Hypericin-induced photosensitization of HeLa cells leads to apoptosis or necrosis

Involvement of cytochrome *c* and procaspase-3 activation in the mechanism of apoptosis

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Abstract Here we report that photoactivated hypericin can induce either apoptosis or necrosis in HeLa cells. Under apoptotic conditions the cleavage of poly(ADP-ribose) polymerase (PARP) into the 85-kDa product is blocked by the caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) and benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk). Both inhibitors protect cells from apoptosis but cannot prevent hypericin-induced necrosis. Conversely, HeLa cells overexpressing the viral cytokine response modifier A (CrmA), which inhibits caspase-1 and -8, still undergo hypericin-induced apoptosis and necrosis. Evidence is provided for the release of mitochondrial cytochrome *c* in the cytosol and for procaspase-3 activation in the hypericin-induced cell killing.

Key words: Photodynamic therapy; Hypericin; Apoptosis; Necrosis; Caspase; Cytochrome *c*

1. Introduction

Cell death can be induced via two mechanisms: apoptosis or necrosis. Necrosis, which occurs in response to chemical and physical insults, leads to an increase in cytoplasmic volume, swelling of mitochondria and loss of plasma membrane integrity. The consequential release of the intracellular content into the extracellular milieu evokes inflammatory reactions. In contrast, apoptosis is a gene regulated cell death, which is characterized by cytoplasmic and nuclear shrinkage, membrane blebbing, chromatin condensation and genomic DNA fragmentation [1]. Recent work from many laboratories has validated the critical role of caspases in the execution of apoptosis. In the TNF- and Fas-induced apoptosis, caspase-8 is directly activated upon recruitment to either the TNF-R1 or Fas receptor signalling complex and initiates a cascade of proteolytic events, eventually leading to the activation of procaspase-3, one of the downstream executors of the apoptotic process [2]. Alternatively, procaspase-3 can be activated via receptor-independent pathways [3].

Photodynamic therapy (PDT) is a relatively new therapeutical approach for the treatment of superficial skin and mucosal cancers. The therapy involves the use of a photosensitizer, which is activated by local irradiation, and subsequently produces reactive oxygen species (ROS), ultimately leading to cell death [4]. PDT with several photosensitizers has been shown to induce both apoptosis and necrosis, in vitro as well as in vivo [4]. Hypericin is a photoactive pigment synthesized by members of the plant genus *Hypericum*. Hypericin exhibits in vitro antineoplastic activity [5,6] and we have recently reported that certain human tumors can be successfully treated with photoactivated hypericin [7,8]. We considered it of major interest to characterize the molecular mechanism(s) underlying hypericin-mediated cell photokilling.

Here, we report that photoactivated hypericin can induce either apoptosis or necrosis in HeLa cells. Both the broad-spectrum caspase inhibitor z-VAD-fmk and the caspase-3-like caspase inhibitor z-DEVD-fmk [9] efficiently protected cells from hypericin-induced apoptosis while neither one of them had any effect on the hypericin-mediated necrosis. In contrast, HeLa cells stably transfected with the viral cytokine response modifier A (CrmA), a serpin-like specific caspase-1 and -8 inhibitor [9], were still sensitive to both hypericin-induced apoptosis and necrosis. In addition, we found that cytochrome *c* is released in the cytosol of cells photosensitized by hypericin and that procaspase-3 becomes activated.

2. Materials and methods

2.1. Cell culture

HeLa cells (American Type Culture Collection, Rockville, MD, USA) and transfected HeLa cells expressing CrmA (CrmA-HeLa) or the empty vector (Hyg-HeLa) obtained as described in [10], were grown at 37°C in Dulbecco's Modified Eagle Medium containing L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% fetal calf serum. All the culture medium components were obtained from Gibco-BRL (Paisley, Scotland, UK).

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Abbreviations: PDT, photodynamic therapy; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; z-DEVD-fmk, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; CrmA, cytokine response modifier A; PARP, poly(ADP-ribose) polymerase; TNF, tumor necrosis factor-α; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; Hyg, hygromycin

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2.2. Cell photosensitization

Hypericin was prepared and used in cell culture experiments as described earlier [11,12]. Cells were incubated in subdued light conditions with hypericin for 16 h and irradiated with a light dose of 4 J/cm^2 in hypericin free medium.

2.3. Assessment of cell viability and morphology

Cells were seeded onto Lab-Tek Chamber slides (Nalge Nunc International, Naperville, IL, USA). Cell viability was estimated by trypan blue exclusion. Dead cells were counted in at least 10 microscopic fields with a minimum of 50 cells per field and expressed as percentage of dead cells over the total number of cells.

2.4. Nuclear fragmentation

Subconfluent cells, grown on Lab-Tek Chamber slides and treated as described before, were fixed in 100% cold methanol for 20 min, washed with PBS and stained with Hoechst 33342 (Sigma). The sample was then examined using a fluorescence microscope (Nikon). Apoptotic nuclei were counted in at least 10 fields with a minimum of 50 cells per field and expressed as a percentage of apoptotic nuclei over the total number of nuclei.

2.5. DNA fragmentation

Following photosensitization, 2.5×10^6 cells were washed with ice cold PBS, scraped and lyzed in 1 ml lysis buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 8, 25 mM EDTA and 0.5% SDS. After a centrifugation step, the supernatant was incubated for 2 h at 56°C with 0.2 mg/ml proteinase K. The DNA was precipitated with 2 volumes of absolute ethanol, resuspended in TE-buffer (10 mM Tris, pH 8, 1 mM EDTA) and treated for 1 h with 1 mg/ml DNase-free RNase prior to loading onto a 1.5% agarose gel. After electrophoresis, the DNA was stained with ethidium bromide and visualized under UV.

2.6. Western blotting

Following photosensitization, cells (1.5×10^6) were collected and washed with ice-cold PBS. For PARP or procaspase-3 analysis, cells were lyzed in buffer A containing 1% Triton X-100 [13]. Cell lysates for cytochrome *c* analysis were made in PBS with 0.02% (w/v) digitonin (Boehringer Mannheim, Germany) and 1 mM EDTA. After lysis, cells were collected, homogenized and centrifuged. Pellets of cell lysates made in digitonin-containing lysis buffer were resuspended in 200 μl lysis buffer A and re-processed as described. These samples were used as positive controls for the detection of mitochondrial cytochrome *c*. Samples were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The blots were blocked overnight at 4°C in TBST buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk and then incubated for 2 h at room temperature with either anti-PARP (Biomol Research Labs., Plymouth, PA, USA), anti-cytochrome *c* (Pharmingen, San Diego, CA, USA) or anti-caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Detection was carried out with the enhanced chemiluminescence detection system (ECL, Amersham International, UK).

2.7. Cell proliferation

Cells were seeded onto 24-well tissue culture plates (Falcon) at 2×10^4 cells per well, and incubated at 37°C. After adherence, cells were treated with hypericin as described before. The caspase inhibitors z-VAD-fmk and z-DEVD-fmk (Enzyme Systems Products, Livermore, CA, USA) were added under subdued light conditions 2 h before irradiation and re-added every 24 h. At each time point, cell proliferation was determined by quantification of the cellular protein content using naphthol blue black (Acros, Beerse, Belgium) as described in [14]. The absorbance was measured at 620 nm using a microtiter plate reader (SLT, Salzburg, Austria).

3. Results

3.1. Hypericin can induce either apoptosis or necrosis in HeLa cells

Recently, we reported that hypericin causes photocytotoxicity in different human cancer cell lines [6,12]. Using HeLa cells as a model system, we investigated in more detail the type of the hypericin-induced cell death, i.e. apoptosis or necrosis. HeLa cells were incubated for 16 h under strictly subdued light conditions with different concentrations of hypericin ranging from 80 nM to 10 μM. Cells were subsequently irradiated and all further incubations were done in dark conditions. The type of cell death was evaluated over a 72-h period, using different criteria. Cells incubated with 80 to 250 nM photoactivated hypericin showed distinctive apoptotic features as shown in Fig. 1. As indicated by the incorporation of trypan blue (Table 1), most of the cells lost their membrane integrity only 24 h after irradiation. Cell death was strictly light- and hypericin-dependent, since HeLa cells treated with similar amounts of hypericin and protected from light were not affected (data not shown). Nuclear staining with Hoechst 33342 confirmed that cells treated with 125 nM photoactivated hypericin were apoptotic, as demonstrated by their condensed and fragmented nuclei (Fig. 1). The percentage of apoptotic nuclei was 50% 24 h after irradiation and further increased up to 74% within 48 h (Table 1). In contrast, HeLa cells exposed to ≥1 μM hypericin died very fast after irradiation, as shown by trypan blue uptake (Table 1). This type of

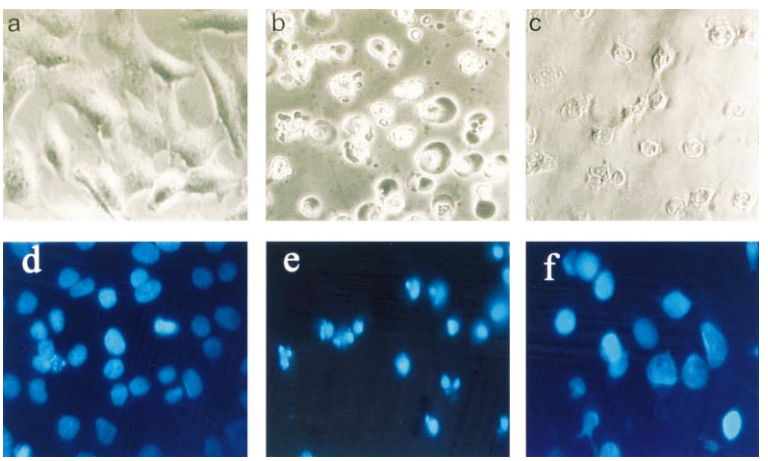

Fig. 1. Photoactivated hypericin can induce apoptosis or necrosis in HeLa cells. Phase-contrast microscopic analysis (a–c) and nuclear Hoechst 33342 staining (d–f) of control cells (a, d) and cells treated with either 125 nM (b, e) or with 1 μM hypericin (c, f) for 16 h. Cells were then exposed to light for 15 min at 4.5 mW/cm^2, to give a total dose of 4 J/cm^2 and analyzed 24 h post-irradiation as described in Section 2.

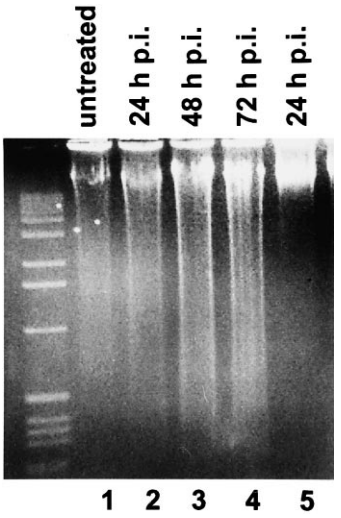

Fig. 2. Time-dependent DNA fragmentation following hypericin photosensitization of HeLa cells. Control cells (lane 1) and cells treated with either 125 nM (lanes 2–4) or 1 μM (lane 5) photoactivated hypericin (4 J/cm^2) were processed for DNA fragmentation analysis at the indicated time periods post-irradiation (p.i.).

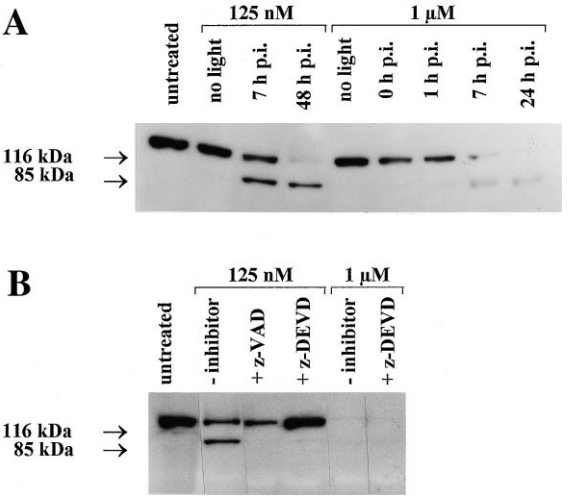

Fig. 3. Induction of PARP cleavage by photoactivated hypericin. A: Time-dependent proteolytic PARP cleavage by 125 nM (lanes 2–4) or 1 μM (lanes 5–9) photoactivated hypericin (4 J/cm^2). Controls are: lane 1, untreated cells; lanes 2 and 5, cells treated with 125 nM and 1 μM hypericin, respectively, without irradiation. Cell lysates were prepared at the indicated time post-irradiation (p.i.), subjected to SDS-PAGE and transferred to PVDF membranes. Blots were then probed with polyclonal anti-PARP antibody and revealed by ECL. B: PARP cleavage induced by apoptotic concentration of photoactivated hypericin is blocked by z-VAD-fmk and z-DEVD-fmk. Cells were treated with 125 nM hypericin (lanes 2–4) or with 1 μM hypericin (lanes 5 and 6). z-VAD-fmk (100 μM) and z-DEVD-fmk (100 μM) were added 2 h prior to light exposure (4 J/cm^2). Cells were lyzed 24 h after irradiation and processed as in A.

hypericin-induced cytotoxicity was considered as necrotic cell death since cells appeared swollen and nuclei were clearly enlarged with no sign of nuclear fragmentation (Fig. 1). Although some kind of membrane blebbing could be observed immediately after irradiation (not shown), this was clearly stopped by the progressive cell swelling (Fig. 1). DNA fragmentation analysis by agarose gel electrophoresis showed a significant time-dependent increase in the amount of low molecular weight DNA extracted from cells treated with 125 nM photoactivated hypericin. No fragmentation was observed when 1 μM photoactivated hypericin was used (Fig. 2). It should be mentioned that despite numerous efforts, using different protocols, no clear DNA laddering could be visualized in apoptotic HeLa cells, an observation which has also been reported by others using different apoptotic stimuli [15].

3.2. Mechanism of hypericin-induced cell death

Caspase-mediated poly(ADP-ribose) polymerase (PARP) cleavage is considered to be a hallmark of the execution of the apoptotic program [16]. Fig. 3A shows a Western blot analysis of the time course of PARP cleavage in apoptotic and necrotic cell death induced by photoactivated hypericin. Untreated control cells and cells exposed to hypericin but not irradiated revealed only a single protein band at 116 kDa, which corresponds to the intact PARP protein (Fig. 3A, lanes 1, 2 and 5). Cells exposed to photoactivated hypericin under apoptotic conditions (Fig. 3A, lanes 3, 4) showed the characteristic 85-kDa PARP cleavage product which started to accumulate between 3 and 7 h after irradiation. The cleavage was complete 48 h after irradiation, a time point at which also a profound DNA fragmentation was observed (Fig. 2). In lysates of cells treated with 1 μM hypericin the full size PARP protein could be still detected 1 h post-irradiation (Fig. 3, lane 7), albeit after that time-period approximately 90% of the cells incorporated trypan blue (Table 1). After prolonged post-irradiation periods, both the intact PARP protein and its cleaved product became hardly detectable by the anti-PARP antibody (Fig. 3A, lanes 8, 9). Although an equal amount of proteins was loaded for Western blot analysis, it is possible that the deficient PARP detection is due to selective protein losses resulting from cell lysis during necrosis.

Since caspases are activated by hypericin treatment, as demonstrated by the specific PARP cleavage, we investigated whether the cell-permeable synthetic caspase inhibitors z-VAD-fmk and z-DEVD-fmk and the serpin-like inhibitor, viral cytokine response modifier A (CrmA), could influence the hypericin-induced cell death. Microscopic evaluation of the cells treated with either z-VAD-fmk or z-DEVD-fmk in com-

Table 1
Time course of cytotoxicity induced by photoactivated hypericin in HeLa cells

Time post irradiation	125 nM hypericin		1 μM hypericin	
	% dead cells	% apoptotic nuclei	% dead cells	% apoptotic nuclei
1 h	1.9 ± 0.7	n.d.	91 ± 2	0
7 h	7.2 ± 3	n.d.	100	0
24 h	32 ± 5	50 ± 4	100	0
48 h	70 ± 32	74 ± 7	100	0

The percentage of dead cells was evaluated by trypan blue uptake and apoptotic nuclei were scored after Hoechst 33342 staining as described in Section 2. Results are means of independent determinations ($n > 3$) and the standard deviation is indicated. n.d. = not determined.

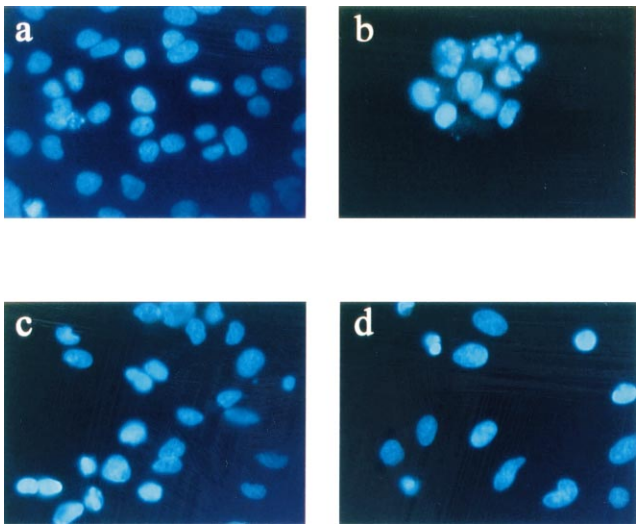

Fig. 4. The caspase inhibitors z-VAD-fmk and z-DEVD-fmk protect HeLa cells from photoactivated hypericin-induced apoptosis. Fluorescent microscopic analysis of HeLa cell nuclei stained with Hoechst 33342 24 h post-irradiation. Untreated cells (a) and cells treated with 125 nM hypericin (b–d) for 16 h. Two hours before light exposure (4 J/cm^2) cells were incubated with either 100 μM z-VAD-fmk (c) or 100 μM z-DEVD-fmk (d).

bination with 125 nM photoactivated hypericin showed an increased survival of cells as compared to control cells. Addition of either z-VAD-fmk or z-DEVD-fmk prior to irradiation reduced the number of apoptotic nuclei, as judged by Hoechst 33342 staining (Fig. 4), from approximately 80% to less than 25%. The pretreatment with z-DEVD-fmk particularly affected the apoptotic morphological changes by drastically counteracting membrane blebbing (not shown). These observations together with the inhibitory effect on the hypericin-induced PARP cleavage (Fig. 3B, lanes 3 and 4), suggest that the anti-apoptotic or protective action of z-VAD-fmk and z-DEVD-fmk is the result of caspase inhibition. No effect of z-VAD-fmk or z-DEVD-fmk was observed when cells were exposed to necrotic concentrations of photoactivated hypericin (not shown). Moreover, the loss or degradation of full size PARP under necrotic conditions was not affected by the presence of caspase-inhibitors (Fig. 3B, lane 6).

The role played by the caspases-1 and -8 in the hypericin induced cell death, was addressed using CrmA-overexpressing HeLa cells. Cells expressing the empty vector, as selected by hygromycin (Hyg-HeLa), were used as control cells. In contrast to what was found with the peptide caspase inhibitors, the CrmA-overexpressing HeLa cells were still killed by apoptotic concentrations of photoactivated hypericin, while being resistant to TNF- and Fas-induced apoptosis (not shown).

As shown in Fig. 5A, both z-VAD-fmk and z-DEVD-fmk efficiently counteracted cell death and completely restored cell growth 24 h post-irradiation, but they only partially prevented cell growth inhibition 48 and 72 h after irradiation. It should be noted that the inhibitors themselves did not affect the level of cell proliferation (data not shown). As shown in Fig. 5B, there were no major differences in the proliferation pattern of CrmA-overexpressing and Hyg-HeLa cells upon treatment with photoactivated hypericin.

Taken together, these observations suggest that the hypericin-induced apoptosis is not mediated by caspase-1 or caspase-8, but involves a z-VAD-fmk and z-DEVD-fmk-inhibitable caspase activity. This would also suggest that the death domain receptors, such as Fas or TNF-R1, are less likely to be involved in hypericin-induced apoptosis since these cytotoxic activities are sensitive to CrmA inhibition (data not shown and [17,18]).

3.3. *Photoactivated hypericin induces release of cytochrome c and activation of procaspase-3*

Apoptosis induced by different agents coincides with the release of cytochrome *c* from mitochondria into the cytosol, where it acts as a cofactor in the activation of procaspase-9, eventually leading to procaspase-3 activation [3]. Our observation that z-DEVD-fmk does inhibit hypericin-induced apoptosis suggests that a caspase-3-like activity could be involved. As shown in Fig. 6 (lanes 2–4), apoptotic concentrations of photoactivated hypericin triggered the mitochondrial release of cytochrome *c* into the cytosol of HeLa cells in a time-dependent manner. The cytochrome *c* accumulated in the cytosol of photosensitized cells as early as 1 h post-irradiation and remained detectable for up to 24 h. The release of cyto-

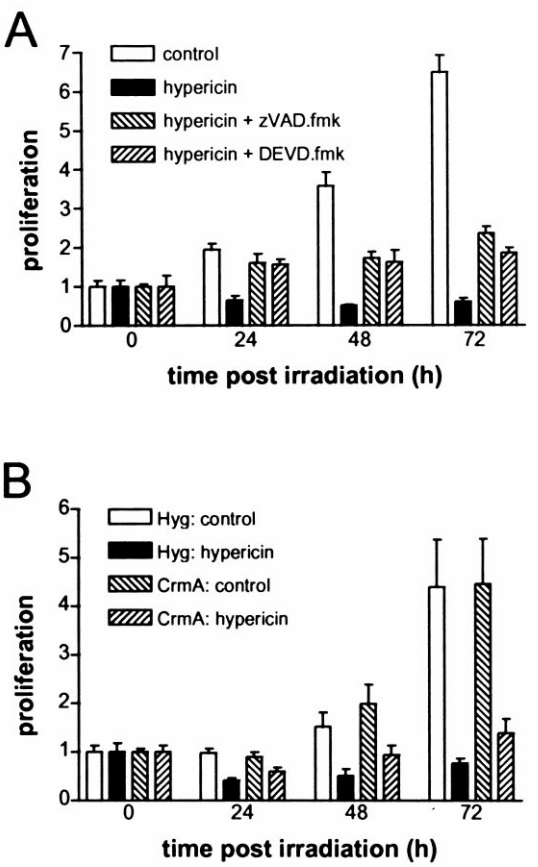

Fig. 5. Proliferation pattern of photoactivated hypericin-treated HeLa cells in the presence of caspase inhibitors. Panel A: HeLa cells were incubated or not (control) with 125 nM hypericin for 16 h. z-VAD-fmk (100 μM) and z-DEVD-fmk (100 μM) were added 2 h before exposing the cells to light (4 J/cm^2). Following irradiation, cells were incubated in the dark and cell proliferation determined at the indicated time periods. Panel B: Hyg-HeLa cells (Hyg) and CrmA-overexpressing HeLa cells (CrmA) were incubated or not (controls) with 250 nM hypericin for 16 h, irradiated and processed as in A. Results are the mean values of 3 independent determinations and vertical bars indicate the standard deviation.

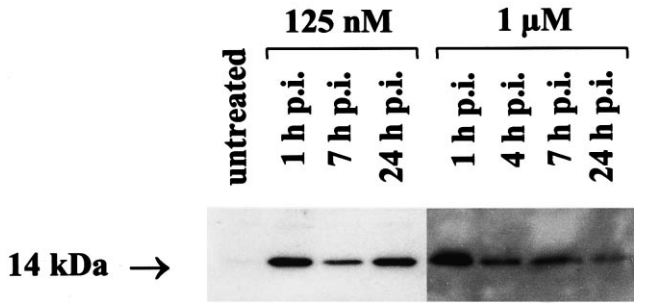

Fig. 6. Photoactivated hypericin induces a fast release of cytochrome *c* in the cytosol. Lysates of HeLa cells photosensitized (4 J/cm^2) by 125 nM (lanes 2–4) or 1 μM (lanes 5–8) hypericin and prepared at the indicated time post-irradiation (p.i.) were subjected to SDS-PAGE, blotted to PVDF membrane, probed with anti-cytochrome *c* antibodies and revealed by ECL.

chrome *c* was observed between 1 and 7 h following irradiation, clearly preceding the onset of PARP cleavage (Fig. 3A). Surprisingly, when we increased the concentration of hypericin to 1 μM to produce the necrotic response, we still could detect a very fast cytochrome *c* release (Fig. 6, lanes 5–8). Under these conditions, the released cytochrome *c* was observed immediately following cell photosensitization (not shown), thus before necrotic destruction of the cell had already occurred.

We next investigated whether the release of cytochrome *c* in the cytosol was accompanied by the activation of procaspase-3. As shown in Fig. 7 apoptotic concentrations of photoactivated hypericin induced the cleavage of the 32-kDa procaspase-3 into its 11-kDa active fragment. This observation together with the inhibitory effect of z-DEVD-fmk on the apoptotic program (see Figs. 3–5), suggests that caspase-3 is a key mediator of the hypericin-induced apoptosis in HeLa cells.

4. Discussion

In this study we report that the type of hypericin-induced cell photokilling can be shifted from apoptosis to necrosis by increasing the concentration of hypericin applied to the cells (Figs. 1 and 2). The cellular response to 125 nM photoactivated hypericin is largely, if not exclusively, apoptotic. Increasing the hypericin concentration to 1 μM leads to necrosis. A shift from an apoptotic to a necrotic response, can be produced by increasing the light dose and by changing the

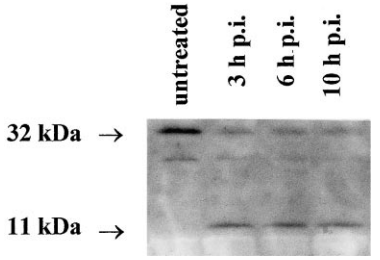

Fig. 7. Photoactivated hypericin induces procaspase-3 cleavage. HeLa cells were treated (lanes 2–4) or not (lane 1) with 125 nM hypericin for 16 h. Cells were lyzed at the indicated times following irradiation (4 J/cm^2). Cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane, probed with polyclonal anti-caspase-3 antibodies and revealed by ECL.

preincubation time, as it has been reported for the widely used photosensitizers Photofrin and chloroaluminum phthalocyanine [19,20]. Similarly, we have also observed that a conversion from apoptotic to necrotic cell death can be produced by increasing the light doses applied to the cells in the presence of nanomolar hypericin concentrations (results not shown). Hypericin-induced apoptosis has been reported in studies dealing with its inhibitory effect on protein kinase C [21–24]. Unfortunately, in these studies little attention was paid to light and photosensitization conditions, making the experimental setups described very difficult to reproduce.

Apoptotic concentrations of photoactivated hypericin induce a time-dependent cleavage of PARP into the 85-kDa fragment (Fig. 3A). PARP is one of the nuclear substrates of the effector caspase-3 and -7 [16]. Fig. 3B illustrates that the addition of either the broad spectrum caspase inhibitor z-VAD-fmk or the caspase-3-like inhibitor z-DEVD-fmk prior to light activation completely prevents the hypericin-induced PARP fragmentation. These data suggest that caspase-3 and/or caspase-7 might participate in the apoptotic pathway triggered by photoactivated hypericin in HeLa cells. In necrotic conditions, the intact PARP protein and its proteolytic fragment were only faintly immunodetectable and the addition of the former caspase inhibitors could not reverse this effect (Fig. 3A and B).

Interestingly, in two recent studies where the photosensitizer chloroaluminum phthalocyanine was used in different cell lines, either preservation of the intact PARP protein [20] or complete conversion into the 85-kDa fragment [25] was observed with high (necrotic) PDT doses. Thus it is possible that excessive cell photosensitization still allows the initiation of the apoptotic program which is then overruled by the extensive oxidative stress produced and necrosis occurs either before or after a caspase-dependent PARP cleavage takes place.

Besides counteracting PARP cleavage, both caspase inhibitors z-VAD-fmk and z-DEVD-fmk efficiently antagonize hypericin-induced apoptosis (Fig. 4) and particularly z-DEVD-fmk prevents the appearance of membrane blebbing. Conversely, HeLa cells overexpressing the serpin-like inhibitor CrmA, which preferentially targets caspase-1 and -8 [9], are not protected from the apoptotic action of hypericin. Since CrmA overexpression efficiently blocks the TNF and Fas-induced apoptotic cell death in this cell line, we can conclude that hypericin induces apoptosis independently from the TNF-R1- or Fas-mediated caspase pathway. The proliferation results shown in Fig. 5 illustrate that the anti-apoptotic effect of both z-VAD-fmk and z-DEVD-fmk is particularly evident 24 h post-irradiation, while the CrmA overexpressing cells do not exhibit significant protection at any time point examined. However, cells treated with photoactivated hypericin in the presence of z-VAD-fmk and z-DEVD-fmk seem to have lost their proliferative potential, as shown by the cytostatic effect of these inhibitors on cell proliferation at later time points.

Collectively taken, these results clearly show that caspase function is required for photoactivated hypericin-induced apoptosis and suggest that a downstream DEVD-caspase activity is a key mediator in this process. Moreover, the fact that none of the three caspase inhibitors used has an effect on hypericin-mediated necrosis, supports the view that the two ways of hypericin-induced cell death do not share similar proteolytic activities.

Recent reports have revealed that the release of the cytochrome *c* from the mitochondria into the cytosol in response to different apoptotic insults, is involved in the proteolytic activation of procaspase-3 [3]. Very little information is available on the release of cytochrome *c* and other mitochondrial activity changes in cells undergoing necrosis. Fig. 6 shows that cytochrome *c* accumulates rather fast and remains detectable up to 24 h in the cytosol of cells photosensitized by 125 nM hypericin, but also by 1 μM hypericin. While in both conditions the release of cytochrome *c* precedes the onset of PARP cleavage (Fig. 3), nuclear fragmentation occurs only in the apoptotic condition (Figs. 1 and 2). This suggests that photoactivation of 1 μM hypericin might initially trigger the apoptotic program (see also Fig. 3A) which is later overruled by excessive photodamage. Alternatively, the necrotic cell damage could also lead to the aspecific release of cytochrome *c*.

The result depicted in Fig. 7 clearly confirms that photoactivated hypericin induces the cleavage and activation of procaspase-3. Interestingly, procaspase-3 activation was also observed with the photosensitizer benzoporphyrin derivative monoacid ring A [26]. Since all the apoptotic activities reported are preferentially blocked by z-DEVD-fmk, we can conclude that the induction of the apoptotic program by photoactivated hypericin is most likely dependent on caspase-3 activity. Furthermore our data suggest that the release of the mitochondrial cytochrome *c* in the cytosol might be the triggering factor upstream of procaspase-3 activation.

Recently it has been reported that 1 μM photoactivated hypericin induces a marked and irreversible decrease in the total cellular ATP levels and in the mitochondrial respiratory function [27]. This observation suggests that mitochondria are major targets of hypericin-induced cellular photodamage. The intracellular ATP level has been recognized as a crucial factor in determining the cell death fate, and in some instances apoptotic signals may be converted in necrotic signals when cellular ATP depletion reaches a critical level [28]. Obviously, further studies will be required to identify the upstream events leading to procaspase-3 activation and the precise role of mitochondria in determining the nature of hypericin-induced cell death.

During PDT in vivo both necrosis and apoptosis are likely to be induced and it can be argued that necrosis will occur at the surface of the tumor where cells will be exposed to a high light dose whereas interior areas will receive light doses sufficient to induce apoptosis. For treatment of small size tumors as in the case of micrometastases, the possibility to discriminate between these two types of cell death might be of therapeutic relevance.

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