Sustained Activation of the Extracellular Signal-regulated Kinase Pathway Protects Cells from Photofrin-mediated Photodynamic Therapy

Zhimin Tong, Gurmit Singh, and Andrew J. Rainbow

ABSTRACT

Photodynamic therapy (PDT) is a cancer therapy in which a photosensitizer selectively accumulates in tumor cells and is subsequently activated by light of a specific wavelength. The activation of the photosensitizer leads to cytotoxic photoproducts that result in tumor regression. PDT can lead to several cellular responses including cell cycle arrest, necrosis, and apoptosis, as well as trigger many signaling pathways. It has been suggested that extracellular signal-activated protein kinases (ERKs), one subfamily of mitogen-activated protein kinases, play a crucial role in the cellular response to radiation therapy and chemotherapy. However, the role of ERKs in the cell survival after PDT is less clear. We have examined the response of the extracellular signal-regulated kinase ERK1/2 in PDT-resistant (LFS087) and PDT-sensitive (GM38A) cells after Photofrin-mediated PDT. ERK1/2 activity was induced rapidly in both cell types after PDT. The PDT-induced ERK1/2 activity was transient in GM38A cells and by 3 h had returned to a level significantly lower than basal levels, whereas the induction of ERK1/2 was sustained in LFS087 cells and lasted for at least 11 h. Blocking of the sustained ERK activity with PD98059, an inhibitor of mitogen-activated protein kinase/ERK kinase, significantly decreased cell survival of LFS087 after PDT. PDT also induced the expression of mitogen-activated protein kinase phosphatase, MKP-1, but reduced Raf-1 protein levels in both cell types. In GM38A cells, the substantially induced levels of MKP-1 correlated with the transient activation of ERK1/2 by PDT, and both basal and induced levels of MKP-1 were substantially greater in GM38 compared with Li Fraumeni syndrome cells. These observations suggest that sustained ERK1/2 activation protects cells from Photofrin-mediated phototoxicity and that the duration of ERK1/2 activation is regulated by MKP-1. In addition, the activation of ERK1/2 by Photofrin-mediated PDT is Raf-1 independent.

INTRODUCTION

PDT is a novel treatment that has been applied to both neoplastic and non-neoplastic disease (1). The application of PDT in the treatment of neoplastic disease involves the accumulation of a photosensitizer in tumor cells and the localized delivery of light to activate the photosensitizer in the presence of molecular oxygen, which leads to cell death through the generation of several chemically reactive molecular species (2). There is evidence that both damage to tumor vasculature as well as direct tumor cell killing play a role in the tumoricidal effects of PDT (1). Photofrin-mediated PDT has been used in many countries throughout the world and shows considerable promise in the treatment of several solid tumors (1). Although the molecular mechanisms of tumor regression induced by PDT are not fully understood, there is evidence that the ROS generated by PDT can lead to apoptosis and/or necrosis of cells, and are responsible for tumor destruction (2).

ROS, including the ROS induced by PDT, have been shown to be very efficient in triggering a variety of cellular signaling pathways (reviewed in Ref. 3). Photofrin-mediated PDT induces the expression of early response genes including c-jun, c-fos, and c-Myc (4), and several stress proteins (5, 6). The former are involved in the regulation of cell proliferation, differentiation, and survival, whereas the latter are thought to act as protection signals (7). Photofrin-mediated PDT causes an increase in intracellular second messenger calcium Ca2+ by releasing it from mitochondria and the endoplasmic reticulum (8). In addition, PDT using various photosensitizers activates nuclear factor κB (9) and releases cytochrome c that leads to apoptosis (10). The activation of MAPKs by PDT has been reported in several different cell models using several different photosensitizers (11). However, the biological functions associated with PDT-induced activation of MAPKs are somewhat controversial (12) and suggest that the cellular signaling responses associated with PDT are complex. The interaction between various signaling pathways may play an important role in the efficiency of PDT.

The MAPK pathway is an evolutionary conserved signaling cascade that plays a critical role in cell growth, differentiation, and cell survival through the activation of intracellular substrates including transcription factors, such as Elk-1, c-jun, and activating transcription factor 2 (13). MAPKs constitute a superfamily of three related kinases that are activated by a diverse array of extracellular stimuli. They include ERKs, JNKs, and p38 protein kinases. ERKs are mainly activated by a variety of mitogenic or stress stimuli and lead to the production of proteins required for cell proliferation and/or differentiation (14). In contrast, JNK and p38 are activated primarily by environmental stresses such as UV light (15), heat shock (16), and other cellular stress (16), and participate in cell apoptosis. The best-characterized pathway leading to activation of MAPK is the growth factor-induced ERK pathway. The binding of growth factor to the receptor tyrosine kinase leads to sequentially activate Ras and Raf. Raf then phosphorylates MEK, which in turn activates ERKs (reviewed in Ref. 13). A critical role for ERK activation in cell proliferation and cell survival has been suggested in many cell types. Blocking of the ERK pathway using dominant-negative mutants of ERK or using the MEK inhibitor PD98059 abolishes cell proliferation (17), enhances cell sensitivity to cisplatin treatment (18), and increases cytosome arabinoside-induced apoptosis (19).

The activities of ERKs are regulated by MKPs. MKPs are dual-specificity phosphatases that inactivate MAPKs by dephosphorylation of both Thr and Tyr residues within their signature sequence (13). MKP-1, one member of this family, is the product of an immediate early gene and shows selectivity for ERK1/2 in vitro (20, 21). MKP-1 inactivates growth factor-induced ERK and suppresses cell proliferation (22). Thus, MKP-1 plays an important role in the regulation of the dynamic activation of ERKs.

Despite an expanded interest in studying the importance of ERKs in cell proliferation and survival, little is known regarding the possible role of the ERK pathway in cellular survival after PDT, especially, for...
Photofrin-mediated PDT. Hypericin-mediated PDT has been shown to result in an inhibition of ERK2 activity in several cancer cell lines (11). In contrast, using phthalocyanine Pc 4 as a photosensitizer, Xue et al. (12) reported a transient activation of ERK2 in Chinese hamster ovary cells but not in LY-R cells. However, neither of these reports presented evidence for a connection between cytotoxicity and the induction of ERK pathways after PDT.

We have reported previously that immortalized LFS cells are significantly more resistant to Photofrin-mediated PDT compared with NHF. The D37 value (Photofrin concentration resulting in 37% colony survival after light exposure) was ~3-fold greater for LFS cells compared with NHF (23). In the present report we have examined the role of ERKs in the sensitivity of LFS and NHF cells to Photofrin-mediated PDT. We show that Photofrin-mediated PDT at equivalent cellular Photofrin levels resulted in increased phosphorylation of ERK1/2 detectable in both LFS and NHF cells at 30 min after PDT. For the NHF cells, the increased phosphorylation of ERK1/2 was transient and decreased to levels lower than that in untreated cells by 3 h after PDT. In contrast, LFS cells showed a prolonged activation of ERK1/2 for at least 11 h after PDT. Therefore, the duration of PDT-induced ERK activity correlated with the cellular resistance to PDT. Blocking the sustained activation of ERK1/2 with PD98059, a MEK1/2 inhibitor increased the sensitivity of LFS cells to PDT. In addition, PDT increased MKP-1 expression in both LFS and NHF cells, and the levels of MKP-1 inversely correlated with the activation of ERK1/2 induced by PDT in both cell types. We also show that the activation of ERKs by Photofrin-mediated PDT is independent of Raf-1 activation, because Photofrin-mediated PDT actually reduced Raf-1 expression. This suggests that the prolonged up-regulation of ERK1/2, through a Raf-1-independent pathway, contributes to the resistance of LFS cells to PDT.

MATERIALS AND METHODS

Cells and Culture Conditions. The NHF stains GM38A and GM9503 were obtained from the National Institute of General Medical Sciences Repository (Camden, NJ). The immortalized LFS cell lines were obtained from Dr. Michael A. Tainsky, M. D. Anderson Cancer Center, Houston, TX. All of the cells were grown in monolayer culture in α-MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Life Technologies, Inc.), and were cultured in a humidified atmosphere at 37°C and 5% CO2.

Reagents. Photofrin was a gift from QLT Inc. (Vancouver, BC). The MEK1/2 inhibitor, PD98059, and antibodies to phosphorylated ERK1/2 or total ERK1/2 were purchased from New England Biolabs, Inc. (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies and antibodies to Raf-1, actin, or MKP-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [α-32P]dCTP was purchased from MANDEL Scientific Company Ltd. (Guelph, Ontario, Canada) and random primer labeling kits from Life Technology (Burlington, Ontario, Canada).

Photodynamic Treatment. Cells were incubated with Photofrin at equivalent cellular Photofrin levels (10 μg/ml for LFS cells and 7.5 μg/ml for NHF cells) for 18 h and followed by replacement with fresh culture medium before exposure to red light. Light exposure to cell monolayers was performed as described previously (23). The light source was light box illuminated by a parallel series of fluorescent tubes (Phillips type TL83), and the light was filtered with red acetate filters (Roscolux, Rosco, CA). The energy fluence rate was 7 mW/cm2 at a wavelength of 630 nm. In the experiment where PD98059 was used, cells were incubated with 75 μM PD98059 (dissolved in DMSO; final concentration in medium was <0.15%) for 2 h before exposure to red light.

Clonogenic Assay. Exponentially growing LFS cells were plated at about 100–200 cells/well and incubated for 4 h to allow cells to adhere before treatment with PDT as described above. After PDT, cells were allowed to grow for 7 days, and colonies were stained with a solution containing 0.5% methylene blue and 70% ethanol. Colonies >30 cells were counted. The surviving fraction was calculated as the percentage of the PDT-treated samples compared with the non-PDT-treated samples that were treated with drug alone (without light exposure).

RNA Isolation and Northern Blot Analyses. Northern blot analyses were performed for detection of specific MKP-1 transcripts. Total RNA was isolated from cells that were harvested at various times after PDT according to the procedure described by the manufacturer using RNeasy mini kit (Qiagen, Inc., Mississauga, Ontario, Canada). Equal amounts of total RNA (10–15 μg) were electrophoresed on 1% agarose gels and transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, England). The cDNA was synthesized by reverse transcription-PCR using primers that were designed according to the sequence of MKP-1 or GAPDH gene. CDNA probes were labeled with [α-32P]dCTP using a random primer labeling kit according to the procedure described by the manufacturer. Membranes were hybridized with the cDNA probes of MKP-1 overnight at 42°C in a solution containing 5× SSC, 50% formamide, 1× Denhardt’s reagent, and 1% SDS. After the membranes were washed three times with 1× SSC and 0.1% SDS, they were subjected to autoradiography with an intensifying screen and quantified by PhosphorImager analysis. The membranes then were stripped and reprobed with a CDNA probe of GAPDH, which was used to normalize for differences in loading and transferring among the samples.

Western Blotting Analysis. After PDT, cells were washed twice with ice-cold PBS buffer and lysed in a buffer containing 50 mM of Tris (pH 8.0), 150 mM of NaCl, 0.5% NP40, 2 mM of EDTA, 100 mM of NaF, 10 mM of sodium orthovanadate, and a protease inhibitor mixture (Boehringer Mannheim, Mannheim, Germany) for 30 min on ice. The debris of cells then was pelleted by centrifugation (>10,000 × g) for 15 min and discarded. The protein concentration of the cell lysate was determined using the Bradford microassay procedure (BioRad, Munich, Germany). Equal amounts of protein (15–30 μg) were resolved on 12% SDS-PAGE and electrophoretically transferred to an enhanced chemiluminescence membrane (Amersham Pharmacia Biotech). The blots were blocked in 5% skim milk in a Tris-buffered saline with 1% Tween 20 for 1 h at room temperature and then incubated at 4°C overnight with corresponding antibodies in the same buffer as the block buffer. Specific antibody-labeled proteins were detected by using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence plus Western blotting detection system (Amersham Pharmacia Biotech). Thereafter, blots were stripped and reprobed with antibodies to actin or total ERK1/2. The data were quantified using PhosphorImager analysis and normalized with actin or total ERK1/2.

RESULTS

Effects of Photofrin-mediated PDT on the Activation of ERKs. To assess the effect of Photofrin-mediated PDT on the activation of ERKs, LFS087 cells and GM38A cells were incubated with Photofrin at equivalent cellular Photofrin levels (10 μg/ml for LFS087 cells and 7.5 μg/ml for GM38A cells) for 18 h. At various times after a red light exposure of 270 mJ/cm2, the phosphorylation of ERK1/2 was examined by Western blot analysis using antibodies that only recognize phosphorylated ERK1/2. Cells were alive at all times after PDT used to harvest for ERK activity as determined by their continued attachment to the culture plate throughout the duration of the experiment. The Photofrin drug concentrations used were based on uptake studies reported previously (23). The clonogenic survival levels for this PDT treatment were ~3 × 10−3 for GM38A cells after 7.5 μg/ml Photofrin and red light and ~10−1 for LFS087 cells after 10 μg/ml of Photofrin and red light (23). As shown in Fig. 1, Photofrin-mediated PDT resulted in a rapid induction of ERK1/2 phosphorylation in both cell types, as early as 30 min after PDT. The maximum phosphorylation of ERK was 1.5-fold and 3.5-fold over the control value (without drug and light) in GM38A and LFS087 cells, respectively, and was detected in both cell types at 30 min after PDT. However, the kinetics of the ERK1/2 phosphorylation induced by PDT was different in GM38A cells compared with LFS087 cells at later times. For GM38A cells, the phosphorylation of ERK1/2 declined after 30 min, and by 3 h after PDT, the level of ERK1/2 phosphorylation was...
significantly lower than that of controls and remained at this lower level for at least 8 h. In contrast, LFS cells exhibited a prolonged activation of ERK1/2 phosphorylation, which was maintained at a substantially enhanced level of ERK1/2 phosphorylation up to at least 11 h after PDT.

Studies concerning MAPK activity after exposure of human skin cells to red light alone have been reported previously. Exposure of human skin fibroblasts to up to 20 J/cm² of red light alone resulted in no detectable activation of the stress kinase p38 (24), and no activation of JNK, p38, or ERK1/2 was detected in human skin cells exposed to 4.5 J/cm² of red light alone (25). Thus, the red light exposure alone of 270 mJ/cm² used in the experiments reported here would have no effect on ERK 1/2 activation.

Photofrin without photoactivation had no effect on the phosphorylation of ERK1/2 in GM38A cells, whereas Photofrin alone induced a small increase of ERK1/2 phosphorylation in LFS087 cells that may protect cells from the toxicity of Photofrin. The increase of ERK1/2 phosphorylation by PDT was not attributable to an increase in the total amount of ERK, because the total levels of ERK after PDT remained the same as that in the controls. Similar results were observed in GM9503 and LFS041 cells (data not shown). These data suggest that the difference in the kinetics of PDT-induced ERK1/2 phosphorylation contributes to the difference in cell sensitivity to PDT between LFS087 and GM38A cells as assessed by clonogenic assay. The decreased sensitivity of LFS087 cells to Photofrin-mediated PDT might be because of the prolonged activation of ERK1/2 phosphorylation detected in these cells after PDT.

Effects of PD98059 on the PDT-induced ERK1/2 Phosphorylation and Cellular Response to PDT. To further investigate the possible role of the activation of ERK1/2 in the cellular response to PDT, we focused on the LFS087 cells as a model system. A specific inhibitor of MEK, PD98059, was used to treat LFS087 cells at 75 μM for 2 h before exposure to red light. We found the PDT-induced phosphorylation of ERK1/2 was reduced by >50% when cells were treated with 75 μM of PD98059 (Fig. 2). The inhibition of ERK phosphorylation by PD98059 was observed at 30 min after PDT and was maintained for at least 3 h. These data suggest that MEK is the upstream activator of ERK1/2 induced by Photofrin-mediated PDT.

To examine the relationship between the activation of ERK1/2 and cellular response to Photofrin-mediated PDT, LFS087 cells were treated with 10 μg/ml of Photofrin for 18 h and 75 μM PD98059 for 2 h immediately before exposure to red light. Cells treated with
Photofrin for 18 h and DMSO for 2 h, as well as cells treated with Photofrin alone or DMSO alone before red light exposure were used as controls. DMSO alone had no significant effect on cell colony survival either with or without red light exposure (Fig. 3). This indicated that treatment of human fibroblasts with 250 mJ/cm² red light alone had no effect on cell survival as determined by clonogenic assay (Fig. 3). This is consistent with previous studies, which report no detectable toxicity to human skin fibroblasts using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assay 24 h after red light exposures up to at least 45 J/cm² (24).

In the absence of red light exposure, cell colony numbers (cell plating efficiency) for cells treated with Photofrin and PD98059 were not significantly different from those for cells treated with Photofrin and DMSO, or cells treated with DMSO alone, or cells treated with Photofrin alone (Fig. 3, legend). This indicated that PD98059 alone was not toxic to LFS cells under the treatment conditions used. Cells pretreated with Photofrin plus PD98059 did show a significantly reduced viability compared with cells treated with Photofrin plus DMSO and cells treated with Photofrin alone after red light exposure, as determined using a clonogenic assay (Fig. 3). The reduction in PDT-induced ERK1/2 phosphorylation and concomitant increase in sensitivity to Photofrin-mediated PDT in LFS cells pretreated with PD98059 suggests that the ERK pathway plays a role in the cell response to PDT. In particular, the sustained activation of ERK1/2 protects LFS cells from PDT-induced cell death.

Effects of Photofrin-mediated PDT on the Expression of MKP-1. The reduction in ERK1/2 activation to below control levels in GM38A cells after the initial transient increase after PDT suggested that a negative regulation of PDT-induced ERK1/2 occurs in NHF cells a few hours after PDT. MKP-1 has been shown to be a specific in-activator of ERK1/2 by dephosphorylation of ERK1/2 in vitro (20, 21). MKP-1 is a product of immediate early genes and can be rapidly induced by cellular stresses such as UV (26) and heat shock (27) mainly at the transcriptional level. It was considered of interest to see whether MKP-1 is involved in the regulation of PDT-induced ERK1/2 phosphorylation. We first examined the alteration of MKP-1 mRNA after PDT. Total RNA isolated from LFS087 and GM38A cells at various times after PDT was used to examine the effects of PDT on the MKP-1 expression by Northern blotting analysis. The levels of MKP-1 mRNA were significantly increased in response to Photofrin-mediated PDT in both cell types (Fig. 4A).
mRNA was observed by 1 h after PDT and lasted at least for 1 h. The maximum increase of MKP-1 mRNA was 2.5-fold and 5-fold in LFS087 and GM38A cells, respectively. However, the overall levels of MKP-1 mRNA in LFS087 were significantly lower than that in GM38A cells. The highest levels of MKP-1 mRNA induced by PDT were two times lower than the basal levels of MKP-1 mRNA in GM38A cells. Photofrin itself had no effect on the expression of MKP-1.

To additionally confirm these differences in MKP-1 expression, the protein levels of MKP-1 were evaluated by Western blot analysis with antibodies to MKP-1. Consistent with Northern blotting, the protein levels of MKP-1 in GM38A cells increased rapidly in response to PDT by as early as 30 min and remained at increased levels for at least 5 h (Fig. 4B). However, the protein levels of MKP-1 in LFS087 cells were undetectable using the same conditions as used in GM38A cells (data not shown). These results together with those of the Northern blot indicated that the levels of MKP-1 were inversely correlated with the activity of ERK1/2, suggesting that MKP-1 participates in the regulation of PDT-induced ERK1/2 phosphorylation.

**Effect of Photofrin-mediated PDT on the Expression of Raf-1.** Raf protein kinases have been reported to participate in the highly conserved Ras/Raf/MEK/ERK intracellular signaling pathway (28). A variety of biochemical and genetic data point to the importance of Raf-1 in the regulation of ERK1/2 activity. For instance, activation of an estradiol inducible Raf-1 results in the rapid activation of MEK and ERK1/2 (29), and dominant-negative Raf-1 mutants inhibit growth factor-induced ERK activity in fibroblasts (30). In contrast, there are some reports that suggest that the endogenous Raf-1 protein is not an important MEK activator because ERK activity can be induced in cell lacking *raf-1* genes (31, 32). Therefore, it was considered of interest to investigate whether Raf-1 is required for the activation of ERK1/2 by Photofrin-mediated PDT. The expression of Raf-1 was determined by Western blot analysis using anti-Raf-1 antibodies after PDT under conditions shown previously to activate ERK1/2. We found PDT significantly reduced the Raf-1 protein by >60% compared with control values in LFS 087 cells (Fig. 5). The reduction of Raf-1 protein by PDT was observed as early as 30 min after PDT and remained at a reduced level for at least 5 h. Similar results were observed in GM38A cells (data not shown). No effect of Photofrin alone on the expression of Raf-1 was observed. These results suggest that Raf-1 is not the upstream activator of the PDT-induced ERK1/2 phosphorylation.

**DISCUSSION**

There are three major new findings in the present study. First, we show that Photofrin-mediated PDT caused ERK1/2 activation, and the duration of ERK1/2 activation induced by Photofrin-mediated PDT correlates with cell resistance to PDT. Exposure of cells to Photofrin-mediated PDT at equivalent Photofrin levels resulted in the activation of ERK1/2 in either a transient or a sustained manner. The cells with lower sensitivity (LFS087 and LFS041) showed a prolonged ERK1/2 activation, whereas cells with high sensitivity (GM38A and GM9053) showed a transient activation of ERK1/2 after PDT. Treatment of LFS cells with 75 μM of PD98059 significantly inhibited the sustained activation of ERK1/2 after PDT. PD98059 is a specific inhibitor of the MAPK cascades (33), and similar concentrations of PD98059 have been reported to inhibit ERK1/2 activation in other *in vitro* cell systems (34). The exquisite specificity of PD98058 may result from its unique mechanism of action. PD98059 binds to the inactive form of MEK1, preventing its activation by "upstream" protein kinases but does not inhibit the phosphorylated (fully activated) form of MEK1 or any other protein kinase tested (33, 34). The inhibition of ERK1/2 activation by PD98059 reported here suggests that MEK is the upstream activator of ERK1/2 induced by Photofrin-mediated PDT. In addition we found that treatment of LFS cells with PD98059 also resulted in an increased cellular sensitivity to Photofrin-mediated PDT at a concentration of PD98059, which alone was not toxic to the cells. As far as we know, this is the first direct evidence of a role for the ERK pathways in the sensitivity of human cells to PDT.

Although the activation of ERK pathways was originally associated with the stimulation of growth factors (35), there is accumulating evidence suggesting that activation of ERK1/2 can be induced by a variety of cellular stress including ROS (36, 37). ROS have been shown to act as secondary messengers capable of activating a number of signaling pathways (38–40). PDT induces ROS and results in the activation of a series of signaling pathways including both cell death and cell survival signals. Treatment of human keratinocytes or skin fibroblasts with methylene blue photosensitization stimulates the nuclear factor κB (41), which is thought to be mainly involved in the promotion of cell survival. JNK and p38 have been shown to be activated by PDT using several different photosensitizers (11, 12). Photofrin-mediated PDT induces early response genes such as *c-fos*, *c-jun*, and *c-myc* (4, 42), which are downstream substrates of ERK1/2 (43). In the present study, we found that Photofrin-mediated PDT caused a rapid and significant increase in ERK1/2 activity in both LFS and NHF cells. In contrast, no or only slight activation of ERK pathways has been reported in cells treated with PDT using photosensitizers other than Photofrin (11, 12). This difference might be because of the difference in cell line and/or photosensitizer used.

Activation of ERK is critical not only in cell proliferation and differentiation (43, 44), but also as a survival signal (45, 46). Inhibition of ERK1/2 activation by PD98059 results in enhanced cisplatin cytotoxicity in ovarian carcinoma cell lines (47), as well as increases...
H$_2$O$_2$-induced apoptosis in cardiac myocytes (48) and hyperoxia-induced apoptosis in alveolar epithelial cells (49). Our data clearly show that the ERK pathway is involved in the cell survival after Photofrin-mediated PDT. In particular, the duration of PDT-induced ERK activation determined the cell fate as evidenced by the transient activation of ERK1/2 in NHF cells and the sustained activation of ERK1/2 in LFS cells, which, when blocked by PD98059, significantly decreased LFS survival. The influence of the ERK pathway on the cellular response has been reported to depend on the duration and intensity of ERK activation in a number of cell types. For example, sustained activity of ERKs induced by thrombin and basic fibroblast growth factor results in cell proliferation in airway smooth muscle, whereas endothelin-1 that induces a transient activation of ERK does not promote cell proliferation (50). Sustained ERK activation resulting from constitutively active MEK mutation shows an enhanced resistance to H$_2$O$_2$ in NIH 3T3 cells (51). In the same cell model, expression of a constitutively active form of ERK2 protects cells from doxorubicin-induced cell death (52). Furthermore, the sustained activation of ERK is required for a proliferation response to the stimuli of extracellular calcium in human osteoblasts, whereas a transient activation of ERK is not sufficient (53). However, sustained activation of ERK has also been shown to inhibit cell growth in some systems (54). Apparently, the sustained ERK activation induced by Photofrin-mediated PDT protected cells from phototoxicity. Our data additionally supports the concept that PDT can trigger cellular pathways involved in both cell survival and cell death.

It is possible that differences in the duration of ERK activation could induce different downstream targets. In Madin-Darby canine kidney cells, hepatocyte growth factor induces sustained ERK activation and in turn increases the expression of integrin 2, whereas epidermal growth factor, which induces a transient activation of ERK, only slightly increases the expression of integrin 2 (55). Similar results are observed in the growth factor-induced expression of serum and glucocorticoid-inducible kinase (56). Such a mechanism may also apply to PDT-induced ERK activity. However, the downstream substrates for PDT-induced ERK pathways are unknown and remain to be clarified. The LFS cells used in the present study express only mutant p53, whereas the NHF cells express wild-type p53. Although we are not aware of any reports that examine the relationship between p53 and ERK, we cannot exclude a possible effect of p53 on the ERK pathway. Therefore, it is possible that the difference in ERK activation between LFS and NHF cells results from some involvement of p53 in the PDT-induced up-regulation of ERK1/2.

The second major new finding of the present study is that Photofrin-mediated PDT induced MKP-1 expression. MKP-1 expression was rapidly induced by PDT in both sensitive and resistant cells. Previous studies have demonstrated that MKP-1 can be induced by a variety of cellular stimuli, as well as by MAPKs, and negatively regulates ERK activity by dephosphorylating ERKs (25, 57). The time course of MKP-1 induction by PDT paralleled the kinetics of ERK inactivation in GM38A cells. Photofrin-mediated PDT caused a significant increase in MKP-1 expression in GM38A cells that showed a transient ERK activation. The increase in MKP-1 expression occurred between 30 min and 1 h after PDT, and remained at increased levels for at least another hour, overlapping with the transient increase and subsequent decrease of PDT-induced ERK activity in NHF cells. Thus, MKP-1 is a PDT-inducible gene, and the increased MKP-1 may be responsible for the transient activation of ERKs in NHF cells. Although the induction of MKP-1 transcription was also observed in LFS cells, the PDT-induced MKP-1 mRNA levels were 10 times lower than that in GM38A cells and 2 times lower than the basal levels of MKP-1 mRNA of GM38A cells. Moreover, the MKP-1 protein was undetectable. Therefore, the sustained activation of ERK by PDT in LFS087 cells might result from levels of MKP-1 too low to reach the threshold required for dephosphorylation of ERK1/2. The results presented here may suggest that prolonged ERK activation induced by PDT in LFS cells may because of the low level of MKP-1 expression in LFS cells. Thus, MKP-1 may serve as a negative feedback regulator of PDT-induced ERK activity and play an important role in the determination of cell fate after PDT. Increasing the level of MKP-1 expression in tumor cells may be a possible approach to improving the efficiency of PDT in the treatment of cancer.

Multiple pathways are involved the MKP-1 expression. MKP-1 mRNA and protein levels have been shown to be regulated by ERK1/2 (58, 59). ERK phosphorylates MKP-1 and in turn stabilizes the MKP-1 protein by reducing its proteolytic degradation (60). We found that PDT-induced MKP-1 mRNA expression occurred after the onset of ERK activation, and the increase of MKP-1 protein occurred earlier and lasted longer compared with the induction of MKP-1 mRNA. This suggests that the expression of MKP-1 in response to PDT is regulated by ERK1/2 at both transcriptional and post-translational levels. It is also possible that the PDT-induced expression of MKP-1 might result from PDT-induced changes in the intracellular concentration of Ca$^{2+}$. In support this idea, Cook et al. (61) have reported that lysophosphatidic acid and fibroblast growth factor-induced MKP-1 expression was blocked by Ca$^{2+}$-chelating agents and resulted in a sustained ERK1 activity. Furthermore, the promoter region of the human MKP-1 gene contains cis-acting elements for AP-1 and activating transcription factor/cyclic AMP-responsive element binding protein sites, which can respond to Ca$^{2+}$/cyclic AMP and ERKs (62). Moreover, Photofrin-mediated PDT results in an increase of intracellular Ca$^{2+}$ by releasing Ca$^{2+}$ from the mitochondria, endoplasmic reticulum, or extracellular medium.

The third major new finding of our study is that Photofrin-mediated PDT inhibited the expression of Raf-1 in both LFS and NHF cells. Several studies have demonstrated that the classical pathway of Ras$\rightarrow$Raf$\rightarrow$MEK-mediated ERK activation occurs in response to cellular stresses (48, 63). In contrast, the activation of ERKs after heat shock was via a Raf-1-independent route (64). It was also reported that normal ERK activation was induced by growth factors in cells in which the Raf-1 gene was knocked out (31, 32). In the present study, Raf-1 rapidly decreased in response to PDT treatment. Thus, PDT-induced ERK activity occurs through some other upstream signal that is independent of Raf-1 activation.

A rapid reduction of Raf-1 in response to cellular stimuli has been demonstrated in cells treated with sodium arsenite (65), treated with complex I inhibitor of the electron transport chain (66), and infected with salmonella (67). These reports suggested that the rapid reduction of Raf-1 results from degradation or cleavage. In the case of PDT-reduced Raf-1, it is possible that PDT-induced ROS resulted in Raf-1 destabilization, because the loss of Raf-1 occurred rapidly, in <30 min after PDT. Furthermore, PDT-induced ROS could directly damage proteins (68) and disturb the electron transport chain. However, the importance of the reduction in Raf-1 in the cellular response to PDT is not clear.

In summary, we have shown that Photofrin-mediated PDT causes either a transient or a sustained increase of ERK1/2 activity in cell type-dependent manner. The duration of ERK induction by PDT correlates with cell resistance. Photofrin-mediated PDT also induces MKP-1 expression and reduces Raf-1 protein. The MKP-1 protein may play a role in the regulation of the kinetics of PDT-induced ERK activity. Thus, the duration of ERK activation may determine the cell fate, e.g., survival or death after Photofrin-mediated PDT (Fig. 6). In addition, we have demonstrated that MEK is the upstream activator of the PDT-induced ERK pathway, but Raf-1 is not. The precise nature...
20. Franklin, C. C., and Kraft, A. S. Constitutively active MAP kinase kinase (MEK1) shows activity of the upstream signal of the PDT-induced MEK/ERK pathway will require additional investigation.

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Fig. 6. A model for the possible role of ERK activation in the cellular response to PDT. The balance of activity between MKP-1 and MEK1/2 regulates the kinetics of ERK1/2 activity induced by PDT. Transient ERK induction leads to cell death, whereas sustained ERK activation supports cell survival.

of the upstream signal of the PDT-induced MEK/ERK pathway will require additional investigation.
ERK AND CELL SENSITIVITY TO PDT


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