Myeloproliferative Disorders

Expression and inducibility of cytoprotective heat shock proteins in the bone marrow of patients with myelodysplastic syndrome: correlation with disease progression

We determined the intracellular expression and inducibility of heat shock proteins (Hsps) 72, 73 and 27 in the bone marrow of patients with myelodysplastic syndrome (MDS) and controls. Hsps were overexpressed in MDS marrow especially in advanced disease, providing resistance to induction of apoptosis. These data suggest that Hsps could be implicated in the progression of MDS to acute myeloid leukemia.

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Myelodysplastic syndrome (MDS) is a neoplastic disorder of the bone marrow (BM) in which a gradually emerging malignant clone leads to disease progression. Abrogation of control of apoptosis leads to subsequent evolution to acute myeloid leukemia.[1] Heat shock proteins (Hsps) are fundamental for cell life and death decisions and their abnormal expression is linked to oncogenesis.[2,3] The constitutively expressed Hsp73 and the stress-inducible Hsp72 and Hsp27 are three essential Hsps that inhibit apoptosis and confer strong cytoprotection.[4] Hsp72 and Hsp27 are overexpressed in several human cancers and hematologic malignancies.[5-7]

We determined the expression of Hsp27, Hsp72 and Hsp73 in MDS BM and CD34$^+$ cells, and tested their inducibility by heat shock or pro-apoptotic cytokine stimulation *in vitro*, to investigate whether a link exists between Hsps expression and MDS subtype, blast percentage and apoptosis. BM samples from 34 MDS patients at diagnosis, before treatment and not receiving red cell transfusions, were studied. The patients suffered from (i) refractory anemia (RA, n=11), (ii) RA with ringed sideroblasts (RARS, n=5), (iii) RA with excess of blasts (RAEB, n=13), (iv) RAEB in transformation (RAEB-t, n=3) and (v) chronic myelomonocytic leukemia (CMML, n=2).

Fifteen age- and sex- matched patients with normal BM served as controls. Informed consent was obtained from all study subjects and the study was approved by the ethical and scientific committees of Patras University Hospital. Hsp expression and apoptosis were determined in freshly isolated BM mononuclear cells (BMMC) incubated overnight under the following conditions: (i) unstimulated (constitutive expression), (ii) after heat shock (30 min at 43°C + 4h rest at 37°C), (iii) after incubation for 24h with tumor necrosis factor (TNF)-α (10 ng/mL)+ interferon-γ (IFN-γ) (1000 IU/mL). Preliminary experiments showed that overnight incubation does not affect Hsps expression, and determined the optimal conditions for apoptosis-induction retaining >85% cell viability. Apoptosis was assessed by the annexin-V/propidium iodide assay.

Intracellular Hsp expression was determined by flow cytometry after intracellular staining with indirect immunofluorescence±initial cell-surface labeling for the CD34 antigen, when appropriate. Data sets were compared by two-tailed independent Student's t-tests or paired t-test for paired observations. A p value <0.05 was considered statistically significant. Linear relationships between samples were assessed by Pearson's correlation-

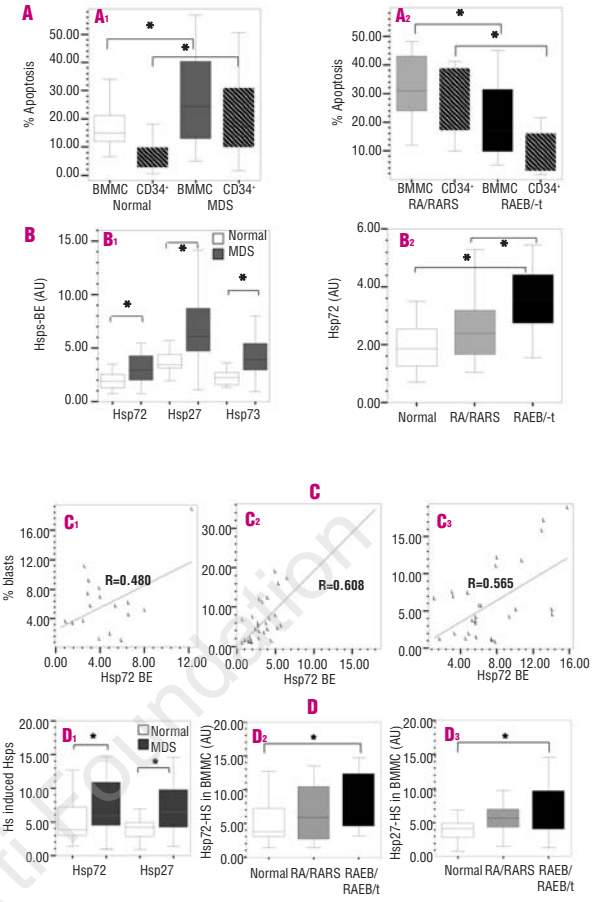

Figure 1. A. Apoptosis in BMMC and CD34$^+$ cells from MDS patients and controls, assessed by annexin-V. **A1.** Apoptotic levels in MDS-derived BMMC and CD34$^+$ progenitors, compared to controls. **A2.** Apoptosis in BMMC and CD34$^+$ cells from patients with early disease (RA/RARS) compared to those with advanced MDS (RAEB/RAEB-t). **B.** Basal intracellular expression of Hsps in BMMC from patients with MDS in comparison to controls. **B1.** Hsp72, sp27 and Hsp73 basal expression (BE) in BMMC from MDS patients compared to controls. **B2.** Hsp72 BE varied among MDS subtypes; RAEB and RAEB-t patients expressed significantly higher levels of Hsp72 in BMMC than did RA and RARS patients or controls. **C.** Correlation between Hsps expression and blast percentage. **D.** Heat shock (HS) induction of Hsp72 and Hsp27 in BMMC of patients and controls. **D1.** HS induction of Hsp72 and Hsp27 in BMMC from MDS patients and controls. **D2** and **D3.** HS induction of Hsps in RAEB/RAEB-t patients, RA/RARS patients and controls. (*) denotes statistical significance; AU: arbitrary units.

test. The expression of the Hsp studied did not correlate with the degree of anemia, lactate dehydrogenase concentration or ferritin levels and cytogenetics in MDS or age in MDS and controls (*not shown*). Apoptosis was significantly increased in MDS BMMC (p=0.01) and CD34$^+$ cells (p=0.001) compared to controls (Figure 1A1) and in patients with early (RA and RARS) vs advanced disease (RAEB and RAEB-t) (p=0.049 for BMMC, p=0.007 for CD34$^+$ cells) (Figure 1A2).

All Hsps studied were overexpressed in MDS BMMC compared to controls (p=0.001 for Hsp73, p<0.001 for Hsp27, p=0.04 for Hsp72) (Figure 1B1), but not in MDS CD34$^+$ cells (*not shown*). Hsp72 expression was found to correlate with disease progression (Figure 1B2). The expression of all Hsps correlated significantly with the percentage of blasts (Figure 1C1-3). Heat shock efficiently induced Hsp72 and Hsp27 in MDS and control BM, but resulted in significantly higher final levels in MDS BM

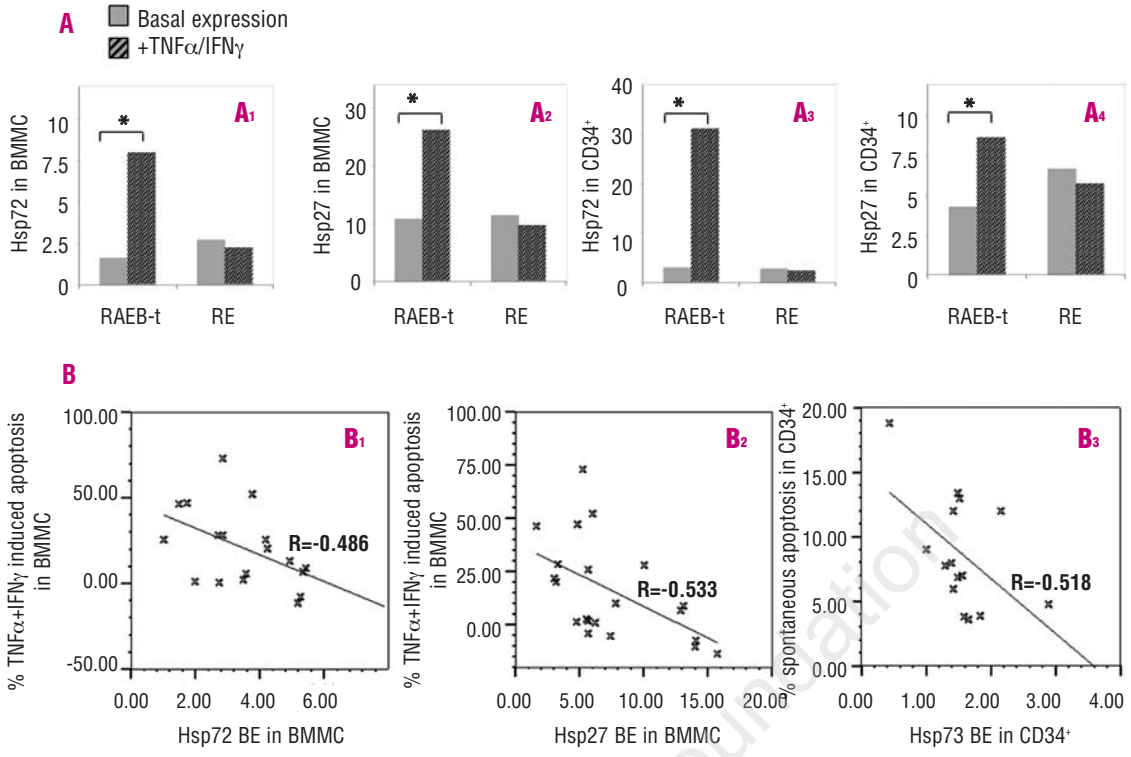

Figure 2. A. Individual response to combined TNF-α+IFN-γ treatment in one patient with RAEB-t subtype and an abnormal karyotype (trisomy 8), at diagnosis and after AML transformation and remission accomplishment. Striking increases in both Hsp72 and Hsp27 were noted in BMMC (A_1, A_2) and CD34$^+$ cells (A_3, A_4) under the TNF-α+IFN-γ pressure when the patient's marrow was tested at diagnosis (RAEB-t stage). Rapid evolution to AML followed within a month and the patient's marrow was re-examined after administration of chemotherapy and achievement of remission (RE), exerting a completely different response (mild decrease). **B.** Correlation between basal or induced expression of Hsp and spontaneous or induced apoptosis: an inverse correlation was demonstrated between apoptosis induction by TNF-α+IFN-γ-and basal expression levels of Hsp27 (B_1) and Hsp72 (B_2) in BMMC of MDS patients. A negative correlation was also demonstrated between spontaneous apoptosis and basal Hsp73 expression in CD34$^+$ cells of MDS patients, at the same time (B_3). Note that induced apoptosis or induced Hsp expression is represented as the percentage of increase or decrease from basal levels after application of proapoptotic treatment. (*) denotes statistical significance; BE: basal expression.

(p=0.022, p=0.002, respectively) and in advanced vs early disease (p=0.02 for Hsp72, p=0.012 for Hsp27) (Figure 1D). TNF-α+IFN-γ treatment reduced Hsp72 expression in control BMMC; no significant effect was observed in MDS BMMC and CD34$^+$ cells (*not shown*). Interestingly, in one RAEB-t patient, a significant increase in Hsps was noted in TNF-α+IFN-γ-treated BMMC and CD34$^+$ cells. This was followed by rapid progression to AML and was not reproduced after remission (Figure 2A$_{1-4}$). Constitutive Hsp72 and Hsp27 levels were inversely correlated with TNF-α+IFN-γ-induced apoptosis in MDS BMMC (Figure 2B$_1$ and $_2$), whereas Hsp73 expression correlated negatively with spontaneous apoptosis in MDS CD34$^+$ progenitors (Figure 2B$_3$). In this study we demonstrated increased constitutive and heat shock-induced expression of Hsp27, Hsp72 and Hsp73 in MDS BM. Despite the initial view, increased Hsp expression is not always beneficial for cell survival, as too much Hsp may even be harmful to cells.[8,9] Therefore, excessive chaperone levels in early disease may support the process of transformation through mutations gathering silently in clonal cells.

The positive correlation of Hsps expression with blast count and disease stage implies that the overexpressing mature cells along with their abnormal progenitors are selected during disease progression, since they are more resistant to the stressing pro-apoptotic MDS marrow and have a survival advantage over normal cells. This could result in the favored selection and expansion of clonal cells, further depression of hematopoiesis and disease progression. The prominent induction of Hsps in the RAEB-t patient with rapid transformation suggests that TNF-α, abundant in myelodysplastic marrow may, if delivered below a threshold, elicit cytoprotection instead of apoptosis in a resistant cell population overexpressing Hsps.[3,10]

In conclusion, overexpression of Hsps in MDS marrow may render a certain cell population resistant to the induction of apoptosis as the disease evolves. On the background of non-susceptibility to apoptosis, a secondary event blocking differentiation could lead to leukemic transformation.

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