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ORIGINAL ARTICLE

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P-glycoprotein expression does not change the apoptotic pathway induced by curcumin in HL-60 cells

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Abstract *Purpose*: One of the mechanisms responsible for the multidrug resistance (MDR) phenotype of cancer cells is overexpression of so-called ATP-dependent drug efflux proteins: the 170-kDa P-glycoprotein (P-gp) encoded by the *MDR1* gene and the 190-kDa multidrug resistance-associated protein 1 encoded by the *MRP1* gene. The purpose of the present study was to verify the hypothesis postulating that P-gp expression, apart from enabling drug efflux, confers on the cells resistance to apoptosis by inhibiting caspase-8 and caspase-3. *Materials and methods*: Human HL-60 cells, either drug-sensitive or with the MDR phenotype caused by overexpression of P-gp (HL-60/Vinc) or MRP1 (HL-60/Adr), were treated with the natural dye curcumin at 50 μ*M* or with UVC to induce apoptosis. Symptoms of cell death were assessed by morphological observation after Hoechst staining, DNA fragmentation was measured by flow cytometry and the TUNEL method, and caspase-8 and caspase-3 activation and cytochrome *c* release from mitochondria were measured by Western blotting. *Results*: Curcumin induced cell death in HL-60 cells, both sensitive and with the MDR phenotype, which could be classified as caspase-3-dependent apoptosis, together with cytochrome *c* release, activation of caspase-3 and oligonucleosomal DNA fragmentation. No active caspase-8 was detected. Also UVC caused caspase-3 activation in both the sensitive and the MDR HL-60 cells. *Conclusions*: Our findings show that there was no correlation between P-gp expression and resistance to caspase-3-dependent apoptosis induced by curcumin and UVC, at least in HL-60 cells. However, we cannot exclude the possibility of parallel P-gp expression and caspase-3 inhibition in some other cell lines, as cancer cells can acquire many different apoptosis-resistance mechanisms.

Keywords HL-60 cells · P-gp · Multidrug resistance · Apoptosis · Curcumin

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Introduction

Apoptosis is currently a subject of intense research, one of the reasons being that we now recognize that tumor cells are susceptible to death by apoptosis in response to drugs and radiation treatment [6, 10]. Many chemotherapeutic drugs function in a caspase-dependent manner, that is by activating the main effector caspase-3 [22]. Caspase-3 can be activated via two main apoptotic pathways described sometimes as intrinsic and extrinsic. The intrinsic (or mitochondrial) pathway, which is mainly triggered by stress and cytotoxic drugs, involves the release of cytochrome *c* from the mitochondria. The stimulation of the mitochondrial membrane in this process is not yet fully understood. Cytochrome *c* in synergy with ATP allows a conformational change of Apaf-1 to occur. Apaf-1 binds caspase-9 which in turn activates caspase-3.

Activation of caspase-3 via the extrinsic pathway is triggered by ligation of death receptors which are members of the tumor necrosis factor (TNF) superfamily (i.e. Fas receptor) and are characterized by an intracellular death domain. The death domain attracts the intracellular adaptor protein FADD which in turn recruits procaspase-8 to the death-inducing signaling complex (DISC). At the DISC, procaspase-8 is cleaved and yields active caspase-8, activating in turn caspase-3. In some cells the amount of active caspase-8 is sufficient to initiate apoptosis directly, but in others the amount is too small and mitochondria are activated by BID protein which belongs to the Bcl-2 family. Caspase-3, as the effector caspase active in both pathways, cleaves nuclear

and cytosolic substrates to induce morphological changes such as DNA fragmentation, membrane blebbing, that are characteristic of apoptosis [12, 13].

Besides these prototypic caspase-dependent apoptosis pathways, there are also pathways that do not require caspase activation, and share some characteristics of the classical apoptotic pathways [2]. As cancer cells frequently acquire resistance to caspase-3-dependent apoptosis causing serious clinical problems in cancer therapy, the ability to induce caspase-3-independent cell death in such cells would be of tremendous therapeutic value. One of the mechanisms responsible for the resistance to apoptosis is the development of multidrug resistance (MDR). In tumor cell lines, MDR is often associated with overexpression of ATP-dependent drug efflux proteins belonging to the superfamily of ATP-binding cassette (ABC) transporters: the 170-kDa P-glycoprotein (P-gp) encoded by the *MDR1* gene and the 190-kDa multidrug resistance-associated protein 1 encoded by the *MRP1* gene [14]. These proteins, bind to and transport various structurally unrelated compounds to maintain their intracellular concentrations below the cytotoxic level.

Recently, it has been postulated that P-gp, apart from actively effluxing drugs from cells, may protect them against apoptosis by inhibiting caspase-8 and caspase-3, as P-gp-positive cells have been found to resist cell death induced by UV irradiation, and ligation of the cell surface death receptors Fas and TNF [7, 23, 24]. Johnstone et al. have postulated that successful treatment of P-gp$^+$ MDR tumors might be enhanced using chemotherapeutic agents that can function in the absence of caspase-3 activation [8]. Our previous results indicate that a dye from the rhizome of *Curcuma longa*, curcumin, induces caspase-3-independent cell death in many human and rodent lymphoid cells, both normal and cancer [1, 20, 21]. The only exception is the human myeloblastic leukemia HL-60 cell line which upon curcumin treatment, at the same 50 μ*M* concentration, underwent cell death with caspase-3 activation [1].

Now, using HL-60-sensitive and MDR cells, the latter expressing either P-gp (HL-60/Vinc) or *MRP1* (HL-60/Adr), and curcumin as cell death inducer, we wanted to verify the hypothesis postulating a correlation between P-gp overexpression and caspase-8 and caspase-3 inhibition. Provided that P-gp overexpression is strictly connected to inhibition of caspase-3-dependent apoptosis, our expectation was that curcumin would be able to induce cell death in the absence of caspase-3 activation in the P-gp$^+$ HL-60/Vinc cells as we had observed previously in LoVo$^{P\text{-}gp+}$ and CEM$^{P\text{-}gp+}$ cells [21]. We also wanted to determine whether P-gp overexpression also influences caspase-8 which is upstream of caspase-3.

Materials and methods

Cell culture and cell treatment

Human leukemia cell lines HL-60-sensitive, 170-kDa P-gp-positive HL-60/Vinc selected for resistance to vincristine, and HL-60/Adr with high expression of 190-kDa non-P-gp membrane-bound protein selected for resistance to Adriamycin, previously described by McGrath et al. [15, 16, 17], were kindly provided by Dr. M.S. Center, Division of Biology, Kansas State University, Kansas. All cells were cultured in complete medium (RPMI-1640 supplemented with 10% fetal calf serum, antibiotics, 2 m*M* L-glutamine) and kept at 37°C in a humidified atmosphere (5% CO_2 in air). HL-60/Vinc cells were cultured in the presence of 0.8 μ*M* vincristine and HL-60/Adr cells in the presence of 2 μ*M* Adriamycin. All antibiotics were from Sigma-Aldrich, Poznan, Poland. Cells were seeded 1 day before experimentation to a density of 0.5×10^6/ml and grown to a density of 10^6/ml.

To induce apoptosis cells were treated with curcumin (Merck, Darmstadt, Germany) or irradiated with UVC. Curcumin was dissolved in 96% ethanol and added to the medium to a final concentration of 50 μ*M* for the times indicated in the Results. For UVC irradiation, cells were resuspended at 10^6/ml in phosphate-buffered saline (PBS) and placed in a large culture dish. They were then exposed to a pulse (several seconds) of ultraviolet light (254 nm) at an energy output of 100 J/m^2 using a Stratalinker 2400 (Stratagene, La Jolla, Calif.). After irradiation the cells were washed, resuspended in complete medium and incubated for 5 or 24 h.

RT-PCR

Total RNA was prepared using a QIAshredder homogenizer and an Rneasy Mini Kit (QIAGEN, Hilden, Germany). Total RNA (1 μg) was then used in reverse transcription reactions using a First Strand cDNA Synthesis Kit for RT-PCR (Roche Molecular Biochemicals, Mannheim, Germany) as described by the manufacturer. The resulting total cDNA was used in PCR performed in a total volume of 25 μl using a Taq PCR Core Kit (QIAGEN) at 94°C for denaturation for 45 s, 60°C for annealing for 60 s, and 72°C for amplification for 80 s for 30 cycles, followed by a final extension at 72°C for 10 min. Primers for MDR1 and *β*-actin used in the PCR were as described by Ogretmen and Safa [19]:

- MDR1 (forward): 5′-CCCATCATTGCAATAGCAGG (3017–3036)
- MDR1 (reverse): 5′-GTTCAAACTTCTGCTCCTGA (3173–3154)
- *β*-actin (forward): 5′-CAGAGCAAGAGAGGCATCCT (216–235)
- *β*-actin (reverse): 5′-TTGAAGGTCTCAAACATGAT (405–424)

All primers were synthesized by Microsynth (Balgach, Switzerland). The *β*-actin mRNA levels were used as internal controls in RT-PCR. *β*-Actin primers were used at a 1:2 ratio to the MDR1 primers to achieve linear amplification conditions. The amplified fragments were separated on 1% agarose gel and visualized by ethidium bromide staining. For each RT-PCR, a negative control without template was performed (not shown).

Functional test for P-gp

Functional analysis of ATP pump was done using the flow cytometry according to the method of Koizumi

et al. [11] with slight modification. Cells (0.5×10^6) were incubated with 0.2 μ*M* Rh-123 (Sigma-Aldrich) for 30 min at 37°C in RPMI-1640 medium. For efflux studies, the cells after Rh-123 incorporation were washed twice with RPMI-1640, then incubated in Rh-123-free medium for 1 h at 37°C. For some experiments cyclosporine A (Sandimmun; Sandoz, Vienna, Austria) was added to the medium (40 μ*M* final concentration) first, then after 15 min Rh-123 was added, and incubation was continued for an additional 30 min. The cells were washed twice with PBS and a total of 10,000 events for each sample were recorded and analyzed on a FACSCalibur flow cytometer using Cell-Quest software (Becton-Dickinson, Warsaw, Poland).

Apoptosis measurement

Cell death was monitored by DNA content analysis on a flow cytometer, by the TUNEL (TdT-mediated dUTP-biotin nick end-labeling) assay and by thorough morphological observation after Hoechst staining, as described previously [1]. Briefly, for the flow cytometric analysis of DNA content, the cells were fixed in 70% ethanol overnight at 4°C. Fixed cells were washed twice with PBS and resuspended for 1 min in 0.1 *M* sodium citrate, pH 7.8. After washing the cells were stained in PBS containing 50 μg/ml propidium iodide and 0.1 mg/ml RNAse A for 30 min. For the TUNEL assay the MEBSTAIN apoptosis kit (Immunotech, Marseille, France) was used as described by the manufacturer. Morphological observation was performed after Hoechst 33258 staining (Molecular Probes, Eugene, Ore.). Cells ($0.2–0.3\times10^6$) were centrifuged on cytospin, fixed with 70% ethanol, washed in PBS and stained for 10 min in 1 m*M* Hoechst 33258 dye. Caspase-3 activation was measured by flow cytometry using FITC-conjugated anti-active caspase-3 mAb (BD Pharmingen, San Diego, Calif.) as described by the manufacturer or by Western blotting as described below.

Western blotting for caspase-8, caspase-3, PARP and cytochrome *c*

For Western blotting of caspase-8, caspase-3 and PARP, total cellular proteins were extracted with a Triton-X buffer, run on 12% SDS-PAGE at 40 μg protein per lane and electrotransferred to a nitrocellulose membrane Hybond C (Amersham Buchler, Braunschweig, Germany) for 1 h at 400 mA. Membranes were blocked for 1 h with 10% dried milk in PBS-Tween 20 (0.1%) and probed overnight at 4°C with a polyclonal antibody to caspase-8 (1:500), caspase-3 (1:2000) or monoclonal anti-PARP (1:500) (BD Biosciences, Warsaw, Poland). For cytochrome *c* assay, soluble cytosolic fraction was prepared by digitonin permeabilization. Cells (5×10^6) were washed in PBS and resuspended in import buffer (110 m*M* KCl, 20 m*M* Hepes, pH 7.4, with 0.5 m*M* EGTA and 70 m*M* mannitol). Then digitonin was added to 40 μg/ml and the cells were incubated for 20 min on ice. The insoluble material was separated by centrifugation for 3 min at 900 *g* and discarded. The

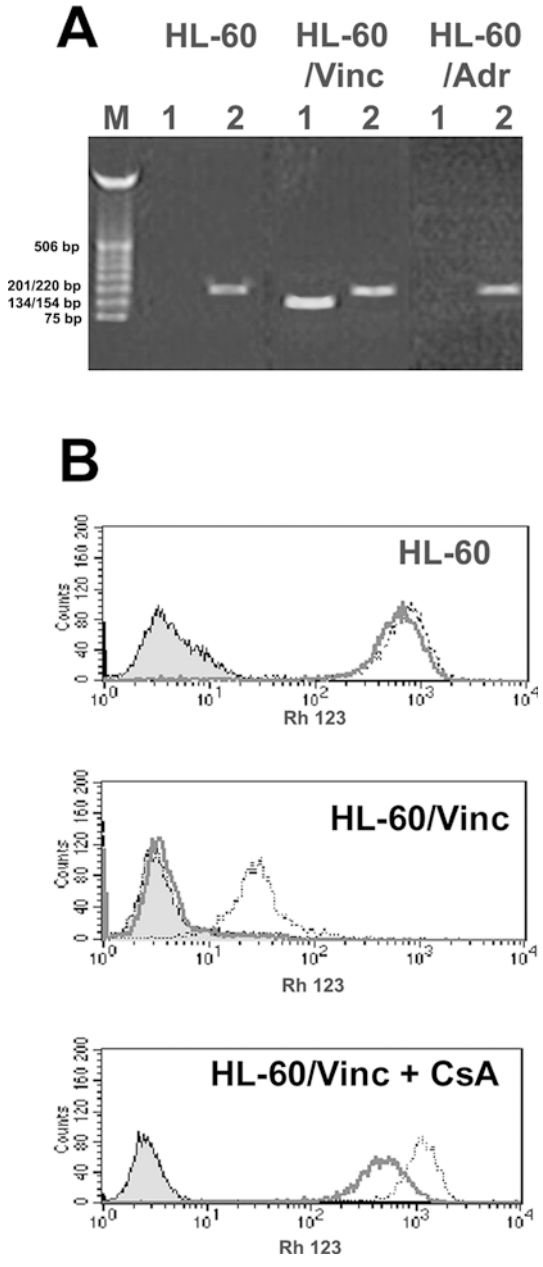

Fig. 1 **A** Level of MDR1 mRNA in HL-60, HL-60/Vinc and HL-60/Adr cells. RT-PCR was performed using primers for MDR1 and *β*-actin (used as internal control): *lane 1* MDR1 (156 bp), *lane 2* *β*-actin (208 bp), *lane M* 1 kb DNA ladder (Gibco-BRL). **B** The functionality of P-gp assessed by flow cytometry: *top* HL-60 cells incubated in the presence of Rh-123, *center* HL-60/Vinc incubated in the presence of Rh-123, *bottom* HL-60/Vinc incubated in the presence of Rh-123 and cyclosporine A (*CsA*) a blocker of P-gp; *shaded histograms* auto-fluorescence of cells not incubated with rhodamine-123, *unshaded histograms* fluorescence of cells incubated with rhodamine-123 and analyzed before (*broken line*) and after (*solid line*) a 1-h recovery. A representative of three experiments is shown

supernatant was centrifuged for 10 min at 20,000 *g* and the cytosolic fraction was collected. Proteins were subjected to SDS-PAGE (as described above) and probed with a mouse monoclonal anti-cytochrome *c* antibody (1:500, Pharmingen), clone 7H8.2C12. As a marker cytochrome *c* from horse heart (Sigma) was used. Detection was carried out by incubating the membranes with an enhanced chemiluminescence reagent (ECL) kit (Amersham Pharmacia Biotech).

Results

MDR1 expression and P-gp function in HL-60/Vinc cells

The presence of P-gp, the product of the *MDR1* gene in HL-60/Vinc, but not in HL-60/Adr cells, was verified at the mRNA level by RT-PCR (Fig. 1A). To determine whether the P-gp expressed by HL-60/Vinc cells was functional, incorporation and efflux of Rh-123 was determined. The results showed that Rh-123 was incorporated into the sensitive cells as well as into their MDR counterparts (HL-60/Vinc) (Fig. 1B). In contrast to the sensitive cells, during the 1-h recovery the drug was completely extruded from the HL-60/Vinc cells. This indicates that the HL-60/Vinc cells have an active extrusion mechanism, presumably the P-gp ATP pump. This was further verified using cyclosporine A, a known inhibitor of ABC transporters. As expected, the efflux of Rh-123 was blocked by cyclosporine A (Fig. 1B, lower images).

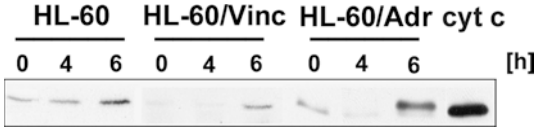

Fig. 2 Cytochrome *c* (cyt c) release from mitochondria. The presence of cytochrome *c* in the cytoplasm of HL-60, HL-60/Vinc and Hl-60/Adr was measured by Western blotting in cells untreated (*0*) or treated with curcumin as indicated. Lane *cyt c* contains cytochrome *c* marker (horse heart). A representative of three experiments is shown

Apoptosis of HL-60 sensitive and MDR cells induced by curcumin

In all the tested cells, both sensitive and with the MDR phenotype, as early as 6 h after curcumin treatment cytochrome *c* was observed in the cytosol (Fig. 2). By 24 h after curcumin treatment the cells displayed DNA breaks and changed morphology, as shown by the TUNEL assay and Hoechst staining, respectively (Fig. 3). Flow cytometric analysis revealed the formation of sub-G_1 fractions in these cells (Fig. 4). The percentages of sub-G_1 (M_1) cells measured 24 h after curcumin treatment from three independent experiments

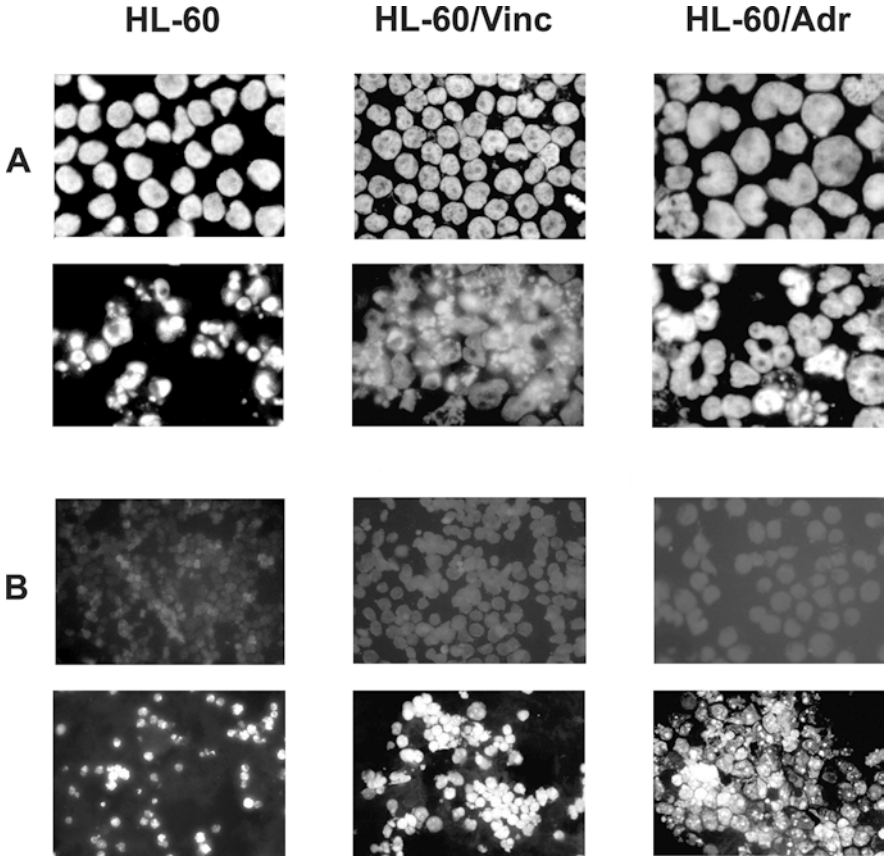

Fig. 3A, B Cell morphology and DNA fragmentation. **A** Morphology of HL-60 sensitive and MDR cells (*HL-60/Vinc* and *HL-60/Adr*), either control (*upper images*) or curcumin-treated (24 h, *lower images*). Cells were stained with Hoechst 33258 (magnification ×400). A representative of three experiments is shown. **B** DNA breaks were assessed by the TUNEL method in control cells (*upper images*) and curcumin-treated cells (24 h, *lower images*) with MDR phenotype (*HL-60/Vinc* and *HL-60/Adr*) (magnification ×200). A representative of three experiments is shown

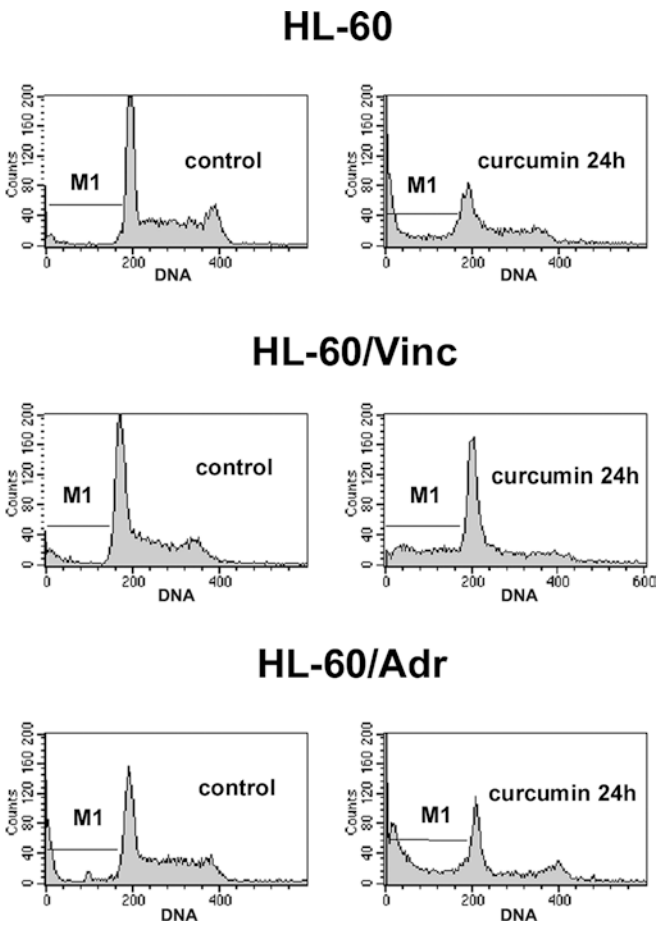

Fig. 4 DNA content in HL-60 and MDR HL-60 cells. DNA content was assessed by flow cytometry of the sub-G_1 (M_1) fraction in HL-60, HL-60/Vinc and HL-60/Adr cells untreated (*control*) or treated with curcumin for 24 h (*curcumin 24h*). A representative of three independent experiments is shown

were as follows (means ± SEM): HL-60 control, 7.3 ± 1.6%; HL-60 curcumin-treated, 29.6 ± 2.9%; HL-60/Vinc control, 10.6 ± 2.6%; HL-60/Vinc curcumin-treated, 23.7 ± 3.6%; HL-60/Adr control, 8.0 ± 2.0% and HL-60/Adr curcumin-treated, 30.5 ± 6. 0%. It is known that such DNA fragmentation occurs in the course of caspase-3-dependent apoptosis. Indeed, Western blot analysis using antibodies against caspase-3 and its substrate PARP showed the presence of the active form of caspase-3 as well as hydrolyzed PARP in curcumin-treated HL-60 cells both sensitive and MDR. The proteolysis of both proteins was clearly visible at 24 h after curcumin treatment (Fig. 5).

The triggering of the apoptotic events by curcumin was not connected with Fas receptor activation as we did not observe activation of the downstream initiator caspase-8 in any of the studied cells (not shown).

UVC-induced caspase-3 activation in HL-60 sensitive and MDR cells

HL-60 cells, both P-gp positive (HL-60/Vinc) and negative (HL-60 and HL-60/Adr), were very sensitive to UVC radiation, as revealed by the flow cytometric DNA content assay (not shown). The appearance of active caspase-3 in all tested cells as early as 5 h after UVC

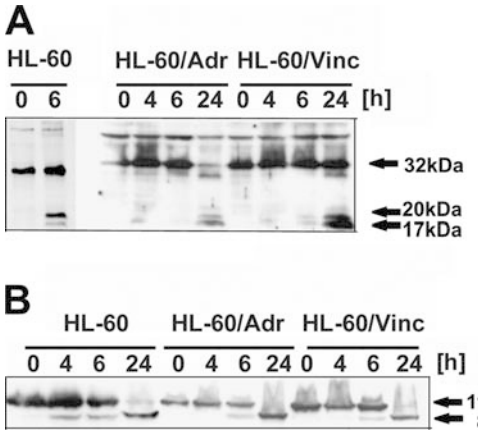

Fig. 5A, B Caspase-3 activity after curcumin treatment. The activity of caspase-3 was assessed by procaspase-3 (**A**) and PARP (**B**) proteolysis measured by Western blotting in HL-60, HL-60/Adr and HL-60/Vinc cells. Cells were untreated (*0*) or treated with curcumin as indicated. Unprocessed (32 kDa) and hydrolyzed forms of caspase-3 (20 kDa and 17 kDa) and two bands of its product PARP (116 kDa intact and 85 kDa hydrolyzed) are indicated. A representative blot of three independent experiments is shown

treatment, as revealed by flow cytometry, proved that the cells were undergoing caspase-3-dependent apoptosis (Fig. 6).

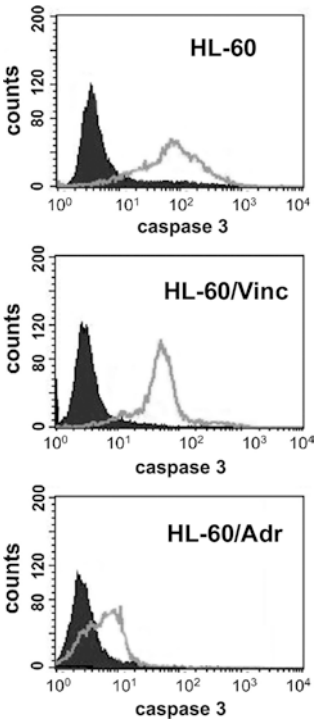

Fig. 6 Caspase-3 activity after UVC irradiation. The activity of caspase-3 was measured by flow cytometry in HL-60, HL-60/Vinc and HL-60/Adr cells untreated or irradiated with UVC at 100 J/m^2 and collected 5 h after treatment (*shaded histograms* control cells with inactive caspase-3, *unshaded histograms* cells with active caspase-3). A representative histogram of three independent experiments is shown

Discussion

Cancer therapy has improved in recent years owing to the better understanding of the mechanisms of cell death and the development of better-designed chemotherapy strategies. However, overcoming resistance to apoptosis, an intrinsic feature of many cancer cells causing a serious clinical problem, has become a real challenge. Development of the MDR phenotype, including the expression of P-gp and MRP1 protecting cells against drug-induced cell death, seems not to be connected with apoptosis per se. However, recently it has been postulated that functional P-gp might play a role in regulating cell death not only by removing drugs from the cell, but also by inhibiting the activation of proteases involved in apoptotic signaling (caspase-8) and execution (caspase-3) [9]. Thus it seems that successful treatment of P-gp$^+$ MDR tumors might be enhanced using chemotherapeutic agents that can function in the absence of caspase-3 activation. Indeed, Johnstone et al. have shown in several studies that P-gp$^+$ cells are resistant to apoptosis induced by agents known to act in a caspase-3-dependent way but that are not substrates for ABC cassette proteins, to which P-gp belongs [7, 8, 9]. On the other hand, they have shown that P-gp-expressing cells are not resistant to caspase-independent cell death mediated by pore-forming proteins and granzyme B as well as by other factors [23, 24]. Curcumin is an agent which at a concentration of 50 μM induces caspase-3-independent apoptosis in many normal and cancer lymphoid cells [1]. In agreement with the data obtained by Johnstone et al, we have recently shown that curcumin also causes cell death without caspase-3 activation in P-gp-expressing colon LoVo and lymphoid CEM cells [21].

As myeloid HL-60 cells, in contrast to other cells, respond to curcumin, by caspase-3-dependent apoptosis [1], we wanted to know how MDR HL-60 cells would respond to curcumin treatment. To this end, we used HL-60 cells expressing either P-gp or the *MRP1* gene product. For comparison, we treated cells with UVC which is a known caspase-3 inducer. In all the tested cells, curcumin caused cell death as shown by the TUNEL assay. Characteristic morphological changes of the nuclei, reflecting chromatin condensation and fragmentation and DNA fragmentation shown by flow cytometry, indicated that the cells had undergone apoptosis. Activation of caspase-3 and hydrolysis of PARP proved that it was caspase-3-dependent apoptosis. The lack of caspase-8 activation and the presence of cytochrome *c* in the cytosol indicated that the apoptotic signal was transduced via the mitochondrial pathway. Taken together our results convinced us that overexpression of P-gp in HL-60 cells did not influence the apoptotic pathway leading to activation of caspase-3.

Similarly, Cullen et al. have shown for many leukemia cell lines that MDR mediated by MRP and P-gp does not correlate with resistance to Fas-mediated cell death or resistance to caspase-3 activation [5]. However, taking into account the diversity of intrinsic factors which can modulate apoptotic pathways in different cancer cells, we cannot exclude the possibility that in some P-gp-expressing cell lines, parallel to P-gp expression, there may be other factors that block caspase-3. Indeed, Campone et al. have shown that P-gp-expressing HL-60 cells are resistant to caspase-3-dependent apoptosis due to the presence of XIAP in these cells. XIAP belongs to the family of inhibitor apoptosis proteins (IAP), and it has been shown to inhibit caspase-3 by binding to the predomain, thereby preventing the activation of procaspase-3 [4]. Increased basal levels of mRNA for some IAPs (c-IAP-2, NAIP and survivin) in P-gp-overexpressing HL-60 cells in comparison with sensitive HL-60 cells have also been observed by others, although no decrease in their levels under apoptotic inducers has been detected [18]. It has recently been shown that curcumin suppresses IAPs in human melanoma and renal Caki cells [3, 26].

Thus, it is possible that, even if there is a correlation between P-gp and IAP overexpression, curcumin can bypass it by IAP suppression. However, our preliminary unpublished data show that curcumin, in spite of decreasing XIAP expression in normal human T lymphocytes, is not capable of inducing caspase-3-dependent apoptosis. Thus, the situation still seems to be far from clear. This is additionally highlighted by the fact that UVC also induced caspase-3-dependent apoptosis

in MDR HL-60 cells with a mechanism unlikely be the same as in the case of curcumin. Altered expression or mutation of many genes encoding key apoptotic proteins may provide cancer cells with inherent resistance to apoptosis, but each cancer cell type is characterized by a specific phenotype which differs from others in its apoptotic regulation [6]. The best examples are lymphoid Jurkat and myeloid HL-60 cells which, upon treatment with 50 μM curcumin, follow completely different apoptotic pathways. Moreover, curcumin protects Jurkat cells from caspase-3-dependent apoptosis induced by UVC and renders them susceptible to caspase-3-independent cell death [20, 25]. On the other hand, we observed a synergism between curcumin and UVC in inducing apoptosis of HL-60 cells (data not shown).

Summing up, our results show yet again the complexity and diversity of the cell death process, especially of cancer cells, which makes any generalization next to impossible. It is imperative that many complementary experimental approaches are used, together with a wide variety of cell lines. Only then will it be possible to approach an understanding of cell death regulation.

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