Promotion of photodynamic therapy-induced apoptosis by stress kinases

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Abstract

Photodynamic therapy (PDT), a cancer treatment that employs a photosensitizer and visible light, induces apoptosis in murine LY-R leukemic lymphoblasts and in CHO cells, but the rate and extent of apoptosis are much greater in LY-R cells. Three MAPK family members, ERK1/ERK2, SAPK/JNK, and p38/HOG, are important intermediates in signal transduction pathways. To ascertain whether activation of one or more MAPKs could mediate PDT-induced apoptosis, Western blot analysis has been performed on the proteins of LY-R and CHO cells at various times following lethal (90–99% cell kill) doses of PDT photosensitized by the phthalocyanine Pc 4. The blots were probed with antibodies to each of the proteins as well as antibodies specific for the activated (phosphorylated) forms of each kinase. Of the three MAPK types, only the p46 and p54 SAPK/JNKs were found to be activated by PDT in LY-R cells, with a maximum ~threefold increase in the content of the phosphorylated forms reached in 30–60 min. An even larger relative activation was observed in CHO cells. PDT did not affect ERK and p38/HOG activation in LY-R cells. In the case of CHO cells, however, ERK2 was slightly activated at 5 min post-PDT, then declined, and p38/HOG was strongly activated from 5 to 60 min post-PDT. A specific inhibitor (PD98059) of MEK1, the kinase that activates ERK, had little or no effect on PDT-induced apoptosis in either LY-R or CHO cells. In contrast, a specific inhibitor of p38/HOG (SB202190) blocked PDT-induced apoptosis in LY-R cells with a lesser effect in CHO cells. The results suggest that both the SAPK and p38/HOG cascades can be stimulated by PDT and that the latter participates in both rapid and slow PDT-induced apoptosis. Furthermore, the high level of constitutively active p38/HOG in LY-R cells may poise those cells for rapid activation of apoptosis following PDT.

Keywords: photodynamic treatment; phthalocyanine; Pc 4; apoptosis; mitogen-activated protein kinases; PD98059; SB202190

Abbreviations: CHO, Chinese hamster ovary; ECL, Enhanced chemiluminescence; ERK, extracellular regulated kinase; FITC, fluorescein isothiocyanate; LY-R, L5178Y-R; MAPK, mitogen-activated protein kinase; p38/HOG, p38/hyperosmotic glucose-regulated kinase; PDT, Photodynamic therapy; PI, propidium iodide; PMA, phorbol myristate acetate; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; SDS, sodium dodecyl sulfate

Introduction

Photodynamic therapy (PDT) employs a tumor-localizing photosensitizer and visible light to produce singlet oxygen and other reactive oxygen species,^{1–3} leading to lipid peroxidation and membrane damage and finally cell death and tumor ablation. PDT induces apoptosis in mouse L5178Y-R (LY-R) lymphoma cells⁴ as well as a variety of other cell types.^{5–7} In our previous investigation into the mechanism of PDT-induced apoptosis in LY-R cells, we found that PDT stimulates a series of membrane-associated reactions, including activation of phospholipases C and A₂,⁸ the release of intracellular Ca²⁺,⁸ and the increased synthesis of the lipid second messenger ceramide.⁹

Apoptosis is a prominent mechanism of cell death following the exposure of many cell types to toxic agents.¹⁰ In order to elucidate the signaling pathways responsible for the regulation of apoptosis, the roles of the members of the MAPK family have been widely studied, because these kinases are important intermediates that convert extracellular signals into intracellular responses. Xia *et al.*¹¹ first reported the opposing effects of the MAPK family members, ERK1 and ERK2 vs SAPK/JNK and p38/HOG, on apoptosis. They found that SAPK and p38 were activated and ERKs were inhibited in PC-12 pheochromocytoma cells when apoptosis was induced by the withdrawal of nerve growth factor. The opposing effects of SAPK and ERK on apoptosis induced by other stimuli, including ceramide, adriamycin, ionomycin, nitric oxide and Fas, as well as oxidative stresses, e.g., tumor necrosis factor, X-rays, H₂O₂, and UVC, have also been reported.^{12–16} Recently, the role of p38 in the control of apoptosis has been studied using specific inhibitors of this kinase. Frasch *et al.*¹⁷ reported that stresses, such as UV, hyperosmolarity, or sphingosine, activated p38 and that the resultant apoptosis could be inhibited by the p38 inhibitor SK&F86002. Two related pyridinyl imidazoles, SB203580 and SB202190, inhibit both the α and β isoforms of p38.^{18–20} SB203580 has been shown to inhibit BCR (B cell receptor)-induced apoptosis in human B cell lines²¹ and apoptosis of neural cells induced by either trophic factor withdrawal or glutamate treatment and sodium salicylate-induced apoptosis in fibroblasts.^{22–24}

In spite of expanded interest in studying the importance of the MAPK family on apoptosis, little is known concerning the potential involvement of these kinases in apoptosis induced by PDT. The activation of SAPK and p38/HOG by PDT has been reported for both murine²⁵ and human²⁶ keratinocytes and other human cells.²⁶ However, neither group presented evidence concerning the level of cell death achieved by the PDT doses studied, whether the cells died by apoptosis or necrosis, or whether the stimulated pathways mediated cell death or were epiphenomena.

There is now strong evidence that PDT with photosensitizers that localize to mitochondria, such as Pc 4,²⁷ promotes the prompt efflux of cytochrome c that initiates the caspase-9 and -3-dependent pathway of apoptosis.^{28–30} However, it is clear that the rate and extent of apoptosis can be modulated by certain signaling pathways. For example, we recently showed that the non-receptor tyrosine kinase Etk provides an anti-apoptotic signal in prostate cancer cells.³¹ In order to determine whether the MAPKs are involved in the regulation of PDT-induced apoptosis, we have studied two cell lines: LY-R cells, that undergo rapid induction of apoptosis beginning as early as 15 min post-PDT, and CHO cells, that initiate apoptosis more slowly. The level of phosphorylation of ERK1/ERK2, SAPK/JNK, and p38/HOG was examined in untreated and PDT-treated cells.

The relationship between the activation of these MAPK family members and apoptosis was investigated.

Results

Photodynamic therapy (PDT) is a potent inducer of apoptosis in many types of cells.⁷ In our previous studies, it was found that PDT with the phthalocyanine photosensitizer Pc 4 induces apoptosis in both LY-R and CHO cells, as measured by oligonucleosomal DNA fragmentation, morphological changes, and flow cytometric analysis.^{4,8,32} However, the two cell lines differed in both the rate and extent of apoptosis in response to PDT. Apoptosis occurred rapidly in LY-R cells, beginning as early as 10–20 min post-PDT⁸ and ultimately involving all cells in the culture. The response was slower and less extensive in CHO cells.³²

In order to confirm the time course and extent of apoptosis in PDT-treated LY-R and CHO cells, the TUNEL assay and flow cytometry were employed (Figure 1). Apoptotic cells were labeled by addition of fluorescein isothiocyanate-dUTP (FITC-dUTP) to DNA ends using terminal deoxynucleotidyl transferase, and DNA was further labeled with the intercalating dye propidium iodide (PI). The apoptotic cells appear as a second population with high fluorescein fluorescence. Figure 1 displays

Figure 1 PDT-induced apoptosis in LY-R and CHO cells as measured by flow cytometry. For PDT, cells were incubated with 0.5 μ M Pc 4, then irradiated with 3 kJ/m² and 10 kJ/m² for LY-R and CHO cells, respectively. Controls were exposed to Pc 4 but not light. At the indicated post-PDT time, cells were stained with FITC-dUTP and PI and analyzed by flow cytometry, as described in Materials and Methods. The upper and lower series of histograms are for LY-R cells (A) and CHO cells (B). The number under each histogram is the % apoptotic cells calculated as the percentage of cells with high FITC staining (box in each histogram)

representative flow cytometry histograms and the estimated percentage of apoptotic cells in LY-R (Figure 1A) and CHO cells (Figure 1B) which were either untreated or treated with isotoxic ($\sim$ LD 99) PDT doses. As shown in Figure 1A, 29% of the LY-R cells were undergoing apoptosis by 45 min after PDT, and the apoptotic cell population reached 72% by 2 h post-PDT. In contrast, only 6% of the CHO cells were observed to be in apoptosis 2 h post-PDT, and the content of apoptotic cells increased to 21% by 6 h post-PDT (Figure 1B). These time courses are in agreement with previous more extensive analyses.^{9,32,33} The results confirmed that apoptosis induced by PDT in CHO cells was slower and less extensive than in LY-R cells.

We have reported^{8,9,33} that PDT stimulates a series of membrane-associated signal transduction reactions. We have also found that PDT causes a rapid stimulation of the tyrosine phosphorylation of two major proteins of Mr $\sim$ 80 000 and $\sim$ 55 000 in LY-R cells; the response was PDT dose-dependent and occurred as early as 20 s after the initiation of light exposure of Pc 4-preloaded cells.³³ By immunoprecipitation and Western blot analysis, one of the Mr $\sim$ 80 000 phosphorylated proteins has been identified as HS1, a substrate of non-receptor-type src protein tyrosine kinases. To further elucidate the signaling pathways responsible for the regulation of PDT-induced apoptosis, the ability of PDT to activate three major MAPK sub-types (ERK, SAPK and p38/HOG) was investigated. Figure 2 presents Western blot analyses of proteins from control and PDT-treated LY-R and CHO cells using a highly sensitive phospho-specific anti-p44/42 ERK antibody which recognizes only the phosphorylated forms of these proteins. As a positive control, some cultures were incubated with 100 nM phorbol myristate acetate (PMA) for 20 min. As shown in the top panels of Figure 2A,B, ERK2 (42 kDa) was found to be partially activated in untreated cells of both LY-R and CHO cultures, and PMA produced a strong activation of ERK2 and a lesser response of ERK1 (44 kDa). In the case of LY-R cells, doses of PDT that result in 90 or 99% loss of clonogenicity (0.5 μ M Pc 4 and 2.1 kJ/m² or 3 kJ/m², respectively) produced no detectable change in the level of phosphorylation of either ERK1 or ERK2 at any time up to 1 h post-PDT. In contrast, equitoxic doses of PDT to CHO cells resulted in a slight elevation of the level of phospho-ERK2 at 5 min post-PDT (top and bottom panels of Figure 2B).

The bottom panel of Figure 2A and the middle panel of Figure 2B show Western blot results using the control anti-ERK antibody to correct for unequal protein loading. As shown by the densities of the ERK1 bands, there was approximately equal loading among these samples. However, the densities of the ERK2 bands in samples from PMA-treated cells and from CHO cells 5 min after PDT were weaker than in other samples. One possible explanation is that the control anti-ERK antibody is less sensitive to phosphorylated ERK2 than to non-phosphorylated ERK2. Based on the above observations and the results of staining gels with Coomassie blue to confirm sample loading (data not shown), the ERK1 band, rather than ERK2, was used as a standard to calculate the relative amount of phosphorylated ERK2 in Figure 2B. The

Figure 2 ERK phosphorylation in PDT-treated LY-R and CHO cells. Cells were untreated or treated with 0.5 μ M Pc 4 for 18 h, then exposed to 2.1 or 3 kJ/m² for LY-R cells (**A**) and 3 or 6.5 kJ/m² for CHO cells (**B**). Cells were collected at the indicated times post-PDT. Equal amounts of protein were loaded on minigels, electrophoresed and transferred to PVDF membranes and probed with a phospho-specific ERK1/ERK2 antibody (top panels) and a control ERK1/ERK2 antibody (lower panel of **A**; middle panel of **B**). The left lanes contained proteins from cells treated with 100 nM PMA at 37°C for 20 min. In (**A**), the apparent slight increase in intensity of the 42 kDa band at 5 min post-PDT was not reproduced in two other experiments. To quantify the results for CHO cells (**B**), the bands from this and two other independent experiments were scanned by densitometry. The ratio of densities of each phospho-ERK2 band (upper panel) and the corresponding non-phosphorylated ERK1 (middle panel) was calculated (see text), normalized to the control ratio, and the mean $\pm$ standard deviation plotted (bottom panel of **B**)

bottom panel of Figure 2B presents the results for ERK2 activation, calculated as the ratio of the densities of the phosphorylated ERK2 band to that of the non-phosphorylated ERK1 band for each sample, normalized to the control, and averaged from three independent experiments. This analysis demonstrates that ERK2 phosphorylation in CHO cells increases about 50% over the baseline level by 5 min following either an LD90 or an LD99 dose of PDT and falls thereafter. For cells treated with the higher PDT dose, ERK2 phosphorylation decreased to about 10% of the untreated control 45 and 60 min after treatment.

The possible involvement of SAPK/JNK in PDT-induced apoptosis was investigated (Figure 3A,B) by Western blot analysis with antibodies recognizing the phosphorylated (Figure 3A,B, panels a) and total (Figure 3A,B panels b) SAPK/JNK. For both LY-R and CHO cells, the panels b of Figure 3A,B indicate approximately equal loading of SAPK protein from each sample, whereas the panels a of Figure 3A,B reveal a marked increase in the phosphorylation of both p54 and p46 SAPKs, beginning as early as 15 min post-PDT and continuing for at least 1 h. For both cell lines, the data for this and two additional experiments were quantified by densitometric scanning of the bands. The data for LY-R cells (Figure 3A) were normalized to the untreated control cells. In contrast, because of the very low level of phosphorylated SAPK in control CHO cells, the

Figure 3 PDT-induced SAPK activation in LY-R and CHO cells. The same samples from control and PDT-treated LY-R and CHO cells as described in Figure 2 were separated on minigels, transferred to PVDF membranes, and probed with a phospho-specific anti-SAPK antibody (panel a) and a control anti-SAPK antibody (panel b). (A) SAPK activation in PDT-treated LY-R cells. The left lane contains proteins from UVC-treated 293 cells as a positive control. (B) SAPK activation in PDT-treated CHO cells. The lanes marked P contain proteins from cells treated with anisomycin (20 μ g/ml for 30 min). The positions of p54 and p46 SAPKs are indicated by arrows. Panels c and d represent the relative levels of phosphorylated p54 and p46, calculated as the ratio of the density in panel a to that in panel b for each sample and further normalized to the control (A) or not (B). Each datum in panels c and d is the mean $\pm$ s.d. of results from three independent experiments

data presented in panels c and d of Figure 3B were not normalized. As revealed in panels a, c and d of Figure 3A, the earliest response in PDT-treated LY-R cells was observed at 15 min, and the activation continued for at least 1 h. Similar time courses were observed for the LD90 and LD99 PDT doses. For CHO cells, a strong response was observed in PDT-treated CHO cells that reached a maximum level by 15 min, and the high level of phosphorylation of both p54 and p46 persisted for at least 1 h (Figure 3B).

Figure 4 shows the effect of PDT on p38/HOG activation. In LY-R cells, the level of phosphorylated p38/HOG was constitutively high, and PDT did not appear to increase it further over the first hour after either dose (Figure 4A). In contrast, little or no p38/HOG was found to be phosphorylated in untreated CHO cells (Figure 4B). However, PDT resulted in marked p38/HOG activation that was both time- and dose-dependent (Figure 4B). The

Figure 4 The response of p38/HOG to PDT in LY-R cells (A) and CHO cells (B) detected by a phospho-specific p38/HOG antibody. The same samples described in Figure 2 were separated on minigels, transferred and blotted with a phospho-specific p38/HOG antibody (top panels) and a control anti-p38 antibody (bottom panel in A; middle panel in B), then detected by ECL. The left lane in B represents anisomycin-treated (20 μ g/ml for 30 min) cells. In (A) the slightly greater intensity of the phospho-p38 band at 5 min post-PDT was not observed in two other experiments. In (B) the changes in phosphorylation were quantified by densitometry and calculated as the ratio of the densities of the phosphorylated p38 to that of non-phosphorylated p38. Each datum is the mean $\pm$ s.d. of results obtained from three independent experiments

response began as early as 5 min after either PDT dose. The bottom panel of Figure 4B shows p38 activation calculated as the ratio of the densities of the phosphorylated p38 band to that of non-phosphorylated p38 for each sample. For the control sample, the ratio is nearly 0. For the lower (LD90) PDT dose, a ratio of ~ 3 was achieved in 15 min after which the level declined. For the higher (LD99) PDT dose, the level of phosphorylated p38 reached a higher maximum (ratio of ~ 6), was maintained until 30 min post-PDT, and declined more slowly (bottom panel, Figure 4B).

A characteristic feature of apoptosis is proteolytic cleavage of specific cellular proteins, including poly(ADP-ribose) polymerase (PARP), by one or more cysteine-requiring proteases, now termed caspases.^{34–36} Cleavage of PARP at the DEVD motif results in the generation of an Mr 90 000 fragment from the Mr 116 000 native enzyme. In order to better understand the involvement of MAPK family members in PDT-induced apoptosis, we measured PARP cleavage in cells treated with PDT in the presence or absence of an inhibitor of ERK or p38/HOG. Figure 5A,B show the effects of SB202190, a specific inhibitor of p38/HOG, on the activation of p38/HOG and on PARP cleavage in PDT-treated LY-R and CHO cells. In Figure 5A, DNA fragmentation was also determined. Preincubation with SB202190 for 30 min (LY-R) or 60 min (CHO) partially reduced the level of p38 phosphorylation below that of untreated control cells but did not induce PARP cleavage. When LY-R cells were treated with 0.5 μ M Pc 4 and 3 kJ/m², the Mr 90 000 PARP fragment was apparent by 30 min after PDT, and the cleavage of PARP was nearly complete

Figure 5 The effects of the p38/HOG inhibitor SB202190 (SB) on PDT-induced apoptosis and p38 activity in LY-R (**A,C,D**) and CHO (**B**) cells. Cells were treated with 0.5 μ M Pc 4 for 18 h, then incubated in 15 μ M SB202190 for 30 min (**A,B**) or 5 min (**C,D**) before irradiation. Cells were exposed to 3 kJ/m² for LY-R cells and 15 kJ/m² for CHO cells. The inhibitor remained in the cultures during and after irradiation. At various time points, cells were collected for DNA fragmentation assay (bottom panel of **A**) and for Western blot analysis with the phospho-specific p38 antibody (top panel) and with the anti-PARP antibody (middle panel in **A** and bottom panel in **B**). Other samples were collected for assay of % apoptotic (TUNEL-positive) cells by flow cytometry (**C**) or caspase-3 and caspase-9 activity (**D**). The height of the bars in **D** represents the average of results from two independent experiments with the error bars indicating the range of values

by 1 h at which time DNA fragmentation was extensive. PDT itself did not alter the level of p38/HOG activation, in agreement with the results in Figure 4A. However, when LY-R cells were preincubated in SB202190 for 30 min before red light irradiation, the level of p38/HOG activation was reduced, and both PARP cleavage and DNA fragmentation were markedly inhibited (Figure 5A). In order to further define the effect of the p38 inhibitor on PDT-induced apoptosis, LY-R cells were treated with SB202190 and/or PDT and apoptosis was quantified by flow cytometry. As shown in Figure 5C, incubation with SB202190 for 50 or 125 min did not induce apoptosis (Figure 5C, top panel). For PDT-treated cells, samples were collected at 45 min and 120 min post-PDT. Because the postincubation period up to 2 h was longer than in previous experiments (e.g. 1 h in Figure 5A), SB202190 was added to the culture 5 min rather than 30 min before irradiation. The bottom panels of Figure 5C indicate that preincubation in SB202190 for 5 min significantly reduced the induction of apoptosis by 60–70% in the 2 h post-PDT period. Figure 5D shows the effect of the p38 inhibitor on caspase-3 and caspase-9 activation in PDT-treated LY-R cells. It is clear that SB202190 markedly inhibited the activation of both caspases. In conclusion, although p38 activation was constitutively high and not affected by PDT in LY-R cells (Figures 4A and 5A), the results with the p38 inhibitor (Figure 5A) suggest that p38, in fact, is required for PDT-induced apoptosis.

The effects of SB202190 on p38 activity and PARP cleavage were also studied in PDT-treated CHO cells. As shown in Figure 5B, pretreatment with SB202190 partially

inhibited both PDT-induced p38 activation and PARP cleavage, although both responses may recover at later times. The fact that PARP cleavage was at least partly reduced in cells in which p38 activity was inhibited implies that the p38/HOG pathway is involved in PDT-induced apoptosis in CHO cells.

A specific inhibitor of MEK1 (MAPK kinase) was also investigated in LY-R and CHO cells (Figure 6A). In the absence of PDT, PD98059 was found to have no effect on either ERK activation or PARP cleavage in either cell line (data not shown). Furthermore, we found no retardation of PARP cleavage when cells were preincubated with PD98059 for 1 h before red light irradiation. Because of these negative results and our earlier observations of little or no activation of ERKs by PDT, we questioned whether or not the inhibitor was active in our cells. Therefore, we took advantage of the strong activation of ERK1 and ERK2 by PMA in LY-R cells to test the effectiveness of PD98059 (Figure 6B). As observed previously (Figure 2A), PDT alone did not increase ERK activation. When LY-R cells were treated with PMA for 20 min, there was a modest activation of ERK1 and a stronger response of ERK2. PDT did not further increase ERK activation in PMA preincubated cells, implying that ERK activation could be entirely attributed to PMA. In the presence of PD98059, the PMA-promoted ERK activation was partially inhibited. As shown in the bottom panel of Figure 6B, PARP was completely cleaved when cells were treated with PDT alone. For cells treated with PDT and PMA, ERKs were strongly activated, and PARP cleavage was slightly slowed. The further addition of PD98059, although partially inhibiting ERK activation, had little or no effect on PARP cleavage.

Discussion

In the present study, we have compared two cell lines with respect to their constitutive levels of phosphorylation of three MAPK family members, ERK1/ERK2, SAPK/JNK and p38/HOG, and the levels induced by lethal doses of PDT. For PDT-treated LY-R cells, although apoptosis occurs rapidly, and DNA fragmentation can be observed as early as 10–20 min post-PDT, neither ERK1/ERK2 nor p38/HOG were activated by PDT. However, SAPK was activated in response to PDT in LY-R cells. In contrast, for CHO cells, that undergo a slower and less extensive apoptotic response, ERK2 phosphorylation was slightly elevated at 5 min post-PDT. Both SAPK and p38/HOG were activated by PDT in CHO cells, and the extent of the activation depended on PDT dose and post-treatment time.

Numerous studies^{12,13,15,16} have implicated SAPK as a mediator of apoptosis following various agents. The results in this study showed that SAPK activity was increased by PDT (LD90 and LD99 doses) in both LY-R and CHO cells, in spite of the differences in their rate and extent of apoptosis. PDT may directly activate one of the approximately ten isoforms of SAPK³⁷ or interact with upstream regulators, such as SAPK kinase, p21-activated kinase or the Ras-regulated small GTP-binding proteins.³⁸ Verheij *et al.*¹⁵ provided evidence that

Figure 6 The effects of the MEK1 inhibitor PD98059 (PD) on PDT-induced PARP cleavage in LY-R (left panel, **A**) and CHO (right panel, **A**) cells and on both ERK activation and PARP cleavage in LY-R cells (**B**). Cells were treated with 0.5 μ M Pc 4 for 18 h, then preincubated in 50 μ M PD98059 for 1 h before exposure to 3 kJ/m² (LY-R cells) or 15 kJ/m² (CHO cells). The inhibitor remained in the cultures until the cells were harvested. The samples were analyzed by Western blot with anti-PARP antibody (**A**) and both anti-PARP and anti phospho-specific antibodies (**B**). In (**B**) some cells were preincubated with 20 nM PMA for 20 min alone or before irradiation to 3 kJ/m²

oxidative stress-induced release of ceramide was an upstream signal activating SAPK and apoptosis. PDT causes the release of ceramide from both LY-R and CHO cells, as well as a variety of other cell types, with a time course and dose response consistent with such a role.^{9,39} Thus, although a selective chemical inhibitor of SAPK has not yet been found, indirect evidence suggests the involvement of this stress kinase in promoting PDT-induced apoptosis.

According to one hypothesis,¹¹ a dynamic equilibrium between ERK1/ERK2 and SAPK/p38 determines whether cells survive or undergo programmed cell death. However, ERK1/ERK2 and SAPK/p38 may not always have opposing effects, and they could be activated either separately or simultaneously depending on the cell type and stimulus.^{40–42} Although the induction of apoptosis by PDT is fast and complete in LY-R cells, neither ERK1 nor ERK2 phosphorylation were affected by PDT. However, pretreatment with PMA slightly inhibited PDT-induced PARP cleavage, and this protective effect could be partially abolished by preincubation of cells with PD98059. Thus, activation of ERK1/ERK2 may be a negative regulator of PDT-induced apoptosis. For PDT-treated CHO cells, in which apoptosis was slower and less extensive, ERK2 phosphorylation was slightly enhanced by 5 min after PDT. It is possible that the induction of apoptosis in PDT-treated CHO cells may be delayed by the early activation of ERK2.

SB202190 is reported to be an inhibitor of p38 kinase activity.^{19,43} p38 activation requires phosphorylation on threonine-180 and tyrosine-182 by a dual specificity kinase, such as MKK3, MKK6, or JNKK1.^{44–46} Our results show that SB202190 also inhibits p38 phosphorylation. This secondary action is likely due to inhibition of the activating kinase (or activation of a p38 phosphatase). In addition, the inhibitory effect appears to be reversible with time.

In LY-R cells, SB202190 significantly inhibited PDT-induced PARP cleavage, DNA fragmentation, activation of caspases-3 and -9, and appearance of apoptotic cells, suggesting that blockade of the p38 pathway is sufficient to prevent apoptosis. Although no p38 activation was observed after PDT-treatment in LY-R cells, inhibition of p38 by SB202190 is consistent with a role for p38 in PDT-induced apoptosis. In contrast, p38 was strongly activated in PDT-treated CHO cells and SB202190 markedly blocked p38 phosphorylation; however, the inhibitor had only a modest effect on PARP cleavage. One possible explanation for the observed difference in response of the two cell lines to SB202190 derives from differences in the constitutive levels of p38 phosphorylation. Thus, the high level of constitutively phosphorylated p38 in untreated control LY-R cells may contribute to the prompt induction of apoptosis in these cells by PDT, a response which is highly sensitive to depletion and inhibition of the phospho-p38 pool. In contrast, CHO cells maintain their p38 in an unphosphorylated form and require several minutes to convert p38 to phospho-p38 following PDT. Possibly, the step in PDT-induced apoptosis that is promoted by phospho-p38 in LY-R cells is less dependent upon p38

activation in CHO cells. On the other hand, p38 may play a different role in apoptosis induced by various stimuli. Frasnich *et al.*¹⁷ found that anti-Fas induced or spontaneous apoptosis is independent of p38 activation and not blocked by the inhibitor. Roulston *et al.*⁴⁷ reported that SB203580 increased TNF α -induced apoptosis. Nemoto *et al.*²⁰ observed that SB202190 potentiated apoptosis induced by Fas ligand or UV irradiation. It appears that the involvement of p38 in apoptosis may be dependent on cell type, as well as stimulus. The function of p38/HOG is complex and may trigger repair processes following cellular injury or initiate a program of cell death.^{48,49}

There is now considerable evidence that PDT with several different photosensitizers activates the mitochondrial pathway of apoptosis. We³⁰ and others^{28,29} have observed the rapid release of cytochrome c from mitochondria and activation of caspases-9 and -3 with lesser activation of other caspases. It would thus appear that the cytochrome c/caspase-3 pathway may be sufficient to carry out apoptosis in PDT-treated cells. However, the results of the present study suggest that a product(s) of one or more of the MAPK cascades can modulate apoptosis, possibly by intersecting with the cytochrome c pathway.

Because activation of caspase-9, as well as caspase-3, was inhibited by SB202190, the primary step that is affected may be upstream of these caspases, perhaps at the level of formation of the Apaf-1:cytochrome c:dATP:procaspase-9 complex. Although we do not yet know what the MAPK pathway products might be that modulate PDT-induced apoptosis, the present data allow the development of hypotheses to be tested in further work. The p38 kinase is responsible for phosphorylating several transcription factors such as ATF-2, MAX, CHOP/GADD153 and CREB^{50–52} that in turn lead to the elevated expression of a set of genes, including IL-6.⁵³ The constitutively active p38 of LY-R cells may ensure the presence of a high steady-state level of a gene product(s) that promotes prompt entry into apoptosis upon damage from PDT. When p38 phosphorylation is inhibited by SB202190, the steady-state level of the essential, but unstable, gene product may decline rapidly, limiting the ability of PDT-damaged cells to undergo apoptosis.

A modified model may explain the results in CHO cells. In the case of CHO cells, there is little or no constitutively active p38 and thus a low level of the putative essential factor. The post-PDT time lag for p38 activation and synthesis of the essential factor may explain at least in part the lag in the entry of CHO cells into apoptosis. Furthermore, the modest inhibition of apoptosis (PARP cleavage) afforded by SB202190 may reflect the delayed generation of active p38 during the period the inhibitor is present or a very strong PDT-induced activation of p38 that may be less susceptible to inhibition. It is also possible that CHO cells have more of the β isoform of p38 than do LY-R cells. It has been proposed²⁰ that p38 β is a suppressor whereas p38 α is a mediator of apoptosis. Therefore, inhibition of p38 β could actually increase cell susceptibility to apoptosis. It is also possible that the SAPK pathway, that is not inhibited by SB202190, contributes to PDT-induced apoptosis in CHO cells to a greater extent than in LY-R

cells. Additional work will be required to elucidate the precise role of these signaling pathways in regulating PDT-induced apoptosis.

Materials and Methods

Cell culture

Mouse lymphoma L5178Y cells (strain LY-R) were grown in suspension culture in Fischer's medium containing 0.1% pluronic F68, 2 mM sodium pyruvate, and 10% heat-inactivated horse serum.⁴ Monolayer cultures of CHO cells (line 5A100) were originally obtained from Dr. D. Green, La Jolla Cancer Institute, and were maintained in McCoy's 5A medium containing 10% fetal bovine serum and 20 mM N-2-hydroxyethylpiperazine N'-2-ethane-sulphonic acid (pH 7.4). The cultures were grown in a humidified atmosphere at 37°C with 5% CO₂.

Photodynamic treatment

The phthalocyanine photosensitizer Pc 4, HOSiPcOSi-(CH₃)₂(CH₂)₃N(CH₃)₂,⁵⁴ was provided by Drs. Ying-syi Li and Malcolm E. Kenney, Case Western Reserve University Department of Chemistry. It was dissolved in dimethyl formamide to 0.5 mM. Cells were loaded with Pc 4 by addition of an aliquot of the stock solution to the culture medium ~18 h before irradiation. In preparation for irradiation of CHO cells, the dye-containing medium was removed and replaced with 3 ml of Hank's balanced salt solution with Ca²⁺ and Mg²⁺. LY-R cells were irradiated in their Pc 4-containing conditioned medium. The light source was a 500 W tungsten-halogen lamp placed 29 inches below the surface of a glass exposure tray and filtered (600-nm long-pass filter). All irradiations were performed at room temperature, and the temperature during irradiation did not exceed 34°C.

Flow cytometry

Cell fixation and staining were performed based on the APO-DIRECT[®] Kit (Phoenix Flow Systems, Inc.),⁵⁵ as we have previously described.³² Analysis was carried out in the Flow Cytometry Facility of the Case Western Reserve University/Ireland Cancer Center. Fluorescence measurements were made on an EPICS ESP flow cytometer (Coulter Corp.), activating at 488 nm and monitoring fluorescence emission at 520 nm (Fluorescein) and 623 nm (PI). Data analysis was supported by version 4.0 of the instrument software.

Western blot analysis

For study of MAPKs, control and PDT-treated cells were lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% w/v bromophenol blue) and boiled for 5 min. Equivalent amounts of protein were loaded onto an 8% polyacrylamide gel, subjected to electrophoresis, transferred to a PVDF membrane and incubated with phospho-specific p44/42 ERK, SAPK, or p38/HOG antibodies (New England BioLabs, Inc. Beverly, MA, USA). The immune complexes were detected by ECL system (Amersham, Arlington Heights, IL, USA). After probing with phospho-specific antibodies, the membrane was washed and reprobed with the corresponding control p44/42 ERK, SAPK (New England BioLabs, Inc., Beverly, MA, USA), or p38/HOG (UBI, Lake Placid, NY, USA) to check the loading and calculate the ratio of activated to non-activated kinases. For study of PARP cleavage, cells were lysed and sonicated

in a solution containing 0.5% sodium deoxycholate, 0.2% SDS, 1% Triton X-100, 5 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethyl sulfonyl fluoride in phosphate-buffered saline. An equal volume of 2×SDS sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% mercaptoethanol, and 20% glycerol) was added to the cell lysate. Proteins were separated and transferred as described above and then probed with a monoclonal anti-PARP antibody (Trevigen, Gaithersburg, MD, USA) and detected by ECL.

DNA fragmentation analysis

Cells were collected at various times after PDT. DNA isolation and gel electrophoresis were performed as previously described.⁴ Briefly, cell pellets were resuspended in 500 µl of a solution containing 1×SSC and 10 mM EDTA, then 1% sodium lauryl sarkosinate and 0.1 mg/ml proteinase K were added, and the mixture was incubated at 50°C for at least 2 h. The DNA was precipitated by addition of 2 volumes of absolute ethanol, resuspended in 200 µl TE, and briefly treated with 1 mg/ml RNase before loading onto a 1.5% agarose gel. After electrophoresis, the gel was stained with 0.5 µg/ml ethidium bromide and photographed under UV light.

Caspase activity

Approximately 2 × 10⁶ cells were collected by centrifugation, and the washed cell pellet was resuspended in 120 µl of lysis buffer (10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 100 µM pepstatin, 100 µM leupeptin, 0.5% Triton X-100 and 20 mM HEPES, pH 7.5), incubated on ice for 20 min, then sonicated and stored at -80°C until use. Aliquots containing 50 µg protein were incubated with 100 µM DEVD-AMC or LEHD-AFC (for assay of caspase-3 and caspase-9, respectively; BIOMOL Research Laboratory, Inc., Plymouth Meeting, PA, USA) in 60 µl of caspase reaction buffer (10% sucrose, 0.1% CHAPS, 1 mM EGTA, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 100 µM pepstatin, 100 µM leupeptin, 25 mM HEPES, pH 7.4) at 37°C for 1 h. The released fluorescent product was measured in a Perkin-Elmer LS50 fluorometer (λ_{ex} 380 nm and λ_{em} 460 nm for caspase-3 assay; λ_{ex} 400 nm and λ_{em} 505 nm for caspase-9 assay).

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