The PI3K/Akt signaling pathway regulates the expression of Hsp70, which critically contributes to Hsp90-chaperone function and tumor cell survival in multiple myeloma

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Online Supplementary Design and Methods

Immunohistochemical analyses

Biopsies from normal human tonsils with a germinal center reaction (n=5) or normal bone marrow biopsies (n=3) from patients without multiple myeloma (MM) were used to analyze the expression of Hsp72 and Hsp73 in different types of non-malignant human B cells. To investigate Hsp72 and Hsp73 expression in transformed plasma cells, bone marrow biopsies from patients with monoclonal gammopathy of undetermined significance (n=3) or MM (n=55; 49 from bone marrow and 6 from extramedullary sites) were selected from the lymph node registry at the Institute of Pathology in Würzburg, Germany. The diagnosis was established according to the criteria of the WHO Classification by morphological and immunophenotypic analyses of paraffin-embedded tissue sections. Seventeen patients had been newly diagnosed with MM and 38 MM patients had already been pretreated. Paraffin-embedded tissue sections were subjected to heat-induced epitope retrieval and used for immunohistochemical or immunofluorescence staining as previously described.[1] Primary antibodies against Hsp72 (SPA-810, clone C92F3A-5, 1:100), Hsp73 (SPA-815 clone 1B5, 1:200), and pan-Hsp70 (anti-Hsp72/Hsp73; SPA-820, clone N27F3-4, 1:100) (all from Stressgen Bioreagents, Ann Arbor, USA) in combination with the LSAB AEC Kit (DakoCytomation, Hamburg, Germany) were used to detect Hsp70 proteins. Cell nuclei were stained with hematoxylin (Merck, Darmstadt, Germany). An anti-CD138 antibody (1:25, Clone MI15, DakoCytomation) together with the anti-Hsp73 antibody in combination with species-specific FITC- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, USA) were used for double-staining. For double-staining against CD138 and Hsp72, the anti-Hsp72 antibody was pre-labeled using the Alexa555-Zenon-antibody-kit (Invitrogen, Carlsbad, USA) and processed further according to the provider's instructions. An anti-Hsp90β antibody (AB3468; Chemicon, Temecula, USA) together with the pan-Hsp70 (anti-Hsp72/Hsp73) antibody combined with species-specific Cy3- or Cy5-conjugated secondary antibodies were used for double-staining. Images were collected with either a BX51 light microscope equipped with a DP50-CCD camera (both from Olympus, Hamburg, Germany) or with a laser scanning TCS SP2 Confocal System equipped with a DMRE microscope and an HCX PLAPO 40x/1.25 NA oil-immersion objective lens, and were processed with Leica Confocal Software version 2.61 (all from Leica Microsystems, Mannheim, Germany). The expression of HSP72 and HSP73 was assessed by a hematopathologist using a scoring system based on the intensity of staining and the number of stained plasma cells. The intensity of HSP73 and HSP72 staining was classified into weak (1), moderate (2) and strong (3). The number of positive plasma cells was semi-quantitatively classified as low (≤10% of all plasma cells), moderate (between 10 and 50%) and high (≥50%). The final score was calculated by multiplying the intensity and number values. Cases with scores equal to or higher than 2 were classified as positive.

Cell culture

Cell culture conditions of human MM cell lines (INA-6, JJN-3, KMS11, L363, MM.1S) have previously been described in detail.[2] MM cell lines were purchased from either the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) or LGC Standards (Wesel, Germany). The interleukin-6-dependent MM cell line INA-6 was a gift from Prof. Martin Gramatzki (Kiel, Germany). On acquisition each cell line was immediately expanded to create a stock bank of 50 frozen aliquots, and from these aliquots working banks were generated as required. To maintain the authenticity of cell cultures, fresh working bank aliquots were thawed and used for experiments every 3-4 months (dead-end cell culture). Culture conditions, isolation of primary MM cells and generation of primary bone marrow stromal cells (BMSC) have been described in detail elsewhere.[3,4] Bone marrow aspirates of MM patients were obtained from routine diagnostic specimens after informed consent. Permission for these studies was granted by the Ethics Committee of the Medical Faculty of the University of Würzburg, Germany (reference number 18/09).

Construction of short interfering RNA expression vectors

pSUPER-derived short interfering (si) RNA expression constructs were designed according to published guidelines.[5] The sequences of the sense oligonucleotides used for construction of the most effective siRNA expression plasmids (sequence derived from the actual gene in bold) were: 5'-dGATCCCC**GGTGATCAACGACGGAGAC**TTCAAGAGA**GTCTCCGTCGTTGATCACC**TTTTTGGAAA-3' (pSUPER/Hsp72; based on positions 279-297 of the human cDNA for *HSP72*), 5'-dGATCCCC**GACCTAAATTCGTAGCAAA**TTCAAGAGA**T**

TTGCTACGAATTTAGGTCTTTTTGGAAA-3' (pSUPER/Hsp73-1; based on positions 1992-2010 of the human cDNA for *HSP73*), and 5'-dGATCCCC**GTCTGAGAATGTTCAAGAT**TTCAAGAGA**ATCTTGAACATTCTCAGAC**TTTTTGGAAA-3' (pSUPER/Hsp73-2; based on positions 1151-1169 of human *HSP73*), 5'-dGATCCCC**GCCAGTACCTCATGGATTAGT**TCAAGAG**ACTAATCCATGAGGTACTGGC**TTTTTGGAAA-3' (pSUPER/PI3Kp110α, based on positions 1452-1471 of human *PIK3CA*).

Transient transfection of INA-6 and MM.1S cells with short interfering RNA expression plasmids

INA-6 or MM.1S cells were transiently transfected with siRNA expression vectors and western blot analysis of the respective target in selected cells was performed after 72 h (pSUPER/Hsp72 or pSUPER/Hsp73) or after 120 h (pSUPER/PI3Kp110α) to verify their efficiency and specificity. The protocol for transient transfection and purification of transfected cells has been described before.[5] In brief, $5x10^6$ MM cells were electroporated with plasmid pCD4Δ [15 μg/mL] and the respective pSUPER constructs. To selectively knock down Hsp72, 20 μg/mL of pSUPER/Hsp72 were used. Sustained Hsp73 knockdown was achieved through a combination of two different siRNA expression vectors: 20 μg/mL each of pSUPER/Hsp73-1 and pSUPER/Hsp73-2.

Western blot analysis

The western blotting procedures were performed as previously described.[4] Co-cultured INA-6 cells were washed off to avoid contamination by BMSC. Following separation by sodium dodecyl polyacrylamide gel electrophoresis, proteins were transferred onto nitrocellulose membranes and stained with antibodies against Hsp72 (SPA-810), Hsp73 (SPA-816) or pan-Hsp70 (anti-Hsp72/Hsp73; SPA-822), pan-Hsp90 (anti-Hsp90α/Hsp90β; SPA-845) (all from Stressgen), B-Raf (sc-166), C-Raf (sc-133; both from Santa Cruz, Heidelberg, Germany), Akt (n. 9272), phospho-STAT3 (Tyr705; n. 9131), STAT3 (n. 3132), p38 (n. 9212), Tyk2 (n. 9312), PI3Kp110α (n. 4249), AIF (n. 4642), caspase 9 (n. 9502), and caspase 3 (n. 9662) (all from Cell Signaling Technology, Frankfurt am Main, Germany), IKKα (n. 556532, Becton Dickinson, Heidelberg, Germany), lamin A (Ab8980, Abcam, Cambridge, UK) or β-actin (A5316, Sigma, Deisenhofen, Germany). Secondary antibodies used were anti-rat (SAB-200, Stressgen), anti-rabbit (NA9340, GE Healthcare, Little Chalfont, UK), and anti-mouse (NA9310, GE Healthcare).

Apoptosis assay

Apoptotic and viable cell fractions were assessed by annexin V-FITC/propidium iodide (PI) staining (Bender MedSystems, Vienna, Austria) as previously described.[4] Briefly, cells were washed in phosphate-buffered saline, incubated for 15 min in 100 μL binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$] containing 2.5 μL annexin V-FITC solution and 1 μg/mL PI and analyzed by flow cytometry (FACSCalibur/CELLQuest; Becton Dickinson).

AlamarBlue viability assay

The AlamarBlue colorimetric assay was performed to measure drug effects on metabolic activity of MM cells. Using a 96-well format, 25000 cells were seeded per well, and each test concentration was prepared in triplicate. The color reaction was measured in a microplate reader and the share of the reduced form of the dye was calculated as described in the manufacturer's manual (MorphoSys, Oxford, UK). Effects were quantified in relation to DMSO-treated controls (100% values).

Drug combination analyses

Combination treatments of MM cell lines INA-6 and MM.1S with NVP-AUY922/VER-155008 or NVP-AUY922/PI103 were performed according to the guidelines discussed by Chou[6] using the AlamarBlue assay to quantify viability. After dose-effect curves had been established for each drug, the respective EC_{50} concentrations were chosen to define the ratio at which to combine the two drugs. In addition to the combination at the EC_{50}, at least three lower and two higher concentrations maintaining the same ratio were chosen (constant ratio – single ray design; simultaneous exposure of MM cells to both drugs for 3 days). A complete experiment comprised the simultaneous determination of the dose-effect curves of two single drugs and of their combination. Only concentrations representing between 2% and 98% of the DMSO-treated control value (i.e. those determining the shape of a dose-effect curve) were considered and at least four values were required to represent this range. An experiment was accepted if the subsequent evaluation with CalcuSyn software (version 2.1; Biosoft, Cambridge, UK) showed correlation coefficients of 0.95 or better for all three of the dose-effect curves involved.

Reagents

Construction of the expression plasmid for human truncated CD4 was previously described.[5] The Hsp90 inhibitor NVP-AUY922 (n. 1542), and the PI3K inhibitor PI103 (n. 1380) were purchased from Axon Medchem BV (Groningen, The Netherlands). The Hsp72/Hsp73 inhibitor VER-155008 (n. 3803) was purchased from Tocris Bioscience/R&D Systems (Wiesbaden, Germany), and the GSK3b inhibitor XXVI (n. 361669-5MG) from Merck Chemicals (Nottingham, UK).

References

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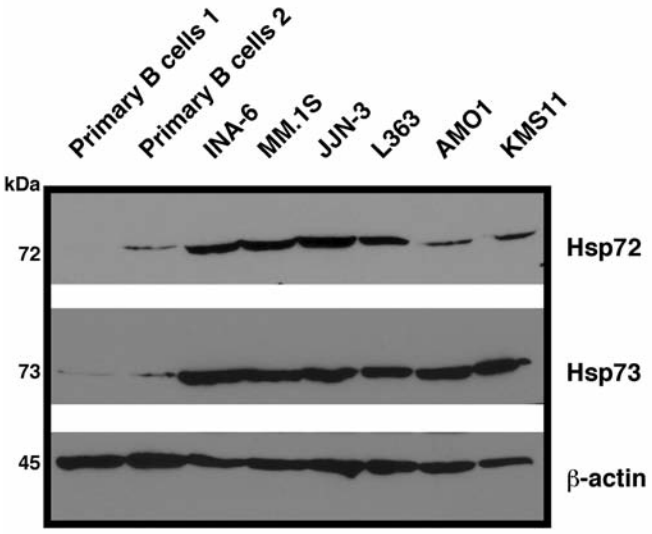
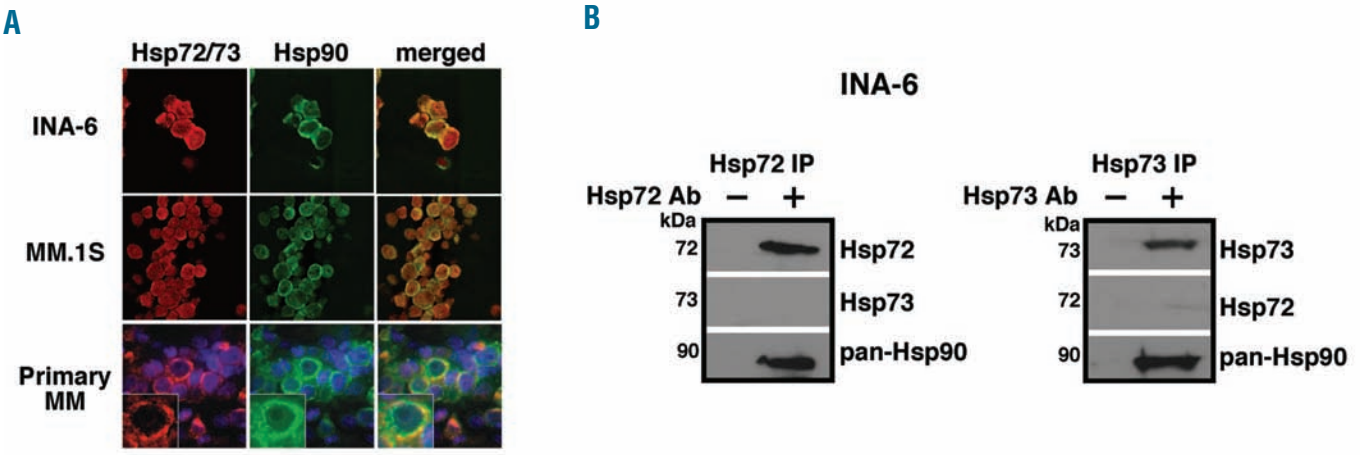


Online Supplementary Figure S1. Overexpression of Hsp72 and Hsp73 in MM cell lines compared to normal B cells. Western blot analyses of constitutive expression of Hsp72 and Hsp73 in six MM cell lines and in normal human B cells derived from the peripheral blood of two healthy donors. In contrast to normal B cells, MM cell lines exhibited elevated Hsp72 and Hsp73 levels. Whereas the protein level of Hsp73 was very similar across the tested MM cell line panel, that of Hsp72 differed markedly. Staining for β-actin served as a loading control.



Online Supplementary Figure S2. Co-localization and interaction of Hsp72 and Hsp73 with Hsp90 in MM cells. (A) Immunofluorescence analysis of Hsp72/Hsp73 and Hsp90 protein co-localization in INA-6, MM.1S (cell line blocks) or primary MM cells (bone marrow biopsy). MM cells were double-stained for pan-Hsp70 (Alexa555, red) and Hsp90β (FITC, green) and analyzed by confocal microscopy. Merged images show Hsp72/Hsp73 in close co-localization to Hsp90. (B) Western blot analysis of the Hsp90/Hsp70 interaction. Hsp72 or Hsp73 was immunoprecipitated from INA-6 or MM.1S cell lysates using isoform-specific antibodies, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by western blotting and staining for Hsp72, Hsp73 and Hsp90. Hsp90 was found co-immunoprecipitated with both Hsp70 isoforms.

Online Supplementary Table S1. Immunohistochemical analyses of Hsp72, Hsp73 or Hsp72/Hsp73 expression in biopsies of MM patients. The table summarizes the data on localization (loc), morphological phenotype (morphology), plasma cell infiltration (PCI), pretreatment (PT), and Hsp70 staining of the 55 MM biopsies in this study. Abbreviations are Hsp72 (%): percentage of Hsp72-positive plasma cells; Hsp72 (I): grade of Hsp72 staining intensity; Hsp72(s): Hsp72 staining score.

No	Loc	Morpho-logy	PT	PCI (%)	Hsp72/ 73 (%)	Hsp72/ 73 (I)	Hsp72/ 73 (S)	Hsp72 (%)	Hsp72 (I)	Hsp72 (S)	Hsp73 (%)	Hsp73 (I)	Hsp73 (S)
1	BM	mature	yes	10	30	2	4	30	1	2	50	2	6
2	EM	anaplastic	yes	80	90	3	9	90	3	9	100	3	9
3	BM	mature	yes	40	10	1	2	10	1	2	10	1	2
4	BM	mature	yes	60	30	2	4	30	1	2	90	2	6
5	BM	mature	yes	90	0	0	0	0	0	0	0	0	0
6	BM	mature	yes	80	80	3	9	80	3	9	90	2	6
7	BM	mature	yes	90	90	3	9	90	3	9	0	0	0
8	BM	mature	yes	90	90	3	9	50	3	9	0	0	0
9	BM	mature	yes	90	80	3	9	80	3	9	90	2	6
10	BM	mature	yes	90	80	2	6	80	2	6	0	0	0
11	BM	mature	yes	85	0	0	0	0	0	0	0	0	0
12	BM	mature	no	90	0	0	0	0	0	0	0	0	0
13	BM	anaplastic	yes	80	90	3	9	90	2	6	90	3	9
14	BM	mature	yes	80	0	0	0	0	0	0	0	0	0
15	BM	mature	no	50	0	0	0	0	0	0	0	0	0
16	EM	anaplastic	yes	100	30	2	4	30	1	2	20	2	4
17	EM	anaplastic	no	100	80	3	9	80	3	9	10	3	6
18	BM	mature	no	95	90	2	6	90	2	6	0	0	0
19	BM	mature	yes	50	10	2	4	0	0	0	90	2	6
20	BM	mature	yes	80	5	1	1	5	1	1	5	1	1
21	BM	mature	no	30	0	0	0	0	0	0	0	0	0
22	BM	mature	yes	70	10	1	2	10	1	2	10	1	2
23	BM	mature	yes	50	90	2	6	90	3	9	0	0	0
24	BM	mature	no	90	80	2	6	50	1	3	90	2	6
25	BM	mature	no	30	0	0	0	0	0	0	0	0	0
26	BM	mature	yes	90	20	2	4	0	0	0	60	2	6
27	BM	mature	yes	15	0	0	0	0	0	0	0	0	0
28	BM	anaplastic	no	90	90	2	6	90	3	9	0	0	0
29	BM	anaplastic	no	10	0	0	0	0	0	0	0	0	0
30	EM	anaplastic	yes	80	90	3	9	100	3	9	5	1	1
31	BM	mature	yes	20	0	0	0	0	0	0	0	0	0
32	BM	anaplastic	yes	90	30	2	4	80	2	6	90	2	6
33	BM	mature	yes	80	90	2	6	30	2	4	0	0	0
34	BM	mature	no	40	10	1	2	10	1	2	0	0	0
35	BM	mature	no	30	0	0	0	0	0	0	0	0	0
36	BM	mature	yes	10	0	0	0	0	0	0	0	0	0
37	BM	mature	yes	90	10	2	4	10	1	2	80	2	6
38	BM	mature	yes	90	50	3	9	50	3	9	100	2	6
39	BM	mature	yes	40	80	2	6	80	1	3	80	2	6
40	BM	mature	no	80	30	3	6	30	3	6	0	0	0
41	BM	mature	no	25	0	0	0	0	0	0	0	0	0
42	BM	mature	yes	95	90	1	3	80	1	3	0	0	0
43	EM	mature	yes	100	90	3	9	90	3	9	0	0	0
44	EM	mature	yes	80	100	3	9	100	3	9	100	3	9
45	BM	mature	yes	20	80	2	6	80	2	6	0	0	0
46	BM	mature	yes	10	0	0	0	0	0	0	0	0	0
47	BM	mature	yes	35	0	0	0	0	0	0	0	0	0
48	BM	mature	no	25	0	0	0	0	0	0	0	0	0
49	BM	mature	yes	80	100	2	6	100	3	9	0	0	0
50	BM	mature	yes	50	30	2	4	30	2	4	30	0	0
51	BM	mature	no	70	10	1	2	30	1	2	10	1	2
52	BM	mature	yes	70	50	2	6	50	2	6	0	0	0
53	BM	mature	yes	40	10	1	2	0	0	0	10	1	2
54	BM	mature	no	20	0	0	0	0	0	0	0	0	0
55	BM	mature	no	90	20	1	2	0	0	0	80	1	3