV [44]. The distribution of the patients according to the Steinbrocker's functional classification was as follows: 96 patients (46.8%) Class I, 87 patients (42.4%) Class II, 20 patients (9.8%) Class III and 2 patients (1%) Class IV. Rtg changes were graded with the classical Steinbrocker's Staging System (I–IV). No rtg changes were found in 94 patients (46.5%). Rtg changes of stage I were detected in 42 patients (20.8%), stage II in 31 patients (15.3%), stage III in 30 patients (14.9%) and stage IV in 5 patients (2.5%). Patients were treated depending on the stage of the disease with non-steroid antirheumatics (NSAIDs), corticosteroids and/or disease-modifying antirheumatics (DMARDs).

We tested 56 patients with less than 2 years' and 153 patients with more than 2 years' duration of the disease (some patients were tested repeatedly; all data were included in the calculation).

In addition, synovial fluid (SF) from 21 JIA patients (9 males, 12 females) aged 4–32 years (mean 17.5, median 15.5) was tested. The underlying diseases were 9 oligoarthritis, 10 RF-negative polyarthritis and 1 RF-positive polyarthritis. A total cohort involved 1 RF-positive and 20 RF-negative; 4 ANA-positive and 17 ANA-negative; 5 HLA B27-positive and 16 HLA B27-negative patients. Concerning functional assessment, the cohort includes 5 class I patients, 12 class II patients and 2 class III patients. No rtg changes were found in 4 patients, stage I changes occurred in 8 patients, stage II in 6 patients and stage III in 1 patient.

Enzyme immunoassays (EIA RF IgA, EIA RF IgG, EIA RF IgM, Test-line, Clinical Diagnostics, Czech Republic) were used for the detection of IgA, IgG and IgM rheumatoid factor in patients' serum or plasma samples.

Local ethics committee approval and informed consent were obtained for all individuals involved in this study. Sera and synovial fluids were stored at −80 °C until used.

2.2. ELISA

ELISA was performed as described previously by Goral et al [45]. Ninety-six-well ELISA plates (Corning-Fisher Scientific, Itasca, IL, USA) were coated with recombinant human hsp60 (rh-hsp60, Lionex, Germany), recombinant hsp65 protein of *Mycobacterium bovis* BCG (*M. bovis* hsp65, Lionex, Germany) and stress-inducible recombinant human hsp70 protein (rh-hsp70, Stressgen, Canada) at a concentration of 0.05 μg/well in 0.05 M bicarbonate buffer, pH 9.5 (Sigma Biosciences, St Louis, MO, USA). After an overnight incubation at 4 °C, plates were washed with PBS, pH 7.4 containing 0.05% Tween-20 (PBST; Sigma Biosciences). PBST was used throughout assays to wash plates. To block non-specific binding, PBST containing 1% bovine serum albumin (BSA; Sigma Biosciences) was used (2 h at room temperature). After washing, 100 μl of serum samples diluted 1:100 in blocking buffer (1% BSA/PBST) were added to the wells in duplicates and incubated overnight at 4 °C. After washing, plates were incubated with peroxidase conjugated secondary antibodies (Sigma Biosciences) for 5 h at the room temperature. To detect all three antibody groups together, rabbit-anti-human immunoglobulins (IgA, IgG and IgM) antibody diluted 1:30 000 was employed. To determine isotype levels of anti-hsp antibodies, goat-anti-human IgG (Fab specific) diluted 1:40 000 and goat-anti-human IgM (μ-chain specific) diluted 1:50 000 were used. The plates were then washed, developed with 0.4 mg/ml of *o*-phenylenediamine dichloride (OPD), 0.4 mg/ml urea hydrogen peroxide in 0.05 M phosphate citrate buffer, pH 5.0 (Sigma FAST OPD, Sigma Biosciences) and incubated in the dark at room temperature for 30 min. Optical density was evaluated at 450 nm using an ELISA plate reader (Dynex Technologies, MRX II, USA).

Semi-quantitative WB was used to confirm the ELISA results [46].

2.3. Statistical analysis

Fisher's exact test and two-tailed Student's *t*-test were used for the comparison of anti-hsp antibody levels between JIA patients and healthy controls.

Correlations between continuous variables including serum and synovial fluid samples derived from the same patients were performed by using linear regression with Pearson's product moment correlation coefficient (r). Significant levels of correlation were set at a P value of less than 0.001. All data were analysed with Excel (Microsoft Windows 2000) and statistical software KyPlot version 2.0 beta 15.

3. Results

We compared the levels of total Ig (IgG, IgM and IgA) antibodies against rh-hsp60, *M. bovis* hsp65 and rh-hsp70 between patients with JIA and healthy controls. The assays were developed by using mouse anti-hsp70 monoclonal and polyclonal antibodies (SPA-810, SPA-812, Stressgen, Canada), anti-hsp65 monoclonal antibody (SPA-882, Stressgen) and anti-hsp highly positive sera from patients with acute infection caused by *Toxoplasma gondii*, *Borrelia burgdorferi* and *Klebsiella pneumoniae*. The absorbance in the wells of all these positive controls was more than 1.00 OD unit. The background of the assay (OD values obtained from the wells with coated hsp in which commercial antibodies and/or patient's sera were substituted with 1% BSA/PBST) was less than 0.05 OD unit. OD values in negative controls involving monoclonal and polyclonal antibodies against another hsp than that one which was coated on the plate (e.g. anti-hsp65 monoclonal antibody used for rh-hsp70) did not also exceed 0.05, which confirmed the specificity of the assay. Intra-assay and inter-assay variability did not exceed 2.7% and 7.0%. Patients' serum samples were considered to be positive if the OD values exceeded the mean plus 2 SD of healthy control sera. None of the JIA patients as well as non-autoimmune controls had suffered a recent infection.

3.1. Anti-hsp60 antibodies (total Ig)

Anti-hsp60 antibodies did not exceed the control level in any patient with JIA (Fig. 1).

Significantly lower anti-hsp60 antibody levels were observed when either a total cohort of JIA patients ($P < 0.001$), JIA oligoarthritis ($P < 0.001$), JIA polyarthritis ($P < 0.001$) and/or JIA systemic disease ($P < 0.001$) were compared with age-matched healthy individuals.

3.2. Anti-hsp65 antibodies (total Ig)

Anti-hsp65 antibodies were found in total JIA cohort (16/209, 7.6%) involving polyarthritis (10/109, 9.2%) and systemic disease (6/17, 35.3%) as well as in healthy controls (4/50, 8%).

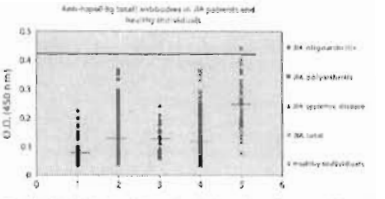
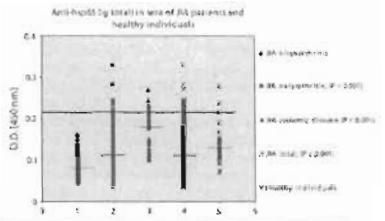


Fig. 1. Antibodies to rh-hsp60 in patients with various subgroups of JIA and healthy individuals. Results are expressed as OD values. The horizontal intermittent line indicates the upper limit calculated as 2 SD above mean OD of the healthy controls.

Significantly lower anti-hsp65 antibody levels were observed when either a total cohort of JIA patients ($P < 0.001$), JIA oligoarthritis ($P < 0.001$) and/or JIA polyarthritis ($P = 0.001$) were compared with age-matched healthy individuals. However, significantly higher anti-hsp65 antibody levels were found in JIA systemic disease ($P < 0.001$) (Fig. 2).

3.3. Total anti-hsp70 antibodies (IgG, IgM and IgA)

Significantly increased levels of total anti-hsp70 antibodies were found in a cohort of patients with JIA (77/209, 36.8%, $P < 0.001$) involving oligoarthritis (35/83, 42.2%, $P < 0.001$)



Anti-hsp65 (Ig total)	OD mean values OD median values JIA patients	OD median values OD median values healthy individuals	P values
JIA oligoarthritis	0.08 0.06	0.13 0.12	$P < 0.001$
JIA polyarthritis	0.11 0.09		$P = 0.001$
JIA systemic disease	0.18 0.19		$P < 0.001$
Total cohort	0.10 0.08		$P < 0.001$

Fig. 2. Antibodies to *M. bovis* hsp65 in patients with various subgroups of JIA and healthy individuals. Results are expressed as OD values. The horizontal intermittent line indicates the upper limit calculated as 2 SD above mean OD of the healthy controls. The table shows the mean and median OD values in each group reflecting that significantly lower anti-hsp65 antibody levels were observed in JIA oligoarthritis, JIA polyarthritis and total cohort of JIA patients when compared with age-matched healthy individuals. However, significantly higher anti-hsp65 antibody levels were found in JIA systemic disease.

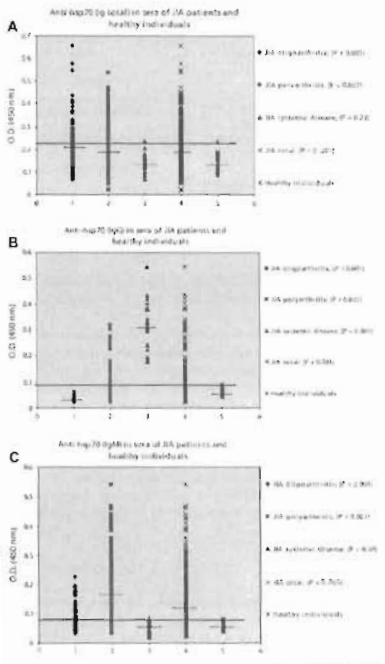


Fig. 3. a–c. Antibodies to rh-hsp70 in patients with various subgroups of JIA and healthy individuals. Results are expressed as OD values. The horizontal intermittent line indicates the upper limit calculated as 2 SD above mean OD of the healthy controls.

and polyarthritis (42/109, 38.5%, $P < 0.001$) when compared with age-matched healthy individuals (1/50, 2%), (Fig. 3a).

No association between anti-hsp70 antibody levels and ANA ($P = 0.52$), HLA B27 ($P = 0.43$) and disease duration (0-2 years × 3–45 years, $P = 0.32$) in a cohort of JIA patients was observed. However, significantly higher anti-hsp70 antibody levels were found in RF-positive JIA patients when compared with RF negative JIA patients ($P < 0.001$) (Table 1a).

The levels of anti-hsp70 antibodies correlated with the severity of the disease evaluated on the basis of Steinbrocker's functional classification and rtg staging system (Tables 1b and 1c).

Further, we determined the frequency of IgG and IgM anti-hsp70 antibodies in a cohort of JIA patients.

3.3.1. IgG anti-hsp70 antibodies

IgG antibodies to stress-inducible rh-hsp70 exceeded the upper limits of the controls in a group of JIA patients (59/209, 28.2%, $P < 0.001$) involving polyarthritis (42/109, 38.5%, $P < 0.001$) and systemic disease (17/17, 100%, $P < 0.001$). However, significantly lower levels of IgG anti-hsp70 antibodies were detected in JIA oligoarthritis ($P < 0.001$) (Fig. 3b). Similarly to previous findings, significantly higher IgG anti-hsp70 antibody levels were found in RF-positive JIA patients ($P < 0.001$) (Table 1a).

The levels of IgG anti-hsp70 antibodies correlated with the severity of the disease evaluated on the basis of Steinbrocker's functional classification and rtg staging system (Tables 1b and 1c).

No association between IgG antibodies to rh-hsp70 and ANA ($P = 0.2$), HLA B27 ($P = 0.25$) and disease duration (0-2 years × 3-45 years, $P = 0.14$) was observed (Table 1a).

3.3.2. IgM anti-hsp70 antibodies

Significantly elevated levels of IgM anti-hsp70 antibodies were found in patients with JIA (120/209, 57.4%, $P < 0.001$) involving oligoarthritis (29/83, 34.9%, $P < 0.001$) and polyarthritis (91/109, 83.4%, $P < 0.001$) (Fig. 3c). No association between IgM anti-hsp70 antibodies and ANA ($P = 0.38$) or disease duration (0–2 years × 3–45 years, $P = 0.4$) was observed. However, significantly higher IgM anti-hsp70 antibody levels were detected in RF-positive ($P < 0.001$) and HLA B27-positive ($P = 0.01$) patients (Table 1a). The levels of IgM anti-hsp70 antibodies correlated with the severity of the disease evaluated on the basis of Steinbrocker's functional classification and rtg staging system (Tables 1b and 1c).

3.3.3. Significant anti-hsp70 antibody level fluctuations during the course of the disease

Significant anti-hsp70 antibody level fluctuations were observed only in 3 out of 120 JIA patients, from whom serum samples were obtained repeatedly, during the course of the disease. In one RF-positive, ANA- and HLA B27-negative patient with systemic JIA (rtg stage III, Steinbrocker's functional class II) evaluated within 4–6 years' duration of the disease, we observed an increase in IgG anti-hsp70 antibody levels from OD 0.43 to 0.54. A significant increase of IgM anti-hsp70 antibody levels (from 0.17 to 0.4) was found in a RF-, ANA- and HLA B27-negative patient with polyarthritis (rtg stage I, Steinbrocker's functional class I) during the third year of the disease. Significant decline of IgM anti-hsp70 antibody levels (from 0.26 to 0.14) was detected in a RF-, ANA- and HLA B27-negative patient with polyarthritis (rtg stage II, Steinbrocker's functional class II) between 8 to 10 years' duration of the disease. In all these cases the status of anti-hsp70 antibody levels corresponded with the actual activity reflecting fluctuation of the disease. Milder fluctuations in anti-hsp70 antibody levels were found in 10 out of 120 re-tested patients. An increase of IgM antibodies was detected in one patient with polyarthritis and a growth of IgG in one polyarthritis and one systemic disease. In other cases we observed a decline of anti-hsp70 antibodies in both classes.

Table 1a
Association of anti-hsp70 antibody levels with RF and HLA-B27 in JIA patients

Total JIA cohort (cut-off = mean + 2 SD of healthy controls)	OD mean values			
	RF pos. × RF neg.	ANA pos. × ANA neg.	HLA-B27 pos. × HLA-B27 neg.	Disease duration 0-2 × 3-45 years
Anti-hsp70 (Ig total) (0.215)	0.31 × 0.18	0.17 × 0.19	0.19 × 0.19	0.19 × 0.19
	$P < 0.001$	$P = 0.52$	$P = 0.43$	$P = 0.32$
Anti-hsp70 (IgG) (0.09)	0.16 × 0.08	0.06 × 0.09	0.07 × 0.09	0.09 × 0.08
	$P < 0.001$	$P = 0.20$	$P = 0.25$	$P = 0.14$
Anti-hsp70 (IgM) (0.08)	0.24 × 0.12	0.13 × 0.13	0.13 × 0.12	0.13 × 0.13
	$P < 0.001$	$P = 0.38$	**$P = 0.01$**	$P = 0.40$

Significantly higher anti-hsp70 antibody levels were found in RF-positive JIA patients. Significantly higher IgM anti-hsp70 antibody levels were detected in HLA B27-positive JIA patients.

3.4 Comparison of anti-hsp antibody levels between sera and synovial fluids in a cohort of patients with JIA

We compared anti-hsp antibody levels in sera and synovial fluids simultaneously drawn from the same patients (11 oligoarthritis and 10 RF-negative polyarthritis). Patients' synovial fluid samples were considered to be positive if the OD values exceeded the mean plus 2 SD of healthy control sera. No sera and synovial fluids were found to be positive for anti-hsp60 and anti-hsp70 antibodies. One patient had both positive serum and synovial fluid for anti-hsp65 antibodies (Fig. 4). Using Pearson's product moment correlation, statistically significant association was identified between anti-hsp antibody presence in patient's serum and synovial fluid samples (Fig. 5a–c).

4. Discussion

In this study, we investigated the presence of antibodies against rh-hsp60, *M. bovis* hsp65 and stress-inducible rh-hsp70 in sera of patients with JIA and age-matched healthy individuals. By using the protocol described above, optimised ELISA assay for the measurement of anti-hsp antibody levels was developed as might be seen from the OD values of positive and negative controls. Other tested protocols did not acquire such good results.

The levels of total anti-hsp60 antibodies more than 2 SD above the mean of healthy control value were not found in a cohort of JIA patients. The numbers of JIA patients who raised total anti-hsp65 antibodies was equal to healthy controls. 36.8% of patients' serum samples contained antibodies against rh-hsp70.

On the basis of these results we investigated the levels of IgG and IgM isotypes of anti-hsp70 antibodies exclusively, which we found significantly increased in a cohort of patients with JIA. These antibodies were predominantly of IgG isotype in systemic disease and IgM isotype in oligoarthritis. In polyarthritis both IgG and IgM antibodies frequently occurred. Detailed analysis revealed significantly higher anti-hsp70 antibody levels in RF-positive JIA patients. The levels of IgG and IgM anti-hsp70 antibodies correlated with the severity of the disease evaluated on the basis of Steinbrocker's functional classification and rtg staging system. We suggest that IgM and IgG anti-hsp70 antibody levels might also reflect an actual disease activity.

The lower levels of IgM and high levels of IgG anti-hsp70 antibodies in most patients with systemic disease might be explained by the regression of systemic features in the course of time and by the strong secondary or memory response since anamnestic antibodies might persist for months, years or even a lifetime. Moreover, a significant increase of IgG antibodies might be caused by the exacerbation of the disease.

Table 1b
Association of anti-hsp70 antibody levels with the severity of the disease evaluated on the basis of Steinbrocker's functional classification

Total JIA cohort (cut-off = mean + 2 SD of healthy controls)	Steinbrocker's functional classification OD mean values			*P* values	
	Class I	Class II	Class III + IV		
Anti-hsp70 (Ig total) (0.215)	0.38	0.19	0.21	I × III + IV	0.19
				I × II + III + IV	0.17
				II × III + IV	0.3
Anti-hsp70 (IgG) (0.09)	0.07	0.08	0.13	I × III + IV	0.001
				I × II + III + IV	0.01
				II × III + IV	0.004
Anti-hsp70 (IgM) (0.08)	0.12	0.13	0.19	II × III + IV	0.009
				I × II + III + IV	0.008
				I × III + IV	0.002

The levels anti-hsp70 antibodies correlated with the severity of the disease evaluated on the basis of Steinbrocker's functional classification.

Table 1c
Association of anti-hsp70 antibody levels with the severity of the disease evaluated on the basis of Steinbrocker's rtg staging system

Total JIA cohort (cut-off = mean + 2 SD of healthy controls)	Steinbrocker's rtg staging system OD mean values				P values	
	Stage 0	Stage I	Stage II	Stage III + IV		
Anti-hsp70 (Ig total) (0.215)	0.17	0.21	0.19	0.21	0 × III + IV	0.03
					I × II + III + IV	0.05
					0 × I	0.006
					II × III + IV	0.21
Anti-hsp70 (IgG) (0.09)	0.07	0.06	0.09	0.12	0 × III + IV	0.007
					I × II + III + IV	0.01
					0 × I	0.02
					II × III + IV	0.06
Anti-hsp70 (IgM) (0.08)	0.1	0.14	0.15	0.18	0 × III + IV	<0.001
					0 × II + III + IV	<0.001
					0 × I	0.006
					II × III + IV	0.11

The levels anti-hsp70 antibodies correlated with the severity of the disease evaluated on the basis of Steinbrocker's rtg staging system.

We tested patients within 1 to 45 years duration of the systemic disease (mean 8.2 years, median 5.0 years). Concerning polyarthritis, growth of IgM of anti-hsp70 antibodies was accompanied by high IgG production. Vice versa, no presence of IgM was associated with lack of IgG anti-hsp70 antibodies. The occurrence of IgM and no presence of IgG anti-hsp70 antibodies in some patients with oligoarthritis might reflect primary immune response.

No association between anti-hsp70 antibody levels and ANA, HLA B27 and disease duration (less than 2 years × more than 2 years) was observed except IgM anti-hsp70 antibody, where significantly higher levels were also detected in HLA B27-positive patients.

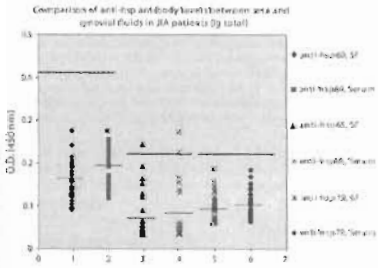


Fig. 4. Comparison of anti-hsp antibody levels between sera and synovial fluids in JIA patients. Results are expressed as OD values. The horizontal intermittent line indicates the cut-off values for positivity calculated as above OD mean plus 2 SD of the healthy control sera. Patient's synovial fluid samples were considered to be positive if the OD values exceeded the mean plus 2 SD of healthy control sera. No sera and synovial fluids were found to be positive for anti-hsp60 and anti-hsp70 antibodies. One patient had positive both serum and synovial fluid for anti-hsp65 antibodies.

To determine isotype levels of anti-hsp antibodies, goat-anti-human IgG (Fab fragment specific) and goat-anti-human IgM (μ-chain specific) conjugates were used. To detect all of the three antibody groups together; rabbit-anti-human immunoglobulins (IgA, IgG and IgM) conjugate was used. We found that IgG antibodies to hsp70 were not visualised in total Ig anti-hsp70 antibody (Fig. 3a) when these data were compared with those achieved from IgG and IgM anti-hsp70 antibody level screening (Fig. 3b, c). We might speculate that the discrepancy could be caused by the lower response against IgG after the immunisation of a rabbit using purified human immunoglobulins, since we later observed a lower response of this conjugate to purified human IgG when compared to purified human IgM in ELISA.

Only a few studies have examined hsp-directed humoral immune response in a large cohort of children with JIA previously. Our results confirmed the data of Ó Nualláin et al., who reported that JCA sera were positive for IgA, IgG and IgM isotypes of anti-hsp70 antibodies [47]. They found significantly elevated levels of IgM antibodies against human hsp70 purified from stressed peripheral blood mononuclear cells in both polyarticular and pauciarticular groups of JIA patients. Also, this group observed no relationship between anti-hsp70 and anti-hsp65 antibody levels and disease duration. Later, Conroy et al. observed the lack of anti-hsp70 antibodies in children with JCA when performing a study using a mixture of mammalian constitutively expressed hsp73 and stress-inducible hsp72 [21].

Several groups detected the humoral immune reactivity against hsp70 in rheumatoid arthritis [48] and other autoimmune disorders like lupus erythematosus [21], immune thrombocytopenic purpura [49], autoimmune inner ear disease [50], thyroid autoimmune processes [51] and autoimmune liver disease [52].

No significant difference in anti-hsp65 and anti-hsp70 antibody levels was found on comparing sera and synovial fluids simultaneously drawn from 21 JIA patients. More data will be necessary to make a final conclusion comparing anti-hsp antibody levels between sera and synovial fluids.

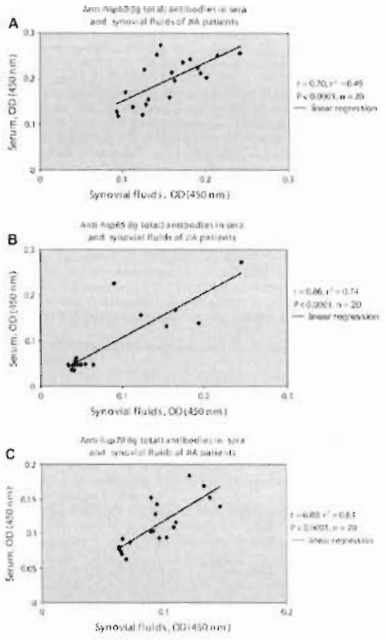


Fig. 5. a–c. Comparison of anti-hsp antibody levels between sera and synovial fluids in JIA patients. The OD values of anti-hsp antibodies in synovial fluids are plotted against the OD values of anti-hsp antibodies in patients' sera. Using linear regression with Pearson's product moment correlation coefficient (r), statistically significant association was identified between anti-hsp antibody presence in patients' serum and synovial fluid samples.

In conclusion, the prevalence of anti-hsp70 antibodies is much higher in JIA patients when compared with healthy controls, suggesting their possible role in pathological mechanism of the disease.

Acknowledgements

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Humoral response against Mycobacterium bovis Hsp65 derived fragments in children and young people under pathologic and physiologic state

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Running title: Antibodies to Hsp65 fragments in humans

Key words: antibodies; juvenile idiopathic arthritis; M. bovis Hsp65; stem cell transplantation; Western blotting.

Abstract

We investigated IgG antibodies against Mycobacterium bovis heat shock protein 65 (M. bovis Hsp65) fragments produced by cleavage with cyanogen bromide (CNBr) in 10 healthy controls, 11 patients with juvenile idiopathic arthritis (JIA) and 10 children with various malignant and non-malignant diseases before allogeneic stem cell transplantation (SCT) by using Western blotting (WB). CNBr cleaved M. bovis Hsp65 to three larger fragments: P1-163, molecular weight (Mw) 17.1 kDa; P191-285, Mw 10.4 kDa and P290-534, Mw 25.3 kDa. Humoral reactivity against M. bovis Hsp65 derived fragments differed on a case-by-case basis. The sera either of JIA patients or those before SCT reacted with individual M. bovis Hsp65 fragments more frequently when compared with healthy controls. While IgG anti-Hsp65 antibody levels showed no significant differences between the small studied cohorts, significantly higher levels of antibodies against M. bovis Hsp65 epitopes were observed in patients before SCT and JIA patients when compared with healthy controls. Comparing WB reflectance densities (DR1 and DR2), significantly elevated antibodies against P1-163 (DR1: p=0.014; DR2: p=0.022) and P290-534 (DR1: p=0.009; DR2: p=0.003) epitopes were found in patients before SCT. Similarly, significantly increased DR1 and DR2 values of antibodies against P1-163 (DR1: p=0.018; DR2: p=0.006) and P290-534 (DR1: p=0.05; DR2: p= 0.04) epitopes were detected in JIA patients. The immune system of each individual would react to different epitopes of M. bovis BCG Hsp65 immunodominant antigen. An increased humoral response against individual Hsp65 derived fragments in a cohort of patients before SCT might be explained by frequent infection in immunocompromised patients suffering from different malignant and non-malignant diseases. Enhanced levels of IgG antibodies to M. bovis Hsp65 derived fragments detected in sera of patients with JIA might reflect anamnestic infection responsible for the onset of the autoimmunity as well as the response against highly expressed Hsp60 in site of inflammation due to a sequential homology between human Hsp60 and M. bovis Hsp65.

Introduction

Heat shock proteins (HSPs) are very immunogenic and abundant intracellular proteins, whose synthesis is upregulated by a variety of stressful stimuli like temperature, hypoxia, irradiation, infection and inflammation (Welch et al 1991). HSPs are categorised into several families that are named on the basis of their approximate molecular mass. They are involved in several intracellular functions, e.g. cytoskeletal stabilisation (small HSP); folding of newly synthesized and denatured proteins, prevention of aggregation of unfolded peptides (HSP60, HSP70, HSP90); thermal tolerance and protein refolding (HSP110) (Pockley 2001). The immune reactivity against different members of HSP families, most frequently HSP60, HSP70 and HSP90, accompanies many infectious diseases (Zugel et al 1999). Extensive sequence homology between microbial and human HSP (Jindal et al 1989) had led to the concept that HSP might be involved in the aetiology and pathogenesis of autoimmune disorders. Enhanced levels of antibodies to HSP have been demonstrated in various autoimmune disorders such as lupus erythematosus (Conroy et al 1996); rheumatoid arthritis (Bahr et al 1988; Hayem et al 1999; Hirata et al 1997); juvenile chronic arthritis (Conroy et al 1996; De Graeff-Meeder et al 1993); Crohn's disease and active ulcerative colitis (Stevens et al 1992); immune thrombocytopenic purpura (Xiao et al 2004); autoimmune inner ear disease (Gottschlich et al 1995; Shin et al 1997); thyroid autoimmune processes (Paggi et al 1995) and autoimmune liver disease (Shingai et al 1995).

Hsp65, a 65kD protein isolated from Mycobacterium bovis BCG, is a member of the HSP60 family (Thole et al 1985; Thole et al 1987). Sequence comparison of Hsp65 from different mycobacterium strains showed that the protein sequence of M. bovis BCG is identical to that of M. tuberculosis, and very similar to that of M. leprae, the pathogens that cause tuberculosis and tuberculoid leprosy, respectively (Thole et al 1985; Shinnick et al 1987). M. bovis BCG Hsp65 was identified as the immunodominant antigen during mycobacterial diseases and vaccination (Van Eden et al 1988).

The mycobacterial Hsp65 is of critical significance in the model of adjuvant arthritis (AA) in rats. Following Mycobacterium tuberculosis immunisation both antibodies and T cell responses to Hsp65 were detected. Arthritogenic and protective T cell clones obtained from arthritic rats recognized the 180-188 sequence of Hsp65. The epitope was also found to react to an epitope of cartilage proteoglycan, suggesting that targeting of inflammation to the joints might be due to cross-reactivity between aa 180-188 of Hsp65 and a self component in the cartilage (van Eden et al 1985). It seems that Hsp65 play a crucial role in the T cell regulatory events involved in both the induction of AA and protection against AA (Hogervorst et al 1992). Even more, a high proportion of children affected by JIA showed both antibody and T lymphocyte responses to Hsp65 and to its 180-188 peptide in contrast to patients with adult rheumatoid arthritis (Danieli et al 1992).

Recently, by using Western blotting (WB) and ELISA we were able to show that anti-M. bovis Hsp65 antibodies were present even in sera of immunocompromised paediatric patients during conditioning and shortly after stem cell transplantation (SCT) for various malignant and non-malignant diseases; further in patients with juvenile idiopathic arthritis (JIA) and healthy controls (Nguyen et al 2006; Zlacka et al 2006a, 2006b). Next, we demonstrated a significantly high proliferative response of peripheral blood mononuclear cells (PBMC) to rh-Hsp60 as well as M. bovis Hsp65 in a cohort of pre-transplant patients with anamnestic and/or actual infection when compared to a cohort of patients without infection as well as healthy individuals (Sedlackova et al 2006a). The elevated proliferative response to rh-Hsp60, M. bovis Hsp65, P562-571 human Hsp60 and P180-188 M. bovis Hsp65 derived peptides were also found in JIA patients, mainly in patients with polyarthritis with established disease lasting more than 2 years (Sedlackova et al 2006b).

In this study we used WB to examine a humoral reactivity of paediatric and young patients with various malignant and non-malignant diseases before SCT and patients with JIA against M. bovis Hsp65 derived fragments generated by cyanogen bromide (CNBr) digestion. Thus, we were able to determine whether there were qualitative

and quantitative differences in the epitopes recognized by the antibodies in different patients´ cohorts when compared to healthy controls.

Material and methods

Patients

Ten patients undergoing stem cell transplant (SCT) for various malignant and non-malignant diseases, eleven patients with juvenile idiopathic arthritis (JIA) and ten healthy controls were included in the study.

The first tested cohort consisted of 10 paediatric patients (8 males, 2 females; age range 1-17 years, mean 9.4, median 8) treated with allogeneic SCT in the Bone Marrow Transplant Unit in the Department of Paediatric Haematology and Oncology at University Hospital Motol in Prague. The underlying diseases in transplanted patients were acute lymphoblastic leukaemia (ALL, n = 2), acute myeloid leukaemia (AML, n = 3), severe aplastic anaemia (SAA, n = 1), Wiskott - Aldrich syndrome (WAS, n = 2) and myelodysplastic syndrome (MDS, n = 2). Patients´ sera were collected before conditioning (range D-65 - D-4). Patients received peripheral blood stem cell (PBSC, n = 4) or bone marrow (BM, n = 6) grafts from unrelated donors (n = 9) and/or HLA identical siblings (n = 1).

The further cohort consisted of eleven patients (8 males, 3 females; age range 12-30 years, mean 18.6, median 18) from the Outpatient Department of Rheumatology at University Hospital Motol in Prague with definite JIA lasting more than two years (range 2-25 years). The underlying diseases, using the Idiopathic Arthritides of Childhood Classification criteria, were 3 oligoarthritis, 6 polyarthritis and 2 systemic arthritis (Petty et al 1998). All patients were RF negative. A total cohort involved 1 ANA positive and 10 ANA negative; 3 HLA B27 positive and 8 HLA B27 negative patients. Patients met the standard American College of Rheumatology (ACR) criteria for disease activity measures and were divided into two groups depending on disease activity 1) complete or near remission with or without on-going treatment (n =4) and 2) active disease (n = 7).

Sera of ten age-matched, healthy individuals (4 males, 6 female; age range 2 - 30 years, mean 16, median 16) were also tested.

Local ethics committee approval and informed consents was obtained for all individuals involved in the study.

Sera were aliquoted and stored at - 80° C until used.

Cleavage of MB- Hsp65 by CNBr

M. bovis Hsp65 fragments were produced by digesting 1 mg of purified lyophilised M. bovis Hsp65 (Lionex, Braunschweig, Germany) with 600 µL of 0.5 M CNBr/70% TFA (Trifluoric Acetic Acid), (Sigma Biosciences, St Louis, MO, USA) (Fontana et al 1986). The cleavage was performed at room temperature in the dark for 24 hours. After the cleavage, the sample was lyophilised by evaporation in Speed-Vac for 4 hours. The mixture of fragments was tested using Tricine-Sodium DodecylSulfate-Polyacrylamide Gel Electrophoresis and Western blotting.

Identification of M. bovis Hsp65 derived fragments by Tricine-Sodium DodecylSulfate-Polyacrylamide Gel Electrophoresis (Tricine SDSPAGE)

Tricine SDSPAGE was performed by a modification of the method described by Schagger and von Jagow (1987), with an 18% separating gel and a 5% stacking gel. The sample containing either M. bovis Hsp65 or Hsp65 derived fragments was diluted in sample buffer, heated at 95°C for 4 minutes and electrophoresed for 15 minutes at 50 V followed by 90 minutes at 140V at concentration 2 µg/lane for M.bovis Hsp65 and 5 µg/lane for its fragments using Mini-PROTEAN 3 Cell (BIO-RAD, CA, USA). The gels were stained with 0.25 % Coomassie Briliant Blue solution (BIO-RAD, CA, USA) or by Silver staining kit for proteins (Roti^{R} - Black P, Carl Roth GmbH+Co., Karlsruhe, Germany).

Western blotting (WB)

By using WB each serum sample was tested at least twice to confirm the results.

M. bovis Hsp65 fragments separated by Tricine SDSPAGE were transferred to Polyvinylidene difluoride (Immobilon-P^{SQ}) membranes (Millipore, MA, USA) in a Mini Trans-Blot Electrophoretic Transfer Cell (BIO-RAD, CA, USA) at 100 V, 350 mA

for 90 minutes at RT. The efficiency of the transfer was confirmed by staining the membranes with 0.1 % Amido black solution (Sigma Biosciences, St Louis, MO, USA). The membranes were rinsed with PBS-0.1% Tween 20 (PBST) (BIO-RAD, CA, USA) and blocked with 5% non-fat dry milk (BIO-RAD, CA, USA) in PBS for 2 h at RT. Patients´ sera were diluted 1:50 in blocking buffer and incubated with the membranes for 2 h at RT with gentle agitation. After washing, horseradish peroxidase conjugated goat anti-human IgG antibody (172-1050, BIO-RAD, CA, USA) diluted 1:3000 in blocking buffer was incubated with the membranes for 90 minutes at RT.

Anti-Mycobacterial Hsp65 monoclonal antibody (SPA-882, Stressgen, Victoria, Canada) together with goat anti-mouse IgG horseradish peroxidase conjugated antibody (170-6516, BIO-RAD, CA, USA) was used as relevant control for WB assay. Simultaneously, omission of the incubation with sera or monoclonal antibody had always been performed to confirm the specificity of the assays.

After washing, bound anti-Hsp65 antibodies were detected by using an Amplified Opti-4CN Substrate Kit (BIO-RAD, CA, USA) according to the manufacturer's instructions.

Analysis of the results was accomplished by capturing strip images, locating bands using pre-stained molecular weight marker (Precision Plus Protein standards 10-250 kDa, Bio-Rad, CA, USA), measuring the reflectance density (DR1, after subtraction of background value) of bands with AlphaEaseFC Stand Alone software (Alpha Innotech, San Francisco, USA). To increase the reproducibility of measurements we calculated the ratio of antibody concentrations (DR2) in each experiment: DR2 = DR1 of antibodies against M. bovis Hsp65 epitope/ DR1 of antibody against M. bovis Hsp65.

Statistical analysis

Two-tailed Student's t-test was used for the statistical analysis. P values of less than 0.05 were regarded as significant.

Results

Cleavage of M. bovis Hsp65 by CNBr and separation of fragments by Tricine SDSPAGE

Using tool "peptide cutter" (www.expasy.com), that predicts potential protease and cleavage sites and sites cleaved by chemicals in a given protein sequence, we predicted that CNBr cleaving proteins at methionine should digest M. bovis Hsp65 into four larger fragments: Fragment No.1 (Fr-1): amino acid position (aa) 1-163, molecular weight (Mw) 17.1 kDa; fragment No.2 (Fr-2): aa 164-190, Mw 2.9 kDa; fragment No.3 (Fr-3): aa 191-285, Mw 10.4 kDa; fragment No.4 (Fr-4): aa 290-534, Mw 25.3 kDa.

The staining either with Coomassie Briliant Blue solution or by the much more sensitive Silver staining kit for proteins revealed three major bands corresponding to the prediction: Fr-1 (17.1 kDa), Fr-3 (10.4 kDa), Fr-4 (25.3 kDa). A number of partial digestion products were also present in the mixture (Figure 1).

Western blotting

Representative WB results are shown in figure 2 and table 1 includes all detailed results.

M. bovis Hsp65 reacted with anti-mycobacterial Hsp65 monoclonal antibody (DR1=118). However, none of the fragments derived from M. bovis Hsp65 showed reactivity with anti-mycobacterial Hsp65 monoclonal antibody. The background of the assay was 4 (DR1= 4). Similarly as we reported previously, IgG antibodies against a whole molecule M. bovis Hsp65 were detected in all tested sera including paediatric patients with various malignant and non-malignant diseases before SCT, JIA patients and healthy controls (Nguyen et al 2006). IgG antibodies against P1-163 epitope (Fr-1, 17.1 kDa) were obviously detected in 7/10 (70 %) of patients before SCT, 7/11 (63.6 %) of JIA patients and none of the 10 healthy controls. Positivity of antibodies against P191-285 epitope (Fr-3, 10.4 kDa) occurred in 7/10 (70 %) of patients before SCT, 7/11 (63.6 %) of JIA patients and 4/10 (40 %) of healthy controls. The presence of antibodies against P290-534 epitope (Fr-4, 25.3 kDa) was observed in

9/10 (90 %) of patients before SCT, 8/11 (72.7 %) of JIA patients and 5/10 (50 %) of healthy controls.

While IgG anti-Hsp65 antibody levels showed no significant differences between these small studied cohorts, significantly higher levels of antibodies against M. bovis Hsp65 epitopes were observed in patients before SCT and JIA patients when compared with healthy controls (Tables 2, figure 3). Comparing DR1 (9.6 vs 4.8, p=0.014; 12.2 vs 6.6, p=0.009) and DR2 (0.24 vs 0.09, p=0.022; 0.3 vs 0.13, p=0.003) values, significantly elevated antibodies against P1-163 (Fr-1, 17.1 kDa) and P290-534 (Fr-4, 25.3 kDa) epitopes were found in a cohort of patients before SCT. Significantly increased DR1 (9.0 vs 4.8, p=0.018; 10.9 vs 6.6, p=0.05) and DR2 (0.19 vs 0.09, p=0.006; 0.21 vs 0.13, p=0.04) values of antibodies against P1-163 (Fr-1, 17.1 kDa) and P290-534 (Fr-4, 25.3 kDa) epitopes were also detected in a cohort of JIA patients.

The levels of antibodies against P191-285 epitope (Fr-3, 10.4 kDa) were higher in both cohorts of patients (patients before SCT 10.0 vs 5.1; p=0.05 and JIA 8.5 vs 5.1; p=0.08) when compared with healthy controls where the difference nearly reached the statistical significance. However, no statistical difference between the studied groups was observed when the ratio of antibodies against P191-285 epitope (Fr-3, 10.4 kDa) and antibodies against M. bovis Hsp65 was calculated (patients before SCT 0.22 vs 0.18; p=0.35 and JIA 0.21 vs 0.18; p=0.40).

Discussion

In this study, we examined humoral response of paediatric and young patients with different malignant and non-malignant diseases before SCT and patients with JIA to M. bovis Hsp65 as well as its fragments obtained after cleavage by CNBr.

Similarly as we reported previously, anti-Hsp65 IgG antibodies were detected in all tested sera (Nguyen et al 2006). The antibody levels against the whole molecule of M. bovis Hsp65 did not differ between either patients' or healthy controls' groups. However regarding antibodies against various M. bovis Hsp65 fragments we could observe the qualitative and quantitative difference between the studied cohorts. Concerning healthy controls, the humoral reactivity against M. bovis Hsp65 derived

fragments differs on a case by case basis despite the fact that all individuals were vaccinated with the same BCG tuberculosis vaccine during childhood. We observed evincible positivity of antibodies against Fr-4 in 3 cases, against Fr-3 in 2 cases and against both of them in 2 cases. That might suggest that the immune system of each individual would react to different epitopes of M. bovis BCG Hsp65 immunodominant antigen.

The sera either of JIA patients or those before SCT reacted with individual M. bovis Hsp65 fragments more frequently (table 1). 7 patients altogether showed reactivity against all of the fragments, 5 patients had antibodies against Fr-1 and Fr-4, 3 patients against Fr-3 and Fr-4, 2 patients against Fr-1 and Fr-3 and 2 patients against Fr-3 or Fr-4.

Significantly elevated levels of IgG antibodies to Fr-1 and Fr-4 were observed in cohorts of pre-SCT and JIA patients when compared with healthy controls. Fr-2, which contains well-known T-cell epitope P180-188, was not detected in Tricine SDSPAGE, probably due to its low molecular weight (Karopoulos et al 1995).

HSPs are highly conserved during evolution, which has resulted in extensive amino acid sequence identities between mammalian and microbial HSPs. In spite of this homology microbial HSPs have been found to be strong immunogens. Immune reactivity against different members of HSP families, most frequently Hsp60, Hsp70 and Hsp90, accompanies many infectious diseases. An increased humoral response against individual Hsp65 derived fragments in a cohort of patients before SCT might be expected, since immunocompromised paediatric patients suffering from different malignant and non-malignant diseases had been frequently affected by infection including various bacteria, viruses and fungi. In our tested cohort, seven out of ten patients suffered from anamnestic or actual infection such as Escherichia coli sepsis, fungal pneumonia or recurrent infections caused by Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter and Burgholderia species (table 1). Most patients were also at present colonized with various bacteria.

In the previous study, we examined peripheral blood mononuclear cell (PBMC) responses to HSPs in relation to infection in paediatric patients with various lympho-hemopoietic malignancies as well as non-malignant disorders subjected to SCT. We demonstrated significantly high proliferative response to rh-Hsp60 as well as M. bovis Hsp65 in a cohort of patients with anamnestic and/or actual infection when compared to a cohort of patients without infection and healthy individuals. Strong PBMC cell responses to HSPs were found in patients who were at present colonized with Escherichia coli and Klebsiella pneumoniae or had previously K. pneumoniae infection with subsequent sepsis (Sedlackova et al 2006a)

As well, we observed that anti-HSP antibodies might be produced even after SCT in relation to infection depending on aetiological agents. We demonstrated de novo humoral response to HSPs in a cohort of patients with actual infection caused by Klebsiella pneumoniae (anti-Hsp60, anti-Hsp65 and anti-Hsp70), Pseudomonas aeruginosa (anti-Hsp60, anti-Hsp70) and Aspergillus fumigatus (anti-Hsp65).

An increased humoral response against individual Hsp65 derived fragments in a cohort of patients with JIA might be explained by several means. Infection is one of the well-known mechanisms responsible for the induction of autoimmune inflammation after which HSP synthesis is greatly increased as a response to a variety of stressful stimuli. Due to a very high interspecies sequence homology between HSP the immune response to microbial HSP may cross-react with self-HSP. Furthermore, the cross reactivity between HSP and other self-proteins might intensify autoimmune process. Hence, enhanced levels of IgG antibodies to M. bovis Hsp65 derived fragments detected in sera of patients with JIA might reflect anamnestic infection responsible for the onset of the autoimmunity as well as the response against highly expressed Hsp60 in site of inflammation due to a sequential homology between human Hsp60 and M. bovis Hsp65. It was reported that synovial lining cells of patients with JIA show an increased expression of endogenously produced Hsp60 (Boog et al 1992) and IgG antibodies to human Hsp60 can be detected in both serum and synovial fluid from patients with JIA (De Graeff-Meeder

et al 1993). Danieli et al. showed elevated titres of circulating antibodies to mycobacterial Hsp65 in high proportion of children affected by JIA (Danieli et al 1992). Enhanced proliferative response of PBMC derived from JIA patients after stimulation with M. bovis Hsp65 was also reported (De Graeff-Meeder et al 1991; Danieli et al 1992; Sedlackova et al 2006).

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Figure 1: Prediction and result of M. bovis Hsp65 cleavage with CNBr

1-A: According to the tool "peptide cutter" (www.expasy.com), CNBr cleaving proteins at methionine should digest M. bovis Hsp65 to four larger fragments: Fragment No.1 (Fr-1): amino acid position (aa) 1-163, molecular weight (Mw) 17.1 kDa; fragment No.2 (Fr-2): aa 164-190, Mw 2.9 kDa; fragment No.3 (Fr-3): aa 191-285, Mw 10.4 kDa; fragment No.4 (Fr-4): aa 290-534, Mw 25.3 kDa.

1-B: M. bovis Hsp65 (0.5 μg/lane) and M. bovis Hsp65 fragments gained after cleavage with CNBr (5 μg/lane) were run on 18% Tricine SDSPAGE and stained by Silver staining kit for proteins. Lane 1: Precision Plus Protein standards - BIO-RAD (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa). Lane 2: M. bovis Hsp65. Lane 3: the separation of three main larger fragments of M. bovis Hsp65 after cleavage with CNBr.

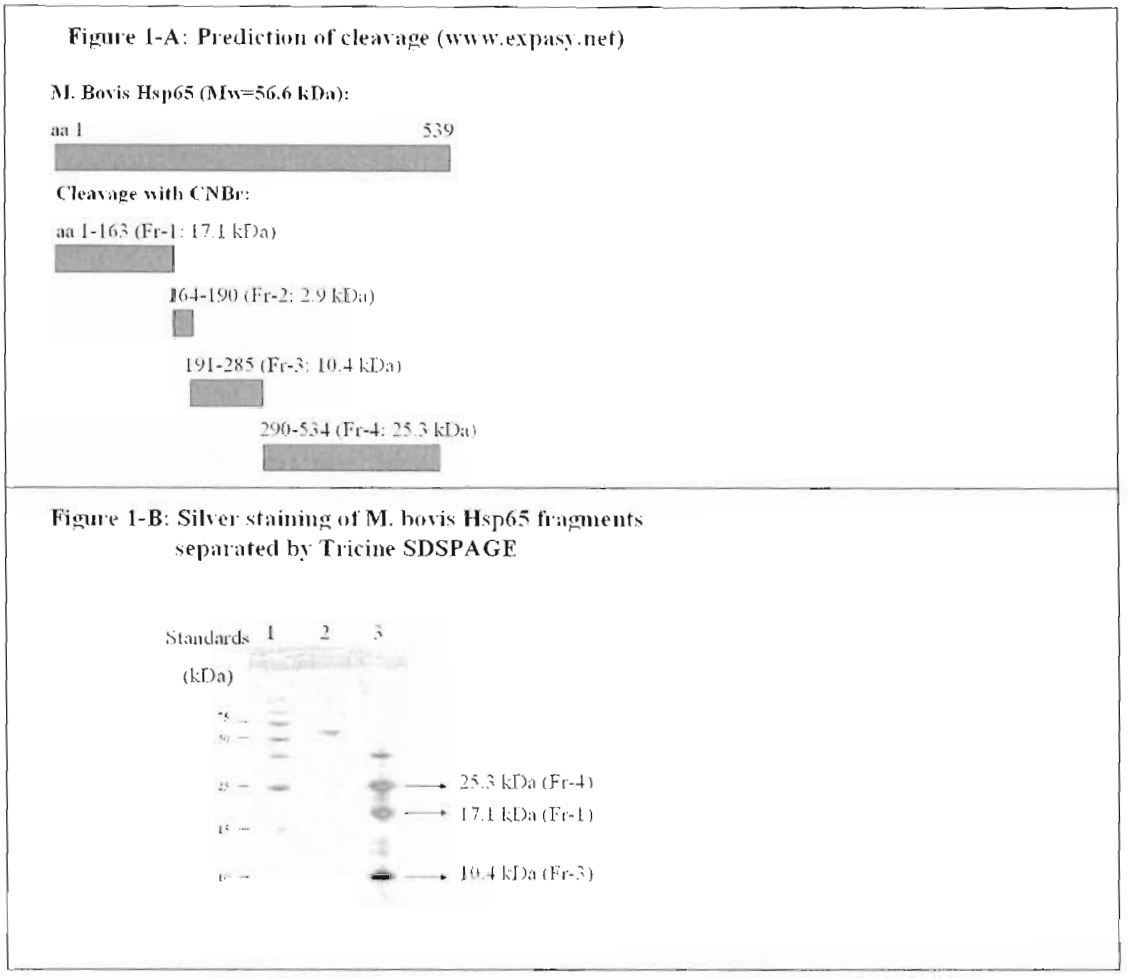


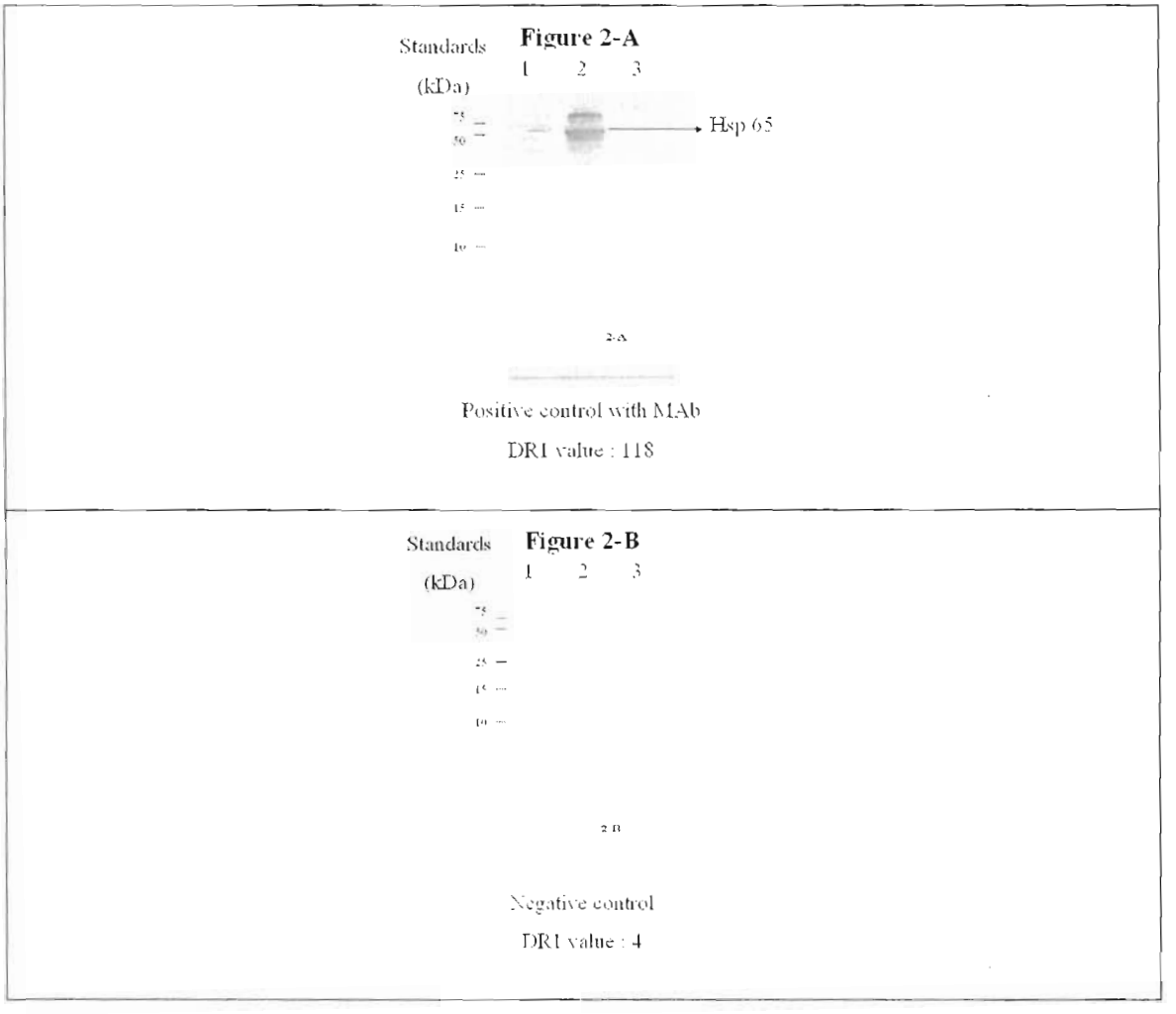
Figure 2: Representative WB results

M. bovis Hsp65 (2 µg/lane) (lanes 2) and a mixture of M. bovis Hsp65 derived fragments (5 µg/lane) (lanes 3) were run on 18% Tricine SDSPAGE and blotted against anti- mycobacterial Hsp65 monoclonal antibody (SPA-882, Stressgen, Victoria, Canada)(MAb) diluted 1:1000 (2-A) or sera in dilution 1:50 in a blocking buffer (5% non-fat dry milk in PBS) (2-C).

M. bovis Hsp65 reacted with anti-mycobacterial Hsp65 monoclonal antibody. The reflectance density (DR1, after subtraction of background value) was 118. However monoclonal antibody did not react with any fragments derived from M. bovis Hsp65

(DR1 =5). The background of the assay (DR1 value obtained from the lanes in which serum samples were substituted with 5% non-fat dry milk in PBS) was 4 (2-B).

Lanes 1: Precision Plus Protein standards - BIO-RAD (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa). Lanes 2: M. bovis Hsp65 reacted with either monoclonal antibody or patient's serum. Lanes 3: the positivity of antibodies against three fragments derived from M. bovis Hsp65 in serum of patient with WAS.



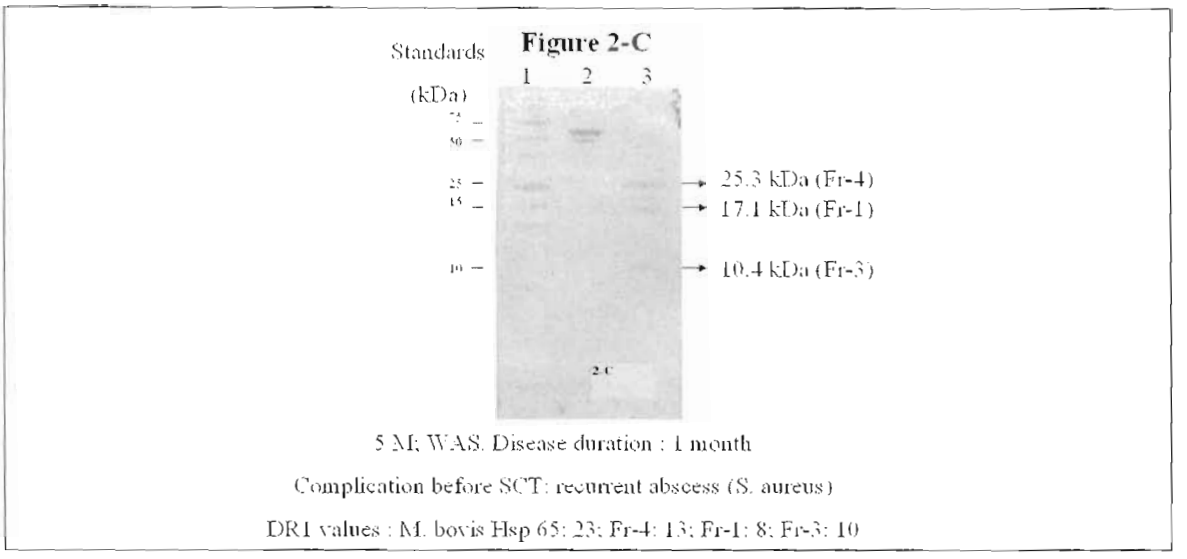
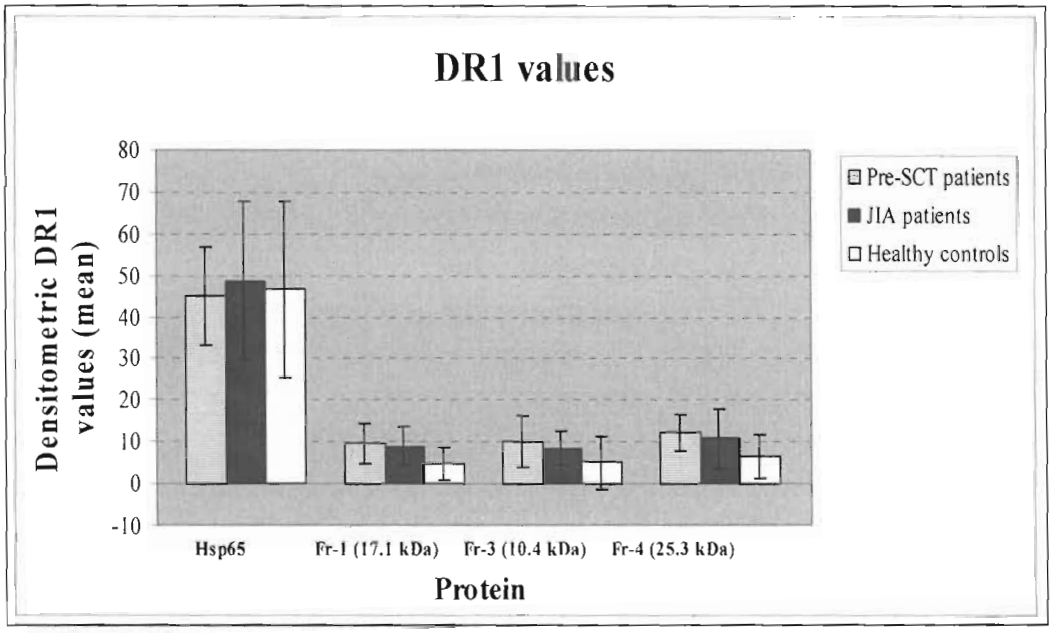


Figure 3: The comparison of densitometric values (DR1 and DR2) of IgG antibodies against M. bovis Hsp65 and M. bovis Hsp65 derived fragments between patients with malignant and non-malignant diseases before SCT, JIA patients and healthy controls

Significant differences were observed between DR1 and DR2 mean values of antibodies to Fr-1 (17.1 kDa) and Fr-4 (25.3 kDa) between both patient cohorts and healthy controls.

In the case of Fr-3 (10.4 kDa), there were elevated levels of antibody DR1 values in both cohorts of patients when compared with healthy controls and the difference nearly reached the statistical significance. However, no statistical difference between the studied groups was observed when the ratio of antibodies against Fr-3 (10.4 kDa) and antibodies against M. bovis Hsp65 was calculated (DR2 values).

3-A


DR1 values
Densitometric DR1 values (mean)
80
70
60
50
40
30
20
10
0
-10
Hsp65
Fr-1 (17.1 kDa)
Fr-3 (10.4 kDa)
Fr-4 (25.3 kDa)
Protein
Pre-SCT patients
JIA patients
Healthy controls

3-B

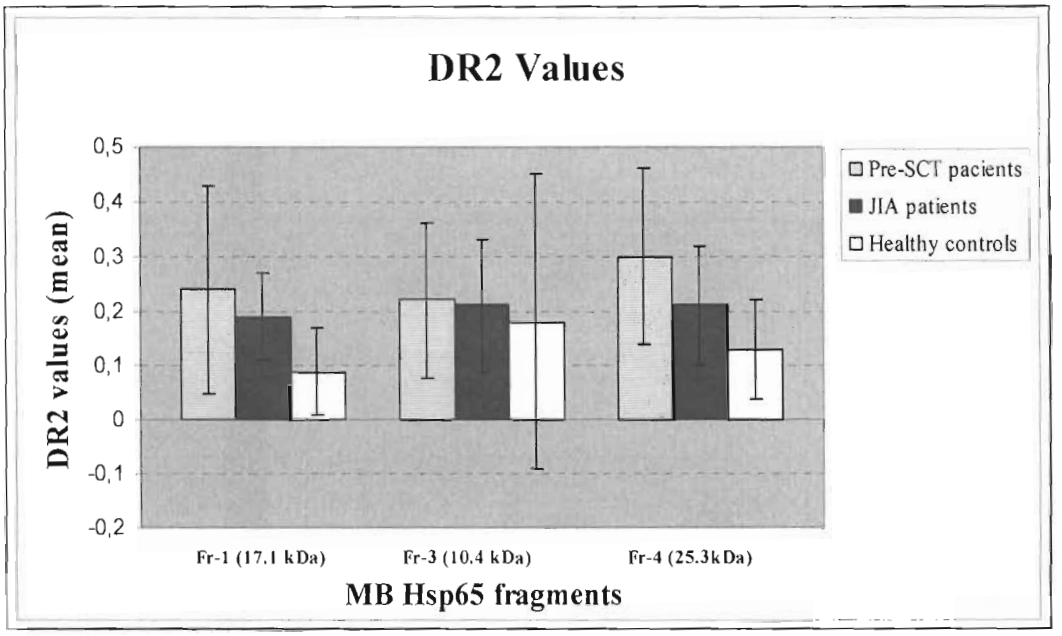

DR2 Values
DR2 values (mean)
0,5
0,4
0,3
0,2
0,1
0
-0,1
-0,2
Fr-1 (17.1 kDa)
Fr-3 (10.4 kDa)
Fr-4 (25.3kDa)
MB Hsp65 fragments
Pre-SCT pacients
JIA patients
Healthy controls

Table 1: Detailed patient clinical characteristics and positivity of IgG antibodies against M. bovis Hsp65 and M. bovis derived Hsp65 fragments

Patients pre-SCT							
UPN	**Diagnosis**	**Age/Sex**	**Clinical characteristics** (Disease duration in months; infection around the time of serum sampling)	**Anti-Hsp65**	**Anti-Fr-1 (17.1kDa)**	**Anti-Fr-3 (10.4 kDa)**	**Anti-Fr-4 (25.3 kDa)**
SCT 1	AML - CR2	17/M	15 m; E.Coli sepsis 4 months before	+	+	+	+
SCT 2	AML - CR1	13/M	5 m; w/o	+	+/-	+	+
SCT 3	AML - CR3	7/M	22 m; lung aspergilossis 1 month before	+	+	-	+
SCT 4	MDS	17/F	7 m; lung aspergilossis and S. epidermidis sepsis 1 month before	+	+	+/-	+
SCT 5	MDS	16/M	6 m; w/o	+	+	+	+
SCT 6	SAA	5/M	36 m; fungal pneumoniae (Rhizopus) 3 months before	+	-	+	+
SCT 7	WAS	4/M	36 m; without complication	+	+/-	+	+/-
SCT 8	WAS	5/M	1 m; recurrent abscess (S. aureus)	+	+	+	+
SCT 9	ALL - CR1	1/F	5 m; recurrent infections (Acinetobacter, Pseudomonas aeruginosa, Burgholderia) 2 months before	+	+	+	+
SCT 10	ALL - CR2	9/M	47 m; w/o	+	+	-	+
Number of sera reactive with M. bovis Hsp65 and M. bovis Hsp65 fragments				**10/10**	**7/10**	**7/10**	**9/10**

JIA patients							
UPN	**JIA subtype**	**Age/Sex**	**Clinical characteristics** (Laboratory parameters; disease duration in years; disease activity and therapy at the time of serum sampling)	**Anti-Hsp65**	**Anti-Fr-1 (17.1kDa)**	**Anti-Fr-3 (10.4 kDa)**	**Anti-Fr-4 (25.3 kDa)**
JIA 1	Systemic	18/M	RF (-); ANA (-); HLA B27 (-); 3 y; active disease; DMARD	+	+	+	+/-
JIA 2	Systemic	12/M	RF (-); ANA (-); HLA B27 (-); 3 y; remission; C/DMARD	+	+	+	+
JIA 3	Polyarthritis	22/F	RF (-); ANA (-); HLA B27 (-); 5 y; remission; C/DMARD	+	+	+	-
JIA 4	Polyarthritis	13/M	RF (-); ANA (-); HLA B27 (-); 3 y; remission; DMARD	+	+/-	+/-	+
JIA 5	Polyarthritis	24/F	RF (-); ANA (-); HLA B27 (+); 2 y; active disease; DMARD	+	+	+	+
JIA 6	Polyarthritis	30/M	RF (-); ANA (-); HLA B27 (-); 25 y; active disease; C/DMARD	+	+	+	+
JIA 7	Polyarthritis	22/M	RF (-); ANA (-); HLA B27 (-); 7 y; active disease; C/DMARD/NSAID	+	+	-	+
JIA 8	Polyarthritis	21/M	RF (-); ANA (-); HLA B27 (+); 14 y; active disease; C/DMARD	+	+	-	+
JIA 9	Oligoarthritis	13/F	RF (-); ANA (-); HLA B27 (-); 2 y; active disease; C/DMARD	+	-	+	-
JIA 10	Oligoarthritis	18/M	RF (-); ANA (+); HLA B27 (-); 13 y; remission; without therapy	+	+/-	+	+
JIA 11	Oligoarthritis	12/M	RF (-); ANA (-); HLA B27 (+); 2 y; active disease; C/DMARD	+	+/-	+/-	+
Number of sera reactive with M. bovis Hsp65 and M. bovis Hsp65 fragments				**11/11**	**7/11**	**7/11**	**8/11**
Healthy controls							
UDN	**Age/Sex**			**Anti-Hsp65**	**Anti-Fr-1 (17.1kDa)**	**Anti-Fr-3 (10.4 kDa)**	**Anti-Fr-4 (25.3 kDa)**
HD 1	14/F			+	+/-	-	+

HD 2	14/M	+	+/-	-	+
HD 3	17/M	+	+/-	-	+/-
HD 4	2/F	+	-	+	-
HD 5	19/M	+	-	+	+
HD 6	18/F	+	+/-	-	+
HD 7	18/F	+	+/-	-	-
HD 8	13/M	+	-	+	-
HD 9	15/F	+	-	+	+
HD 10	30/F	+	+/-	+/-	+/-
Number of sera reactive with M. bovis Hsp65 and M. bovis Hsp65 fragments		10/10	0/10	4/10	5/10

Table 2: Statistical analysis of DR1 (densitometric values) and DR2

DR1 values (mean and range)				
	M. bovis Hsp65	**Fr-1 (17.1 kDa)**	**Fr-3 (10.4 kDa)**	**Fr-4 (25.3 kDa)**
Patients before SCT	45.1 23 - 62	9.6 0 - 20	10.0 0 - 17	12.2 6 - 20
JIA patients	48.7 15 - 86	9.0 0 - 18	8.5 0 - 17	10.9 0 - 25
Healthy controls	46.7 9 - 96	4.8 0 - 12	5.1 0 - 23	6.6 0 - 18
P values (DR1) – T test				
	M. bovis Hsp65	**Fr-1 (17.1 kDa)**	**Fr-3 (10.4 kDa)**	**Fr-4 (25.3 kDa)**
Patients before SCT x controls	0.421	0.014	0.05	0.009
JIA patients x controls	0.411	0.018	0.08	0.05
DR2 values (mean and range)				
		Fr-1 (17.1 kDa)	**Fr-3 (10.4 kDa)**	**Fr-4 (25.3 kDa)**
Patients before SCT		0.24 0 – 0.74	0.22 0 – 0.43	0.3 0.13 – 0.57
JIA patients		0.19 0 – 0.33	0.21 0 – 0.41	0.21 0 – 0.40

Healthy controls	0.09 0 – 0.21	0.18 0 – 0.89	0.13 0 – 0.26
P values (DR2) – T test			
	Fr-1 (17.1 kDa)	**Fr-3 (10.4 kDa)**	**Fr-4 (25.3 kDa)**
Patients before SCT x controls	0.022	0.35	0.003
JIA patients x controls	0.006	0.40	0.04

Abbreviations used in tables 1 and 2:

ALL: acute lymphoblastic leukaemia; ANA: antinuclear antibody; AML: acute myeloid leukaemia; C: corticosteroids; CR: complete remission; DMARDs: disease modifying antirheumatics; E. Coli: Escherichia Coli; F: female; HD: healthy donor; HLA: human leukocyte antigen; JIA: juvenile idiopathic arthritis; M: male; m: month; MDS: myelodysplastic syndrome; NSAIDs: non-steroid antirheumatics; RF: rheumatoid factor; SAA: severe aplastic anaemia; S. epidermidis: Staphylococcus epidermidis; SCT: stem cell transplant; UDN: unique donor number; UPN: unique patient number; WAS: Wiskott - Aldrich syndrome; y: year.

Heat shock protein 70 membrane expression on fibroblast-like synovial cells derived from synovial tissue of patients with rheumatoid and juvenile idiopathic arthritis

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Objective: To screen fibroblast-like synovial cells derived from synovial tissue of rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) patients for the membrane expression of the heat shock protein Hsp70.

Methods: We performed flow cytometric (fluorescence-activated cell sorting, or FACS) analysis on fibroblast-like synovial cells of 15 RA patients and three JIA patients to investigate Hsp70 membrane expression. Skin fibroblasts derived from the operation wound (n=4) and peripheral blood mononuclear cells (PBMC) of seven RA and three JIA patients were also tested. Peripheral blood lymphocytes (PBL) and skin fibroblasts of 10 healthy individuals were used as negative controls.

Results: A significantly higher percentage of Hsp70 membrane expression was found on fibroblast-like synovial cells derived from arthritis-affected joints in RA patients (mean 47.7%) when compared with autologous skin fibroblasts (mean 9.5%, $p<0.001$) and control skin fibroblasts (mean 5.6%, $p<0.001$) or autologous PBL (mean CD45/Hsp70-positive 10.4%, $p<0.001$) and control PBL (mean CD45/Hsp70-positive 7.7%, $p<0.001$). A high percentage of Hsp70 membrane expression was also observed on fibroblast-like synovial cells derived from three patients with JIA (mean 35.2%) when compared with autologous PBL (mean CD45/Hsp70-positive 10.4%). Synovial cells derived from non-affected joints in a patient with RA who underwent synovectomy for trauma showed low expression of Hsp70 (10.9%).

Conclusion: Fibroblast-like synovial cells derived from patients with severe course of RA and JIA are strongly positive for membrane-expressed Hsp70.

Current evidence suggests that heat shock proteins (HSPs) could play an important role in the pathogenesis of autoimmune diseases. The HSP70 family, heat shock proteins with a molecular weight of about 70 kDa, has been implicated in the pathogenesis of both experimental and human arthritis. By using an enzyme-linked immunosorbent assay (ELISA) we could frequently detect antibodies against Hsp70, the major heat-inducible form of the HSP70 group, in sera of patients with juvenile idiopathic arthritis (JIA) (data not shown). In rheumatoid arthritis (RA), physical interactions between Hsp70 and MHC class II shared epitopes suggest that Hsp70 participates in the autoimmune response (1). Hayem et al reported elevated levels of antibodies to a constitutive HSP70 (HSC70, HSP73) in RA (2). Martin et al detected dramatically increased level of Hsp70 in RA synovial fluid versus normal human sera, RA sera, osteoarthritis, and gout synovial fluid (1). Schett et al reported an enhanced cytoplasmic expression of Hsp70 in RA synovial tissue using Western blotting, immunohistochemistry, and immunofluorescence (3).

In the present study we screened fibroblast-like synovial cells derived from synovial tissue of RA and JIA patients for the membrane expression of the Hsp70 protein.

Materials and methods

Patients and cell preparation

Local ethics committee approval and informed consents were obtained for all patients and healthy individuals involved in the study. The cohort of RA patients who fulfilled the American College of

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Rheumatology criteria for RA (4) consisted of 21 patients (16 females, five males; age range 26–73 years) with disease duration ranging from 2 to 39 years. Eighteen out of 21 patients were rheumatoid factor (RF) seropositive. Hsp70 cell-surface expression was investigated on synovial cells, skin fibroblasts, and peripheral blood mononuclear cells (PBMC) derived from patients with RA.

On the basis of preliminary results in RA patients, the Hsp70 cell-surface expression study was initially extended to six JIA patients (three females, three males; age range 10–33 years) with established disease. The underlying diseases, using the Idiopathic Arthritides of Childhood Classification criteria (5), were selected to incorporate patients from various JIA subgroups (oligoarthritis n=1, polyarthritis n=4, systemic disease n=1). All JIA patients were RF seronegative and one of them had anti-nuclear antibodies (ANA). Three out of six JIA patients were human leucocyte antigen (HLA) B27 positive. Hsp70 cell-surface expression was investigated on synovial cells and PBMC derived from the patients with JIA.

Patients were treated, depending on the stage of the disease, with non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and/or disease-modifying anti-rheumatic drugs (DMARDs).

Synovial cells derived from a non-affected joint (elbow) of a patient with RA, who underwent synovectomy for trauma, were also studied for Hsp70 cell-surface expression.

Peripheral blood lymphocytes (PBL) and skin fibroblasts of 10 healthy individuals (seven females, three males; age range 20–40 years) were used as negative controls. On the basis of results from previous screening of normal cells and tissues (PBL and skin fibroblasts) by flow cytometry, a cut-off value for Hsp70 of 10% was chosen (6).

Synovial cells were derived from synovial tissues and skin fibroblasts from the operation wound during the synovectomy. Initially, the skin tissue was trimmed of epidermis, then both synovial and skin tissues were cut to 2×2 mm sections and cultured in HMEM medium (Sevapharma, Prague, Czech Republic) containing 10% foetal bovine serum (Sigma Biosciences, St Louis, MO, USA), L-glutamine (Invitrogen-Gibco, Paisley, UK), and antibiotics (Sigma Biosciences) at 37°C with 5% CO_2 for 2 months on average. The medium was replaced twice a week. Upon reaching confluence, cells were trypsinized using 0.05% trypsin/0.02% EDTA in phosphate-buffered saline (PBS) (PAN Biotech GmbH, Aidenbach, Germany) for 30 s, after which 1 mL of cell culture medium was added. The suspended cells were centrifuged, resuspended, and all of the cells derived from these primary cultures were used directly for flow cytometric analysis (fluorescence-activated cell sorting, or FACS). Remaining tissue sections were cultivated again (overall two times) and the newly gained cells were used to repeat the FACS analysis. We observed mild changes in Hsp70 membrane expression (less than 10%) when comparing the data (data shown in Table 1 represent average values from all FACS analyses). FACS analysis was not performed on cells expanded over the passages.

Cell viability was determinated first by the Trypan blue exclusion method. All cells were viable after trypsinization.

Adherent synovial cells were defined as fibroblast-like synovial cells (type B) (7). Cells were identified by their unique growth pattern, morphology, and by using monoclonal mouse anti-human vimentin V9 IgG1 antibody (Immunotech, France) for cells of mesenchymal origin (8, 9).

Flow cytometry

Flow cytometry was performed as described by Farkas et al (6) using a standard direct immunofluorescence technique and mouse anti-human monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) on a FACStrak flow cytometer (Becton Dickinson, San Jose, USA). After washing in PBS containing 10% foetal calf serum (FCS, Sigma Biosciences), a single-cell suspension of 0.2×10^6 cells was incubated with anti-Hsp70 (cmHsp70.1-FITC, IgG1, Multimmune, Regensburg, Germany) and anti-major histocompatibility complex (MHC) class I (IgG2a, Cymbus Biotechnology, Chilworth, Southampton, UK), which was used as a positive control, for 30 min at 4°C in the dark. After one washing step, 7-amino-actinomycin D (7-AAD, Becton Dickinson)-negative viable cells with intact cell membranes were analysed. The percentage of specifically stained cells was defined as the number of positively stained cells minus the number of cells stained by the isotype-matched control antibody. Mouse IgG1-FITC (Becton Dickinson) was used as an isotype-matched control antibody for the detection of Hsp70 and mouse IgG2a-FITC (Becton Dickinson) as an isotype-matched control antibody for the detection of MHC I.

After whole-blood lysis, PBMC were analysed by flow cytometry (10, 11). In brief, 100 µL of EDTA-anticoagulated peripheral blood samples were dual stained with phycoerythrin (PE)-labelled monoclonal antibodies for human CD45 (IgG1), CD3 (IgG1), CD14 (IgG2a) (all from Becton Dickinson), and FITC-labelled anti-Hsp70 monoclonal antibody (Multimmune) for 15 min at room temperature (RT). Corresponding isotype controls such as mouse IgG1-PE- and mouse IgG2a-PE-labelled monoclonal antibodies (Becton Dickinson) were used. After washing, 2 mL of FACS lysing solution (Becton

Table 1. Clinical details and the percentage of cells positive for membrane-expressed Hsp70.

Examined groups	UPN	Patients' characteristics: sex/age (years); RF status; disease duration (years); therapy	Percentage of cells positive for Hsp70
RA fibroblast-like SC	1	F/64; RF(+); 21; C/NSAID/DMARD	25.8
	2	F/55; RF(+); 20; C/DMARD	49.2
	3	F/27; RF(+); 5; C/NSAID/DMARD	26.4
	4	F/73; RF(+); 5; C/NSAID	40.3
	5	F/59; RF(+); 18; C/NSAID	30.0
	6	F/43; RF(+); 12; C/DMARD	49.4
	7	M/61; RF(+); 6; C/NSAID/DMARD	66.1
	8	F/33; RF(+); 2; C/NSAID/DMARD	42.2
	9	F/71; RF(+); 7; C/DMARD	57.3
	10	F/48; RF(+); 5; NSAID/DMARD	75.1
	11	F/56; RF(+); 5; DMARD	47.3
	12	M/52; RF(−); 15; C/NSAID/DMARD	47.4
	13	F/72; RF(+); 22; C/NSAID/DMARD	58.4
	14	F/35; RF(+); 7; C/NSAID/DMARD	63.6
	15	M/48; RF(+); 39; C/NSAID	37.0
			range 25.8–75.1%; mean 47.7%
RA skin fibroblasts	1	F/64; RF(+); 21; C/NSAID/DMARD	10.2
	3	F/27; RF(+); 5; C/NSAID/DMARD	7.3
	5	F/59; RF(+); 18; C/NSAID	17.3
	7	M/61; RF(+); 6; C/NSAID/DMARD	3.3
			range 3.3–17.3%; mean 9.5%
RA PBMC	Percentage of cells positive for Hsp70 PBL: CD45/Hsp70-positive, CD3/Hsp70-positive, PBM: CD45/Hsp70-positive, CD14/Hsp70-positive		
	12	M/52; RF(−); 15; C/NSAID/DMARD	PBL: 18%, ND; PBM: 97.7%, ND
	13	F/72; RF(+); 22; C/NSAID/DMARD	PBL: 3.2%, ND; PBM: 82.3%, ND
	16	F/26; RF(−); 2; NSAID/DMARD	PBL: 8.2%, 7.1%; PBM: 55.6%, 45.8%
	17	F/58; RF(+); 5; NSAID	PBL: 16%, ND; PBM: 87.1%, ND
	18	M/48; RF(+); 6; DMARD	PBL: 4.5%, ND; PBM: 79.1%, ND
	19	F/56; RF(+); 8; C/NSAID/DMARD	PBL: 9.3%, 5.4%; PBM: 85.0%, 69.0%
	20	F/28; RF(−); 8; C/NSAID	PBL: 13.4%, 4.0%; PBM: 88.3%, 70.8%
	PBL: CD45/Hsp70: range 3.2–18.0%; mean 10.4%; CD3/Hsp70: range 4.0–7.1%; mean 5.5% PBM: CD45/Hsp70: range 55.6–97.7%; mean 82.2%; CD14/Hsp70: range 45.8–70.8%; mean 61.9%		

Examined groups	UPN	Patients' characteristics: sex/age (years); JIA subgroup; RF status; ANA status; HLA-B27 status; disease duration (years); therapy	Percentage of cells positive for Hsp70
JIA fibroblast-like SC	1	M/22; polyarthritis; RF(−); ANA(−); HLA B27 (−); 7; C/NSAID	46.7
	2	F/12; oligoarthritis; RF(−); ANA(−); HLA B27 (−); 2; C/DMARD	28.3
	3	F/20; systemic JIA; RF(−); ANA(−); HLA B27 (−); 11; C/DMARD	30.5
			range 28.3–46.7%; mean 35.2%
JIA PBMC	Percentage of cell positive for Hsp70 PBL: CD45/Hsp70-positive, CD3/Hsp70-positive, PBM: CD45/Hsp70-positive, CD14/Hsp70-positive		
	4	M/29; polyarthritis; RF(−); ANA(−); HLA B27(+); 20; C/DMARD	PBL: 14.9%, ND; PBM: 91.3%, ND
	5	F/33; polyarthritis; RF(−); ANA(−); HLA B27(+); 20; C/NSAID/DMARD	PBL: 10.4%, 2.7%; PBM: 96.2%, 73.4%
	6	M/10; polyarthritis; RF(−); ANA(+); HLA B27(+); 8; C/NSAID/DMARD	PBL: 5.8%, 3.0%; PBM: 97.5%, 85.0%
	PBL: CD45/Hsp70: range 5.8–14.9%; mean 10.4%; CD3/ Hsp70: range 2.7–3.0%; mean 2.9%; PBM: CD45/Hsp70: range 91.3–97.5%; mean 95.0%; CD14/Hsp70: range 73.4–85.0%; mean 79.2%		

Table 1. (Continued.)

Negative controls	UDN	Donor's characteristics, percentage of cells positive for Hsp70
Fibroblast-like SC	21	From RA non-affected joint (synovectomy carried out due to the trauma) M/52, RF(+); 8; C/DMARD, 10.9%
Healthy individuals PBMC	1–10	Seven females, three males; age range 20–40 years PBL: CD45/Hsp70: range 1.3–9.6%; mean 7.7%; CD3/Hsp70: range 0.1–0.4%; mean 0.3% PBM: CD45/Hsp70: range 31.8–99.3%; mean 80.3%; CD14/Hsp70: range 47.8–82.4%; mean 72.6%
Healthy individuals skin fibroblasts	1–10	Seven females, three males; age range 20–40 years range 3.0–7.0%; mean 5.6%

Data for fibroblast-like synovial cells and skin fibroblasts represent average value from all FACS analyses. ANA, anti-nuclear antibodies; C, corticosteroids; DMARD, disease-modifying anti-rheumatic drug; F, female; HLA, human leucocyte antigen; M, male; NSAID, non-steroidal anti-inflammatory drug; PBL, peripheral blood lymphocytes; PBM, peripheral blood monocytes; RF, rheumatoid factor; SC, synovial cells; UDN, unique donor number; UPN, unique patient number.

Table 2. Statistical analysis of the data.

	p-value (t-test)
RA SC × RA skin fibroblasts	<0.001
RA SC × RA PBL (CD45/Hsp70-positive)	<0.001
RA PBL (CD45/Hsp70-positive) × RA skin fibroblasts	0.41
RA SC × control skin fibroblasts	<0.001
RA SC × control PBL (CD45/Hsp70-positive)	<0.001
RA skin fibroblasts × control skin fibroblasts	0.14
RA PBL (CD45/Hsp70-positive) × control PBL (CD45/Hsp70-positive)	0.12
RA PBM (CD45/Hsp70-positive) × control PBM (CD45/Hsp70-positive)	0.42

PBL, peripheral blood lymphocytes; PBM, peripheral blood monocytes; SC, synovial cells.

Dickinson), diluted 1:10 in water, was added and incubated for 10 min at RT. After discarding the supernatant, the cells were washed again and resuspended in 300 μL of PBS containing 10% FCS (Sigma Biosciences). Hsp70 membrane expression was assessed separately on peripheral blood lymphocytes (PBL) and monocytes (PBM) in relevant gates, which were identified by using not only forward scatter (FSC) and side scatter (SSC) but also monoclonal antibodies against specific markers (CD3 for lymphocytes and CD14 for monocytes).

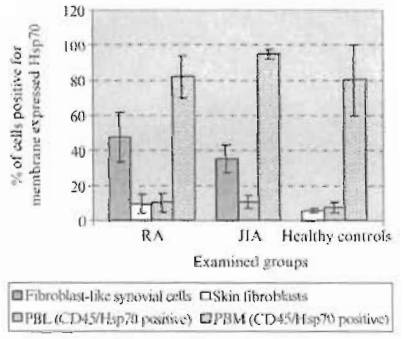

Figure 1. The percentage of cells positive for membrane-expressed Hsp70 on fibroblast-like synovial cells, skin fibroblasts, and peripheral blood mononuclear cells derived from patients with RA, JIA, and healthy controls (mean values).

K562 cells, a human myeloid leukaemia cell line (American Type Culture Collection, Rockville, MD, USA), were used as positive control for screening of Hsp70 membrane expression. K562 cells were cultured in RPMI-1640 medium (Cambrex Bio Sciences Verviers, Verviers, Belgium) supplemented with heat-inactivated 10% FCS (Sigma Biosciences), sodium pyruvate (Sigma Biosciences), L-glutamine (Invitrogen-Gibco), and antibiotics (Sigma Biosciences), and the cells were dual stained with anti-Hsp70 (cmHsp70.1-FITC, IgG1, Multimmune) and anti-CD45 (PE, IgG1, Becton Dickinson) or anti-CD33 (PE, IgG1, Becton Dickinson). After cultivation for 1 month, 61.9% of K562 cells co-expressed CD33/Hsp70 and 3% of them were dual positive for CD45/Hsp70.

Statistical analysis

The two-tailed Student's t-test was used for statistical analysis. p-values of less than 0.05 were regarded as significant.

Results

Tables 1 and 2 and Figure 1 show detailed patients' clinical characteristics and the results of Hsp70 membrane expression on fibroblast-like synovial cells, skin fibroblasts, and PBMC of patients with RA and JIA, as well as healthy controls.

A significantly higher percentage of Hsp70 membrane expression was found on fibroblast-like synovial cells derived from arthritis-affected joints in RA patients (range 25.8–75.1%, mean 47.7%) when compared with autologous skin fibroblasts (range 3.3–17.3%, mean 9.5%, $p<0.001$) and control skin fibroblasts (range 3.0–7.0%, mean 5.6%, $p<0.001$) or autologous PBL (CD45/Hsp70-positive: range 3.2–18.0%, mean 10.4%, $p<0.001$) and control PBL (CD45/ Hsp70-positive: range 1.3–9.6%, mean 7.7%, $p<0.001$).

Figure 2 shows a representative profile of Hsp70 staining of fibroblast-like synovial cells derived from synovial tissue of a 55-year-old female with RF-seropositive RA lasting for 20 years (unique patient number 2, UPN 2).

A representative flow cytometric analysis of skin fibroblasts derived from the operation wound of a 61-year-old male with RF-seropositive RA lasting for 6 years is shown in Figure 3 (UPN 7).

A high percentage of Hsp70 membrane expression was also observed on fibroblast-like synovial cells derived from three patients with JIA (range 28.3–46.7%, mean 35.2%) when compared with autologous PBL (CD45/Hsp70-positive: range 5.8–14.9%, mean 10.4%).

Synovial cells derived from a non-affected joint in a patient with RA, who underwent synovectomy for trauma, showed low expression of Hsp70 (10.9%).

No statistical significance was found when Hsp70 membrane expression on PBL of patients with RA and healthy controls was compared in lymphocyte gate ($p=0.12$). PBL derived from patients with JIA were also found not to express Hsp70 on the cell surface, as for the healthy controls. When CD3-positive T cells were analysed for Hsp70 membrane expression in lymphocyte gate (FSC/SSC), we found no positivity in both RA and JIA patients (CD3/Hsp70-positive: range 2.7–7.1%, mean 4.5%).

PBM were always found to be Hsp70 membrane positive in patients with RA (CD45/Hsp70-positive: range 55.6–97.7%, mean 82.2%) or JIA (CD45/Hsp70-positive: range 91.3–97.5%, mean 95.0%), or in healthy controls (CD45/Hsp70-positive: range 31.8–99.3%, mean 80.3%). FACS analysis of CD14-positive cells confirmed that Hsp70 membrane expression occurred on monocytes (in monocyte gate) of patients with RA (range 45.8–70.8%, mean 61.9%), JIA (range 73.4–85.0%, mean 79.2%) or healthy controls (range 47.6–82.4%, mean 72.6%). This might be explained by binding of soluble Hsp70 present in peripheral blood onto the HSP receptors such as CD14, CD91, and Toll-like receptors expressed on the surface of monocytes.

Discussion

The present study supports the suggestion that the HSP70 family may be involved in the pathogenesis of

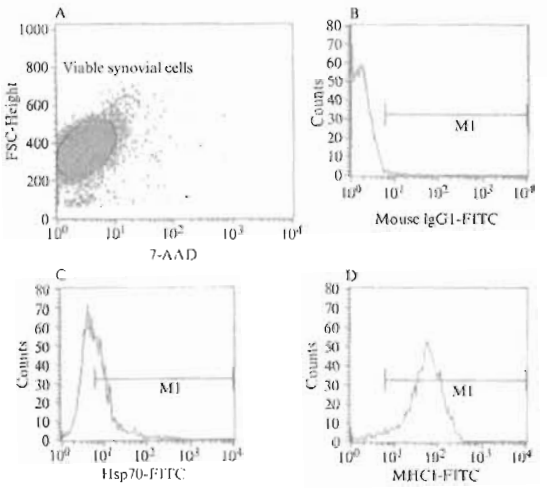

Figure 2. Representative flow cytometric analysis of fibroblast-like synovial cells derived from synovial tissue of a 55-year-old female with RF-seropositive RA lasting for 20 years (therapy: C/DMARD) (UPN 2). Synovial cells derived from synovial tissue of a metatarso-phalangeal joint were cultivated for 72 days upon reaching the confluence; the adherent cells were trypsinized, resuspended in cell culture medium, and stained with isotype-matched control (B); monoclonal anti-Hsp70-FITC conjugated antibody (C), or anti-MHC class I-FITC conjugated antibody (D). Only viable synovial cells negative for 7-AAD were gated and analysed (A). The inducible form of Hsp70 and MHC class I membrane expression was detected on 41.3% and 98% of analysed cells, respectively.

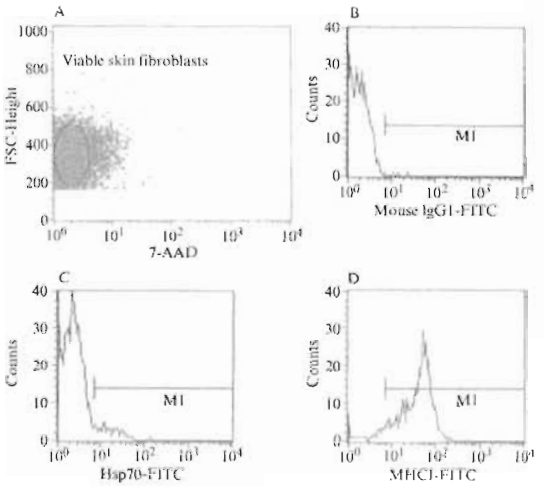

Figure 3. Representative flow cytometric analysis of skin fibroblasts derived from the operation wound of a 61-year-old male with RF-seropositive RA lasting for 6 years (therapy: CJNSAID/DMARD) (CPN 7). Skin fibroblasts derived from the operation wound from the knee joint were cultivated for 42 days upon reaching the confluence; the adherent cells were trypsinized, resuspended in cell culture medium, and stained with isotype-matched control (B), monoclonal anti-Hsp70-FITC conjugated antibody (C), or anti-MHC class I-FITC conjugated antibody (D). Only viable skin fibroblasts negative for 7-AAD were gated and analysed (A). The inducible form of Hsp70 and MHC class I membrane expression was detected on 4.7% and 93.3% of analysed cells, respectively.

autoimmune diseases such as RA and JIA. Although the immunological microenvironment in the joints of patients with JIA is different from that of patients with RA, the synovial tissues in both cases are affected by a chronic inflammatory process leading to an alteration of cellular homeostasis followed by an increased expression of the HSPs (12). By using different methods, several investigators have shown that a variety of HSPs and chaperones are intracellularly overexpressed in the RA synovial membrane, including human HSP60, BiP, and human homologues of the bacterial DnaJ chaperone, in order to protect cells from apoptosis (13).

HSPs have been shown to protect cells against several toxic conditions characteristic of inflamed rheumatoid joints, including hypoxia, production of large amounts of reactive oxygen species, and inflammatory cytokines such as tumour necrosis factor (TNF-α) and interleukin-1 (IL-1) (13).

The HSP70 family includes highly stress-inducible and constitutively expressed proteins that are generally considered to be intracellular in location (14, 15). Scheet et al showed an enhanced intracellular expression of Hsp70 in synovial cells of RA patients and speculated that this overexpression might influence processing and presentation of synovial cell antigens, possibly leading to a quantitatively and qualitatively altered antigenic repertoire in rheumatoid synovia (3). Cell-surface localization of Hsp70 has been documented on human immunodeficiency virus (HIV)-infected cells (16), certain tumour cells (14, 17), myeloid dendritic cells (DCs) in RA synovial fluid (1), retro-ocular fibroblasts derived from patients with autoimmune Graves' ophthalmopathy (15), but not on normal tissues and cells including the brain, colon, fibroblasts, umbilical vein-derived endothelial cells, and PBL (17). Cell-surface expression of Hsp70 on human tumour cells increases their sensitivity to lysis mediated by natural killer (NK) cells (18). Multhoff et al reported that N-terminal-extended 14-mer peptide (TKD, aa 450–463) was able to stimulate the cytolytic and proliferative activity of NK cells, similar to the full-length Hsp70 protein (19). In addition, Krause et al demonstrated the anti-tumour activity of *ex vivo* Hsp70-peptide-activated autologous NK cells in the treatment of colon and lung cancer in clinical Phase I trial (20).

This study is the first demonstration of Hsp70 membrane expression on fibroblast-like synovial cells derived from synovial tissues of RA and JIA patients. Like Martin et al, in the case of myeloid DCs in RA synovial fluid, we speculate that Hsp70 might be translocated to the cell surface from the cytosol in response to sustained stress and/or that Hsp70 might be captured on the cell surface from the extracellular space through HSP receptors (1).

Some authors have speculated that trypsinization might influence the expression of membrane molecules. However, we observed that only fibroblast-like synovial cells derived from arthritis-affected joints of patients with RA and JIA showed high Hsp70 membrane expression, although they were detached from tissue culture flasks by trypsinization like synovial cells derived from non-affected joint and skin fibroblasts derived from the operation wound, which displayed in all cases baseline and/or borderline Hsp70 expression. The expression of

MHC I molecules on skin fibroblasts and synovial cells exceeds 90% in most cases.

Hsp70 might bind autoantigens released from chronically affected synovial tissue and contribute to the autoantigen processing in RA and JIA. Hsp70–autoantigen peptide complexes released from stressed and dead cells may be endocytosed by professional antigen-presenting cells (synovial fluid dendritic cells) through binding to HSP receptors (e.g. CD91, CD14) and represented by MHC molecules. Fibroblast-like synovial cells in rheumatoid synovial membrane show an activated phenotype with increased expression of MHC class II and adhesion molecules. It has been suggested that they also act as antigen-presenting cells involved in the activation of T cells (21).

Subsequent investigations will be performed on a larger cohort of RA/JIA patients to study the association of plasma membrane Hsp70 expression with the clinical characteristics. In summary, these data will be confronted with Hsp70 expression (hsp70 mRNA) in various cells and tissues of patients and controls by multiplex real-time polymerase chain reaction (RT-PCR) by the comparative threshold method, comparing the relative amount of the target sequence and any of the chosen references, which are being developed in our laboratory. However, we note that Botzler et al reported that Hsp70 cell-surface expression on human carcinoma cells is independent of the cytoplasmic amount of Hsp70 (22).

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Abstract:

We examined the membrane expression of inducible Hsp70 and HSP receptors like TLR2, TLR4, CD14, CD36, CD40 and CD91 on fibroblast-like synovial cells (SC) derived from synovial tissue in 23 patients with rheumatoid arthritis (RA) who underwent synovectomy by using flow cytometric analysis. For the comparison, autologous skin fibroblasts (SF) derived from the operation wound were tested.

Significantly higher Hsp70 expression was found on synovial cells than on skin fibroblasts (median SC 21.4 % x SF 5.0 %, $p<0.001$).

Both synovial cells and skin fibroblasts expressed high levels of cell surface CD91 (median SC 80.2 % x SF 79.2 %), however no or low levels of CD14, CD40, TLR2, TLR4 and CD36. Further, we observed high co-expression of CD91 and Hsp70 on RA synovial cells (median 18.6 %), while skin fibroblasts showed only background Hsp70 expression (median 3.9 %, $p<0.001$).

Since we demonstrated the high prevalence of inducible Hsp70 in RA synovial fluids, we speculate that Hsp70 might be captured onto the membrane of synovial cells from the extracellular space via CD91 receptor. The significance of the Hsp70 interaction with synovial cells via CD91 remains undefined but may mediate other non-immune purposes.

Key words: CD91; inducible Hsp70; rheumatoid arthritis; synovial cells

Introduction

Current evidence suggests that the HSP70 family, heat shock proteins with a molecular weight of about 70 kilodaltons (kDa), may play a role in the pathogenesis of various autoimmune diseases involving rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA).

By using enzyme-linked immunosorbent assay (ELISA) we could frequently detect antibodies against Hsp70, the major heat - inducible form of the HSP70 group, in sera of patients with JIA [1].

We also demonstrated that fibroblast-like synovial cells derived from synovial tissues of patients with severe course of RA and JIA were strongly positive for membrane expressed Hsp70 [2]. Alike Schett et al. reported an enhanced cytoplasmic expression of Hsp70 in RA synovial tissue using Western blotting, immunohistochemistry and immunofluorescence [3].

Martin et al. detected dramatically increased level of Hsp70 in RA synovial fluid versus normal human sera, RA sera, osteoarthritis and gout synovial fluid. Moreover, the authors demonstrated high levels of Hsp70 on the surface of myeloid dendritic cells (DCs) in synovial fluids of patients with RA that occurred concurrently with CD91 and CD14 [4].

Members of the HSP70 family interact with a wide variety of antigenic peptides from pathogens as well as self peptides discussed to be involved in autoimmunogenic processes [5]. It has been reported that HSP-chaperoned antigenic peptides can be presented via MHC class I and II molecules and thus enhanced activation of antigen-specific cytotoxic lymphocytes and CD4+ T cells [5]. Extracellular Hsp70: peptide complexes can be detected in MHC class II-enriched compartments after receptor-mediated endocytosis [6]; complexes from the cytosol may reach the MHC class II presentation pathway via autophagic processes [7].

HSP70 molecules were found to bind peptide sequences comprising the shared epitope (SE) sequences ^{70}QKRAA^{74}, ^{70}QRRAA^{74}, ^{70}RRRAA^{74}, a highly conserved motif of similar amino acid sequences found in HLA-DRB1 molecules associated with an increased risk for rheumatoid arthritis [8]. It was reported that HSP70 molecules do not exclusively interact with the shared epitope sequences but also with most other sequences found in the HV-3 region of HLA-DR molecules, with the exception of the amino acid sequence ^{70}DERAA^{74} (SE), a sequence exclusively found in RA-protective HLA-DR molecules. It suggested a possible association of non-binding of Hsp70 to HLA-DR molecules or its 70-74 fragments and protection from RA [9].

It has been speculated that Hsp70 might be translocated to the cell surface from the cytosol in response to sustained stress and/or that Hsp70 might be captured onto the cell surface from the extracellular space via HSP receptors [2, 4].

The goal of the current study was to estimate the expression of the most common HSP receptors such as Toll-like receptor (TLR) 2 and 4, CD14 (a receptor for endotoxin - lipopolysaccharide), CD36 (collagen type I and thrombospondin receptor), CD40 (a receptor molecule on the cell surface of B cells, endothelial and epithelial cells) and CD91 (α2-macroglobulin/low density lipoprotein receptor) on fibroblast-like synovial cells derived from synovial tissue and skin fibroblasts derived from the operation wound in patients with RA who underwent synovectomy.

Materials and Methods

Patients

Local ethics committee approval and informed consent were obtained for all individuals involved in this study. The cohort consisted of 23 patients (22 females, 1 male) aged 29 - 79 years (mean 56.6, median 57 years) who fulfilled the American College of Rheumatology criteria for RA [10] with disease duration ranging from 3 to 32 years (median 16 years). Twenty two patients suffered from rheumatoid factor

(RF) positive polyarthritis and one of them had RF negative polyarthritis. Clinical disease activity was assessed using the physician's global assessment of overall disease activity. All patients had an active disease at the time of testing. The patients were treated depending on the stage of the disease with non-steroid anti - rheumatics (NSAIDs), corticosteroids (C) and/or disease modifying antirheumatics (DMARDs). Synovial cells were derived from RA affected synovial tissues (finger joint n=1, metacarpophalangeal joint n=3, elbow joint n=1, shoulder joint n=1; metatarsophalangeal joint n=10, ankle joint n=2, knee joint n=1 and hip joint n=4) and skin fibroblasts from RA non-affected operation wound.

ELISA

Using ELISA we investigated the levels of inducible Hsp70 in synovial fluid and serum samples of patients with RA.

Sera of 24 age-matched healthy controls were included in the ELISA assays as a control.

Inducible Hsp70 was measured using a commercial quantitative sandwich ELISA (Stressgen, Canada) according to manufacturer's instructions. Optical density was measured at 450 nm using ELISA plate reader (Dynex Technologies, MRX II, USA). Hsp70 concentrations from synovial fluid and serum samples were quantitated by interpolating absorbance readings from a standard curve and expressed as nanograms per millilitre.

Cell cultivation and preparation for fluorescent- activated cell sorting (or flow cytometry, FACS) analysis

Initially, the skin tissue was trimmed of epidermis, then both synovial and skin tissues were cut to 2x2 mm sections and cultured in AmnioMax medium (Gibco, Invitrogen Corporation, N.Y., USA) containing 1 % antibiotics (Penicillin-Streptomycin, Sigma Biosciences, St Louis, MO, USA) and 1 % Fungizone (Antimycotic, Gibco, Invitrogen Corporation, N.Y., USA) at 37°C with 5 % $C0_2$ for 2 months in average. The medium was replaced twice a week. Upon reaching confluence, cells were trypsinised using 0.05 % trypsin/0.02 % ethylenediamine

tetraacetic acid (EDTA) in phosphate buffered saline (PBS) (PAN Biotech, GmbH, Germany) for 30 seconds, after which 1 ml of medium was added. The suspended cells were washed and resuspended again in 1 ml of PBS. The approximate cell number and viability was determinated by Trypan Blue Exclusion method. All cells were viable after trypsinisation. $0.1x10^6$ of the cells per tube was the minimal amount used for the FACS analysis.

The adherent synovial cells were defined as fibroblast-like synovial cells (type B). Cells were identified by their unique growth pattern, morphology, and by using monoclonal mouse anti-human vimentin V9 IgG1 antibody (Immunotech, France) for cells of mesenchymal origin [2].

FACS

The adherent cells at primary cultures to third passages were used for FACS analysis. Flow cytometry was performed as it was previously described by Farkas B et al. using a standard direct immunofluorescence technique and mouse anti-human monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) and/or phycoerythrin (PE) on a FACStrak flow cytometer (Becton Dickinson, San Jose, USA) [11]. After washing in PBS containing 10% fetal calf serum (FCS, Sigma Biosciences), single-cell suspension of 0.1 x 10^6 cells per tube was dual-stained with anti-Hsp70 (cmHsp70.1-FITC, IgG1, Multimmune, Regensburg, Germany) and PE-conjugated monoclonal antibodies against CD91 (IgG1), CD14 (IgG2a), CD40 (IgG1), CD36 (IgM) (all these antibodies were purchased from Becton Dickinson, San Jose, USA), TLR2 and TLR4 (IgG2a, eBioscience, San Diego, CA, USA) for 30 minutes at 4°C in the dark. After one washing step, 7-amino-actinomycin D (7-AAD, Becton Dickinson) - negative, viable cells with intact cell membranes were analysed. The percentage of specifically stained cells was defined as the number of positively stained cells minus the number of cells stained by the isotype-matched control antibody. Anti-major histocompatibility complex (MHC) class I (IgG1, Becton Dickinson) conjugated with FITC as well as PE was used as a positive control. Mouse IgG1-FITC, IgG1-PE, IgG2a-

PE and IgM-PE (Becton Dickinson) were used as isotype-matched control antibodies for the detection of Hsp70 and Hsp70 receptors.

Jurkat cells, a human T-ALL cell line (American Type Culture Collection, Manassas, VA, USA; a kind gift from Professor Multhoff, University Hospital Regensburg, Germany) and CCRF-CEM cells, a human T-ALL cell line (ECACC, Porton Down, UK; a kind gift from Professor Dickinson, University of Newcastle upon Tyne, UK) were used as positive controls for screening of Hsp70 membrane expression. Cell lines were cultured in RPMI-1640 medium (Cambrex Bio Sciences Verviers, Verviers, Belgium) supplemented with heat-inactivated 10% FCS (Sigma Biosciences), sodium pyruvate (Sigma Biosciences), L-glutamine (Gibco, Invitrogen Corporation), Fungizone (Gibco, Invitrogen Corporation) and antibiotics (Sigma Biosciences) and the cells were dual stained with anti-Hsp70 (cmHsp70.1-FITC, IgG1, Multimmune) and anti-CD45 or anti- MHC I (PE, IgG1, Becton Dickinson).

A cut-off value for Hsp70 of 10 % was chosen based on the results from previous screening of normal cells and tissues (peripheral blood lymphocytes and skin fibroblasts) by flow cytometry [11].

Statistical analysis

Two-tailed Student's t-test was used for the statistical analysis. P values of less than 0.05 were regarded as significant.

Results

A high prevalence of soluble Hsp70 detected in RA synovial fluids

Hsp70 positivity was detected in 100 % RA synovial fluids (range 474.5 - 1078.9, mean 713.0, median 550.1 ng/ml) in contrast to control sera (range 8.0 - 53.4, mean 18.2, median 15.8 ng/ml; $p<0.001$) and RA sera (range 12.0 - 44.6, mean 24.5, median 27.7 ng/ml; $p<0.001$) which were both shown to be negative.

Expression of Hsp70 and HSP receptors on RA synovial cells and autologous skin fibroblasts

We examined the expression of inducible Hsp70 and HSP receptors like TLR2, TLR4, CD14, CD36, CD40 and CD91 on fibroblast-like synovial cells derived from synovial

tissue and skin fibroblasts derived from the operation wound in patients with RA who underwent synovectomy.

Human leukaemia cell lines, which were used as positive controls for the detection of membrane - bound Hsp70, expressed inducible Hsp70 on the cell surface continuously from the beginning till the end of the short-term culture (Jurkat cells - d+3 - d+11, range 74.8 %-97.7 %, mean: 87.4 %, median: 87.1 %; CCRF - CEM cells - d+5 - d+47, range 70.6 % - 90.7 %, mean: 81.5 %, median: 81.8 %).

Similarly as in our previous study, significantly higher Hsp70 membrane expression was found on fibroblast-like synovial cells (SC) than on autologous skin fibroblasts (SF) (SC: mean 26.8 %, median 21.4 % x SF: mean 5.5%, median 5.0 %; $p<0.001$).

Both synovial cells and skin fibroblasts expressed relatively high levels of cell surface CD91 (SC: mean 77.1 %, median 80.2 % x SF: mean 68.6 %, median 79.2 %). No or low expression of CD14 (SC: mean 0.9 %, median 0.5 % x SF: mean 0.5 %, median 0.2 %), CD40 (SC: mean 2.0 %, median 0.6 % x SF: mean 0.5 %, median 0.2 %), TLR2 (SC: mean 4.3 %, median 3.2 % x SF: mean 1.4 %, median 1.0 %), TLR4 (SC: mean 4.9 %, median 1.7 % x SF: mean 1.2 %, median 0.6 %) and CD36 (SC: mean 5.0 %, median 3.6 % x SF: mean 7.9 %, median 5.3 %) was detected on these cells of mesenchymal origin derived from arthritis affected synovial tissue and non-affected skin. The expression of MHC class I molecules exceeds 90 % in most cases.

Alike, we observed high co-expression of CD91 and Hsp70 on RA synovial cells (mean 19.4 %, median 18.6 %), while skin fibroblasts showed only background Hsp70 expression (mean 4.8 %, median 3.9 %; $p<0.001$).

Figure 1 shows a representative flow cytometry analysis of RA fibroblast-like synovial cells and autologous skin fibroblasts.

Hsp70 membrane expression was found on fibroblast-like synovial cells derived from arthritis-affected joints when compared with autologous skin fibroblasts either when single staining (Hsp70) or dual staining with particular HSP receptor (CD91/Hsp70) was used.

Figure 2 shows cell surface expression of inducible Hsp70 and HSP receptors on fibroblast-like synovial cells derived from RA-affected joints (2-A) and skin fibroblasts derived from the operation wounds (2-B).

Discussion

RA is a disorder that has its origin in unfettered growth and activation of type B (fibroblast-like) synovial cells [12, 13]. It is this cell type that mediates the initial joint damage [13]. Only in the later stages, after the initial damage has taken place, do migrating cells of the immune system play a role in accelerating disease progression.

Fibroblast-like synovial cells are among the most versatile cells with the potential to activate an array of genes that are able to initiate and propagate inflammation in RA-affected joints [14]. Also of importance is the notion that fibroblast-like synovial cells are in command of a surprisingly extensive array of resources to respond to activation. Not only are fibroblast-like synovial cells able to activate a series of cytokines such as tumor necrosis factor (TNF) α, interleukin (IL) IL-1β, IL-1α, IL-6, and IL-8, they are also able to activate molecules such as tissue factor, PAI-1, MCP-1 as well as a number of matrix metalloproteinases that are involved in tissue degradation. Furthermore, fibroblast-like synovial cells have at their disposal also the ability to activate a series of adhesion molecules such as VCAM and ICAM: these genes that have been implicated in cell migration and cell activation [15, 16].

Patients with rheumatoid arthritis (RA) are confronted with a multitude of stressful events during the course of their disease such as mechanical stress, heat stress, cytokine stress and oxidative stress [17]. Heat is one among physical stress factors present in the inflamed synovial membrane. Flares of disease activity based on an increased inflammatory activity lead to hyperaemia and the release of pyrogenic substances. Thus, hyperthermia is a frequent and long-known symptom of arthritis. Heat stress can induce matrix metalloproteinases [18, 19], for example, which participate in tissue destruction during RA. The function of HSPs is to protect the folding of nascent proteins, the refolding of denatured proteins and the solubilization of protein aggregates especially under conditions of heat stress [20]. Although heat is

the most typical inducer of HSPs, other stress factors such as shear stress [21], oxidative stress [22] and proinflammatory cytokines [23] can also induce certain types of HSP.

In our studies we repeatedly observed high membrane expression of Hsp70 on cell surface of fibroblast-like synovial cells derived from RA patients [2]. It was reported that Hsp70 protects cells against a variety of toxic conditions such as oxidative stress, TNF-α, heat shock, heavy metals and cellular damage after ischaemia. In addition, Hsp70 overexpression has been shown to be protective against apoptotic death; synovial cells expressing elevated Hsp70 levels might therefore develop a certain resistance to apoptosis [24, 25]. This would be in line with the observation of the low frequency of apoptosis in rheumatoid synovium despite the abundance of apoptosis-inducing factors [26, 27].

Extracellular stress proteins including HSPs and glucose regulated proteins (Grp) are emerging as important mediators of intercellular signaling and transport. Release of such proteins from cells is triggered by physical trauma and behavioral stress as well as exposure to immunological "danger signals". Stress protein release occurs both through physiological secretion mechanisms and during cell death by necrosis. After release into the extracellular fluid, HSP or Grp may then bind to the surfaces of adjacent cells and initiate signal transduction cascades as well as the transport of cargo molecules such as antigenic peptides [28]. Many of the effects of extracellular stress proteins are mediated through cell surface receptors.

Previous studies suggest that extracellular Hsp70 can initiate a potent innate and adaptive immune response [29-31]. HSPs interacts with antigen presenting cells (APCs) through surface receptors such as scavenger receptors LOX-1 [32, 33]; CD94 [34] and SR-A [35]; the LDL-receptor-related protein/α2-macroglobulin CD91 receptor [36, 37]; the Toll-like receptor (TLR) 2 and 4 [31, 33]; CD14 [29]; CD36 [33] and CD40 [38, 39]. Formation of Hsp70-HSP receptor complex is associated with the induction of the pro-inflammatory response including a cytokine production (IL-1β, TNF-α, IL-6, etc.), expression of MHC class II [40] and nitric oxide (NO) release [41].

In addition to APCs, Hsp70 can avidly bind to non-APC cell lines, especially those from epithelial or endothelial background [42].

Since we observed simultaneously a high membrane expression Hsp70 on RA synovial cells and a high prevalence of soluble Hsp70 in RA synovial fluids, we examined whether extracellular Hsp70 might be bound to the surface of RA synovial cells via any HSP receptors mentioned aboved.

We report the high membrane expression of CD91 on the cells of mesenchymal origin derived from RA affected and non-affected tissues. Further, we observed high simultaneous membrane co-expression of CD91 and Hsp70 in cell cultures derived from RA synovial tissues, while autologous skin fibroblasts showed only background Hsp70 expression.

It was proposed that the oxidized LDL binding protein CD91/LRP found on antigen presenting cells and other cell types [36] could be the common receptor for all immunogenic HSP, including Hsp60, 70, Gp96 and calreticulin [36]. However, its role as a direct high/medium affinity HSP binder is still not clear. Theriault et al. examined the ability of Hsp70 in free solution to bind cells with, or without CD91 expression and observe minimal differences [42].

The large group of HSP receptors may reflect the large and heterogeneous group of proteins often with radically different cellular effects. In addition, stress proteins likely recognize different receptors on different cell types [42]. The multiplicity of receptors may also indicate specialization for individual functions: receptors such as the TLR, CD40 and CCR5 may be adapted for transmembrane signaling while CD91 and SR may play more important roles in internalization of HSP.

Indeed, it has been found that even between quite closely related members of the Hsp70 family there were differences in interactions with individual receptors [28].

Since we simultaneously demonstrated the high prevalence of inducible Hsp70 in RA synovial fluids and a high membrane co-expression of Hsp70-CD91 on RA synovial cells, we speculate that Hsp70 released from inflamed synovial tissue might be captured onto the cell surface of synovial cells from the extracellular space via CD91

receptor. The significance of the Hsp70 interaction with synovial cells via CD91 remains undefined but may mediate other non-immune purposes like development of a higher resistance to stress-induced apoptosis as was described e.g. in adjacent neuronal cells taking up extracellular Hsp70 released from glial cells in normal conditions or during stress [43].

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Figure 1: Representative flow cytometry analysis on fibroblast-like synovial cells and skin fibroblasts derived from 54 years old female with RF-seropositive RA lasting for 20 years; therapy: C/DMARD.

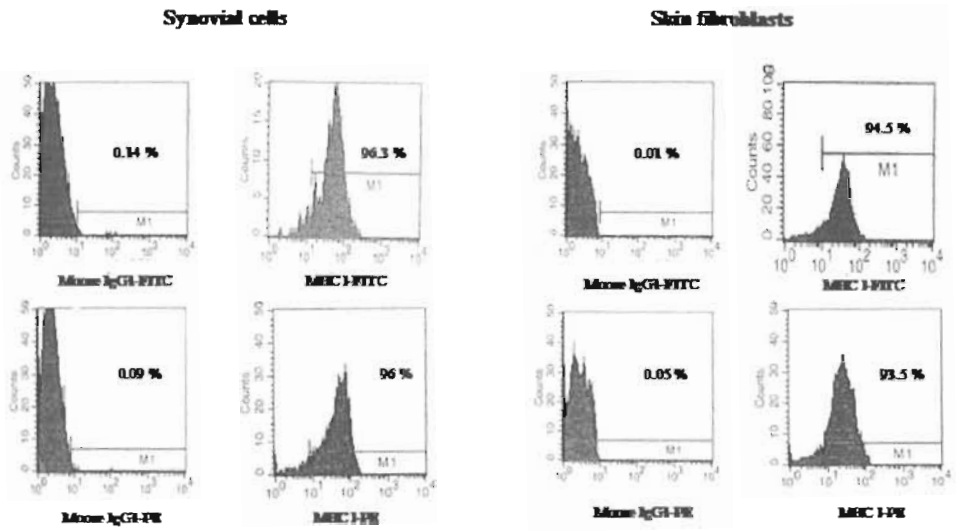
Legend to Figure 1: Synovial cells derived from synovial tissue of hip joint were cultivated for 40 days and skin fibroblasts derived from the operation wound for 49 days upon reaching the confluence. The adherent cells were trypsinised, resuspended in cell culture medium and single stained with monoclonal anti-IgG1 and anti-MHC class I antibodies, both conjugated with FITC and/or PE (1-A); single stained with anti-Hsp70-FITC conjugated antibody (1-B) and dual stained with anti-Hsp70-FITC and CD91-PE conjugated antibodies (1-C).

Only viable cells negative for 7-AAD were gated and analysed. MHC class I, used as a positive control, was detected on most of analysed cells (1-A).

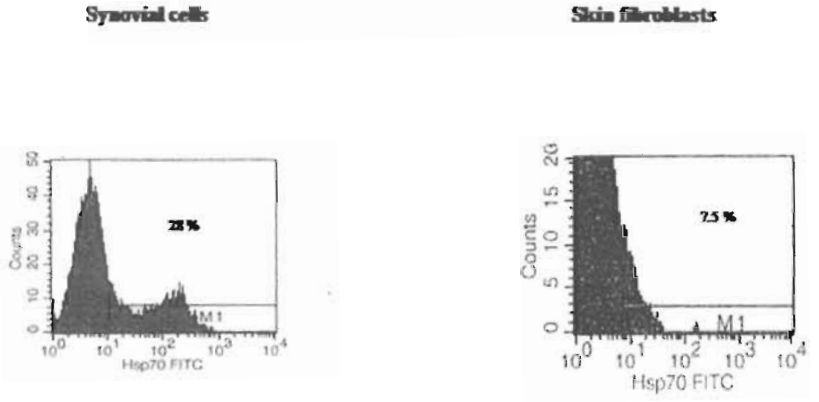
Figure 1-B shows the high percentage of Hsp70-expressing cells among synovial cells (Hsp70: 28 %), whereas skin fibroblasts showed only background expression (Hsp70: 7.5 %).

Both synovial cells and skin fibroblasts expressed high levels of CD91 on the cell surface (synovial cells: 88 %; skin fibroblasts: 73.4 %). 23% of CD91 positive synovial cells carried also Hsp70 while only 2.9 % of CD91 positive skin fibroblasts were simultaneously positive for Hsp70 (1-C).

I-A: isotype controls and positive controls



I-B: Hsp70 membrane expression (single staining)



1-C: Hsp70 membrane expression (dual staining with CD91)

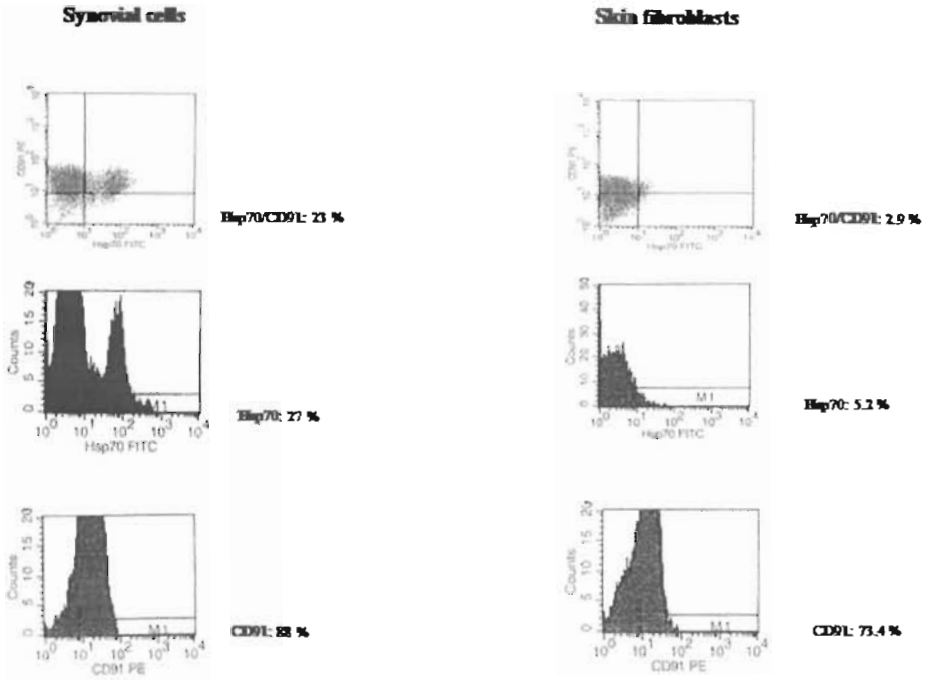


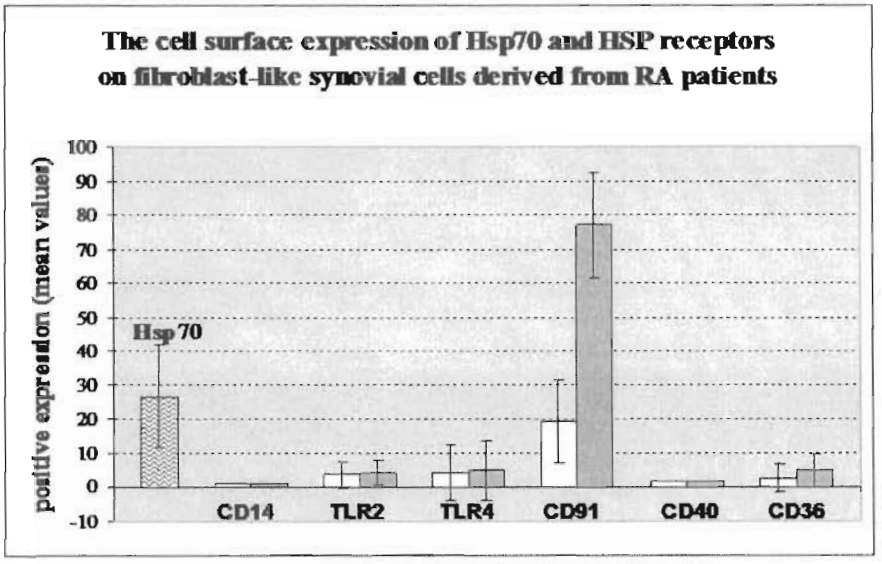
Figure 2: The surface expression of inducible Hsp70 and HSP receptors on fibroblast-like synovial cells derived from RA-affected joints (2-A) and skin fibroblasts derived from the operation wounds (2-B).

Legend to Figure 2: The first bar, marked Hsp70, represents the percentage of Hsp70 expressing cells of all R1 events.

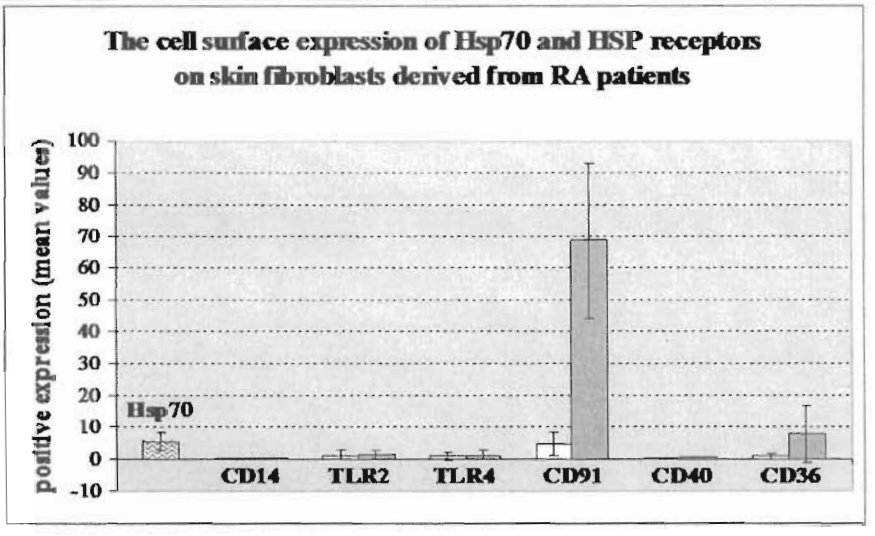
The first of each pair of staples (the light bar) represents a mean value of the percentage of Hsp70-bound cells among fibroblasts expressing so-called HSP receptors. The second of each pair of staples (the dark bar) indicates the percentage of fibroblasts expressing the different receptors.

Both synovial cells (SC) and skin fibroblasts (SF) expressed relatively high levels of cell surface CD91 (mean SC: 77.1 % x SF: 68.6 %). No or low expression of CD14 (mean SC: 0.9 % x SF: 0.5 %), CD40 (mean SC: 2.0 % x SF: 0.5 %), TLR2 (mean SC: 4.3 % x SF: 1.4 %), TLR4 (mean SC: 4.9 % x SF: 1.2 %) and CD36 (mean SC: 5.0 % x SF: 7.9 %) was detected. Significantly higher percentage of Hsp70 membrane expression was found on fibroblast-like synovial cells derived from arthritis-affected joints than on autologous skin fibroblasts (Hsp70: mean SC: 26.8 % x SF: 5.5%, $p<0.001$).

Alike, high co-expression of CD91 and Hsp70 on RA synovial cells (mean 19.4 %) was observed, while skin fibroblasts showed only background Hsp70 expression (mean 4.8 %; $p<0.001$).


The cell surface expression of Hsp70 and HSP receptors
on fibroblast-like synovial cells derived from RA patients
positive expression (mean values)
100
90
80
70
60
50
40
30
20
10
0
-10
Hsp70
CD14
TLR2
TLR4
CD91
CD40
CD36

2-A


The cell surface expression of Hsp70 and HSP receptors
on skin fibroblasts derived from RA patients
positive expression (mean values)
100
90
80
70
60
50
40
30
20
10
0
-10
Hsp70
CD14
TLR2
TLR4
CD91
CD40
CD36

2-B