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HSP27 protects AML cells against VP-16-induced apoptosis through modulation of p38 and c-Jun

Hein Schepers[a,b,*], Marjan Geugien[a,*], Marco van der Toorn[c], Anton L. Bryantsev[d], Harm H. Kampinga[e], Bart J.L. Eggen[b], and Edo Vellenga[a]

[a]*Division of Hematology, Department of Medicine, University Medical Center Groningen, Groningen, The Netherlands;* [b]*Departments of Developmental Genetics;* [c]*Allergology, and* [e]*Radiation and Cell Stress Biology, University of Groningen, Groningen, The Netherlands;* [d]*Molecular and Cellular Cardiology Lab, Institute of Experimental Cardiology, Cardiology Research Centre, Moscow, Russia*

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***Objective*. To investigate 1) the signal transduction pathways affected by heat shock protein 27 (HSP27) expression; and 2) the expression and regulation of HSP27 in acute myeloid leukemia (AML).**

***Materials and Methods*. RNA interference studies for HSP27 in leukemic TF-1 cells were used to investigate the effects on downstream signal transduction and apoptosis after VP-16 and CD95/Fas treatment. HSP27 expression and activation was investigated in AML blasts through Western blot analysis.**

***Results*. RNA interference for HSP27 resulted in a twofold increase in VP-16-induced apoptosis, which was preceded by enhanced p38 and c-Jun phosphorylation and a twofold increased cytochrome c release into the cytoplasm. DAXX co-immunoprecipitated with HSP27, suggesting an inhibitory role of HSP27 in VP-16-mediated activation of the ASK1/p38/JNK pathway. CD95/Fas-induced apoptosis, however, was unaffected by HSP27 siRNA, due to upregulation of HSP27. Although HSP27 was highly expressed and phosphorylated in primitive monocytic AML blasts (M4-M5, 91%, n = 11) and undetectable in myeloid blasts (M1-M2, n = 5), VP-16-mediated apoptosis correlated moderately with HSP27 expression. This is likely due to the co-expression of $p21^{Waf1/Cip1}$, which is in the majority of the monocytic AML M4-M5 blasts constitutively localized in the cytoplasm. Overexpression of cytoplasmic p21 inhibited the enhanced p38 phosphorylation after HSP27 RNAi, suggesting a predominant anti-apoptotic role of p21 over HSP27.**

***Conclusion*. 1) HSP27 inhibits VP-16-mediated phosphorylation of p38 and c-Jun, cytochrome c release, and subsequent apoptosis; 2) HSP27 is expressed and activated in monocytic AML blasts; 3) cytoplasmic expression of p21 compensates for the lack of HSP27. © 2005 International Society for Experimental Hematology. Published by Elsevier Inc.**

Introduction

Acute myeloid leukemia (AML) is characterized by an accumulation of immature cells in the bone marrow, resulting in the disruption of normal hematopoiesis [1–3]. The leukemic population possesses a growth advantage that is in part linked to the constitutive activation of intracellular proteins that trigger the activation of anti-apoptotic proteins [4–7]. The small heat shock protein 27 (HSP27) is a member of the heat shock protein (Hsp) family, whose expression is transiently induced in response to stress. It has been demonstrated that HSP27 levels change during cellular stress [8] and differentiation [9–11], both on the transcriptional and posttranslational level [8]. In normal cells, HSP27 mainly exists in large oligomeric units up to 800 kDa in size. Stress leads to changes in the multimeric status of the protein due to phosphorylation of HSP27 on three serine residues [12] that can change the activity of the protein [13]. HSP27, besides being involved in cytoskeletal stability, cell motility, and its function as a chaperone [8,13–15], has been implicated in apoptosis [11,16–20].

Induction of apoptosis can occur via an intrinsic as well as an extrinsic signal transduction pathway. The classical

Offprint requests to: Edo Vellenga, M.D., Ph.D., Division of Hematology, Department of Medicine, University Medical Center Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands; E-mail: e.vellenga@int.umcg.nl

*Both authors contributed equally to this work.

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intrinsic pathway is initiated through the release of cytochrome c from mitochondria. Cytochrome c interacts with apoptosis protease activating factor-1 (Apaf-1), which oligomerises and binds to pro-caspase-9 [12]. The formation of this caspase-activating complex, termed the apoptosome, results in the activation of caspase-9, which in turn triggers the proteolytic cleavage of pro-caspase-3, leading to apoptosis [12].

Binding of ligands to death receptors on the cell surface (e.g., CD95/Fas) not only results in the formation of the death-inducing signaling complex (DISC) via the recruitment of the adapter molecule FADD, but also to the recruitment of DAXX to the cytosolic end of the CD95/Fas receptor [21,22]. DAXX then binds apoptosis signal regulating kinase-1 (ASK1), which in turn activates c-Jun N-terminal kinase (JNK) and p38, leading to cytochrome c release and activation of caspases [16,17,23,24].

The mode of HSP27 action in apoptosis is still unclear but could exert its effect at the level of mitochondrial stability [19,25] or DAXX signaling [16,17].

Since HSP27 expression has been observed in multiple malignancies [26,27] including AML [28,29] and HSP27 expression is frequently correlated with unfavorable prognosis [30], we questioned whether HSP27 plays an important role in the protection of AML blasts against CD95/Fas- or VP-16-induced apoptosis; both have been described to activate the DAXX pathway [7,16,17].

Here we demonstrate that HSP27 is predominantly expressed and phosphorylated in AML blasts of the monocytic lineage. RNA interference indicates that HSP27 affects the VP-16-induced DAXX pathway through the modulation of JNK and p38 [7,12] activation, resulting in the enhanced cytochrome c release and induction of apoptosis. Additionally, our results also indicate that in monocytic leukemia, HSP27 is not the determining anti-apoptotic factor, since cytoplasmic localized $p21^{Waf1/Cip1}$ is able to reverse the enhanced VP-16-induced phosphorylation of p38 after RNAi for HSP27.

Experimental procedures

Patient population and isolation of AML cells

Peripheral blood cells or bone marrow cells from 16 adult untreated patients with AML were studied after informed consent. The AML cases were defined according to the classification of the French-American-British (FAB) committee as M0-M6 [31]. AML blasts were isolated by density-gradient centrifugation. The cells were cryopreserved in aliquots of 20 to 30×10^6 cells in RPMI 1640 (Biowhittaker, Verviers, Belgium) supplemented with 10% dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA) and 10% fetal bovine serum (FBS; Bodinco, Alkmaar, The Netherlands), employing a method of controlled freezing and storage in liquid nitrogen. After thawing, T lymphocytes were depleted by 2-aminoethylisothioronium bromide (AET)-treated sheep red blood cell (SRBC) rosetting. The cell population consisted of more than 98% AML blasts as determined by May-Grünwald-Giemsa staining. Fluorescence-activated cell sorting (FACS) analysis demonstrated less than 1% CD3 (Becton-Dickinson, Sunnyvale, CA, USA)-positive cells.

Preparation of monocytes, granulocytes, and $CD34^+$ BM cells

Peripheral blood cells were obtained from healthy volunteer blood donors, and mononuclear cell suspensions were prepared by Ficoll-Hypaque density-gradient centrifugation. T lymphocytes were depleted by AET-treated SRBC rosetting. Monocytes were further enriched by plastic adherence (1 hour, 37°C, 5% CO_2) and demonstrated a purity greater than 95% detected by FACS analysis with anti-CD14 antibody (Becton-Dickinson, Sunnyvale, CA, USA).

Peripheral blood from healthy volunteers, anti-coagulated with 0.32% sodium citrate, was used to isolate granulocytes as described by Fuhler et al. (and references therein) [32].

$CD34^+$ cells were isolated from bone marrow from healthy donors by first making mononuclear cell suspensions followed by incubation of the suspension with phycoerythrin-labeled anti-$CD34^+$ antibody for 30 minutes at 4°C and subsequent FACS sorting using the MoFlo (DakoCytomation, Carpinteria, CA, USA). $CD34^+/CD36^-$ and $CD34^-/CD36^+$ cell populations were obtained by incubating the AML blasts with phycoerythrin-labeled anti-$CD34^+$ and FITC-labeled anti-$CD36^+$ antibodies for 30 minutes at 4°C. Subsequently, the different cell populations were isolated by FACS sorting using the MoFlo.

Cell culture, viral vectors, and transfections

AML blasts were cultured at 37°C, 5% CO_2 at a density of 1×10^6/mL in RPMI 1640 media supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (ICN Biomedicals, Aurora, OH, USA), and 10% FBS. Isolated CD34+ cells were cultured in IMDM medium (ICN Biomedicals) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin. Monocytes were cultured at 37°C, 5% CO_2 at density of 1×10^6/mL in RPMI 1640 and 10% FBS. The human cell lines U937 (ATCC, Manassas, VA, USA, Product No. CRL-1593.2), THP-1 (ATCC, Product No. TIB-202), and HL-60 (ATCC, Product No. CCL-240) were cultured in RPMI 1640 supplemented with 10% FBS. The human cell line TF-1 (ATCC, Product No. CRL-2003) was cultured in RPMI 1640 supplemented with 10% FBS and 10 ng/mL GM-CSF (Genetics Institute, Cambridge, MA, USA).

pMSCV-p21dNLS was constructed by removing the C-terminal bipartite nuclear localization signal (NLS; RKRR, Amino acids 140–143) via polymerase chain reaction (PCR) from pCMV-p21 (kindly provided by Prof. Dr. R.H. Medema and Dr. P. Coffer) and ligating the XhoI-EcoRI fragment into pMSCV-iGFP (kindly provided by Dr. J.J. Schuringa). TF-1 cells were transduced with viral particles collected from

293T cultures transfected with pCL-AMPHO and pMSCV-p21dNLS using FuGENE6 (Roche, Almere, The Netherlands). GFP^+ TF-1 cells were isolated by FACS sorting using the MoFlo.

Reagents and antibodies

An antibody against phosphorylated p38 was obtained from New England Biolabs (Beverly, MA, USA). Antibody against HSP27 was purchased from Stressgen (Victoria, BC, Canada); anti-actin (C4) was obtained from ICN Biomedicals (Aurora, OH, USA). Antibodies against c-Jun, cytochrome c, and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), pan-serine from Zymed Laboratories (San Francisco, CA, USA). Anti-FLAG (M2) was obtained from Sigma. Antibody against p21 was bought from Transduction Laboratories (Lexington, KY, USA). Anti-CD34-HPCA2-PE was obtained from Becton-Dickinson. And CD36 FITC was obtained from IQ Products (Groningen, The Netherlands). Recombinant human (Rh) interleukin (IL)-1β was obtained from Mekesson HBOC Bioservices (Rockville, MD, USA). Rh granulocyte-monocyte colony-stimulating factor (GM-CSF) were purchased from Genetics Institute (Cambridge, MA, USA). The VP-16 was obtained from TEVA (Haarlem, The Netherlands) and the apoptosis-inducing monoclonal antibody CD95/Fas (7C11) was obtained from Immunotech (Marseille, France).

Preparation of protein extracts and Western blotting

The amount of HSP27, GAPDH, $p21^{Waf1/Cip1}$, and actin and the degree of phosphorylated p38, c-Jun, and pan-serine were determined by SDS-PAGE analysis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) on whole-cell extracts. Cells were harvested and total cell extracts were prepared by resuspending the cells in lysis buffer (20 mM Tris HCl pH 7.6, 100 mM NaCl, 10 mM EDTA, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 2 mM PMSF, 1 μM pepstatin, and 1 mM DTT) and kept for 15 minutes on ice. Binding of each antibody was detected by HRP-labeled secondary antibodies using enhanced chemiluminesence (ECL) according to the manufacturer's recommendations (Amersham Life Sciences, Buckinghamshire, UK).

For cytoplasmic and nuclear extracts, cells were harvested and were prepared according to the "mini extracts" method [33]. The extracts were normalized for protein content prior to SDS-PAGE. Proper fractionation and lack of leakage of nuclear proteins to the cytosol was determined by Western blotting for the nuclear protein retinoblastoma (Rb).

RNA interference

Short interfering RNA (siRNA) duplexes for HSP27 were made using the Silencer siRNA construction kit from Ambion (Austin, TX, USA) according to the manufacturer's protocol. The HSP27 target sequence used is: AAGCTGCAAAATCCGATGAGA. GAPDH control siRNA duplexes are provided within the kit. Two × 10^6 TF-1 cells were transfected with a final concentration of 25 nM of RNAi duplexes using oligofectamine according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). Lysates were prepared at the indicated time points in lysis buffer and equal amounts of protein were subjected to Western blot analysis.

Combined annexin V/PI staining procedure

Viability was assessed using an annexin V staining kit (IQ Products, Groningen, The Netherlands) according to the manufacturer's recommendations. Briefly, after 6 hours of culture in RPMI 1640 medium supplemented with 10% FBS, with or without addition of VP-16 (20 μg/mL) or CD95/Fas (2 μg/mL), cells were harvested, resuspended in 100 μL calcium buffer containing 5 μL of annexin V, and incubated for 20 minutes at 4°C in the dark. Cells were washed with 5 mL calcium buffer and subsequently incubated in 300 μL calcium buffer containing 2.5 μL of propidium iodide (PI) for 10 minutes in the dark at 4°C. Finally, binding of fluorescein-conjugated annexin V and PI was measured by fluorescence-activated cell sorting.

Immunoprecipitation

A total of 10^7 AML blasts were cultured for 16 hours in RPMI 1640 medium supplemented with 10% FBS. Cells were harvested, washed with ice-cold phosphate-buffered saline (PBS) containing 1 mM sodium orthovanadate, and subsequently lysed in 500 μL lysis buffer (20 mM Tris HCl pH 7.6, 100 mM NaCl, 10 mM EDTA, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 2 mM PMSF, 1 μM pepstatin, and 1 mM DTT) for 15 minutes on ice. Cell lysates were clarified at 10,000*g* for 20 minutes and incubated with 5 μL HSP27 antibody. After 4 hours rotating, protein A sepharose beads were added and incubated overnight at 4°C. Immunocomplexes were washed 3 times with lysis buffer and separated by SDS-PAGE. Binding of anti-pan-serine antibody was detected using ECL (Amersham, UK).

Twenty × 10^6 TF-1 cells were electroporated with 20 μg of pCDNA3 MYC-FLAG-DAXX and 20 μg of pCDNA3 HA-ASK1 at 240 V and 960 μF. After 24 hours cells were harvested, and co-immunoprecipitations were performed as described above. Binding of anti-FLAG antibody was detected using ECL.

Cytochrome c ELISA

The Quantikine human cytochrome c immunoassay from R&D Systems (Minneapolis, MN, USA) was used to measure cytochrome c levels in the cytoplasm in response to VP-16 or CD95/Fas treatment, according to the manufacturer's recommendations. Cytoplasmic and mitochondrial fractions were separated using the mitochondria isolation kit from Sigma (Zwijndrecht, The Netherlands).

Statistical analysis

The Student's *t*-test for paired samples was used to determine statistical significance of the data about apoptosis induction in the TF-1 cell line. The 2 × 2 contingency test was

used to determine association between FAB classification and HSP27 expression.

Results

HSP27 expression in hematopoietic cells

To determine whether HSP27 is expressed in different hematopoietic cells, we isolated granulocytes, monocytes, mononuclear bone marrow cells, and CD34$^+$ cells from healthy donors. Mononuclear cells from different donors express HSP27 at equal levels, as well as monocytic cells and CD34$^+$ cells (Fig. 1A). In terminally differentiated granulocytes, however, HSP27 could not be detected. A differentiation-dependent expression was also observed in cell lines. HSP27 was highly expressed in the monocytic cell lines THP-1 and U937; moderate expression was observed in the erythroleukemic cell line TF-1; and in the myeloid cell line HL-60 HSP27 could not be detected (Fig. 1B).

RNA interference for HSP27 enhances apoptosis after VP-16 treatment

To investigate whether HSP27 plays a role in the apoptotic process in leukemic cells, RNA interference was used to knock down HSP27 protein levels in TF-1 cells. Figure 2A shows the decrease in HSP27 levels after transfection with siRNAs in time. After two days HSP27 levels were reduced approximately 50% compared to the expression levels in untransfected cells (-). A further reduction in HSP27 protein levels was observed 3, 4, and 5 days after RNAi transfection. The siRNAs used were specific, since no effect on GAPDH expression was observed using HSP27 siRNAs and vice versa (Fig. 2B).

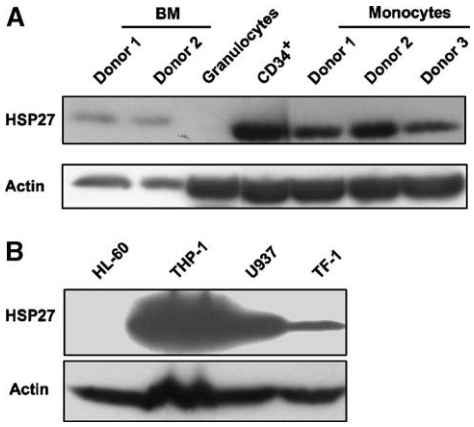

Figure 1. Expression of HSP27 in normal hematopoietic cells and cell lines. **(A):** Bone marrow mononuclear cells, granulocytes, and monocytic cells from healthy donors were isolated and cultured as described in materials and methods. CD34$^+$ cells were isolated from bone marrow from healthy donors by making mononuclear cell suspensions followed by incubation of the suspension with phycoerythrin-labeled anti-CD34$^+$ antibody for 30 minutes at 4°C and subsequent FACS sorting using the MoFlo. HSP27 expression was investigated by Western blot analysis. Actin is shown as loading control. A representative experiment is shown. **(B):** HL-60, THP-1, U937, and TF-1 cells were cultured as described in the materials and methods section, and lysates were analyzed for HSP27 expression using Western blot analysis. Actin is shown as loading control. A representative experiment is shown.

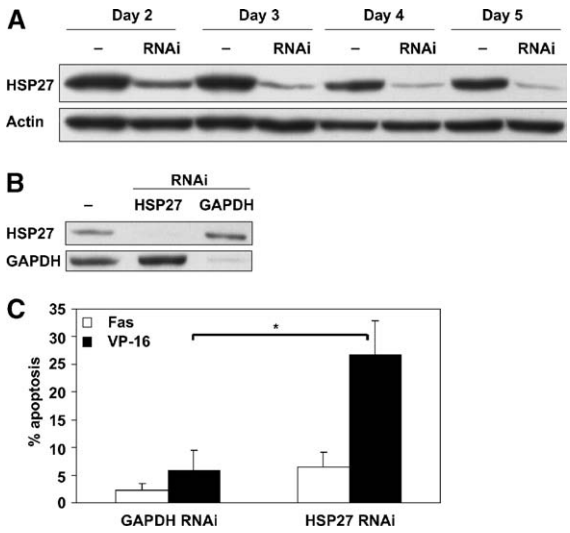

Figure 2. RNA interference for HSP27 specifically knocks down HSP27 expression and enhances VP-16-induced apoptosis. **(A):** 2×10^6 TF-1 cells were transfected using oligofectamine with RNA duplexes targeting HSP27, as described in materials and methods. On the indicated time points, lysates were made and subjected to Western blot analysis for HSP27 expression. Actin is shown as loading control. A representative example of 5 experiments is shown. **(B):** To indicate specificity, TF-1 cells were also transfected with RNA duplexes targeting GAPDH. Western blot analysis on day 4 indicates that both RNA duplexes are specific. A representative example of 5 experiments is shown. **(C):** 4 days after transfection with either HSP27 RNAi or control GAPDH RNAi, TF-1 cells were treated with VP-16 (20 μg/mL) or CD95/Fas (2 μg/mL) for 6 hours. Apoptosis was measured with annexin V/PI staining using flow cytometry as described in materials and methods. A representative example of 5 independent experiments performed in triplicate is shown. ($^* = p < 0.005$).

To investigate whether HSP27 indeed protects against apoptosis, TF-1 cells were transfected with either HSP27 or GAPDH siRNAs and the percentage of apoptotic cells was determined by means of annexin V/PI staining followed by flow cytometry, 6 hours after incubation with VP-16 or CD95/Fas. VP-16-mediated apoptosis was significantly increased in HSP27-RNAi-treated cells compared with the control GAPDH-RNAi-treated cells (26.8% ± 8.7% vs 5.8% ± 3.8%, n = 5, $p < 0.005$, Fig. 2C). HSP27 does not seem to be involved in CD95/Fas-induced apoptosis, since knockdown of HSP27 did not significantly affect the percentage of apoptotic cells upon treatment with CD95/Fas.

RNA interference for HSP27 enhances cytochrome c release from the mitochondria in response to VP-16

Next we investigated at which level HSP27 affected the VP-16-induced apoptotic response. HSP27 has been described

to interact with pro-caspase-3 [25] and cytochrome c [19], ultimately preventing the activation of pro-caspase-3 by caspase-9-mediated proteolysis. Co-immunoprecipitation studies could not detect complex formation between HSP27 and either pro-caspase-3 or cytochrome c in TF-1 cells, which corresponds with observed cleavage of pro-caspase-3 after CD95/Fas treatment (data not shown).

Next we investigated whether HSP27-blocked cytochrome c release from mitochondria [34]. To investigate whether HSP27 inhibits the release of cytochrome c upon VP-16 and CD95/Fas treatment, we performed ELISAs (enzyme-linked immunosorbent assays) on cytosolic extracts from VP-16 and CD95/Fas-treated cells. Control GAPDH siRNA-treated cells showed a twofold increase in cytochrome c release upon VP-16 treatment (Fig. 3A) and a 1.5-fold increase was found after CD95/Fas treatment. Knockdown of HSP27 resulted in a fourfold increase in cytochrome c release upon VP-16 treatment, whereas the effect of CD95/Fas was not altered (Fig. 3A). These results indicate that HSP27 interferes with VP-16-induced cytochrome c release from mitochondria.

RNA interference for HSP27 influences phosphorylation of p38 and c-Jun after VP-16 treatment

Cytochrome c release from mitochondria has been shown to be involved in JNK-mediated, c-Jun-mediated cell death [24,35]. Therefore we focused on the downstream effectors of VP-16- and CD95/Fas-induced apoptosis, i.e., the stress-activated protein kinase (SAPK, also known as JNK; c-Jun amino-terminal kinase) and the p38 MAP kinase [23]. TF-1 cells transfected with either HSP27 or GAPDH siRNAs were treated with VP-16 or CD95/Fas for 1 and 3 hours. P38 and c-Jun activation were determined using Western blot analysis (Fig. 3B). VP-16 induced a strong activation of p38 and c-Jun in the GAPDH RNAi transfected TF-1 cells after 3 hours, whereas no activation was observed using CD95/Fas (lanes 2 and 3). RNA interference for HSP27 further increased VP-16-induced p38 phosphorylation levels approximately threefold (compare Fig. 3B lanes 5 and 10). In addition, the kinetics of p38 phosphorylation were also changed, since phosphorylation of p38 was already observed after 1 hour of VP-16 treatment in HSP27 RNAi-treated cells (compare lanes 4 and 9). A similar effect was found on c-Jun phosphorylation after 3 hours of VP-16 treatment.

In accordance with the absence of enhanced apoptosis, CD95/Fas had no effect on p38 and c-Jun phosphorylation, although JNK has been reported to be a downstream target of CD95/Fas-induced apoptosis by caspase-independent pathways [17]. This coincided with an upregulation of HSP27 expression after CD95/Fas treatment that was not only observed in the control cells, but also in the HSP27 RNAi transfected cells, despite the presence of the siRNA (Fig. 3B, compare lanes 1, 2, and 3 to 6, 7, and 8 respectively). In nontransfected HL-60 cells CD95/Fas treatment

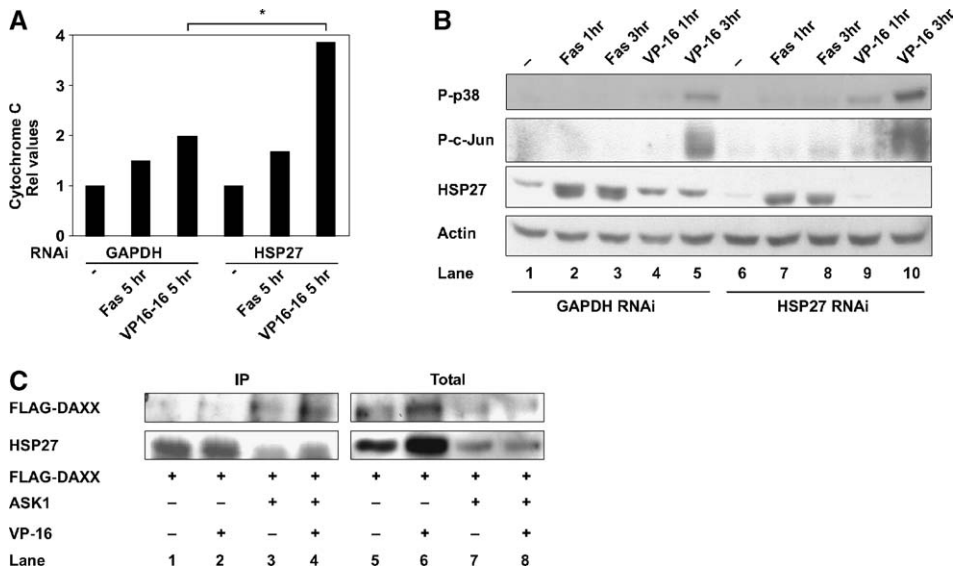

Figure 3. RNA interference for HSP27 enhances the release of cytochrome c and modulates the phosphorylation of p38 and c-Jun in response to VP-16. **(A):** 4 days after transfection with either HSP27 RNAi or control GAPDH RNAi, TF-1 cells were treated with VP-16 (20 μg/mL) or CD95/Fas (2 μg/mL) for the indicated hours and cytochrome c release was measured in cytoplasmic extracts by ELISA, as described in materials and methods. Relative values normalized against untreated cells are shown. A representative case of three individual experiments is shown. **(B):** 4 days after transfection with either HSP27 RNAi or control GAPDH RNAi, TF-1 cells were treated with VP-16 (20 μg/mL) or CD95/Fas (2 μg/mL) for 1 or 3 hours and subjected to Western blot analysis for phosphorylated p38, c-Jun, and HSP27. Actin is shown as loading control. A representative example is shown. **(C):** 20×10^6 TF-1 cells were electroporated with 20 μg of pCDNA3 MYC-FLAG-DAXX with or without 20 μg of pCDNA3 HA-ASK1. After 24 hours cells were lysed in 500 μL lysis buffer. By immunoprecipitation (IP) of HSP27 and Western blot analysis for anti-FLAG, complex formation between HSP27 and DAXX was investigated. Immunocomplexes were detected using ECL. A representative example is shown.

also induced HSP27 expression (data not shown). In conclusion, these data demonstrate that knockdown of HSP27 leads to an enhanced activation of p38 and c-Jun in response to VP-16, indicating a role for HSP27 upstream from mitochondria.

HSP27 forms a complex with DAXX in an ASK-1-dependent manner

Since HSP27 has been described to interact with DAXX [16,17], which is located upstream of ASK1, an activator of p38 and JNK [36,37], we performed co-immunoprecipitation studies to investigate whether HSP27 interacts with DAXX. TF1 cells were transfected with FLAG-DAXX and ASK1 expression plasmids as indicated. In Figure 3C, we show that complex formation between DAXX and HSP27 occurs and that this is ASK1 dependent (Fig. 3B, panel IP). In the absence of ASK1, FLAG-DAXX could not be detected in the precipitates (lanes 1 and 2). Co-expression of both ASK1 and DAXX led to massive cell death (data not shown), resulting in lower amounts of protein in the ASK1/DAXX transfected samples (totals, lanes 3 and 4).

Summarizing, these data indicate complex formation between HSP27 and DAXX in TF-1 cells and suggest a protective effect of HSP27 against VP-16-induced DAXX/JNK/p38 activation.

HSP27 expression in acute myeloid leukemia

In the erythroleukemic cell line TF-1 we demonstrated that knockdown of HSP27 enhances VP-16-mediated apoptosis via modulation of p38 and c-Jun phosphorylation and subsequent mitochondrial cytochrome c release, which eventually results in enhanced apoptosis.

Therefore we questioned whether HSP27 also protects AML blasts against an apoptotic insult. In view of the high expression of HSP27 in $CD34^+$ and monocytic cells (Fig. 1A), we first studied HSP27 expression levels in myeloid (FAB M1/M2, n = 5) and monocytic (FAB M4/M5, n = 11) AML samples. None of the AML M1/M2 cases expressed HSP27, despite the presence of high CD34 antigen levels in 60% of these cases (Fig. 4 and Table 1). In contrast, 10 out of 11 AML M4/M5 patients demonstrated distinct, although variable, HSP27 expression (correlation $p < 0.005$; Fig. 4 and Table 1). Within these AML patients, HSP27 is most prominently expressed within immature AML progenitors ($CD34^+/CD36^-$) as compared to the more differentiated AML cells ($CD34^-/CD36^+$), investigated through FACS sorting and Western blot analysis (n = 2, data not shown). To exclude that experimental procedures modulated HSP27 levels, fresh AML blast and cryopreserved blasts of the same patient were compared, directly and several hours after isolation. No effect of cryopreservation and isolation procedures was observed on HSP27 levels (data not shown).

Table 1. FAB classifications, percentage of apoptotic cells, expression patterns, and CD34 percentages in patients with AML

AML case	FAB	Apoptosis % VP-16	Apoptosis % Fas	HSP27	p21	% $CD34^+$ cells
1	M5	8	16	++	+++	3
2	M1	n.d.*	n.d.*	–	n.d.	1
3	M1/2	27	14	–	–	40
4	M5	9	2	+++	+++	3
5	M4	17	5	++	+++	n.d.
6	M4	16	21	+	+++	n.d.
7	M5	16	42	–	+/–	< 1
8	M4	4	28	+++	++	5
9	M4	28	2	++	–	2
10	M2	16	20	–	–	8
11	M5	11	12	+	++	3
12	M5	8	5	++	++	1
13	M1	25	49	–	–	91
14	M5A	51	8	+	–	n.d.
15	M1	16	1	–	+/–	80
16	M5	14	3	+++	–	64
TF-1		20	10	+	–	

n.d. = not determined, * = spontaneous apoptosis > 90%, FAB = French – American-British classification, VP-16 = percentage of apoptosis induced by VP-16; Fas = percentage of apoptosis induced by CD95/Fas, HSP27 = HSP27 expression levels; p21 = $p21^{waf1/Cip1}$ expression levels, % CD34+ cells = the percentage of CD34 positive cells as determined by FACS analysis.

Expression of HSP27 is not correlated with decreased apoptosis in acute monocytic leukemia

In view of the expression of HSP27 in acute monocytic leukemia, we questioned whether HSP27 expression was related to sensitivity towards apoptotic stimuli. Both myeloid (FAB M1/M2) and monocytic (FAB M4/M5) AML blasts were incubated with VP-16 or CD95/Fas. After 6 hours of incubation, the percentage of apoptotic cells was determined by annexin V/PI staining. In the group without HSP27 expression more than 15% VP-16-mediated apoptosis was found in all cases studied, whereas 60% of the HSP27-expressing cases demonstrated less than 15% apoptosis.

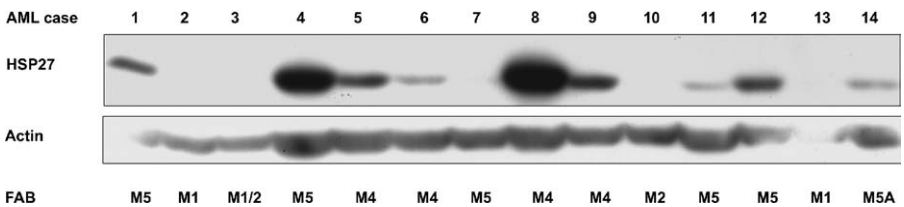

Figure 4. HSP27 is differently expressed in AML samples. Acute myeloid leukemic cells were isolated and cultured as described in materials and methods and HSP27 expression was investigated using Western blot analysis. A representative case of 3 experiments is shown. The 2×2 contingency test was used to determine association between FAB classification and HSP27 expression (correlation, $p < 0.005$).

However, when all samples were included, no absolute correlation was observed between VP-16- and CD95/Fas-induced apoptosis and HSP27 expression levels (Fig. 5A and Table 1).

Phosphorylation and activation of HSP27

Since no distinct correlation between HSP27 expression and decreased apoptosis were detected, we wondered whether HSP27 is phosphorylated and activated in AML cells. To investigate whether the HSP27 protein detected in the monocytic AML samples is phosphorylated, immunoprecipitations (IP) were performed on AML lysates using an anti-HSP27 antibody. Following IP, Western blot analysis was performed with an anti-phosphoserine antibody (Fig. 5B and C). Figure 5B shows faint phosphorylation of HSP27 in untreated TF-1 cells (lane 1). In the absence of HSP27 antibodies, HSP27 was not precipitated (lane 2). Interleukin (IL)-1 treatment, a known activator of HSP27, resulted in a transient increase in HSP27 serine phosphorylation (lanes 3 and 4) [12,38,39]. The finding of phosphorylated and hence activated HSP27 in the TF-1 cell line is in accordance with the finding that it interacts with DAXX in these cells, since this phosphorylated dimer of HSP27 has been described to interact with DAXX [17]. Finally, Figure 5C depicts the results of 6 AML cases. Case 9 shows IL-1-stimulated AML blasts as a positive control. These results demonstrate that HSP27 is phosphorylated in all AML cases studied irrespective of apoptotic sensitivity.

The cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ is co-expressed with HSP27 in monocytic AML blasts and can block the enhanced VP-16-induced apoptosis after HSP27 RNAi

The variability in the results of the sensitivity of monocytic leukemic cells towards apoptotic stimuli might be related to other (more dominant) factors that affect the apoptotic process. As recently described and confirmed in this study (Table 1, Fig. 6A, similar to Fig. 4B, with Western blot for p21), monocytic leukemic cells frequently express the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ in the cytoplasm, which counteracts the effects of VP-16 on the apoptotic program [7]. In 70% of the HSP27-expressing AML cases, p21 was co-expressed, whereas in the AML cases with no HSP27 expression, p21 was expressed in 40% of the cases, indicating that at least two proteins are protective in many monocytic leukemias. We therefore investigated whether p21 could inhibit the enhanced effect on VP-16-induced apoptosis after HSP27 RNAi by using Western blot analysis for phosphorylated p38. TF-1 cells were virally transduced with an expression construct for cytoplasmic p21 (p21dNLS). This p21dNLS lacks the bipartite nuclear localization signal and is therefore retained in the cytoplasm, where it has been shown to inhibit apoptosis [7,40,41]. Figure 6B demonstrates cytoplasmic localization of the p21 dNLS protein in TF-1 cells. These cells were transfected with HSP27 or GAPDH siRNAs and treated with VP-16 for

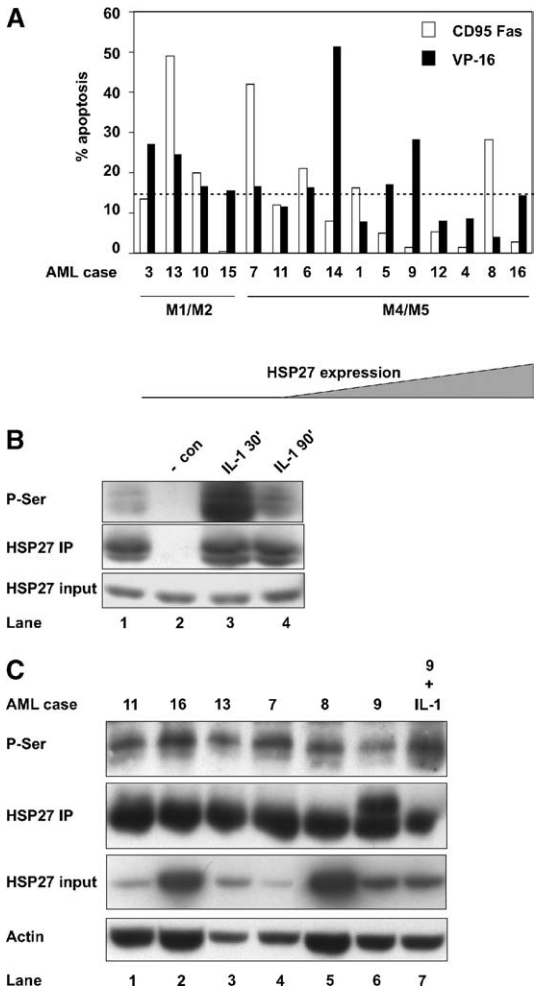

Figure 5. VP-16 and CD95/Fas-mediated apoptosis and HSP27 phosphorylation in AML cells. **(A):** Apoptosis was induced by culturing the AML cells in the presence of VP-16 (20 μg/mL) or CD95/Fas (2 μg/mL). After 6 hours of culture, binding of fluorescein-conjugated annexin V and PI was measured by fluorescence-activated cell sorting. The Mann-Whitney *U*-test was used to analyze differences in apoptosis between the two groups of differentially expressing HSP27 AML blasts (presence or absence of HSP27 expression and M1/M2 vs M4/M5 FAB classification), but no significant differences could be observed. The dotted line represents the 15% apoptosis level. A representative example of two experiments is shown. **(B):** By immunoprecipitation (IP) of HSP27, and Western blot analysis for pan-serine phosphorylation, the activation of HSP27 was investigated. As control for the HSP27 IP and phosphorylation analysis, performed in the AML cases, the procedure was tested in TF-1 cells. The first lane shows untreated TF-1 cells. Lane 2 shows the IP procedure without antibody, indicating specific pull-down of HSP27 in the other lanes. Lane 3 shows TF-1 cells treated for 30 minutes with 10 ng/mL IL-1β and lane 4 shows 90 minutes of treatment with 10 ng/mL IL-1β, indicating the transient phosphorylation in TF-1 cells. The HSP27 input is shown here as loading control and this is the HSP27 in total cell lysates before the IP procedure. A representative example is shown. **(C):** 10^7 AML blasts were cultured for 16 hours in RPMI 1640 medium supplemented with 10% FBS. Cell lysis and IP of HSP27 is performed as described in materials and methods. The activation of HSP27 was investigated as described above. Input indicates HSP27 levels in total lysates before IP. Six representative cases are shown. In order to show that indeed phospho-HSP27 is detected, AML case 9 is also treated with 10 ng/mL IL-1β, a known stimulator of HSP26 phosphorylation. Actin is shown as loading control.

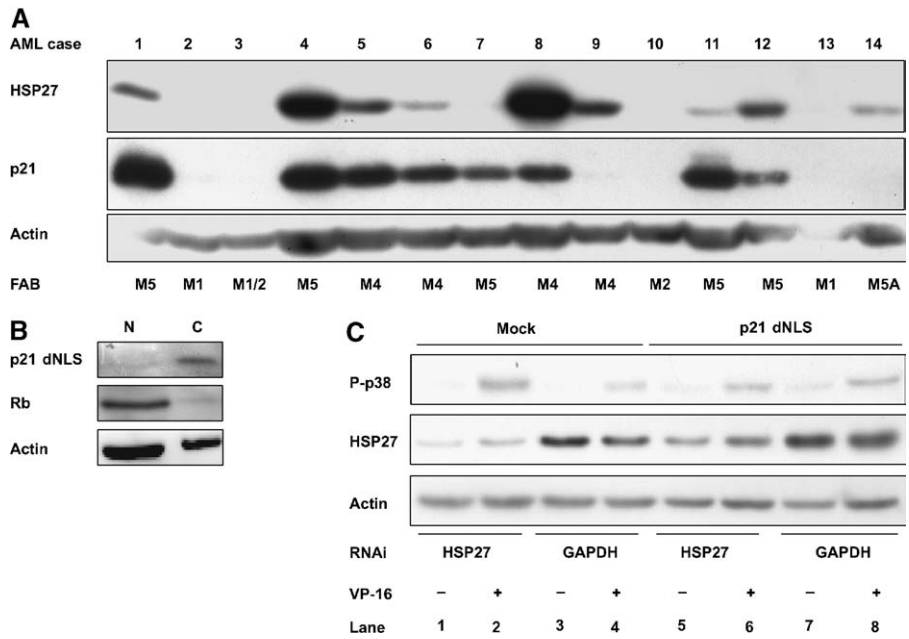

Figure 6. p21 is co-expressed with HSP27 and cytoplasmic p21 (p21dNLS) inhibits enhanced phosphorylation of p38 after RNA interference for HSP27 in response to VP-16. **(A):** Acute myeloid leukemic cells were isolated and cultured as described in materials and methods. HSP27 (same as Fig. 4A) and p21 expression was investigated using Western blot analysis. **(B):** TF-1 cells were virally transduced with pMSCV-p21dNLS and nuclear and cytoplasmic fractions were isolated as described in materials and methods. Western blot analysis was performed on p21 to demonstrate cytoplasmic localization. Rb is shown to indicate proper fractionation. Actin is shown as loading control. **(C):** 4 days after transfection with either HSP27 RNAi or control GAPDH RNAi, TF-1 mock or TF-1 p21dNLS cells were treated with VP-16 (20 μg/mL) for 3 hours and subjected to Western blot analysis for phosphorylated p38 and HSP27. Actin is shown as loading control. A representative example is shown.

3 hours. Cell lysates were prepared and subjected to Western blot analysis for the activation of p38. Figure 6C demonstrates that HSP27 siRNAs reduced HSP27 levels in both mock and p21 infected TF-1 cells and that p38 phosphorylation is only increased in response to VP-16 treatment in mock transfected HSP27 siRNA TF-1 cells. (Compare lanes 2 and 4 with 6 and 8). These data suggest that cytoplasmic p21 can compensate for a lack of HSP27 and block VP-16-induced apoptosis via the p38 pathway.

Discussion

In this study we investigated the potential mechanisms by which HSP27 modulates the apoptotic process in AML blast cells, since co-expression of HSP27 with additional proteins is linked to an unfavorable prognosis [28,29]. Our results demonstrate lineage-restricted expression of HSP27 in normal hematopoietic cells and their malignant counterparts. Normal $CD34^+$ cells and monocytes express high levels of HSP27, whereas in AML cells, HSP27 expression is predominantly restricted to monocytic leukemia. HSP27 appeared to be more expressed in more primitive leukemic progenitor cells, i.e., the $CD34^+/CD36^-$ sorted cell fraction, as has been demonstrated for other anti-apoptotic proteins [42], underscoring a functional relevance of HSP27 in the malignant stem cell compartment.

However, functional studies with VP-16 did not show a uniform response pattern in monocytic leukemia with regard to VP-16-mediated apoptosis, although HSP27 was phosphorylated in all AML cases. An explanation for this apparent discrepancy is the expression of additional anti-apoptotic proteins including $p21^{Waf1/Cip1}$, which is cytoplasmic localized in monocytic leukemia and interferes with the VP-16-mediated p38 and c-Jun phosphorylation [7]. Overexpression of a cytoplasmic form of p21 in the TF-1 cell line reversed the VP-16-induced effect seen on the activation of p38 after HSP27 RNAi, indicating that p21 can substitute for HSP27 as an anti-apoptotic factor. Functional studies demonstrate that HSP27 is an important anti-apoptotic factor in the VP-16-induced apoptotic process. Both VP-16 and CD95/Fas can induce apoptosis through the extrinsic signal transduction pathway [7,12]. Induction of apoptosis through this pathway involves translocation of the nuclear protein DAXX to the membrane, where it can bind to the cytosolic end of the CD95/Fas receptor [43]. Following binding to the CD95/Fas receptor, DAXX binds to ASK1 [12]. ASK1 activation in turn leads to activation of the stress-activated protein kinase (SAPK, also known as JNK; c-Jun amino-terminal kinase) and p38 subgroups of MAP kinases [23,36], which have also been described to occur upon VP-16 treatment [7].

The results presented here indicate that HSP27 RNA interference resulted in enhanced phosphorylation of p38 and c-Jun (a target of JNK) in response to VP-16, but not to

CD95/Fas stimulation. In addition, HSP27 RNA interference resulted in an increased release of cytochrome c to the cytoplasm, which has been shown to require ASK1/JNK activation [24,35,44]. The kinetics of these results suggest that cytochrome c release is downstream of the DAXX/ASK1/JNK pathway. c-Jun and p38 phosphorylation are observed as early as 1 to 3 hours after VP-16 treatment, followed at 5 hours by a release of cytochrome c to the cytoplasm. The sequential activation rather than parallel activation is confirmed by experimental evidence that ASK1-mediated apoptosis can be blocked by the mitochondrial pore opening inhibitor, cyclosporin A, whereas activation of p38 MAPK/JNK is left unaltered [45].

Complex formation between HSP27 and DAXX, as confirmed here, further underscores a role for HSP27 at the level of DAXX [16,17]. Since HSP27-DAXX complex formation is ASK1 dependent, it is likely that HSP27 only interacts with DAXX when it acquires a pro-apoptotic function. This is consistent with a role for HSP27 in blocking DAXX translocation to the cytoplasm [17], where it exerts a pro-apoptotic function rather than a transcriptional regulatory function [46].

Bcl-2 family members are known to play a role in mitochondrial integrity and cytochrome c release [47]. DAXX/ASK1 activation has been shown to inactivate the anti-apoptotic protein *bcl*-2 [48] and activate pro-apoptotic proteins such as Bax, Bim, and Bid [49] (and references therein). HSP27 expression and hence DAXX/ASK1 inactivation might also influence the balance between pro- vs anti-apoptotic signals at this level. Since HSP27 and *bcl*-2 protect against apoptosis with different efficiencies and different pathways [50], more work needs to be done to clarify the similarities and differences in the affected signal transduction pathways.

A different response pattern was observed with CD95/Fas. HSP27 levels were strongly upregulated by CD95/Fas in the studied cases. This elevated level of HSP27 subsequently prevented the phosphorylation of p38 and c-Jun. Despite the absence of p38 and c-Jun activation, CD95/Fas did induce apoptosis in TF-1 cells to some extent, although not comparable to VP-16-induced levels (Table 1).

It is conceivable that CD95/Fas-induced apoptosis in TF-1 cells does not involve the DAXX pathway, but leads to FADD activation and cleavage of pro-caspase-3 [12]. It has been demonstrated that overexpression of HSP27 inhibits DAXX-mediated apoptosis, but not CD95/Fas-induced FADD/caspase-dependent apoptosis [17]. Indeed, cleavage of pro-caspase-3 is observed after CD95/Fas treatment of the TF-1 cells, indicating that CD95/Fas is able to induce apoptosis without p38 and JNK activation.

Overall, these experiments indicate that HSP27 protects leukemic cells from VP-16-induced apoptosis through binding to DAXX and modulation of the activation of the p38/JNK pathways, whereas CD95/Fas-induced apoptosis is not affected, either through the upregulation of HSP27 or because of DAXX-independent signaling through a FADD/caspase-dependent pathway. This report underscores that in AML blasts, multiple anti-apoptotic routes are activated [6,7,28,29,51] and implicates that strategies should be designed in targeting multiple signal transduction pathways in order to treat this highly malignant disorder.

Recently it has been demonstrated that HSP27 confers resistance to the proteasome inhibitor Bortezomib/PS-341 [52] and blockade of HSP27 restores sensitivity towards Bortezomib/PS-341. A similar approach might be relevant in the treatment of monocytic leukemia.

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