Heat shock proteins and Bcl-2 expression and function in relation to the differential hyperthermic sensitivity between leukemic and normal hematopoietic cells

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Abstract A major problem in autologous stem cell transplantation is the occurrence of relapse by residual neoplastic cells from the graft. The selective toxicity of hyperthermia toward malignant hematopoietic progenitors compared with normal bone marrow cells has been utilized in purging protocols. The underlying mechanism for this selective toxicity has remained unclear. By using normal and leukemic cell line models, we searched for molecular mechanisms underlying this selective toxicity. We found that the differential heat sensitivity could not be explained by differences in the expression or inducibility of Hsp and also not by the overall chaperone capacity of the cells. Despite an apparent similarity in initial heat-induced damage, the leukemic cells underwent heat-induced apoptosis more readily than normal hematopoietic cells. The differences in apoptosis initiation were found at or upstream of cytochrome *c* release from the mitochondria. Sensitivity to staurosporine-induced apoptosis was similar in all cell lines tested, indicating that the apoptotic pathways were equally functional. The higher sensitivity to heat-induced apoptosis correlated with the level of Bcl-2 protein expression. Moreover, stable overexpression of Bcl-2 protected the most heat sensitive leukemic cells against heat-induced apoptosis. Our data indicate that leukemic cells have a specifically lower threshold for heat damage to initiate and execute apoptosis, which is due to an imbalance in the expression of the Bcl-2 family proteins in favor of the proapoptotic family members.

INTRODUCTION

Relatively high heat sensitivity of leukemic malignant cells compared with normal hematopoietic cells has been reported for human and murine models (Moriyama et al 1992; Larocca et al 1997; Wierenga et al 2003). This difference suggests a common underlying mechanism leading to the differential heat sensitivity between normal and leukemic hematopoietic progenitors. Until now, the mechanism of this selective toxicity remains unclear. Several studies have shown correlations between the proliferative activity and heat sensitivity of subsets within the normal hematopoietic compartment (Moriyama et al 1992; Wierenga and Konings 1993; Wierenga et al 1995, 2002) and revealed that quiescent primitive stem cells are more resistant to heat stress than the progenitors with higher cell cycle activity. However, this difference in proliferative status could not explain the difference in sensitivity to hyperthermia as seen between normal and leukemic primitive stem cells (Wierenga et al 2003).

Exposure of cells to elevated temperatures leads to changes in nearly all cellular compartments. Protein damage and accumulation of misfolded proteins in cells are considered to be the main cause of heat-induced cell death (Kampinga 1993). The main cellular defense mechanism against thermal stress is mediated by Hsps (Lan-

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Received 19 March 2007; Revised 1 May 2007; Accepted 4 May 2007.

dry et al 1986; Li et al 1995; Nollen et al 1999). By acting as molecular chaperones, Hsps bind to heat-unfolded proteins, preventing their irreversible aggregation and facilitating their poststress handling (refolding or degradation) (Terlecky et al 1992; Frydman and Hartl 1996; Fisher et al 1997). Indeed, close interrelationships between Hsp level, protein damage resistance, and heat resistance have been reported (Kampinga 1993; Nollen et al 1999). So, it can be hypothesized that reduced levels or impaired functionality of Hsps in leukemic cells are responsible for their relatively higher sensitivity to heat stress.

An alternative hypothesis for the differential heat sensitivity between normal and leukemic cells might lie in the threshold for activating cell death pathways upon heat shock. The balance between pro- and antiapoptotic proteins could have been systematically altered in leukemic cells to such an extent that even if the death trigger (ie, thermal damage) is the same, the leukemic cells more readily execute apoptosis. It has been suggested that, again, Hsps could play an inhibiting role in apoptosis execution, in particular after heat shock (Mosser et al 1997; Beere et al 2000; Buzzard et al 1998; Jaattela et al 1998; Meriin et al 1999; Saleh et al 2000; Ravagnan et al 2001). Heat-induced activation and phosphorylation of the Jun N-terminal kinase (JNK) pathway can result in permeabilization of the mitochondrial membrane (Gabai et al 2002). This then initiates the release of apoptosis-inducing proteins like AIF and cytochrome *c* (cyt *c*; Reed 1997), which in turn can trigger downstream cell death effectors. Hsps have been suggested to attenuate JNK phosphorylation after heat shock (Meriin et al 1999) and also act at the level of mitochondrial stability (Samali et al 2001). More clearly, the mitochondria-mediated apoptosis pathway is dependent on a large group of other apoptosis-controlling proteins, the most prominent ones being proteins of the Bcl-2 family (Desagher and Martinou 2000). The ratio of anti- to proapoptotic proteins such as Bcl-2/Bax is an important determinant for triggering this intrinsic apoptotic pathway (Kornblau et al 2000; Del Poeta et al 2003). Interestingly, in many leukemic cells, changes in the expression of proteins of the Bcl-2 family proteins have been noted (Kornblau et al 1995), and it can be hypothesized that this could be related to the systematic differences in thermal sensitivity between normal and leukemic hematopoietic cells.

In this study, we investigated the protective role of Hsp in human and murine cell lines. In contrast to our expectations, Hsp levels, Hsp inducibility, and overall cellular chaperone capacity could not be related to the observed differences in thermal sensitivity. Rather, our data suggest that leukemic cells have a lower threshold specifically for heat damage to initiate and execute apoptosis because

of an imbalance in the expression of the Bcl-2 family proteins in favor of the proapoptotic family members.

MATERIALS AND METHODS

Hematopoietic cell lines

Murine leukemic myeloid cell line L1210 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 10^{-4} M β -mercaptoethanol. The cells were cultured in suspension by rotation (120 rpm) at 37°C.

Normal murine myeloid 32D cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 2% Pokeweed mitogen-stimulated murine spleen cell conditioned medium (Stemcell Technologies, Canada) at 37°C in a 5% CO₂ humidified atmosphere. L5178Y murine lymphoma cells, Jurkat human T-cells, and M1 human myeloblast cells were cultured in RPMI-1640 medium supplemented with 10% FCS; TF1 human erythroid cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF).

Hyperthermia

Cell suspensions were placed in culture tubes at a concentration of $2-4 \times 10^6$ cells/mL in complete medium and heat treated for 0–60 minutes at 43°C. Heat treatments were performed in a precision water bath ($\pm 0.1^\circ\text{C}$) and chilled on ice to terminate heating.

Colony-forming assays

For measuring the clonogenic capacity of the established cell lines, cells were treated in culture medium in suspension. After treatment, appropriately diluted samples were seeded on 35-mm plates containing culture medium supplemented with 1.2% methylcellulose and 30% FCS. Colonies containing >50 cells were scored after 1 week (murine cells) or 2 weeks (human cells).

Cell transfection and cellular chaperone measurements

Cells were transfected with pRSVLL/V encoding luciferase under control of a Rous sarcoma virus long terminal repeat (LTR) promoter (Li et al 1995). The transfections were performed by electroporation (Gene pulser II, BIORAD). Cells (5×10^6 in 0.5 mL of Dulbecco modified Eagle medium) were placed into 0.1-mm gap cuvettes and transfected with 30 μg of pRSVLL/V. The electroporation settings were 280 V and 980 μF capacitance. The cells were resuspended 24 hours after electroporation in medium containing 20 mg/mL cycloheximide and 20

mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7. The cells were divided into culture tubes and heat treated at 43°C followed by a recovery period at 37°C. At several time points, triplicate samples were taken for luciferase measurement. Hereto, cells were cooled and lysed with 500 μ L of lysis buffer (25 mM H_3PO_4 /Tris, pH 7.8, 10 mM MgCl_2 , 1% [v/v] Triton X-100, 15% [v/v] glycerol, 1 mM ethylenediaminetetraacetic acid [EDTA], and 0.5% [v/v] β -mercaptoethanol). The lysates were kept frozen until measurement of luciferase activity with a Berthold Lumat 950 as described before (Michels et al 1995).

Stable Bcl-2-overexpressing L1210 cells were generated with pBcl-2 DNA under control of a CMV promoter (generous gift from Solange Desagher). The clones 32.12 and 32.15 were obtained by picking a single clone and subcloning after culturing for several weeks in medium containing geneticine.

Western blotting

Cells (2×10^6) were washed with cold phosphate-buffered saline (PBS) and resuspended with 100 μ L of cold PBS, diluted with an equal volume of sample buffer (2 $\times$), sonicated, boiled for 5 minutes, and frozen at -20°C . Samples were processed on 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gels and blotted on nitrocellulose membranes.

For detection of Hsps, we used rabbit anti-Hsp25 polyclonal antibody (Stressgen [now Nventa, San Diego, CA, USA], SPA-801), rabbit anti-Hsp40 polyclonal antibody (Stressgen, SPA-400), rabbit anti-Hsp60 monoclonal antibody (Stressgen, SPA-806), rabbit anti-Hsp70 (Hsp72) polyclonal antibody (Stressgen, SPA-812), rat anti-Hsc70 (Hsp73) monoclonal antibody (Stressgen, SPA-815), mouse anti-Hsp90 monoclonal antibody (Stressgen, SPA-830), and anti-Hsp110 (a generous gift from Professor Bensaude).

Apoptosis measurements

To investigate apoptotic signaling, activation of JNK was measured by Western blotting, with a mouse antibody recognizing the activated, phosphorylated JNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cyt *c* release was measured after lyses of 10×10^6 cells under conditions that kept mitochondria intact by incubating the cells for 10 minutes on ice in PBS containing 0.5% CHAPS, phenylmethanesulfonylfluoride (PMSF, 1 mM), and protease inhibitor cocktail (Roche, Woerden, The Netherlands). Hereafter, the cell lysates were centrifuged for 15 minutes at $14000 \times g$. The pellets were dissolved in 0.5% CHAPS, and total cell lysates, the pellet, and supernatant fractions were diluted 1:1 with SDS-

polyacrylamide gel electrophoresis (PAGE) sample buffer (2 $\times$), sonicated, and boiled for 5 minutes. The samples were stored at -20°C until analysis by gel electrophoresis and Western Blotting. Mouse anti-cyt *c* monoclonal antibody (R&D Systems, Minneapolis, MN, USA) was used for detection.

To measure activation of the apoptosis execution phase, whole cell lysates were analyzed by Western blotting for activated caspase-3 (rabbit anti-active caspase-3 monoclonal antibody, BD Pharmingen, Alphen aan de Rijn, The Netherlands), and for proteolytic cleavage of poly(ADP-ribose) polymerase (PARP), a rabbit anti-PARP polyclonal antibody was used (Santa Cruz Biotechnology).

DNA fragmentation was analyzed in total DNA from 6×10^6 cells. Cells were lysed for 20 minutes in 200 μ L of ice-cold lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100) and 50 μ g/mL of freshly added proteinase K. DNA was extracted by phenol-chloroform-isoamylalcohol (Invitrogen, Carlsbad, CA, USA) containing 0.1% hydroxyquinoline and precipitated by ethanol. The pellets were resuspended and incubated in 25 μ L of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 20 μ g of RNase and 25 μ g of proteinase K for 1 hour at 37°C. The optical density at 260 nm (OD_{260}) was measured with a spectrophotometer. A 10- μ g DNA sample in 0.2% loading buffer was analyzed in 2% agarose gel in TAE buffer (0.004 M Tris acetate, 0.001 M EDTA) with 5 μ L of ethidiumbromide.

Finally, the steady-state levels of a number of pro- and antiapoptotic proteins involved in control of mitochondrial cyt *c* leakage were measured by Western blotting employing rabbit anti-Bcl-2 polyclonal antibody (Santa Cruz Biotechnology) and anti-human and -mouse X-linked inhibitor of apoptosis protein (XIAP, R&D Systems).

RESULTS

Hyperthermic sensitivity of hematopoietic cells

Both murine and human leukemic stem cell subsets are more sensitive to heat stress than their normal counterparts (Fig 1A, redrawn after Wierenga et al 2000, 2003). This even holds true when comparing various human samples of normal bone marrow with samples of different leukemic FAB classifications (Wierenga et al 2003), indicating that these differences are systematic and dominant over other existing differences in genetic background. The magnitude of killing is highly relevant for transplantation purposes. Herein, a 4-log depletion of the leukemic burden in an autograft is required to be considered as an effective purging modality (see, eg, Keating 1991). This, of course should coincide with the least possible killing of normal progenitors, which indeed is at

Fig 1. Heat sensitivity of normal and leukemic hematopoietic cells. (A) The sensitivity of human and murine primary normal bone marrow (NBM) cells and human and murine acute myeloid leukemic (AML) cells to 60 minutes of hyperthermia at 43°C (* data derived from Wierenga et al 2002, 2003) was compared with that of established human leukemic cell lines (Jurkat, TF-1 and M1), a normal murine 32D cell line, and 2 murine leukemic cell lines (L1210 and L5178Y). (B) Clonogenic cell survival of Jurkat (closed squares), TF-1 (closed diamonds), M1 (closed triangles), 32D (open squares), L1210 (open triangles), and L5178Y cells (open diamonds) after 0–60 minutes of exposure to 43°C. Data points represent the mean of 5 independent experiments, and the error bars indicate the standard errors of the means.

least 100–1000-fold less than for leukemic cells (Fig 1A; Wierenga et al 2000, 2003).

The above-mentioned data were obtained by (long-term) survival assays employing crude normal bone marrow and bone marrow or peripheral blood samples from leukemic patients. To delineate the underlying molecular mechanism, it is not possible to use fresh samples because they contain only a very small fraction of the relevant progenitor cells. For such a population, adequate isolation techniques to yield sufficiently large cell numbers for molecular and biochemical analysis are not available. Therefore, we turned to established murine and human

hematopoietic cell lines derived from different lineage-specific populations and first asked whether systematic differences in heat sensitivity are also maintained for these in vitro cell lines. As can be seen in Figure 1A, the 3 human cell lines—Jurkat T cell lymphoma cells, TF-1 myeloid leukemic cells, and M1 myeloblast leukemic cells—were equally or slightly more heat sensitive than the primary acute myeloid leukemic (AML) cells; thus, all 3 were more sensitive than the normal bone marrow (NBM) cells for which we had no stable human cell line available. The human leukemic cell lines were more resistant than the 2 murine leukemic cell lines (L1210 myeloid leukemic cells and L5178Y lymphoma cells), again recapitulating the difference between human and murine primary AML cells (Fig 1A). Most importantly, the murine 32D cells, which have been used as a model for normal hematopoietic cells (Valtieri et al 1987; Blandino et al 1995) were as heat resistant as the murine primary NBM cells and more resistant than both the 2 murine leukemic cell line models and the primary murine AML cells. This differential heat sensitivity was already seen after 15 minutes at 43°C and reached a maximum at 60 minutes (Fig 1B). So, our data demonstrate that the differential heat sensitivity between normal and leukemic progenitors is even preserved for established cell lines. This strongly indicates that there must be conserved mechanistic differences, dominant over other existing differences in genetic background, that makes leukemic cells more prone to heat-induced cell lethality than normal bone marrow cells. These data also imply that it is justified to use the in vitro cell lines as adequate models to search for the biochemical mechanism behind these systematic differences in heat sensitivity. We like to emphasize at this point that despite the dramatic loss in survival in terms of clonogenic ability of the various progenitors after heat shock, such biochemical analyses are still feasible because the majority of these nonclonogenic cells remain fully intact up to 48 hours after treatment (data not shown).

Expression, inducibility, and chaperone functionality of Hsps in hematopoietic cell lines

In an attempt to understand the differences in heat response between hematopoietic cells, we first focused on Hsps. Hsps are known to be able to protect cells against thermal stress at the level of attenuating the cell death trigger (ie, protein damage). Therefore, it was hypothesized that the more heat resistant cells contain higher levels of these Hsps than the more heat sensitive progenitors. We first examined the basal expression of the major Hsps using Western blot analysis. All human and murine cells expressed high levels of the constitutive form of Hsc70 (Fig 2). The inducible Hsp70 was only expressed in human cells (Fig 2, lanes 1, 3, and 4). Also, higher

Fig 2. Hsp levels in hematopoietic cells. Western blot analysis of basal Hsp expression in untreated Jurkat (J), 32D (D), TF-1 (T), M1 (M), L1210 (L), and L5178 (LY) cells. Whole-cell extracts (10 μ g) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. For loading control, Hsc70 expression and γ -tubulin were measured in all the samples.

Hsp40 expression (the Hsp70 cochaperone) was observed in the human cell lines when compared with the murine cell lines. However, most intriguingly, the heat-resistant murine 32D cells had identical basal Hsc70, Hsp70, and Hsp40 expression profiles as the heat sensitive murine L1210 and L5178Y cells. So, although high Hsp70/40 levels were associated with the higher relative heat resistance of human vs rodent cells, the large difference in heat sensitivity between the normal (32D) and leukemic (L1210, L5178Y) murine cells could not be explained by

a differential Hsp expression. In addition, when comparing the different human leukemic cells, Hsp expression levels did not correlate with heat sensitivity.

It can be reasoned that the ability to up-regulate the heat shock response, not the basal expression, is responsible for the differential heat sensitivity of leukemic and normal cells. To test this idea, the induction kinetics of the major inducible heat shock proteins was tested in the most heat sensitive murine L1210 cells and in the heat-resistant murine 32D cells. The cells were heat treated for 30 minutes at 43°C, and after different recovery periods at 37°C, samples were taken for immunoblot analysis. Exposure of L1210 and 32D cells to 43°C resulted in a similar increase in Hsp70 levels 2–8 hours after the heat shock (Fig 3A). Comparable data were found for the induction kinetics of Hsp40 and with the use of isotonic heat treatments (not shown). The levels of Hsc70 remained largely constant up to 8 hours after the heat treatment (data not shown), which not only served as a loading control but also indicated that no significant cell loss occurred in either cell line despite major heat sensitivity differences in terms of clonogenicity. Hence, the large difference in heat sensitivity between the 2 cell lines cannot be explained by differences in expression of Hsps, inducibility of Hsps, or both.

As far as heat-induced cell killing is concerned, the protection by Hsps is thought to be related to Hsp-mediated protection against thermal protein denaturation (Kampin-

Fig 3. Hsp inducibility and functionality in L1210 and 32D cells. (A) Western blot analysis of heat-induced Hsp70 expression in L1210 and 32D cells. The expression of Hsp70 was analyzed in untreated (unt) and heat-treated cells for 30 minutes at 43°C after a recovery period of from 0 to 8 hours at 37°C. (B) Inactivation and reactivation of luciferase in L1210 (closed squares) and 32D (closed triangles) cells after heat shock at 43°C. The 32D and L1210 cells were transiently transfected with firefly luciferase. Heat inactivation of luciferase was measured directly after 0–30 minutes at 43°C (left panel). The luciferase activity of untreated cells was taken as 100%. Reactivation of luciferase (right panel) was measured after a heat shock of 30 minutes at 43°C after a recovery period of 0–4 hours at 37°C in the presence of cycloheximide to prevent new protein synthesis. Data points represent the means of 4 independent experiments, and error bars indicate the standard errors of the means.

ga 1993; Nollen et al 1999). This so-called chaperone activity is not just determined by the expression of major Hsps alone but depends on an intricate balance among major chaperones (Hsp27, Hsp60, Hsp70, Hsp90, Hsp110) and their cochaperones, such as, for example, Hsp10 for Hsp60 (Hohfeld and Hartl 1994) and Hsp40 (Michels et al 1997), Bcl-2-associated athonogene, Bag-1 (Takayama et al 1997; Nollen et al 2001a), Hip (Hohfeld et al 1995; Nollen et al 2001b), and CHIP (Ballinger et al 1999; Kampinga et al 2003) for Hsp70, as well as their localization (Nollen et al 2001b). As a putative measure for the overall cellular chaperone capacity, we employed an *in vivo* refolding assay with firefly luciferase as a model reporter protein (Michels et al 1995). This assay has shown significant correlations to thermoresistance (Kampinga 1993; Nollen et al 1999). It was tested to see whether the heat-resistant 32D cells had a higher functional chaperone capacity than the sensitive L1210 cells. L1210 and 32D cells were transiently transfected with firefly luciferase, heat treated, and assayed for luciferase activity either immediately after heat shock or after different recovery periods at 37°C (Fig 3B). Luciferase activity was found to decline at equal rates in both cell lines when the cells were exposed to 43°C (Fig 3B). Reactivation of luciferase activity was slight faster in 32D cells than in L1210 cells. However after 3 hours recovery, both cell lines were equally capable of refolding a similar fraction of heat-denatured luciferase back to an active state (Fig 3B). Thus, apart from Hsp expression and inducibility, the selective cytotoxicity of heat between murine normal and leukemic cells cannot be explained at the level of Hsp functionality in terms of intrinsic chaperone activity.

L1210 cells more rapidly initiate heat-induced apoptosis than 32D cells

Because no differences were found in the functionality of Hsp that could explain the differences in heat sensitivity between normal and leukemic cells, we questioned whether intrinsic apoptotic responses are triggered differentially between the cell lines irrespective of the same heat damage. Hereto, we investigated the extent of PARP cleavage as a late hallmark of classical apoptosis. Clearly, the hypersensitive murine leukemic L5178Y and L1210 cells showed rapid and dramatic PARP cleavage after the heat shock, whereas the heat-resistant murine 32D cells, as well as the human leukemic Jurkat and TF-1 cells, showed no PARP cleavage up to 8 hours after heat shock (Fig 4). Human M1 cells, the most sensitive of the heat-resistant human cell line panel showed minimal, but detectable, PARP cleavage. This differential response was specific for heat shock because all cell lines except the TF-1 cells were highly sensitive to apoptosis induced by staurosporine (Fig 4, STS). The combined data so far thus

Fig 4. Comparison of heat-induced poly(ADP-ribose) polymerase (PARP) cleavage in hematopoietic cell lines. Typical of 1 of 3 independent experiments showing the extent of PARP cleavage by Western blot analysis 0–4 hours after heating for 30 minutes at 43°C (full-length PARP 115 kDa, cleaved product 85 kDa). Staurosporine (sts)-induced PARP cleavage is depicted to show that all cell lines except TF1 can efficiently execute PARP cleavage.

imply that the same death trigger apparently results in a more efficient cell death execution in L5178Y and L1210 compared with 32D cells. One of the early sensors for cellular damage, especially after heat shock, is JNK activation (Meriin et al 1999). Stress severity is related to the accumulation of the activated and phosphorylated form of JNK. Although the basal level of activated JNK was somewhat higher in the L1210 cells, it was found that heat-induced accumulation of activated JNK was similar. Despite the death trigger by heat apparently being the same, L1210 showed not only more PARP cleavage (Fig 4) but also more DNA fragmentation (Fig 5B), as well as more activation of caspase-3 after the same heat treatment than the 32D cells (Fig 5C). These data suggest that the differential heat sensitivity between murine leukemic and normal progenitors is due to a higher activation of the execution phase in the apoptotic pathway specifically after heat.

Differences in apoptotic proneness are often related to alterations in the so-called initiating phase of apoptosis. Many triggers and activators of initiator caspases culminate in changes in mitochondrial permeability, leading to cytochrome *c* leakage and activation of the so-called apoptosome. When the extent of cytochrome *c* release after heat shock in L1210 and 32D cells was analyzed, we found cytochrome *c* in the cytosol fraction of L1210 cells, but not in that of the 32D cells (Fig 5D). Taken together, these data indicate that the differential heat sensitivity between murine normal and leukemic cells is due to a stronger response of the apoptosis-

A. pJNK **B. DNA fragmentation** **C. active caspase-3** **D. Cytochrome-c**

Fig 5. Apoptosis initiation and execution after heat shock in 32D and L1210 cells. L1210 and 32D cells were untreated (unt) or heat stressed for 30 minutes at 43°C after a recovery period of from 0 to 6 hours at 37°C. (A) Western blot analysis of phosphorylated Jun N-terminal kinase (JNK) in L1210 and 32D cells after 0–30 minutes of heat stress at 43°C. (B) DNA fragmentation in L1210 and 32D cells in untreated (lanes 3 and 11) cells and in cells treated with 2 μ M staurosporine for 2 hours (lanes 2 and 10) or heat treated for 30 minutes at 43°C after a recovery period of 0, 1, 2, 4, and 6 hours at 37°C (L1210, lanes 4–8; 32D, lanes 12–16). Lanes 1 and 9 are markers. (C) Western blot analysis of the 17-kDa cleaved protein product of pro-caspase-3. (D) Western blot analysis of cytochrome c in total cell lysate (t), pellet (p), and cytosol (s) fractions in untreated and heat-treated cells. Protein content for every lane was 10 μ g.

initiating program at a level downstream of JNK activation and upstream of cyt *c* release. So, differential control of mitochondrial stability after heat shock could be a more plausible explanation for the higher apoptosis initiation in L1210 cells.

Bcl-2 levels and hyperthermic sensitivity of hematopoietic cells

Mitochondria-mediated apoptosis is mainly dependent on the balance of antiapoptotic (Bcl-2, Bcl-xl, Mck-1) and proapoptotic proteins (Bax, BAK, BAD) of the Bcl-2 family. These proteins regulate the release of cyt *c* from the mitochondria into the cytosol by interfering with the permeability of the mitochondrial membranes. One of the main antiapoptotic proteins is Bcl-2 (Vaux et al 1988). In the cell panel studied here, Jurkat, TF-1, and M1 cells were found to express higher levels of Bcl-2 protein than the heat-sensitive murine L1210 and L5178Y cell lines (Fig 6A). More specifically, the 32D cells expressed higher Bcl-2 levels than L1210 and L5178Y cells. This observation raised the possibility that Bcl-2 expression could be 1 possible factor in preventing heat-induced cell death. To test

this idea, we stably overexpressed Bcl-2 in the heat-sensitive murine L1210 cells. Two stable clones, cl32.12 and cl32.15, expressing 3- and 2-fold higher levels of the Bcl-2 than the parental L1210 cells (Fig 6B) were subsequently heated, and their apoptotic sensitivity was assessed by PARP cleavage. Clearly, both Bcl-2-overexpressing clones showed reduced heat-induced PARP cleavage when compared with the parental cells, and the levels of Bcl-2 expression correlated with the extent of PARP cleavage (Fig 6C). Yet, the transgenic L1210 cells still showed more PARP cleavage than the 32D cells in which PARP cleavage was completely absent after heat shock (Fig 4). Nevertheless, the combined data clearly indicate that the differential heat sensitivity between the murine L5178Y and L1210 cells and the 32D cells is due to altered control of mitochondrial stability upon heat treatment, at least in part related to differential Bcl-2 expression.

DISCUSSION

Several studies in murine as well as in human systems have revealed that leukemic progenitors are more system-

Fig 6. Heat-induced apoptosis is reduced in Bcl-2-overexpressing L1210 cells. (A) Western blot analysis of Bcl-2 expression in Jurkat (J), TF1 (T), M1 (M), 32D (D), L1210 (L), and L5178Y (LY) cells. Protein samples were taken of whole-cell lysate. Expression of γ -tubulin was used as a loading control. (B) Bcl-2 expression levels in wild-type L1210 cells and in Bcl-2 stable transfected L1210 cells (cl32.12 and cl32.15). (C) Western blot analysis and quantification of full-length poly(ADP-ribose) polymerase (PARP) protein (115 kDa) and its cleaved product (85 kDa) in wild-type L1210 and Bcl-2-transfected L1210 cells. Protein samples of untreated cells (c) and cells heat stressed for 10, 20, and 30 minutes at 43°C after by a recovery period of 0–2 hours at 37°C were taken for immunoblot analysis. Percentage of cleaved PARP was calculated after scanning the cleaved and uncleaved PARP band. Expression of γ -tubulin was used as a loading control. Data represent 4 independent experiments.

atically sensitive to heat stress than normal progenitors (Symonds et al 1981; Robins et al 1983; Flentje et al 1993; Okamoto et al 1988; Da et al 1989; Murphy and Richman 1989; Gidali et al 1990, 1994; Iwasawa et al 1991; Moriyama et al 1986, 1990, 1991, 1992, 1993; Wierenga et al 1998, 2003). The underlying mechanism for this systematic hypersensitivity of both human and murine malignant hematopoietic progenitors to hyperthermia has remained unclear so far. In this study, we show that this systematic hypersensitivity is maintained even for in vi-

tro-established cell lines. This conserved difference in heat sensitivity suggests that a fundamental biochemical molecular difference between normal and leukemic cells exists that makes leukemic cells selectively more prone to heat-induced cell death. Because Hsps play a major role in the response to heat shock, we initially sought correlations between expression, inducibility, and functionality of these proteins and their differential heat sensitivities. However, our data were clearly negative in this respect. We found the main difference between the heat-resistant

murine 32D and heat-sensitive murine L1210 cells at the level of stability to heat-induced permeabilization of mitochondria, an effect that—at least in part—could be explained by differential expression of the antiapoptotic protein Bcl-2.

Given the prominent role of Hsps in heat resistance (Kampinga 1993; Nollen et al 1999), we first investigated the possibility that Hsps regulate heat-induced cell death by acting as either molecular chaperones or antiapoptotic proteins. For this, we tested the heat sensitivity of normal vs leukemic progenitors in human and murine cell lines. The expression levels of the inducible form of Hsp70 correlated positively with the heat sensitivity of cells, as previously described by other authors (Kampinga 1993; Nollen et al 1999). Human cells that expressed the inducible form of Hsp70 under normal growth condition were on average more resistant to heat stress than the murine cells that lack the inducible form of Hsp70, although absolute differences in heat sensitivity between the various human cells were not correlated with Hsp70 expression. The latter is consistent with early findings of Anderson et al. (1986) and suggests that, rather than Hsp expression levels per se, a balance between heat-sensitive proteins and Hsp might be more important. Indeed, if the cellular ability to deal with protein damage was sought as a parameter, in general, good correlation with thermal sensitivity was obtained (Kampinga 1993; Roti Roti and Turkel 1994; Stege et al 1995; Kampinga et al 1997; Nollen et al 1999). However, and most important for this study, the differential heat sensitivity between the normal and leukemic counterparts was not paralleled by differences in the capacity to deal with protein damage.

One could still argue that this assay is insufficient to cover the overall chaperone functionality and that other aspects of chaperone function (eg, cytoskeletal stabilization by Hsp27 [Landry and Huot 1995]) could have been overseen. However, our findings that JNK activation as an early marker of cell stress was similar in the heat-resistant murine 32D and heat-sensitive murine L1210 cells support the idea that the handling of protein damage is indeed not significantly different. To our knowledge, this is the first report that shows differential heat sensitivity between cells despite the damage trigger being the same.

The finding that Bcl-2 overexpression could (partially) rescue the sensitivity of L1210 to thermal stress, together with the 5-fold difference in Bcl-2 expression between L1210 and 32D cells, clearly points to an altered control of mitochondrial (heat) stability between both cell types. It is conceivable that the incomplete rescue is connected to the only moderately elevated Bcl-2 protein levels in the transfected L1210 clones compared with the 5-fold difference with 32D cells. The protective effect of Bcl-2 was only observed after heat stress and not after alternative cell death inducers. This raises the question of how

Bcl-2 can specifically protect against heat stress. One possibility could be the dual function of the Bcl-2-associated athonogene, Bag-1. This protein not only can enhance the antiapoptotic effect of Bcl-2 (Takayama et al 1995) but also is known to interact with and modulate the chaperone activity of Hsp70 (Takayama et al 1997; Nollen et al 2001). It has been speculated that Hsp70 could act as a bridging protein between Bag-1 and Bcl-2. One could hypothesize that after heat stress, Hsp70 is recruited to the heat-unfolded proteins, which impairs this bridging function and thus leads to a reduced fraction of functional antiapoptotic Bag-1/Bcl-2 complexes. Because we found no differences in Hsp70 (this report) and Bag-1 (unpublished observations) expression levels between the heat-resistant and -sensitive cell lines, higher levels of Bcl-2 might be required to specifically deal with the heat stress. Also, heat might directly and specifically activate the proapoptotic mitochondrial proteins Bax and Bak, as was recently suggested (Pagliari et al 2005). This leads to an increased release of cyt *c* from mitochondria and hence more apoptosis, unless sufficient Bcl-2 is present to counteract this activation. So, controlling mitochondrial stability after heat stress seems the mechanistic basis for the systematic difference in heat sensitivity between leukemic and normal hematopoietic cells.

ACKNOWLEDGMENTS

We thank S. Desagher for the Bcl-2 plasmid, B.J. Kroeze for anti-PARP and anti-XIAP antibodies, R. Kalicharan for electronic microscopy (EM) support, G. Mesander for fluorescence-activated cell sorting (FACS) support, and G. Kamps for technical expertise. This work was supported by grants from the Dutch Cancer Society (RUG 98-1715 and RUG 01-2360).

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