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Apoptosis-resistant phenotype in HL-60-derived cells HCW-2 is related to changes in expression of stress-induced proteins that impact on redox status and mitochondrial metabolism

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Abstract

The onset of resistance to drug-induced apoptosis of tumour cells is a major problem in cancer therapy. We studied a drug-selected clone of promyelocytic HL-60 cells, called HCW-2, which display a complex resistance to a wide variety of apoptosis-inducing agents and we found that these cells show a dramatic increase in the expression of heat shock proteins (Hsps) 70 and 27, while the parental cell line does not. It is known that stress proteins such as Hsps can confer resistance to a variety of damaging agents other than heat shock, such as TNF-α, monocyte-induced cytoxicity, and also play a role in resistance to chemotherapy. This elevated expression of Hsps is paralleled by an increased activity of mitochondrial metabolism and pentose phosphate pathway, this latter leading to high levels of glucose-6-phosphate dehydrogenase and, consequently, of glutathione. Thus, the apoptotic-deficient phenotype is likely because of the presence of high levels of stress response proteins and GSH, which may confer resistance to apoptotic agents, including chemotherapic drugs. Moreover, the fact that in HCW-2 cells Hsp70 are mainly localised in mitochondria may account for the increased performances of mitochondrial metabolism. These observations could have some implications for the therapy of cancer, and for the design of combined strategies that act on antioxidant defences of the neoplastic cell.
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Keywords: stress proteins; cancer; multidrug resistance; mitochondrial metabolism; apoptosis

Abbreviations: $\Delta\psi$, mitochondrial membrane potential; 2CdA, 2-chlorodeoxy-adenosine; AA, antimycin A; Dau, daunomycin; dRib, 2-deoxy-D-ribose; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; MDR, multidrug resistance; MMC, mitomycin C; MRPs, multidrug resistance proteins; MT, MitoTracker™ Red CMXRos; mtDNA, mitochondrial DNA; MTG, MitoTracker™ Green; MTH, MitoTracker™ CM-H_2X Ros; NAO, 10-N-nonyl acridine orange; QC-PCR, competitive-quantitative polymerase chain reaction; Rh123, rhodamine 123; ROS, reactive oxygen species; STS, staurosporine; TCA, tricarboxylic acid

Introduction

The efficacy of several antineoplastic agents is strictly due to their capacity to induce programmed cell death/apoptosis in tumour cells. As a consequence, the onset of resistance to apoptosis can result either in a metastatic phenotype or in therapy failure.[1–3] In the last years, many mechanisms of resistance to chemotherapy and radiotherapy have been investigated and partially elucidated, including those related to the overexpression of multidrug resistance proteins (MRPs) and products of the Bcl-2 family.[4–10] However, other mechanisms are likely present and may play an important role in the resistance to drug-induced apoptosis. Indeed, it can be hypothesised that cells become refractory to apoptosis because of an increased general resistance to a variety of stresses. In particular, modulation of heat shock proteins (Hsps) is reported to be protective against drug-induced apoptosis[11] and Hsp70 expression is reported to be an indicator of poor therapeutic outcome in breast cancer.[12] Moreover, genes activated by changes in energy metabolism could confer an increased resistance to different stressing agents (such as heat, UV light, oxygen radicals, starvation of glucose, among others) that cause apoptosis.[13] Taking into account that mitochondria: (a) are deeply involved in apoptosis;[14,15] (b) are a primary target of Hsp70;[16] and (c) are the main site of the oxidative metabolism, it is likely that these organelles are crucial targets of antiapoptotic strategies in cancer cells. Recently, it has been demonstrated that a mitochondrial stress (because of mitochondrial DNA (mtDNA)

depletion) can induce *in vitro* an invasive phenotype in otherwise noninvasive tumour cells.[17] In particular, this mitochondrial stress can increase cytoplasmic Ca^{2+} concentration, which in turn is responsible for the induction of genes such as Cathepsin L and TGF-β. This may account, at least in part, for the invasive phenotype.[17]

In our studies, we used an apoptosis-resistant clone of human promyelocytic HL-60 cells, called HCW-2. This clone has been selected from parental HL-60 cells by treatment with a cytotoxic dose of 8-Cl-cAMP, and it resulted resistant to cycloheximide and staurosporine (STS), but still underwent growth arrest.[18] It has been proposed that the deranged expression of Bcl-2 family in HCW-2 cells genes was the cause of such a resistance, and in particular that the lack of Bid could be responsible for apoptosis resistance,[19,20] suggesting an involvement of mitochondria in such a process. Accordingly, we used a variety of cytofluorimetric and molecular biology techniques to detect (a) differences in HCW-2 *versus* HL-60 cells at the level of mitochondrial morphology and mtDNA content; (b) the expression of a variety of pro- and antiapoptotic members of Bcl-2 family; (c) the expression of several stress-related proteins, such as Hsps, that can have mitochondria as a target; (d) parameters correlated to the metabolic activity of these organelles, such as lactate production, glutamine degradation and oxygen consumption. Finally, we checked the activity of pentose phosphate activity, which is a limiting step for the maintenance of glutathione-reducing capability.

Results

HCW-2 cells are highly resistant to different apoptotic stimuli and such resistance is not affected by a collapse in $\Delta\psi$

As reported by other authors,[18] HCW-2 cells showed a dramatic resistance to different apoptotic stimuli such as cycloheximide and STS. We extended this observation to other apoptotic agents which act with different mechanisms, including antitumour drugs (such as daunomycin (Dau), mitomycin C (MMC), 2-chlorodeoxy adenosine (2CdA)), highly reducing sugars (2-deoxy-D-ribose (dRib)) and γ-irradiation. The results are reported in Figure 1. None of the stimuli we used was capable of inducing a significant apoptosis in HCW-2 cells, while the parental HL-60 cells were highly sensitive to all the tested apoptotic agents (in all cases, $P<0.01$).

The resistance to apoptosis of HCW-2 cells is not related to multidrug resistance (MDR) pump activity, since both cell types had similar activity, as detected by the test of rhodamine 123 (Rh123) extrusion (Table 1). Rh123 is a cationic dye, which rapidly accumulates in cells and is actively extruded by means of the activity of MDR pump. HCW-2 cells accumulated much more Rh123 than HL-60 cells, depending on their size, but the rate of Rh123 efflux was similar in the two cell lines, indicating a similar activity of MDR pump. Moreover, the resistance to apoptosis is not correlated to the capability of maintaining a high $\Delta\psi$. Indeed, the addition of an uncoupling agent such as valinomycin (Val), which provokes a collapse

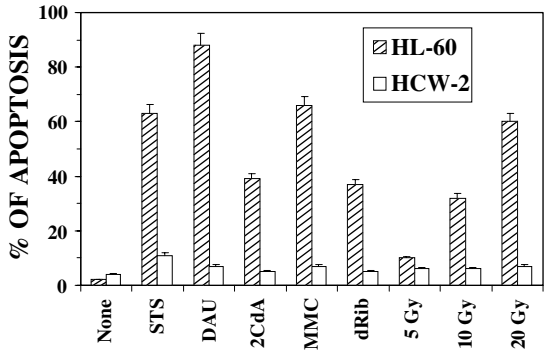

Figure 1 Marked resistance of HCW-2 cells to apoptosis induced by several agents. Bars indicate the percentage of apoptotic cells, as assessed by the cytofluorimetric quantitation of cells with the hypodiploic DNA content, and represent the mean value ($\pm$S.E.) of 4–6 experiments. In all cases but control (none, meaning none stimulus), $P<0.01$

Table 1 MDR pump activity

Time	t_0	15 min	30 min	60 min
HL-60	247 ± 25	231 ± 13	222 ± 18	208 ± 6
	Δ	-16 (-6.5%)	-25 (-10.2%)	-39 (-15.8%)
HCW-2	499 ± 32	477 ± 5	453 ± 12	426 ± 14
	Δ	-22 (-4.4%)	-46 (-9.2%)	-73 (-14.7%)

The activity of MDR pump was assessed as capacity of the cells to extrude the fluorescent dye Rh123. See details in Materials and Methods. Numbers represent the median channel value of fluorescence intensity of Rh123-stained samples to which spontaneous fluorescence has been subtracted, expressed as mean$\pm$S.E. from three separate experiments. Δ represents the difference between the median channel value measured at each time point (minutes) and the t0 value of Rh123 fluorescence. Numbers in bracket represent the decrease of fluorescence intensity expressed as percentage with respect to t0, considered as 100%

$\Delta\psi$, was not able to induce neither apoptosis *per se*, nor to change the sensibility to STS (data not shown).

HCW-2 cells possess a higher number of mitochondria but not a higher amount of mtDNA

We analysed at the single cell level the content of mitochondria, in terms of mitochondrial mass, using flow cytometry and two different fluorescent probes (i.e., NAO and MT green). Both probes indicated that HCW-2 had a higher mitochondrial mass (Figure 2, left part). This is also confirmed by morphological analysis (Figure 3). This finding was not unexpected, because HCW-2 cells display a higher volume, as can be noted by both cytometric analysis, electron microscopy (Figure 3), and protein content determination (protein content ratio between HCW-2 and HL-60 cells was 1.46). The higher mitochondrial content was confirmed even when expressed as a function of cell volume (NAO/FSC parameter ratio were 1.1 ± 0.05 of HCW-2 cells *versus* 0.68 ± 0.03 of HL-60 cells, $P=0.008$). Moreover, ultrastructural observations revealed that HCW-2 cells (Figure 3d, f) possess more mitochondria with elongated and irregular

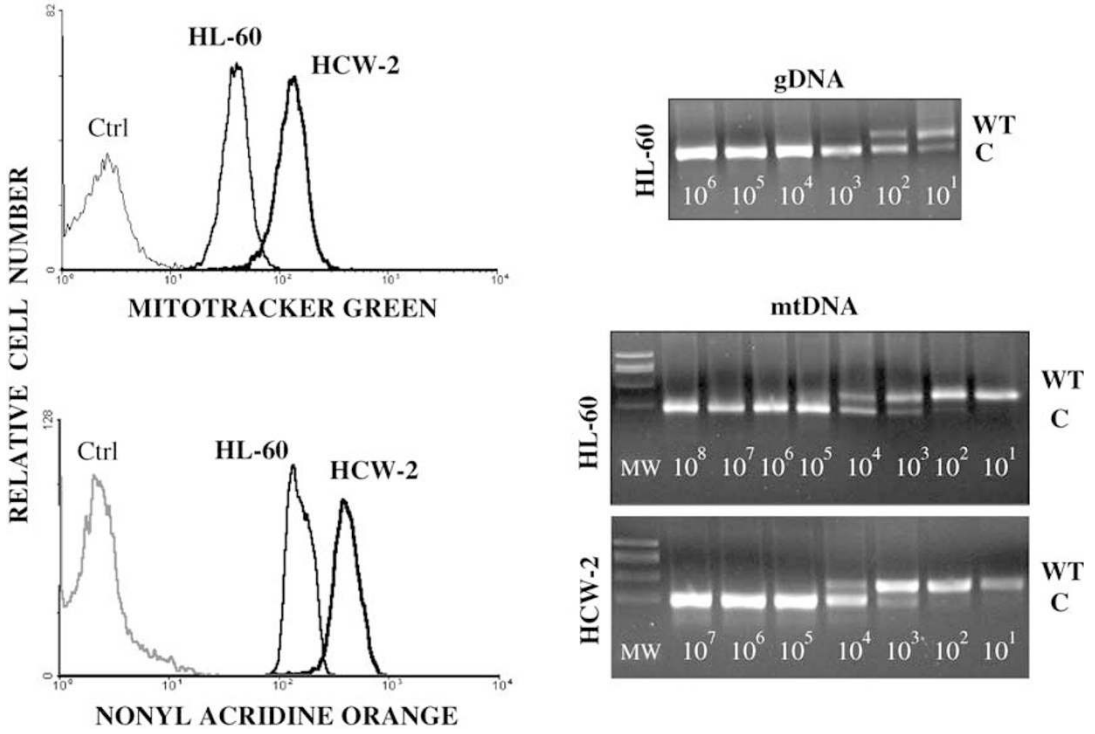

Figure 2 HCW-2 cells have a higher mitochondrial mass than HL-60, but a similar amount of mtDNA. Two different fluorescent probes were used to measure mitochondrial mass, that is, MTG or NAO (left part, upper and lower panels, respectively). With both dyes, HCW-2 had a higher fluorescence intensity than HL-60. Right part: representative example of a QC-PCR for the quantification of the mtDNA content (see text for details). gDNA: genomic DNA, referred to HL-60 cells; mtDNA: mitochondrial DNA; C: competitor; WT: wild type; MW: molecular weight marker. Numbers indicate the copies of competitor used in each lane. Note that the competition for mtDNA occurs at a concentration of competitor, which is about 100-fold higher than that for gDNA

shape, more pronounced and electrondense cristae and reduced matrix with respect to HL-60 cells (Figure 3a–c).

mtDNA content was then measured by using competitive-quantitative polymerase chain reaction (QC-PCR) (Figure 2, right part). Three separate experiments showed that HCW-2 had a mean ($\pm$S.E.) of 591 ± 54 copies of mtDNA per cell, while HL-60 had 661 ± 43 copies; such difference was not statistically significant. However, considering that HCW-2 cells have much more organelles than HL-60 cells, it can be reasonably assumed that mitochondria from HCW-2 cells have a lower number of mtDNA copies than mitochondria from HL-60 cells. It is at present not clear if this putative lower number of mtDNA copies can have some effects on mitochondrial metabolism of HCW-2 cells.

Complex changes in the expression of different members of the Bcl-2 family and Hsps

With a semiquantitative, cytofluorimetric approach, we measured the expression of some major members of Bcl-2 gene family present into mitochondria, such as Bcl-2, Bcl-x_L, Bag-1, having antiapoptotic activity, and Bax, Bad, Bid and Bak, having proapoptotic activity (Table 2). We then expressed the amount of these proteins as a function of mitochondrial mass content, quantified by NAO. In the case of Bax and Bid, which have a cytoplasmatic localisation, FSC parameter, related to cell volume, was used instead of NAO. We thus obtained an index of protein content, which is more informative than the simple value of fluorescence intensity. In agreement with previous reports,[18–20] when compared to parental HL-60 cells, HCW-2 cells display a paradoxical reduction on the levels of Bcl-2 protein, and similar levels of Bax protein. It has been reported that HCW-2 cells overexpress Bcl-x_L and lack Bid proteins, but we could not confirm this observation, either by using both flow cytometry (Table 2) or Western blot analysis (Figure 4). On the contrary, we found a slight (even if not significant) decrease in the expression of Bcl-x_L in HCW-2 cells, and similar levels of Bid. Moreover, Bad and Bak proteins were significantly reduced in HCW-2 cells (see Table 2). This suggests that these cells have a general down-regulation of the expression of Bcl-2 gene family.

Using a similar cytometric approach, we analysed the basal expression of proteins related to energy metabolism, such as the glucose transporter protein Glut-1, the mitochondrial ATP exchange protein ANT and cytochrome *c* (Table 3). HCW-2 and HL-60 cells had a similar content of Glut-1 and cytochrome *c*, while a significantly lower content of ANT was reported for HCW-2 cells.

The basal expression of some stress response genes was then analysed. We have found that the p85 regulatory subunit of PI3 kinase and the hypoxia-inducible factor HIF-1α are not different in the two cell types, while Hsp70 and Hsp27 protein levels are dramatically higher in HCW-2 cells, as shown in Table 3. In particular, this was strikingly evident for Hsp70 protein, as also shown in Figure 5a. We asked whether such

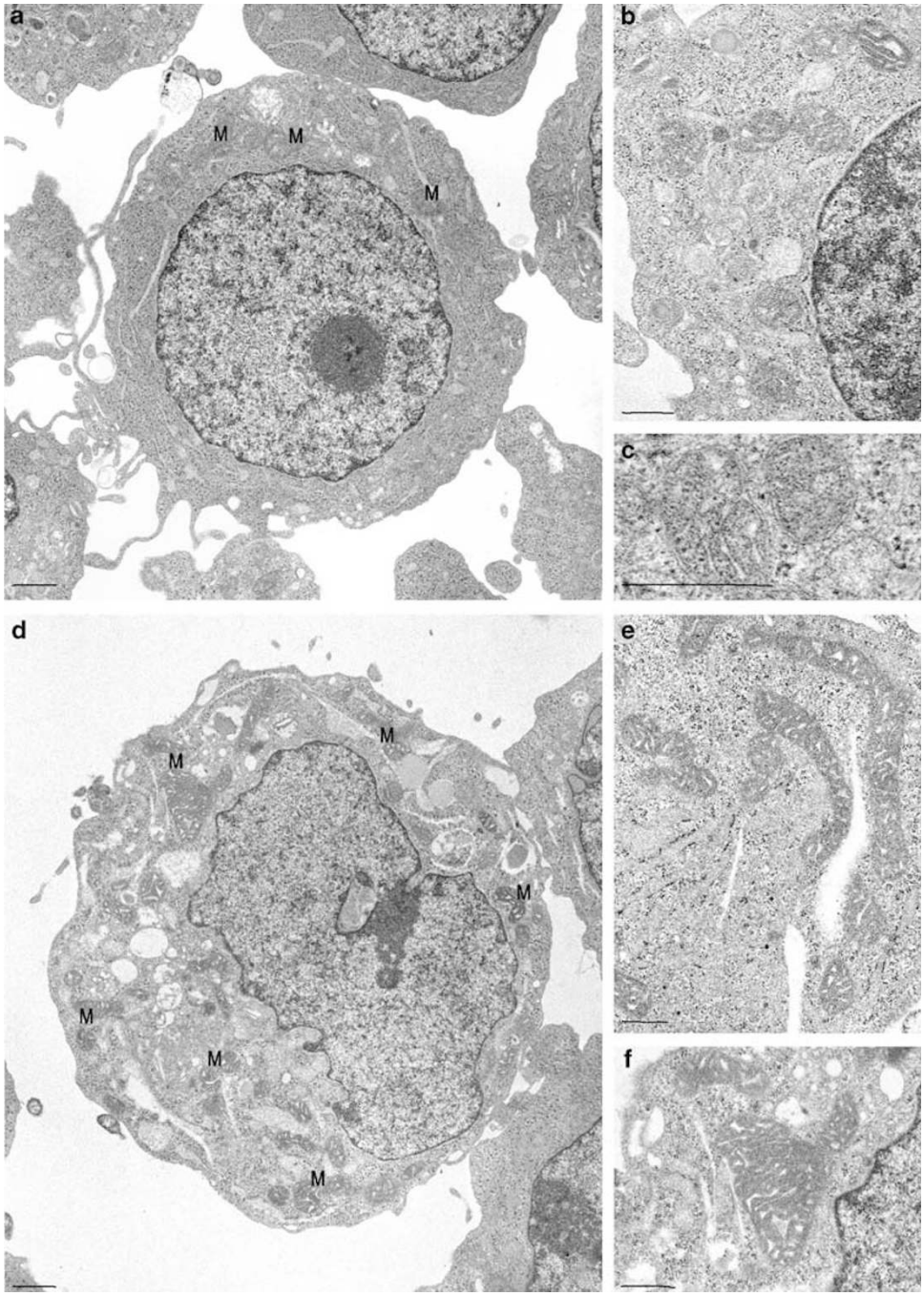

Figure 3 Morphological ultrastructure of HL-60 and HCW-2 cells. HL60 (a–c) and HCW-2 (d–f) cells were analysed by transmission electron microscopy. Compared to HL-60, HCW-2 cells exhibited numerous electrondense mitochondria (M) of heterogeneous size and shape. Bar=1 μm (a and d); 0.5 μm (b, c and e, f).

protein had a peculiar localisation in HCW-2 cells, and performed fractionation experiments in order to answer this question. As showed in Figure 5b, a consistent part of Hsp70 is located into mitochondria, while the nuclear fraction is completely Hsp70-free.

Biochemical measurements

Proliferative capability of HCW-2 cells is higher than that of HL-60 cells (data not shown). This means that a good ATP availability, and thus a high mitochondrial activity, are needed in HCW-2 cells. We measured the total amount of ATP at steady state of the two cell types (Table 4) and we found no difference between HL-60 and HCW-2 cells. This suggests that the availability of ATP in HCW-2 cells is likely because of a faster rate of ATP turnover rather than to a higher concentration of the molecule itself. Moreover, the addition of Val provokes a much higher decrease of ATP concentration in HL-60 than in HCW-2 cells. This suggests that HCW-2 cells have a higher coupled respiration. Interestingly, STS

Table 2 Flow cytometric quantification of Bcl-2 family member expression and its relation with mitochondria content or cell volume

	Δ Median		Δ Median/NAO	
	HL-60	**HCW-2**	**HL-60**	**HCW-2**
Bcl-2	140.3±24.8	70.0±19.0*	0.44±0.10	0.14±0.04*
Bcl-x_L	344.2±59.0	350.4±39.5	1.08±0.20	0.69±0.08
Bag-1	176.5±15.5	270.0±14.0	0.52±0.04	0.46±0.02
Bad	227.7±24.0	165.3±24.1	0.71±0.07	0.32±0.05*
Bax	253.0±29.0	320.0±21.5	0.79±0.09	0.63±0.04
Bak	322.3±34.7	267.7±23.5	1.01±0.11	0.52±0.05*
Bid	171.0±22.0	204.0±2.0	0.34±0.01	0.38±0.04

Upper part: antiapoptotic members; lower part: proapoptotic members. Data are expressed as mean±S.E. of 3–5 separate experiments. The first column reports the difference between the median fluorescence channel of the positive sample and its negative control. The second column reports the correlation with mitochondrial mass, expressed as linear median fluorescence of NAO, a probe stoichiometrically related to the mitochondrial mass, as in Figure 2, or with cell volume (analysed by linear median channel of FSC) for antigens with cytoplasmic localisation (Bax, Bid).*HL-60 *versus* HCW-2: $P<0.02$, by two-tailed unpaired Student's *t*-test.

Table 3 Flow cytometric quantification of proteins involved in energetic metabolism and stress response

		Δ Median/FSC	
		HL-60	**HCW-2**
Energetic metabolism	Glut-1	0.33±0.06	0.34±0.02
	ANT†	0.59±0.03	0.35±0.02*
	Cytochrome c†	0.38±0.05	0.39±0.03
Stress response	P85	0.05±0.01	0.06±0.02
	HIF-1α	0.12±0.04	0.08±0.01
	Hsp 70	0.21±0.15	0.74±0.14*
	Hsp 27	0.15±0.01	0.20±0.01*

Data are expressed as in the second column of Table 2. The linear median fluorescence of irrelevant antibody has been subtracted to that of the specific antibody to give a Δ, that has been therefore divided by the linear median channel of FSC parameters, except where indicated (†: divided by NAO linear median fluorescence). Data represent the mean±S.E. of three separate experiments.*HL-60 *versus* HCW-2 cells: $P<0.05$, by two-tailed unpaired Student's *t*-test.

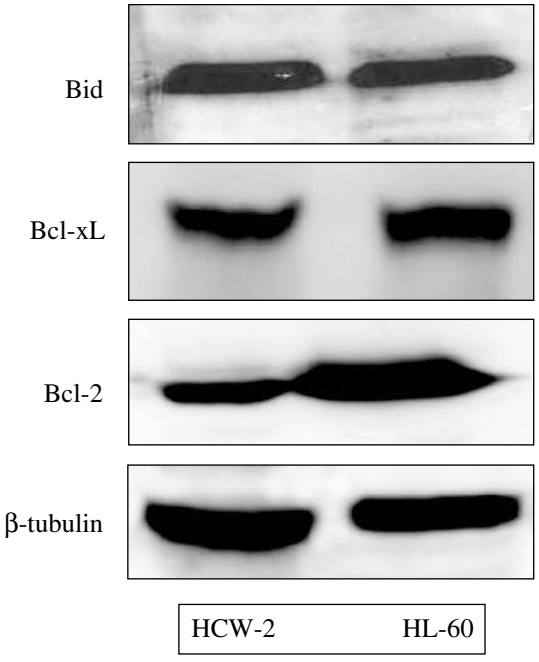

Figure 4 Western blot analysis of Bcl-2 family members. Bcl-2 was much more expressed in HL-60 cells, while Bcl-x_L and Bid were quite similar in the two cell types. β-tubulin was used as control of loading

treatment provokes a significant ATP reduction only in HL-60 cells. When used together, Val and STS have an additive effect on ATP decrease. On the contrary, HCW-2 cells show a marked resistance to decrease their ATP content even in the presence of depolarised mitochondria. This had led us to study different steps of the main metabolic pathways leading to ATP production, that is, glycolytic pathway and mitochondrial activity, measured as lactate production in basal conditions or in presence of antimycin A (AA), an inhibitor of complex III of the respiratory chain. The difference between 'basal' lactate (*i.e.* spontaneously produced) and AA-induced lactate is called 'Δ lactate', and is an index of mitochondrial function[21] (US Patent no. 6 261 796 B1; 17 July 2001 by Bovina *et al.*). The results are summarised in Figure 6. In particular, it can be noted that, when compared to parental HL-60 cells, HCW-2 cells have similar glycolytic activity, but higher mitochondrial activity (Figure 6a). Indeed, treatment with AA, that blocks oxidative phosphorylation and induces an increase of lactate production, produces higher Δ lactate in HCW-2 cells, thus indicating that they have higher mitochondrial activity than HL-60 cells. Moreover, glutamine degradation, measured as $^{14}CO_2$ production from ^{14}C-Glutamine,[22] and phosphorylative oxidation, measured as oxygen consumption[23] were assessed. Surprisingly, HCW-2 cells display a decreased activity of glutamine decarboxylation (Figure 6b). This suggests that the pathway of glutamine degradation is deranged in HCW-2 cells, but, taking into account that glutamine is thought to be only partially oxidised in tricarboxylic acid (TCA) cycle of lymphoid cells,[24] it cannot be concluded from this finding that TCA cycle activity is lower in HCW-2 cells. This is also confirmed by the analysis of oxygen consumption (Figure 6c). Indeed, respiration is higher in HCW-2 cells when measured in a glucose-rich buffer, while, on the contrary, when measured in a glucose-depleted buffer containing glutamine as the sole carbon source, O_2 consumption decreases in HCW-2 cells to a value similar to that of HL-60 cells. These data suggest that HCW-2 cells utilise both glucose and glutamine as energy sources, while HL-60 cells utilise mainly glutamine. Nevertheless, both cell types are able to consume O_2 when suspended in a buffer containing only glucose (data not shown). Moreover, on the basis of respiration data and taking Δ lactate as a measure of mitochondrial ATP production, it is possible to indirectly calculate the degree of coupling as an ATP/O ratio. According to this calculation, the ratio would be 0.7 in HL-60 and 1.6 in HCW-2 cells. This would indirectly confirm the hypothesis that

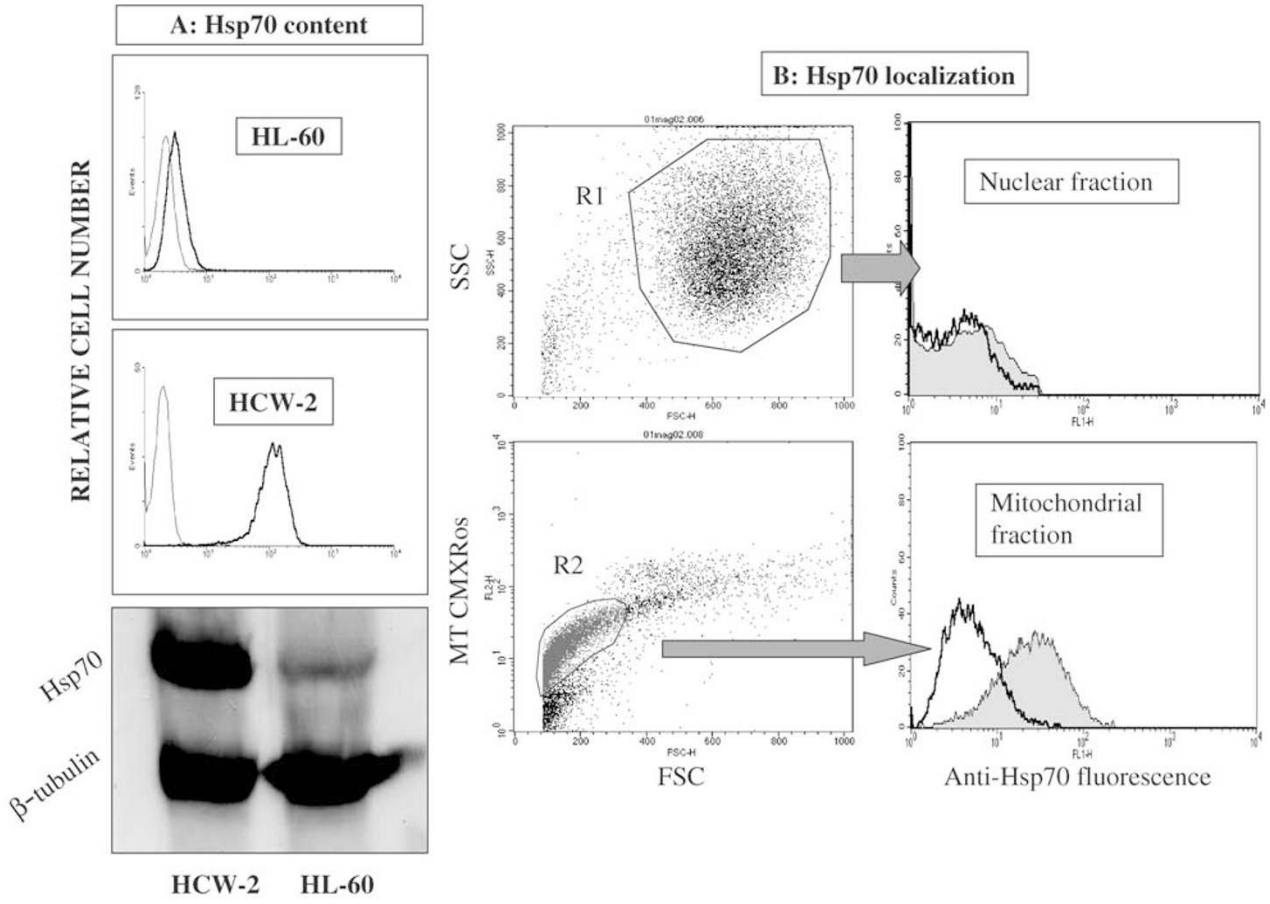

Figure 5 Hsp70 expression and localisation. (a) Expression of Hsp70 in HL-60 and HCW-2 cells. Upper panels: flow cytometric analysis. Grey line: unrelevant antibody, black line: anti-Hsp70 moAb. The intensity of the staining is proportional to the intracellular amount of the protein. Lower panel: Western blot analysis. Note the high amount of Hsp70 present in HCW-2 cells. (b) Localisation of Hsp70 in HCW-2 cells. Cells were stained with the mitochondrial potentiometric probe MT, then lysed with Dounce Homogeneiser. Nuclear and mitochondrial fractions were collected and stained with anti-Hsp70 moAb and analysed with flow cytometer. The fluorescence of anti-Hsp70 moAb was collected from gated events having the scatter of nuclei (upper panels) or having both high MT fluorescence and lower scatter than nuclei (lower panels). Gates are indicated as R1 and R2, respectively. Black line: unrelevant antibody; grey shaded line: anti-Hsp70 moAb. The nuclear fraction was virtually Hsp70-free, while the mitochondrial fraction contains a consistent amount of Hsp70

Table 4 Determination of intracellular ATP content

	Control	Val	STS	Val+STS
HL-60	14.9±2.2	8.2±1.7* −45%	8.3±2.2* −44%	4.2±0.8** −72%
HCW-2	16.2±3.7	11.3±0.4* −30%	15.2±1.8† −6%	9.4±0.5*† −42%

Data are expressed in nmol/mg of proteins and indicate the mean±S.E. of three separate experiments; incubation with Val, STS or both was 2 h *versus* control: *$P<0.05$, **$P<0.01$ (by paired Student's *t*-test); *versus* HL-60 cells: $^{\dagger}P<0.05$ (by unpaired Student's *t*-test).

HCW-2 cells have an higher degree of coupling, as mentioned above.

The activity of pentose phosphate pathway was also measured as $^{14}CO_2$ production from 1-^{14}C-glucose.[22] HCW-2 cells display higher activity of this pathway (Figure 7a). To formally demonstrate an increase in pentose phosphate pathway activity, we studied the expression on the activity of glucose-6-phosphate dehydrogenase (G6PD), the key enzyme of such metabolic pathway (Figure 7b). Both Western blot analysis and enzyme activity assay show that HCW-2 cells have a dramatic increase in such an enzyme, thus confirming metabolic data. Taken together, these data suggest that HCW-2 cells underwent a deep remodelling of energetic metabolism towards a biochemical profile characterised by high rate of NADP reduction. High amounts of reducing power in the form of NADPH can provide the cell with a large antioxidant capacity that can preserve it from many injuries, either of endogenous or exogenous origin. This was confirmed by detecting the amount of glutathione (Figure 7b). We measured the amount of reduced or oxidised glutathione by capillary electrophoresis as described,[25] and found that HCW-2 cells have a two-fold higher content of GSH with respect to HL-60 cells. In both cell types, the amount of GSSG was negligible ($<20\ \mu$M). High amount of reduced glutathione can protect cells from damages induced by reactive oxygen species (ROS) and xenobiotics such as chemotherapic

a **mitochondrial function**

pmoles lactate/s/μg protein	HL-60	HCW-2
basal lactate (glycolytic function)	1.15 ± 0.70	1.71 ± 0.81
lactate with inhibitor AA	1.64 ± 0.99	4.00 ± 2.37
Δ lactate (mitochondrial function)	0.49 ± 0.36	2.29 ± 1.61 *
mitochondrial/glycolytic	0.51 ± 0.37	1.28 ± 0.45 *
* p< 0.05 vs HL-60		

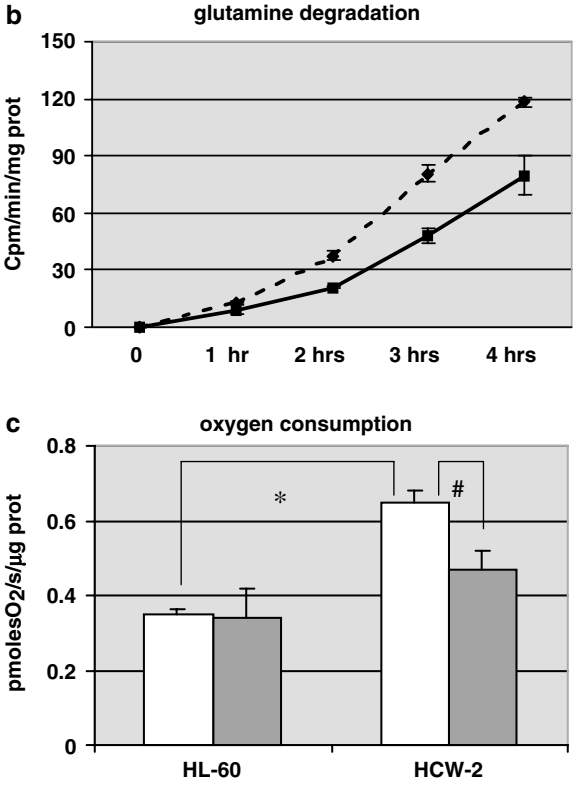

Figure 6 Bioenergetics parameters of HL-60 and HCW-2 cells. (a) Lactate production. The amount of 'basal lactate' produced by cells during incubation stage represents the glycolytic function of cells and is similar in both types of cells. In presence of AA, lactate production is higher in HCW-2 cells and is significantly different also in Δ lactate values, which represent the mitochondrial function of cells. The ratio Δ lactate (oxidative ATP)/basal lactate (glycolytic ATP) is referred as 'mitochondrial/glycolytic'. Statistical analysis was performed by Student's *t*-test. (b) Glutamine degradation, detected as production of $^{14}CO_2$ from of [U-^{14}C] glutamine. HL-60 cells have higher rate of $^{14}CO_2$ from glutamine than HCW-2 cells. Data are expressed in c.p.m. and are the mean ± S.E. of three separate experiments. Full line: HCW-2 cells; dotted line: HL-60 cells. (c) Oxygen consumption. The respiration was assessed using two different buffers, with (white columns) or without glucose (black columns). In presence of glucose buffer, HCW-2 cells showed an increased respiration respect to HL-60 cells (*P=0.012, Student's *t* test). In presence of buffer depleted of glucose, the respiration of HCW-2 cells decreased with respect to the complete buffer (#P=0.013) to a level similar to that of HL-60 cells. Data from all tests were always referred to the total protein content

drugs.[26] We thus measured the production of free radicals in HL-60 and HCW-2 cells in basal conditions and after treatment with H_2O_2. Cells were stained with the reduced probe MitoTracker™ CM-H_2X Ros (MTH), whose oxidation produces red fluorescence, detectable with flow cytometer. The analysis of MTH fluorescence showed that HCW-2 cells have a lower level of free radicals than HL-60 cells, in basal conditions and even after treatment with H_2O_2 (Figure 7c). This strongly indicates that such cells are provided with a sort of radical sponge, likely because of high GSH concentration. Oxidative stress can be the cause of cell death, and resistance to this stress can account, at least in part, for resistance to apoptosis seen in HCW-2 cells. To demonstrate this hypothesis, we treated cells with 0.5 mM diamide, an agent capable to deplete GSH. Diamide induced massive death in HL-60 cells as soon as after 3 h of incubation, while it took 24 h to cause cell death in about one-third of HCW-2 cells (Figure 7c).

Discussion

The extreme resistance to drug-induced apoptosis of HCW-2 cells has been explained until now in terms of deranged expression of Bcl-2 family members. It has been reported that such cells overexpress Bcl-x_L while lacking the expression of Bid.[18,20] We could not reproduce these results; furthermore, we found that several members of such family underwent complex changes, as some of them, with either a pro- or antiapoptotic action, displayed a decreased expression in HCW-2 cells when compared to the parental cell line. The same was noted as far as the mitochondrial ADP/ATP exchanger ANT is concerned. ANT channels can be responsible for the dissipation of $\Delta\psi$ and the induction of apoptosis,[27] and are bound by Bcl-2 and Bax. Nevertheless, it seemed unlikely that the resistance to apoptosis of HCW-2 cells could be because of a general downregulation of ANT and Bcl-2 family. We further investigated metabolic parameters crucial for both apoptosis and stress response at mitochondrial level. It is known that mitochondria play a crucial role in several processes and physiopathological situations, including apoptosis.[28,29] First of all, we found that HCW-2 cells did not have MRPs activity higher than HL-60 cells. Furthermore, HCW-2 cells showed resistance also to physical agents like γ-rays, thus MRPs must be excluded as a possible mechanism of resistance to apoptosis in such cells. In the present study, we show that HCW-2 cells possess mitochondria that are strongly different from those of the parental cell line. Indeed, as shown by cytometric analysis, HCW-2 cells have higher amount of mitochondria than the HL-60 cells, but such organelles possess a lower amount of mtDNA and a different morphology. Moreover, we found that these cells have an increased mitochondrial activity and are capable to utilise both glutamine and glucose as carbon sources. A change in energetic metabolism is thought to be a common feature of tumour cells.[30] A shift towards glycolytic metabolism is usually driven by the necessity of solid tumours to survive in an anaerobic microenvironment, and is related to a more resistant and aggressive phenotype.[30] Nevertheless, also the expression of stress proteins such as Hsps is often found in highly resistant tumours.[31] Accordingly, we found a

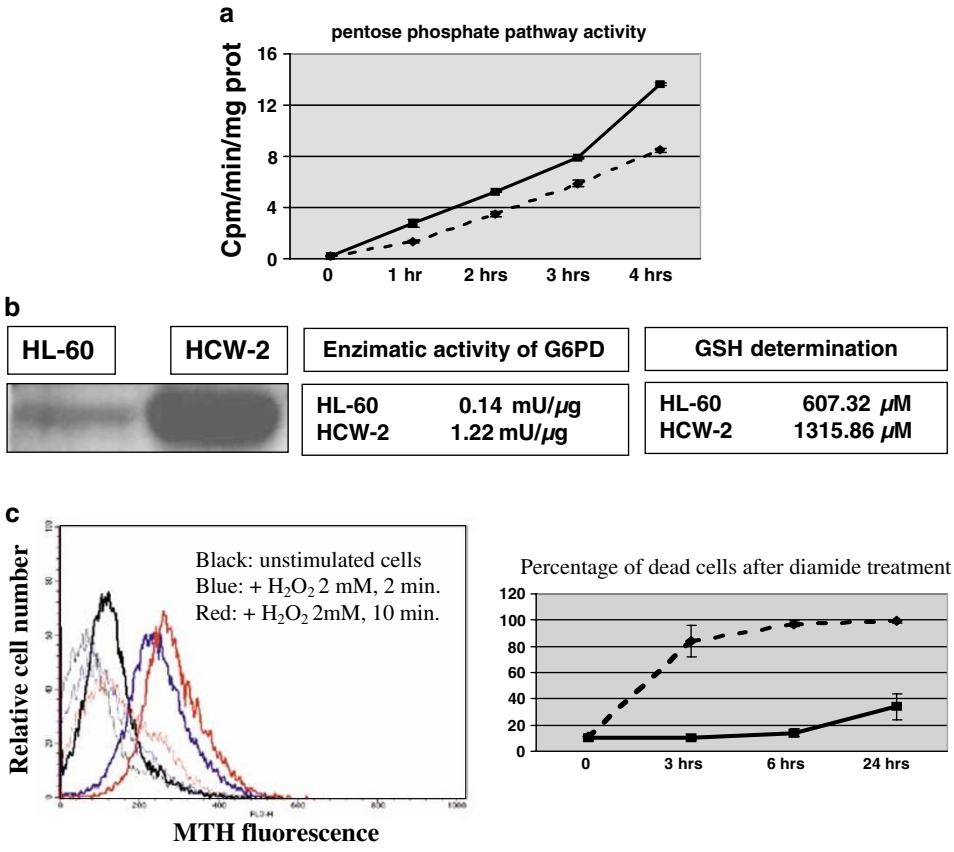

Figure 7 Expression and activity of G6PD. (a) Pentose phosphate pathway activity, detected as production of $^{14}CO_2$ from of [1-^{14}C] glucose. HL-60 cells have lower activity of pentose phosphate pathway than HCW-2 cells. Data are expressed in c.p.m. and are the mean ± S.E. of three separate experiments. Full line: HCW-2 cells; dotted line: HL-60 cells. (b) Expression of the G6PD protein was detected by Western blot analysis; the enzymatic activity was detected by spectrophotometric analysis of NADPH production and expressed as mU/mg of total protein extract, GSH was detected by capillary electrophoresis. Data are referred to normalised protein extracts. (c) Free radical production after H_2O_2 treatment (left) and cell death after diamide treatment (right). Cells were stained with 40 nM MTH and analysed with flow cytometer before and after the addition of 2 mM H_2O_2 leaved to stay for 2 or 10 min. Cell death was detected by flow cytometry as PI incorporation after treatment with 0.5 mM diamide. Data are expressed as percentage of PI+cells and are the mean ± S.E. of two experiments

dramatically increased expression of Hsp70 and Hsp27 in HCW-2 cells. In particular, Hsp70 is a family of key molecules that are involved in the response to a large variety of stressors, and are considered the major mitochondrial import proteins. Thus, it is possible to conclude that the apoptotic-resistant phenotype of HCW-2 cells is related to the abnormally high expression of stress proteins. Interestingly, it is reported that Hsps, in particular Hsp27, can increase the activity of G6PD.[32] This could suggest that the protective effect of Hsps is mediated by the induction of G6PD and by the consequent increase in the concentration and/or turnover of GSH. Accordingly, we found a dramatic increase in G6PD activity and GSH pool in HCW-2 cells. It is also interesting to note that G6PD is considered a stress-induced protein and its expression is increased under stress conditions, such as ionising radiations and oxidative stress.[33,34] The depletion of GSH was able to induce eventually cell death in HCW-2 cells, thus confirming the idea that resistance to apoptosis is mediated by GSH pool, which is maintained by a high pentose phosphate pathway activity. Moreover, Hsp70 can exert their protective effects at the mitochondrial level, as demonstrated by several groups, including ours.[16,35] This in turn may protect mitochondria from ROS-induced damages and allow them to better support the ATP request. This may account for the increased growth rate of HCW-2 cells.

It is known that a certain number of blood malignancies is resistant to several chemotherapic agents, even if they do not express MRPs, which is exactly the feature of our experimental model. It would be of great interest to verify whether the *in vitro* situation we show here is paralleled by *in vivo* observations performed in patients with chemotherapy resistant, MRPs-negative neoplasms. If so, therapeutic strategies should be envisaged to prevent or to cope with this metabolic adaptation of tumour cells. Further studies are needed to investigate this possibility.

Materials and Methods

Materials

Foetal calf serum and RPMI 1640 medium were from Life Technologies Ltd (UK), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), Rh123, MitoTracker™ Red CMXRos (MT), MitoTracker™ Green (MTG), MitoTracker™ CM-H_2X Ros (MTH), and 10-N-nonyl

acridine orange (NAO) fluorescent probes were from Molecular Probes (Eugene, OR, USA) and were dissolved and stored following manufacturer's instructions. Monoclonal and polyclonal antibodies for members of the Bcl-2 family, and for other proteins involved in energetic metabolism (Glut-1, ANT, cytochrome *c*) or stress response (p85, HIF-1α, Hsp27, Hsp70) were from Santa Cruz Technology (Santa Cruz, CA, USA) or from BD Pharmingen (San José, CA, USA). Propidium iodide (PI), STS, Dau, MMC, 2-chlorodeoxy adenosine (2CdA), dRib, Val, AA and other chemicals were from Sigma Chemal (St Louis, MO, USA), and were of analytical grade. *Taq* Polymerase and Moloney murine leukaemia virus reverse transcriptase (MMLV RT) were from Promega (Madson, WI, USA).

Cell culture and drug treatment

Micoplasma-free HL-60 human promyelocytic leukemic cell line and its apoptosis-resistant clone HCW-2 (kindly provided by Drs. JH Wyche and Z Han, Brown University, Providence, RI, USA) were cultured in complete medium (RPMI 1640 supplemented with 10% heat inactivated foetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine) and kept at 37°C in humidified atmosphere (5% CO_2 in air). Cells were collected during the log phase of growth, washed in phosphate-buffered saline (PBS) at room temperature, counted and adjusted to the density of 10^6/ml. As described previously, cells have then been treated for different periods with one of the following apoptosis-inducing agents: 5 μM STS, 4 h;[36] 1 μM Dau, 18 h; 10 μM 2CdA, 18 h;[37] 0.3 μg/ml MMC, 18 h; 20 mM dRib,[38] 48 h; γ-rays (5, 10 or 20 Gy), 24 h.[39] In some experiments, STS has been used also in the presence of 100 nM Val, a compound capable of depolarising mitochondria without inducing apoptosis at such a low dose.[40]

Flow cytometry

Cytofluorimetric analyses were performed using a FACScan or a FACScalibur cytometer (BD, San José, CA, USA) equipped with an Argon ion laser tuned at 488 nm. In all analyses, a minimum of 10 000 cells per sample were acquired in list mode and analysed with Cell Quest and WinMDI 2.8 softwares. The following parameters were assessed:

MDR pump activity

Cells were loaded with 100 ng/ml Rh123 for 15 min at 37°C, then extensively washed with prewarmed, Rh123-free medium. Then cells were allowed to stay at 37°C for 15, 30 and 60 min and analysed for the level of Rh123 fluorescence intensity. At the end of each time point, an aliquot of cells was harvested and checked by flow cytometry. The intensity of Rh123, collected in linear scale, was compared to time 0 for each time point.

Mitochondrial mass

We have used two independent methods to quantify mitochondrial mass. The first uses the fluorescent probe MTG.[41] The second method employs a low dose of the fluorescent dye NAO.[42] For the comparison with the content of Bcl-2 gene family products, the quantification of mitochondrial mass after staining with NAO was performed after linearisation of fluorescence signals.[43]

Changes in mitochondrial membrane potential ($\Delta\psi$)

$\Delta\psi$ has been evaluated by using the potentiometric probe JC-1, which changes reversibly its colour from green to orange as $\Delta\psi$ increases (over values of about 80–100 mV). Briefly, cells were stained with 2.5 μg/ml JC-1 and kept at room temperature for 20 min, washed twice with PBS, resuspended in a total volume of 400 μl PBS and analysed as described.[44,45]

Apoptosis

Apoptosis has been evaluated by the appearance of the hypodiploid peak of PI fluorescence, as described.[46] Briefly, cells were resuspended in hypotonic solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml PI, and kept for 20 min at 4°C. Therefore, cells were analysed with flow cytometer and those with low PI fluorescence were considered containing less DNA and thus apoptotic.

Cell viability (plasma membrane integrity)

We used the classical PI exclusion test, as described.[45] Briefly, cells were resuspended in 400 μl PBS, stained with 5 μg/ml PI, and immediately analysed with flow cytometer. Cells negative for PI fluorescence were considered alive.

Intracellular detection of proteins

Cells were collected and fixed for 10 min in 2% paraformaldehyde, permeabilised with 0.05% Triton X-100 and incubated with specific monoclonal or polyclonal antibodies, following standard procedure for intracellular antigens staining.[47] Briefly, cells were first incubated with complement-deprived human serum, in order to saturate Fc receptors, then incubated with primary antibody for 1 h at 4°C. Cells were washed twice in cold PBS and incubated with FITC-conjugated secondary antibody for 1 h at 4°C, washed in cold PBS and analysed. For each sample a negative control (i.e. sample stained with an irrelevant antibody) was performed. To quantify the amount of antigen present into cells, we subtracted the linearised median value of the negative sample (spontaneous autofluorescence) from the linearised median value of positive samples, as described.[43,48] In Hsp70 localisation experiment, cells were resuspended in buffer A (KCl 10 mM, HEPES KOH 20 mM, $MgCl_2$ 1.5 mM, EGTA and EDTA 0.1 mM, $NaVO_4$ 0.2 mM, saccarose 250 mM, DTT 1 mM and protease inhibitor cocktail, Sigma, St Louis, MO, USA), lysed with Dounce Homogeniser and subcellular fractions were collected after differential centrifugates. Nuclear and mitochondrial fractions were collected and stained following the mentioned procedure. In order to better identify mitochondria, before lysis cells were stained with mitochondrial potentiometric probe MT 40 nM. Events having scatter smaller than that of nuclei and high MT fluorescence were gated and analysed for the fluorescence of anti-Hsp70 moAb.

Electron microscopy

HL-60 and HCW-2 cells were cultured as described above, and harvested during the log phase of growth. Cells were then fixed in 0.5% glutaraldehyde in PBS, centrifuged at 11 000 $\times$ *g* for 5 min at 4°C, fixed in 2.5% glutaraldehyde in PBS pH 7.3 for 12 h. Pellets were further fixed in 1% osmium tetroxide in PBS, pH 7.3, dehydrated in ethanol, in propylene oxide and finally embedded in Spurr resin. Ultrathin sections collected on copper grids were stained with uranyl acetate and lead citrate and observed with a Jeol 1200 EX II electron microscope.

Biochemical determinations

Intracellular ATP determination

Steady-state levels of ATP were detected as follows: cells were collected after 2 h of incubation with different agents, washed, counted and resuspended at the concentration of 5×10^6/ml in distilled water. Cells were then immediately boiled at 100°C for 1 min and spun down to eliminate membranes and DNA. ATP was measured in the supernatant by using the ATP Bioluminescence Assay kit CLS II (Boehringer Mannheim, Germany) and a LKB 1250 Wallac luminometer (Finland).

Oxygraphic measurements

Respiration of cells was measured at 30°C by means of a thermostatically controlled oxygraph apparatus equipped with a Clark's electrode and a rapid mixing device. In a typical experiment, 1×10^6 cells were incubated for 5 min at 30°C in appropriate respiration medium (0.25 M sucrose, 50 mM HEPES, 4 mM $MgSO_4$, 0.5 mM EDTA, 10 mM KH_2PO_4, pH 7.4, final volume 1.6 ml). Uncoupler FCCP (p-trifluormethoxy phenylhidrazone) titrations (50–250 nM) were performed. No differences were detected about percentage of total uncoupling in FCCP titrations between the two cells type (data not shown). Final inhibition with KCN (1 mM) as downstream respiratory chain inhibitor was tested.

Lactate determination

Cells are washed and counted and fixed aliquots are incubated at 37°C in presence or absence of AA, an inhibitor of mitochondrial respiratory chain, as described in[21] (US Patent no. 6 261 796 B1; 17 July 2001 by Bovina *et al.*). After the incubation time, cells are centrifuged and lactate released in the supernatant fraction was collected and measured by a spectrophotometric method. Lactate production by cells is strongly enhanced by AA inhibition of mitochondrial respiration because of Pasteur effect: the Δ lactate production (lactate produced in presence of AA minus lactate produced in absence of AA) represent the oxidative phosphorylation function, whereas the basal lactate production (in absence of AA) represent the glycolytic function. Several experiments were conducted to investigate the optimal concentration of inhibitor AA of each cellular type in order to obtain total inhibition of respiratory chain activity: HL-60 cells were inhibited by 2 μM AA and HCW-2 cells by 7 μM AA (data not shown). Experiments of time courses showed an optimal incubation time of 2 h to analyse significant Δ lactate values (data not shown).

Measurement of the production of $^{14}CO_2$

Cells were preincubated for 1 h in complete medium without sodium pyruvate containing 580 mg/l L-glutamine, 5 mM glucose and 10% dialysed foetal calf serum before the addition 1 μCi/ml of [1-^{14}C] glucose for the assessment of the pentose phosphate pathway activity. For detection of glutamine utilisation, cells were preincubated for 1 h in complete medium with sodium pyruvate containing 2 mM L-glutamine, 5 mM glucose and 10% dialysed foetal calf serum before the addition 1 μCi/ml of [U-^{14}C] glutamine. The measurement of the $^{14}CO_2$ was performed as described by Reitzer,[22] modified by the benzethonium hydroxide capture of the $^{14}CO_2$.[49]

G6PD activity

The enzyme activity in the total cell extracts was measured as previously described.[50] Briefly, an excess of glucose 6-phosphate and NADP was added to a fixed amount of total cellular extract and the absorption at 345 nm was analysed with spectrophotometer, in order to detect the amount of NADPH, expressed as mU/μg of protein at 25°C. Total protein concentration was determined according to Bradford's protein–dye method.[51]

Western blot analysis

Cells were collected as described above, washed twice in PBS and then resuspended in 200 μl of RIPA buffer (Tris buffer pH 8, 50 mM; NaCl 150 mM; sodium deoxycholate 0.5%; SDS 0.1%; Triton X-100 1%; EDTA 5 mM; protease inhibitor cocktail, Sigma). Total protein concentration was determined according to Bradford's protein–dye method.[51] Protein extracts (40 μg) were subjected to electrophoresis in an SDS-polyacrilamide gel (10%) and then transferred onto a nitrocellulose paper by electrophoresis in a Trans-blot chamber (mini Protean II, BioRad, Hercules, CA, USA). Proteins were identified by using specific moAbs as described and Western blotting luminol reagent (Santa Cruz, CA, USA).

Quantification of mtDNA by competitive-PCR

The number of copies of mtDNA per cell was determined by a QC-PCR as described.[52] A 180 bp fragment of the mitochondrial gene encoding for the subunit VI of F_0/F_1 ATPase was amplified using primers called mt192R (5′-GCTCTAGAAAGAGATC-AGGTTCGTCCTTTAGTG-3′) and mt27D (5′-AAAATGAACGAAAATCTGTTC-GCT-3′). An internal deletion of 20 bp was carried out using the primer mt70D (5′-ggaattcaaaatgaac-gaaaatctgttcgctcctacccgccgcagtagtga-3′) that has a tail identical to primer mt27D. This PCR product can be amplified using primers mt27D and mt192R, as well as wild-type DNA, but is 20 bp shorter and can be easily distinguished from wild type on agarose gel. This fragment was then cloned into the pFASL plasmid that contains the competitor for Fas ligand,[53] taking advantage of the consensus sequences for *Eco*RI and *Xba*I restriction enzymes present at both its ends. The construct, named pMito, was extracted from *E. Coli* cells using High Pure Plasmid isolation kit (Roche Biochemicals, Mannheim, Germany) quantified with spectrophotometer and limiting dilution analysis, and then stored at −20°C. This construct was used to evaluate with QC-PCR the number of copies of mtDNA or genomic DNA (gDNA) that are present in a sample, depending on the couple of primers used in the PCR mix. Indeed, mtDNA has been quantified carrying out several PCR reactions with the same amounts of DNA extracted from the sample and scalar amounts of competitor DNA (from 10^8 to 10^2 copies). Each tube contained 25 μl of a PCR mix consisting of dNTPs 2.5 mM, primers mt27D and mt192R 200 nM, $MgCl_2$ 1.5 mM, Tris-HCl 50 mM, pH 9 at 25°C, 1 U of Taq polymerase. Every PCR reaction was of 50 cycles, each cycle consisting of a denaturation step (94°C for 30 s) an annealing step (52°C for 30 s) and an extension step (72°C for 30 s) The first cycle was preceded by a denaturation step of 5 min at 94°C, and the last one was followed by an extension step of 7 min at 72°C.

gDNA was quantified with the same technique, using the same competitor at identical concentrations, and the same PCR mix; however, in this case primers 276R and 60D, instead of mt27D and mt276R, were added to the mix, while PCR cycling was slightly different: each cycle consists of a denaturation step (94°C for 30 s), an annealing step (56.5°C for 35 s) and an extension step (72°C for 40 s). The first cycle was preceded by a denaturation step of 5 min at 94°C, and the last one was followed by an extension step of 7 min at 72°C.

PCR products were loaded on a 3% agarose (FMC, etc.) gel for 40 min at 90 V and stained with EtBr 0.5 mg/ml; relative intensity of the two bands (competitor *versus* wild type) was calculated using a densitometer (GelDoc 2000, BioRad) with relative software (Multianalyst 1.0.1).

Fluorescence intensity of the competitor band was corrected by a factor of 1.10 (for mtDNA) or 1.16 (for gDNA) to compensate minor EtBr incorporation. The number of copies of mtDNA per cell was calculated as the ratio between number of copies of mtDNA, (obtained by the first PCR), and the number of copies of gDNA, obtained by the second PCR, multiplied 2 (as two copies of Fas Ligand gene are obviously present in the gDNA).

Statistical analysis

The analysis of the data was performed by two-tailed unpaired Student's *t*-test, using the software SPSS 10 operating under Windows ME.

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