THE MECHANISMS OF CELLULAR SENSITIVITY TO PHOTODYNAMIC THERAPY

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Title: The Mechanisms of Cellular Sensitivity to Photofrin-mediatd photodynamic therapy.

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Abstract

Photodynamic therapy (PDT) is a cancer treatment in which a photosensitizing drug is retained by tumor cells and activated by visible light to create toxic photoproducts including singlet oxygen and other free radicals. Photofrin-mediated PDT is being applied in many clinics throughout the world. Although PDT is a promising treatment for many types of tumors, the response of cells to PDT varies between different cell types. The mechanisms of cell response to PDT are not fully understood. Therefore, an improved understanding of mechanisms of cell response to PDT may lead to novel strategies for increasing the cellular response to PDT, which has the potential to improve significantly the cure rate of cancers.

Although there is evidence that the p53 tumor suppressor plays a role in tumor response to the chemotherapy and radiation therapy, its role in the response of human cells to PDT is less clear. In the present study, Li-Fraumeni syndrome (LFS) skin fibroblast cells that express only mutant p53 and normal human skin fibroblasts (NHF) strains expressing wild-type p53 were used to elucidate the role of p53 in the

cell response to Photofrin-mediated PDT. NHF cells were found to have a higher sensitivity to Photofrin-mediated PDT compared to LFS cells. Reintroduction of wild-type p53 reduced the viability of LFS cells following PDT. These data suggest a role for p53 in the response of human cells to PDT. Although the treatment of PDT resulted in an increased level of p53 proteins in NHF cells, no apoptosis or any alteration in the cell cycle was found. Whereas LFS cells displayed a prolonged accumulation of cells in G_2/M phase and the cells underwent apoptosis. Thus, the loss of p53 may result in a prolonged G_2/M arrest that contributes to the photoresistance of LFS cells.

In order to see the possible role of p53 in the cellular response to Nile Blue A (NBA), a secondary photosensitizer-mediated PDT, the clonogenic survival assay was used to compare the viability of LFS cells to NHF cells following NBA-mediated PDT. We found an extreme sensitivity of NHF cells compared to LFS and several tumour cells for treatment with NBA alone. In addition, Nile Blue A was found to be unable to produce a significant photo-cytotoxic effect on human cells using NBA concentrations which have relatively low toxicity for normal human fibroblasts. Since the LFS cells

and the human tumour cells examined have some alteration in the expression of p53, these data suggest that p53 contributes to the high sensitivity of NHF cells to Nile Blue A.

PDT eradicates tumor cells through the intracellular generation of reactive oxygen species (ROS). ROS is able to trigger several cellular events including cell apoptosis and several signaling pathways. It has been suggested that mitogen-activated protein kinases (MAPKs) play a crucial role in controlling cell proliferation, differentiation and cell survival. However, the activation of MAPKs by Photofrin-mediated PDT and its role in the cell response to PDT are lacking. We report here that Photofrin-mediated PDT rapidly activated MAPKs including extracellular signal-regulated kinase 1 and 2(ERK1/2), c-jun NH_2-terminal kinase 1(JNK1) and p38 MAPK in both LFS and NHF cells. The more PDT resistant LFS cells exhibited a sustained activation of MAPKs while the activation of MAPKS in the PDT sensitive NHF cells was transient. Blocking of the sustained activation of ERK1/2 pathway in LFS cells led to a reduction in cell viability following PDT. In contrast, blocking of prolonged p38 activation in LFS cells has no effect on the cell sensitivity to PDT. These data suggest that sustained ERK1/2 activation

protects cells from cell killing by Photofrin-mediated PDT. However, the p38 pathway alone does not play a major role in the sensitivity of LFS cells to Photofrin-mediated PDT. The role of JNK1 activation remains to be clarified.

Several studies have suggested the importance of raf-1 in the regulation of ERK1/2 activity, as well as the importance of MAPK phosphatase 1 (MKP-1) in the negative regulation of ERK1/2 activity. In the present study, it was found that Photofrin-mediated PDT increased expression of MKP-1 at both mRNA and protein levels. The levels of MKP-1 were inversely correlated with the kinetics of ERK1/2 activity following PDT. In addition, PDT resulted in a rapid reduction of the raf-1 expression. These data demonstrated a role of MKP-1 in the regulation of PDT-induced ERK1/2 activation, and suggested that PDT-induced ERK1/2 activity was raf-1 independent.

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Finally, I would like to express gratitude to my parents for their encouragement and confidence. I dedicate this thesis to my husband Yuenian and my son Tong.

Preface

This thesis is presented in a series of chapters in the form of journal articles. One of these articles, chapter 3, has already been published. An additional paper, chapter 4, has been accepted and two more, chapters 5 and 6 will be submitted in the near future.

All the work presented in this thesis has been carried out by the author except for the clonogenic survival assay shown in figure 4, chapter 4, which was carried out by Merna Espiritu, Hamilton Regional Cancer Centre.

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Chapter One

Introduction

I. Cancer and cancer treatments

Cancer can be defined as a disorder of cell growth. It is caused by the accumulation of genetic changes that can interfere with cellular growth. Cancer cells have three characteristics: 1) cancer cells grow indefinitely which involves changes in growth control; 2) the growth of cancer cells is independent of factors needed for normal cell growth, such as anchorage as well as serum and contact inhibition. Cancer cells can grow into foci; 3) cancer cells are able to invade normal tissue, so that they can move away from the tissue of origin and establish a new colony somewhere else in the body. This is called metastasis and is one of the most damaging features of cancer to the organism (for review see Silkora 1999).

Three principle modalities of cancer treatment are used in the clinic. These are surgical therapy, radiation therapy and chemotherapy as well as their combinations. Although some cancers have been curable using these therapies, there are limitations. For instance, radiation therapy destroys cancerous tissue as well as the adjacent normal tissue. Therefore, radiation therapy can cause severe complications such as local skin reactions or gastrointestinal toxicity, with nausea, vomiting or diarrhea. In addition, radiation therapy is mutagenic and carcinogenic and is

associated with increased risk of development of both secondary leukemias and solid tumors (for review see Fauci and Longo 1998). Increasing the effectiveness of cancer treatment is a major focus of biomedical research. As a result of fundamental and applied research, several new cancer treatments have been approved for either clinical use or clinical trials. One of such new treatment modality is photodynamic therapy (PDT).

II. Photodynamic therapy

1. History and applications

A. History

PDT is a novel technique for the treatment of cancers in which a photosensitizing drug is retained by tumor cells and activated by visible light to generate toxic photoproducts that in turn damage tumors. The earliest attempts to exploit the phenomenon of photodynamic reaction for potential antitumor therapy were made by Jesionek and Tappeiner in 1903 by exposure of skin cancer to sunlight in the presence of eosin (Tappeiner and Jesionek 1903). Ten years later, Meyer-Betz injected himself with hematoporphyrin and demonstrated that solar photosensitivity, manifest as edema and

hyperpigmentation, could last for 2 months (Meyer-Betz 1913). The modern interest in PDT for cancer therapy was initiated by Lipson and Schwartz in the 1960s (Lipson et al. 1966). Lipson et al. found that hematoporphyrin derivative (HPD), a mixture of porphyrins, selectively localized in tumors. Dougherty made pioneering efforts in clinical photodynamic therapy in the 1970s, and found complete or partial responses in 111 of 113 cutanous or subcutaneous malignant lesions treated with PDT using HPD as a photosensitizer (Dougherty et al. 1978). The discovery of HPD as an effective tumor localizing photosensitizer, and the subsequent development of laser and optical light delivery systems (Dougherty 1980) led to a large number of experimental and clinical studies of PDT in a variety of tumor types world wide.

B. Applications

Photofrin, an active fraction of HPD, was the first photosensitizer approved by the Federal Health Agency in Canada in 1993 for the treatment of early bladder cancers. Subsequently, Photofrin-mediated PDT has been used in many other countries, including France, Germany, Japan and the United States for many types of cancers such as skin cancer, advanced stage esophageal tumors and early stage lung, gastric and cervical cancers (Dougherty et al. 1998; Schuitmaker et

al. 1996). Photofrin-mediated PDT for other types of cancer and PDT with other photosensitizers for cancer treatment have also been studied in clinical trials (Dougherty et al. 1998). In addition, PDT has been also applied to diseases other than cancer. These include psoriasis, macular degeneration of retina (the leading cause of blindness in the elderly), autoimmune conditions, arteriosclerosis and restenosis (for a review see Levy 1995).

A common protocol used for Photofrin-mediated PDT in the clinic is to give a 2.0 mg/kg dose of Photofrin intravenously followed by light treatment with 630 nm red light at 48 hours after the administration of Photofrin. The total light dose varies for different tumor sites and tumor sizes (Gomer et al. 1989a). For example, 15 J/cm^2 is used for transitional cell carcinoma of the bladder while 200-400 J/cm^2 is used for esophagus or bronchus.

2. Photosensitizers and light sources

A. Photosensitizers

HPD, the first generation of photosensitizers, was developed in 1960 by Lipson *et al.* (Lipson and Baldes 1960). It was used in many basic and clinical studies during the 1970s and early 1980s. HPD is a complex mixture of various

porphyrin species including monomers, dimers and oligomers derived from hematoporphyrin, which can be connected with ether and/or ester bonds (for review see Moan 1986). Photofrin (porfirmer sodium) is only partially purified, and has had removal of the less-active porphyrin's monomers (Dougherty 1996). Photofrin has an intense absorption in the blue light region around 400 nm and four additional absorption bands with decreasing intensity from 500 to 650 nm. Although photofrin is the most widely used and a very efficient photosensitizer in cancer treatment, it has certain limitations, such as the complex chemical composition, low absorption of tissue-penetrating red light (e.g. light with long wavelength, >600 nm), and the tendency to accumulate in skin as well as its long half-life. The last two properties result in a prolonged skin photosensitivity that can cause a severe sunburn-type reaction (Razum et al. 1987; Dougherty et al. 1990). These properties provided incentives for developing new photosensitizers.

The second-generation of photosensitizers have rapid plasma and tissue clearance, enhanced tumor to normal tissue selectivity, comparable photoactivation efficiency, and superior absorption of tissue penetrating light. A photosensitizer that has a strong photoactivation at longer wavelengths than Photofrin could yield a greater effective

light penetration into tissue. At 630 nm the effective tumoricidal depth is 3 to 15 mm, depending on tissue type and the way of illumination. The longer the wavelengths of light, the deeper penetration into tissue. An increasing number of second-generation photosensitizers are being developed, including pthalocyanines, chlorins, purpurins, vertaporphin and benzophenoxazine derivatives (Levy 1995; Cincotta et al. 1987; van Hillegersberg et al. 1994).

Benzophenoxazine derivatives are cationic chromophores. Early studies by Lewis et al. demonstrated that various benzophenoxaziniums, such as Nile Blue A (NBA) and Nile Blue 2B (NB2B) selectively stained and inhibited the growth of tumors in mice (Lewis et al. 1946; Lewis et al. 1949). Both NBA and NB2B showed phototoxicity and some dark toxicity. However, the phototoxicity is lower than that of some other photosensitizers (Fowler et al. 1990). Several more efficient benzophenoxazine derivatives with high absorption of longer wavelength light (>650 nm) have been developed. They have low dark toxicity and high phototoxicity both *in vitro* (Cincotta et al. 1987; Lin et al. 1991; Cincotta et al. 1993) and *in vivo* (Frimberger et al. 1998).

5-amino levulinic acid (ALA) is another interesting chemical. It is not a photosensitizer *per se*, but it can be endogenously converted into the photosensitizer,

protoporphyrin IX (PPIX), which is the penultimate product of the heme biosynthesis pathway and is photoreactive (Peng et al. 1997). ALA can be used topically, orally or systemically (Loh et al. 1993;Regula et al. 1995). ALA has been shown to be a efficient photosensitizer for PDT (Kennedy et al. 1990; Calzavara-Pinton 1995). However, the bioavailability of ALA is low.

B. Tumor selectivity

A number of studies have indicated that there is a different localization preference between tumor and normal tissues for a number of photosensitisers. However, the mechanism(s) of photosensitizer delivery and retention has not been completely determined and the retention ratio between normal and tumor tissue is highly variable (Moore et al. 1997). The density of lipoprotein receptors has been suggested as a specific mechanism for increased uptake of photosensitizer in tumors (Kessel 1986). The association of the photosensitizer with lipoprotein, in particular to low-density lipoprotein (LDL), could result in selective or preferential release to tumor cells (Allison et al. 1990; Allison et al. 1991). Many types of tumor cell express a high number of LDL receptors which promote the internalization of the LDL-bound photosensitizer by endocytosis (Soncin et al.

1995). However, some poor tumor localized protoporphyrins are also able to associate with LDL (Kongshaug 1992). Thus, uptake assisted by LDL is only partially explained for the tumor selectivity of photosensitizers. It has been reported that tumor-associated macrophages in animal tumors take up large amount of HPD and Photofrin (Bugelski et al. 1981; Korbelik et al. 1991). Tissue factors of tumors, such as low pH, newly synthesized collagen that binds porphyrin, and high amounts of lipid that have a high affinity for lipophilic sensitizers, have also been suggested to be involved in the preferential distribution of photosensitizers (Moan and Berg 1992). Understanding the mechanism of tumor selectivity is important for the improvement of the efficiency of PDT. The use of carrier molecular or delivery systems such as lysosome delivery systems has been reported to increase the efficiency and selectivity of photosensitizers (Ricchelli et al. 1993; Jori 1996).

C. Light sources

A variety of light sources can be used in PDT (for review see Fisher et al. 1995). Incandescent filament and arc lamps are used for surface illumination, particularly on skin. These light sources are relatively inexpensive and reliable. However, it is necessary to filter the light spectrum emitted

by these light sources, so that a relevant photoactivating wavelength can be achieved. The wavelength of light used for Photofrin-mediated PDT is 630 nm. Although these light sources cannot be efficiently coupled to fiber optics it is still a good light source for both *in vitro* study and treatment of surface lesions.

Most preclinical and clinical PDT studies use laser systems to generate the photosensitizing wavelength, since the laser can be efficiently coupled into single optical fibers that can be inserted into flexible endoscopes and used interstitially. Laser systems used in the clinic are of many types. The most widely used is the Argon ion laser-pumped dye laser (ADL) (Fisher et al. 1995). The ADL system has minimized the requirement for precise optical alignment of the dye laser and is able to produce light of 550-700 nm. However, this system is very expensive to purchase and maintain. This is one factor limiting the clinical application of PDT. In order to increase the clinical application of PDT, it is very important to develop a laser system which has a relatively low cost and is easy to maintain.

3. Photodynamic reaction and tumor destruction

A. Photodynamic reaction

Upon absorption of a photon of light, the photosensitizer is activated to a high-energy singlet state. The singlet photosensitizer can then form a triplet state by intersystem crossing, or it can decay back to the ground state (Pathak 1982). The triplet state is unstable and can undergo two types of reactions. Type I reactions involve a direct interaction of an excited triplet photosensitizer with a biomolecule such as the membrane lipid via electron transfer. This produces radicals that can react with oxygen and result in the generation of oxygen radicals. Type II reactions involve energy transfer between the triplet sensitizer and oxygen, which generates singlet oxygen ($^1O_2$). Singlet oxygen is a highly reactive oxygen species which has a very short half life in biological systems (<40 nanoseconds) and a short radius of action (<0.02 μm) (Vermeersch et al. 1991; Moan and Berg 1991). Oxygen is required in PDT. At low oxygen concentration, PDT efficiency is reduced or abolished both *in vivo* and *in vitro* (Gomer et al. 1984; Henderson and Dougherty 1993). In the presence of oxygen, the photosensitizer can produce $^1O_2$ efficiently (Cincotta et al. 1993; Fernandez et al. 1997; Cincotta et al. 1993) and the highly reactive oxygen species produced by either type I or type II reaction can cause tumor damage (Weishaupt et al. 1976; Buettner and Need 1985).

B. Tumor destruction

The targets of PDT include tumor cells, tumor microvasculature, normal microvasculature and the inflammatory and immune systems. Evidence of both apoptosis and necrosis in tumor biopsies or cell cultures following PDT (Zaidi et al. 1993; Zhou et al. 1996; Noodt et al. 1996) suggest a direct cell killing effect by PDT. However, the direct photodamage is not thought to be sufficient for tumor cure. *In vivo* studies have reported that direct tumor cell killing by PDT is less than a reduction of 2 logs in surviving fraction and in most cases less than 1 log, which is far from the requirement for tumor cure (Henderson and Dougherty 1992). Experimental studies have documented that vascular injury plays an important role in tumor destruction. Photofrin-mediated PDT has been reported to cause vessel construction, leakage, leukocyte adhesion and thrombus formation (Fingar et al. 1993; Fingar et al. 1997). Tumor vascular shutdown has been observed following PDT with porphyrin or 5-ALA (Roberts et al. 1994). Furthermore, tumor cell death occurred secondary to vascular collapse after chlorin and phthalocyanine photosensitization (Nelson et al. 1988). In addition, PDT can induce strong inflammatory and immune reactions and both are thought to be important events in the mechanism(s) of PDT-mediated tumor destruction (for review see Dougherty et al. 1998).

4. Target and biochemical response

Sites of initial cell and tissue damage by PDT are near the location of the photosensitizer due to the limited diffusion of $^1O_2$ from its formation sites. The localization of photosensitizers in culture cells depends upon its characteristics and the method of administration. Porphyrin-related photosensitizers localize mainly to the mitochondria in the conditions used in clinic (for review see Gomer et al. 1989b).

A. Mitochondrial damage

Mitochondria are considered to be a major target for PDT. Fluorescence microscopy has identified that mitochondria are a major subcellular site of porphyrin (Berns et al. 1982). Studies also showed an inhibition of oxidative phosphorylation and respiration, swelling and disruption of the mitochondria, and inactivation of various mitochondria enzymes following PDT (Hilf et al. 1984; Gomer et al. 1988). Photodynamic treatment with benzoporphyrin derivative (BPD) and Pc 4 caused rapid release of cytochrome c from mitochondria to cytosol (Granville et al. 1998; Varnes et al. 1999). A rapid loss of mitochondria membrane potential was observed following PDT in some cases (Kessel and Luo 1999). In addition, photoactivated PPIX has been found to be the endogenous ligands of the

peripheral benzodiazepine receptor (PBR), a protein localized in the outer mitochondrial membrane (Verma et al. 1987). PBR is involved in the mitochondrial membrane permeability transition (MPT) (McEnery et al. 1992; Camins et al. 1994). These results suggest that PDT can cause the release of cytochrome c through activation of MPT. In contrast, studies from isolated rat liver mitochondria demonstrated that $^1O_2$ induced by PDT inactivated MPT function (Salet et al. 1997). Thus the mechanism of PDT-induced release of cytochrome c from mitochondria remains to be elucidated.

Mitochondria play an important role in the controlling of cell apoptosis. Cytochrome c released from mitochondria is able to trigger the final stages of apoptosis (Kroemer et al. 1997; Liu et al. 1996a). Cytochrome c can activate caspase cascades, which in turn participate in the execution phase of apoptosis (Nunez et al. 1998). It has been reported that PDT induces a rapid activation of caspases and apoptosis in several cell types using mitochondrial localized photosensitizers (He et al. 1998; Granville et al. 1997). Overexpression of Bcl-2, an anti-apoptotic protein of the mitochondrial outer membrane, partially protected Chinese hamster ovary cells from Pc 4-induced apoptosis and increased cell survival (He et al. 1996). Inhibition of Bcl-2 with anti-sense Bcl-2 increased apoptosis induced by BPD-mediated PDT.

Moreover, Bcl-2 reduction was observed in human breast epithelial cell line (MCF10A) following PDT using AlPc through direct photodamage (Kim et al. 1999). These data suggest a down stream inhibitory role for Bcl-2 in PDT-induced apoptosis.

B. Membrane damage

The plasma membrane is the target for hydrophobic photosensitizers. The damage to the plasma membrane can be induced within minutes by photodynamic treatment. This type of damage is evidenced by swelling, bleb formation (Moan et al. 1979), reduction of active transport (Moan et al. 1983), a rise in Ca^{2+} (Joshi et al. 1994) and lipid peroxidation (Thomas and Girotti 1989). The activation of membrane associated enzymes such as phospholipase A2 (PLA2) and phospholipase C (PLC) can lead to the initiation of a cascade of cellular events. For instance, PLA2 plays a role in the rescue of T24 cells from HPD-mediated PDT (Penning et al. 1992) while participating in the cell killing by AlPc-mediated PDT in L5178Y cells (Agarwal et al. 1993).

C. Lysosomal damage

A large number of photosensitizers including Photofrin and Nile blue derivatives have been documented to localize in

lysosomes (for review see Berg and Moan 1997). Lysosomal damage-induced enzyme leakage has been documented following PDT with porphyrin (Jori and spikes 1984) and release of lysosomal hydrolases might contribute to photocytotoxicity (Wilson et al. 1987). However the degree of lysosomal damage caused by PDT did not correlate with photocytotoxicity (Lin et al. 1993; Berg and Moan 1994). This may be due to the inactivation of lysosomal enzymes by PDT (Berg and Moan 1997). Therefore, release of the sensitizer from the lysosomes during light exposure followed by PDT-induced damage might contribute to the photocytotoxicity. Several sensitizers including Nile Blue derivatives have been reported to relocate to the extralysosomal area after PDT (Berg and Moan 1997; Georgakoudi and Foster 1998).

D. Nuclei damage

Although many photosensitizers localize in the nuclear membrane and do not enter the nucleus (Kvam et al. 1992), several types of DNA damage can be induced by PDT. PDT with porphyrin caused single-or double-strand breaks and alkali-labile lesions as well as sister chromosome exchanges *in vitro* (Gomer 1980; Gomer et al. 1983; Kvam et al. 1992). In mouse lymphoma L5178Y cells, PDT with chloroaluminum phthalocyanine induced DNA-protein cross-links and DNA degradation

(Ramakrishnan et al. 1989). In addition, the inhibition of DNA and RNA synthesis by HPD-mediated PDT in hamster ovary cells has also been observed (Lin et al. 1986).

PDT is mutagenic in some cell systems. The frequency of PDT-induced mutation depends upon the cell type, photosensitizer and the target gene. For instant, mutation induced by PDT with Photofrin or AlPc in murine L5178Y cell was higher than that in human lymphoblastic cell lines (Evans et al. 1997). The Photofrin-mediated PDT resulted in a higher mutant frequency than PDT with AlPc in human WTK1 cells. The induction of mutation by Photofrin-mediated PDT was similar to that induced by UVC radiation but lower than that induced by X-radiation in WTK1 cells (Evans et al. 1997).

5. Stress response induced by PDT

A. Stress proteins

A number of proteins involved in the cellular stress response may be regulated by PDT. Photofrin-mediated PDT induced glucose-regulated protein (GRP) expression at both the transcriptional and translational levels in RIF-1 cells (Gomer et al. 1991) and similar results were observed in FaDu cells treated with Victoria Blue BO-mediated PDT (Morgan et al. 1998). Although both these sensitizers are located in

mitochondria, the consequence of the induction of GRP is somewhat controversial. Overexpression of GRP by calcium ionophor A-2187 reduced cell sensitivity to Photofrin-mediated PDT in RIF-1 cells but potentiated the PDT effect of Victoria Blue Bo and Photofrin in FaDu cells (Gomer et al. 1991; Morgan et al. 1998). These data suggest that the effect of GRP in cell sensitivity is cell type specific.

Another group of stress-induced proteins is heat shock proteins (hsp). Accumulated evidence demonstrates PDT is able to induce several hsp. Treatment of murine tumor cells with BPD-mediated PDT induced a number of hsp *in vitro* as well as *in vivo* (Curry and Levy 1993). Hsp70 induction was observed in RIF-1 cells treated with several photosensitizers (Gomer et al. 1996). Recently, PDT-induced hsp60, hsp27 and GRP78 (unpublished data) have also been found in HT29 cells. However, Xue et al. reported a down regulation of hsp70 in V79 cells treated with PDT using AlPc as photosensitizer (Xue et al. 1995). Further more, PDT with 1 h incubation of Photofrin had no effect on the expression of hsp70 while PDT with 16 h incubation increased hsp expression in Chinese hamster ovary cells (Fisher et al. 1993). Thus, the cellular targets of photosensitizer may determine the induction of hsp70.

B. Signaling pathways

Photooxidative lesions in the plasma membrane and membranes of cellular organelles can trigger a variety of cellular events. One process initiated at the membrane level involves signal transduction pathways. One of the well-characterized signaling pathways is the extracellular signal-regulated kinase (ERK) pathway, which is generally activated by mitogenic stimuli. PDT with hypericin has been shown to have no effect on the activity of ERK but to inhibit the EGF-induced ERK2 activation (Assefa et al. 1999). PDT prevented the phosphorylation of the EGF receptor (de Witte et al. 1993) as well as the insulin receptor (Agostinis et al. 1995). In other studies, it was shown that PDT has no effect on ERK activity (Tao et al. 1996; Klotz et al. 1998). However, a slight activation of ERK2 was observed in Chinese hamster ovary cells treated with Pc 4 (Xue et al. 1999). Currently, there is no direct evidence for an involvement of the ERK pathway in photocytotoxicity.

In contrast to ERK, c-jun NH_2-terminal kinase (JNK) and p38 pathways are activated by stress stimuli. PDT with porphyrin has been shown to induce several early response genes including c-fos, c-myc and c-jun (Luna et al. 1994). These are the downstream effector molecules of several signal transduction pathways and studies of kinase activation have provided clues regarding the upstream molecules. Using

hypericin as a photosensitizer, PDT induced the JNK and p38 kinase pathway in a variety of cell lines (Assefa et al. 1999). Similar results were also observed in human skin cells treated with 5-aminolevulinate-mediated PDT (Klotz et al. 1998) and in murine LY-R leukemic lymphoblasts and in Chinese hamster ovary cells treated with Pc 4-mediated PDT (Xue et al. 1999). The activation of the JNK and p38 kinase pathways was shown to protect HeLa cells from apoptosis induced by hypericin-mediated photocytotoxicity (Assefa et al. 1999) while the activation of p38 potentiated the apoptosis of LY-R cells following Pc 4-mediated PDT (Xue et al. 1999). The singlet oxygen produced by PDT is thought to participate in the activation of JNK and p38 pathways, although the upstream activators of these pathways remain to be established.

The activation of several signal pathways by PDT is listed in Table I.

6. Sensitivity of cell to PDT

Differential responses to PDT have been studied in several cell types. Using HPD as a photosensitizer, Foultier et al. (Foultier et al. 1989) reported that murine L1210 cells had increased sensitivity compared to normal hematopoietic progenitor cells for the same drug uptake. Similarly, human smooth muscle cells from atherosclerotic plaques exhibited an

Table 1.

Some other signaling reactions activated by PDT

Reaction activated	Photosensitizer
A. Release of second messenger	
Phospholipases C, A_2	AlPc
Ceramide	Pc 4
Prostaglandin E2	Photofrin
Calcium ion	AlPc
Nitric oxide	Pc 4
B. Protein kinase cascade	
NF-κB activation	Photofrin Methylene Blue
HS1 phosphorylation	Pc 4
C. Induction of cytokine expression	
TNF-α	Photofrin Pc 4
IL-6	Photofrin
IL-10	Photofrin
D. Gene involved in cell cyle regulation	
$P21^{waf}$ induction	Pc 4
PRb dephosphorylation	Pc 4

increased response to Photofrin-mediated PDT compared to smooth muscle cells from non-atherosclerotic arteries (Dartsch et al. 1990). In addition, cells resistant to PDT have also been generated (Sharkey et al. 1993). There are many mechanisms that could contribute to the cellular response to PDT (for review see Moore et al. 1997).

Several laboratories have studied the influence of cellular p53 status on the response of cells to PDT, with differing results. Human promyelocytic leukemia HL60 cells expressing none or mutant p53 have been shown to be more resistant to cell killing by Photofrin or tin ethyl etiopurpurin-mediated PDT compared to HL60 cells which express wild-type p53 (Fisher et al. 1997). A similar result was observed in human colon carcinoma cells (Fisher et al. 1998) and human lymphoblastic cells (Evans et al. 1997). In contrast, the SV40-transformed IMR-90 cells in which p53 function is abrogated were more sensitive to PDT compared to their untransformed counterparts using HPD as a photosensitizer (Denstman et al. 1986). Also, the abrogation of p53 expression by human papillomavirus type 16 E6 had no effect on the survival of colon or breast carcinoma cells following Photofrin-mediated PDT (Fisher et al. 1999). The different effects of p53 on the cell sensitivity in these studies may result from confounding effects of additional

genetic alterations present in the tumor cells that could modulate p53 dependent pathways or be due to differences in cell type and the conditions of PDT employed. Cells expressing only mutant p53 should be good models for studying the role of p53 in the cellular response to PDT.

The mitogen-activated protein kinase (MAPK) pathway may be also involved in the regulation of cellular responses to PDT. As indicated in a previous section, the inhibition of p38 activation by Pc 4-mediated PDT decreased apoptosis in LY-R cells (Xue et al. 1999). In contrast, inhibition of the JNK and p38 kinase pathways increased apoptosis in HeLa cells following Hypericin-mediated PDT (Assefa et al. 1999). However, these studies used apoptosis and not a clonogenic assay to determine photocytotoxicity and apoptosis does not always correlate with overall cell killing by PDT (Xue et al. 2001).

III. P53

P53 was initially identified as an oncogene since overexpression of p53 caused oncogenic transformation of cells (Eliyahu et al. 1984) whereas, the normal function of p53 was shown to be anti-oncogenic (For review see Ko and Prives 1996). Over 50% of human tumors show loss of normal p53 function (Hollstein et al. 1991; Levine et al. 1991).

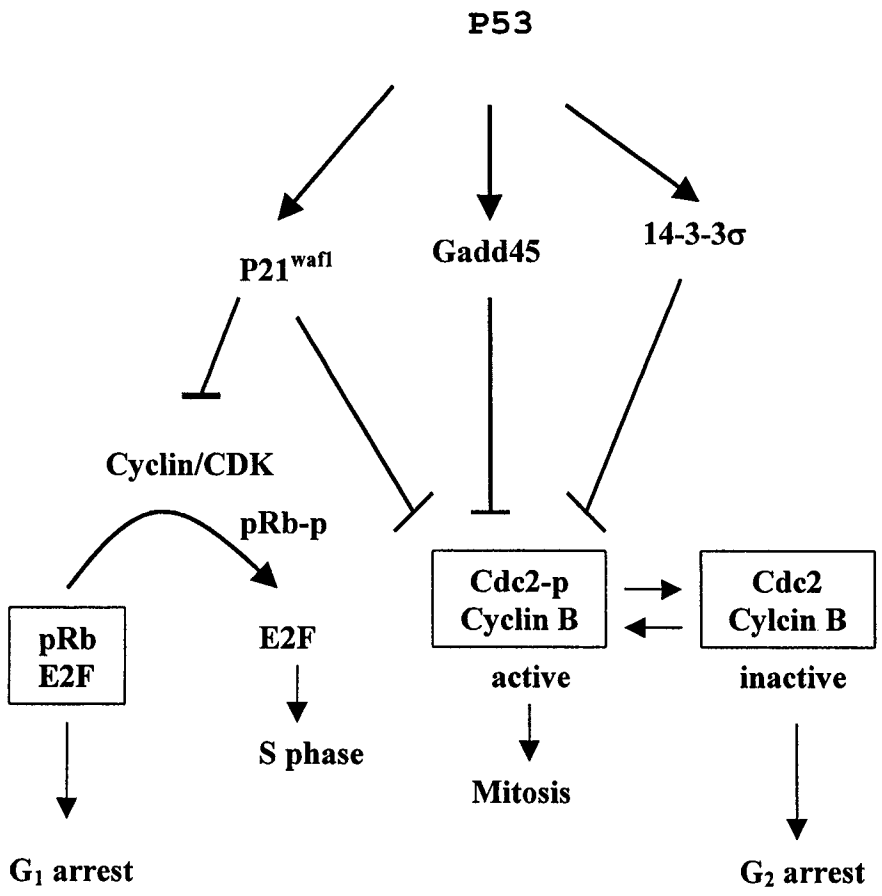
Individuals with Li-Fraumeni syndrome inherit a germ-line mutation on one allele of the p53 gene and are highly susceptible to diverse tumors as a result of the loss of the remaining wild-type p53 gene (Malkin 1994b). P53 is considered as "the guardian of the genome" for its role in preventing the accumulation of genetic alterations, since p53 can induce growth arrest and apoptosis, which allows damaged DNA to be repaired and aberrant cells to be eliminated (For review see Ko and Prives 1996).

1. P53 and cell cycle arrest

p53 plays a role in regulating vital checkpoints during the G_1 and G_2 phase of the cell cycle through its transcriptional activating functions (Figure 1). A number of genes associated with cell cycle regulation have been shown to contain p53-binding sites and/or p53 response elements (for review see Ko and Prives 1996). $p21^{WAF1}$ is the most well studied p53 responsive gene, and it is responsible for G_1 arrest (el Deiry et al. 1994). p21-Mediated p53-dependent G_1 arrest is through the inhibition of cyclin-dependent kinase (CDKs) activity (Harper et al. 1993; Xiong et al. 1993). This in turn prevents the phosphorylation of the retinoblastoma protein (pRb) (Slebos et al. 1994). The hypophosphorylated form of pRb is able to bind and sequester the E2F

Figure 1. Schematic representation for the participation of p53 in regulating cell cycle checkpoints.

P53 mediated G_1 arrest occurs mainly through transcriptional activation of $p21^{waf1}$, while p53 mediated G_2 arrest may be via transcriptional activation of $p21^{waf1}$, Gadd45 and the 14-3-3σ protein.


P53
P21wafl
Gadd45
14-3-3σ
Cyclin/CDK
pRb-p
pRb
E2F
E2F
S phase
Cdc2-p
Cyclin B
active
Cdc2
Cylcin B
inactive
Mitosis
G1 arrest
G2 arrest

transcription factor, resulting in the blocking of the transition into S phase (Hiebert et al. 1992; Helin et al. 1993).

In addition to G_1 arrest, p53 has also been shown to be involved in G_2 arrest (for review see Taylor and Stark 2001). p53 Blocks cell cycle at the G_2 checkpoint, which involves the inhibition of cdc2. Cdc2 is a cyclin-dependent kinase required for entering mitosis. Overexpression of p53 in human fibroblasts inactivates cdc2 by reducing phosphorylation on tyrosine residues (Taylor et al. 1999). Moreover, G_2 arrest accompanied by low levels of cdc2 activity was reported in cells overexpressing p21 (Smits et al. 2000). Some other transcriptional targets of p53 are implicated in regulating G_2/M transition include Gadd45 and 14-3-3σ proteins. Gadd45 is able to dissociate cdc2 from Cyclin B1 and inhibits the activity of cdc2 (Zhan et al. 1999). 14-3-3σ Proteins can bind to cdc2/cyclin B1 and sequester cdc2/cyclin B1 in the cytoplasm (Hermeking et al. 1997).

Cell arrest at the G_1 or G_2 phase can also be induced by one or more p53-independent pathway(s). Many p53-null cell lines can arrest in G_1 or G_2 in response to DNA damage (Kastan et al. 1991; Shao et al. 1997). G_1 or G_2 arrest in response to genotoxic stress provides additional time to allow the cell to repair damage before entering the normal cell cycle (Hwang and

Muschel 1998). If the damage cannot be repaired then apoptotic cell death can be triggered (Oren 1994).

2. p53 and apoptosis

Apoptosis, e.g. programmed cell death, is an intrinsic property of most cell types. It is distinguished from cell death by necrosis by a defined set of morphological changes including chromosome condensation and DNA fragmentation (Allen et al. 1997). Apoptosis can be induced by many kinds of stress stimuli including chemotherapeutic agents (Kaufmann and Earnshaw 2000), and PDT (Xue et al. 2001; Xue et al. 1999). Apoptosis is an important event which removes damaged and unhealthy cells. Accumulated evidence suggests a role for p53 in apoptosis (for review see Ko and Prives 1996). Transcriptional activation appears to be essential for p53 induced apoptosis. For example, apoptosis induced by p53 may be partially mediated via transcriptional regulation of the bcl-2 family member proteins. One of these family members is the proapoptotic protein bax, which is upregulated in response to p53 expression (Miyashita et al. 1994). Bax is able to bind to the anti-apoptotic protein bcl-2 and antagonizes its ability to block apoptosis (for review see Rao and White 1997). Overexpression of bax accelerates apoptosis in some model systems (Sakakura et al. 1996). However, cells from bax

deficient mice are nevertheless able to undergo apoptosis in response to p53 (Brady et al. 1996). These data suggest that there are other p53 independent pathways involved in apoptosis. Tumor cells that possess either a mutation in or a deletion of the p53 gene are still capable of undergoing apoptosis (Strobel et al. 1996; Chang et al. 1996). Since most chemotherapeutic agents and ionizing radiation are strong apoptotic inducers (Kaufmann and Earnshaw 2000; Dewey et al. 1995), it is now believed that apoptosis is a critical mechanism involoved in the cytotoxic effects of chemotherapy and radiation therapy.

3. The regulation of p53 activity

The p53 protein can be activated by several stress conditions including DNA damage, reactive oxygen species and hypoxia (for review see Amundson et al. 1998). The p53 protein is regulated at two main levels: protein stability and biochemical activity. In most cell types, the p53 protein is relatively unstable under normal growth conditions. Its half-life is about 20 minutes (Blagosklonny 1997). Exposure of cells to DNA damaging agents such as anti-cancer drugs, UV and ionizing radiation results in a rapid accumulation of p53 protein without increase in transcription of p53 (for review see Giaccia and Kastan 1998). These data indicate that the

increase of p53 protein in response to DNA damage may be due to protein stabilization rather than increase in the protein production. A key player in this regulation is mdm-2 protein. Mdm-2 is a transcriptional target of p53 (Juven et al. 1993) and able to bind to p53 at its transactivation domain, which in turn results in both transcriptional suppression and degradation of p53 (Momand et al. 1992; Haupt et al. 1997; Kubbutat et al. 1997). Therefore, mdm-2 acts as a negative feed back mechanism controlling p53 levels. In addition, post-translational modification of p53 by phosphorylation has been proposed to be an important mechanism by which p53 stabilization and function are regulated (Meek 1994). Several protein kinases can phosphorylate p53 (Prives 1998). For instance, the JNK pathway has been shown to be able to phosphorylate p53 which leads to the dissociation of p53 from mdm2 and stabilize the p53 protein (Fuchs et al. 1998). However, mutation of some phosphorylation sites of p53 is still sensitive to mdm-2-mediated degradation (Ashcroft et al. 1999).

4. P53 and cell response to cancer therapy

A correlation between apoptosis and cell response to cancer therapy has been reported in several cell types (for review see Bold et al. 1997). p53 has been demonstrated to

play an important role in cell apoptosis (Bold et al. 1997). Both *in vivo* and *in vitro*, loss of p53 or p53 mutations result in the resistance of some tumor cells to chemotherapy and radiation therapy (McIlwrath et al. 1994; Lowe et al. 1994) as well as PDT (Fisher et al. 1997). In contrast, reintroduction of wild type p53 into ovarian tumor cells (which normally express mutant p53) by recombinant adenovirus-based infection increases the sensitivity to radiation therapy (Gallardo et al. 1996). Similarly, reintroduction of wild-type p53 results in an increase of human lung cancer cell sensitivity to chemotherapy (Fujiwara et al. 1994) as well as an increase in the photosensitivity of HT29 colorectal carcinoma cells (Zhang et al. 1999). However, wild type p53 expression is not always positively correlated with the cellular sensitivity to cancer therapy. Negative or no correlation between p53 status and cell responsiveness has also been reported in some cell types (De Feudis et al. 1997; Fan et al. 1995; Fisher et al. 1999). These data suggest that the role of p53 in the cell response to cancer therapy is cell type and treatment dependent.

IV. MAPKs

Cells respond to a variety of environmental stimuli that regulate many aspects of cell function such as proliferation, differentiation and death. Many signaling

pathways are involved in this process. Among these, the mitogen-activated protein kinase (MAPK) pathway has been considered as an important one.

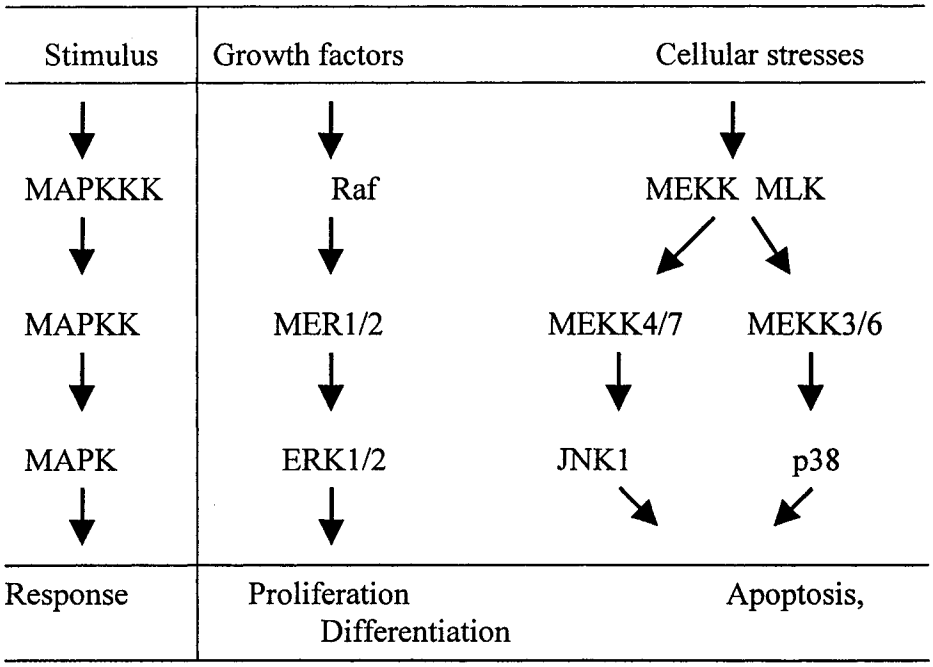
1. Mammalian MAPK pathways

In mammalian cells, genetic studies have identified that there are at least three MAPK pathways (Schaeffer and Weber 1999), including the extracellular signal-regulated kinase (ERK), c-jun NH_2-terminal kinase (JNK) and p38 protein kinase pathways. Each group of MAPKs is activated by protein cascades (Figure 2), which include enzymes that are activated in series: the MAPK is activated by a MAPK kinase (MAPKK), which is activated by a MAPK kinase kinase (MAPKKK) (for review see Lewis et al. 1998). In the JNK subgroup, three genes and ten different splicing variants have been described (for review see Mielke and Herdegen 2000), although the functional differences between these isoforms have not been fully clarified. Similarly, the p38 protein kinase has six isoforms(Mielke and Herdegen 2000) and the ERK has three isoforms including ERK1 and ERK2. In most cases, both ERK1 and ERK2 are activated upon cell stimulation (Cobb 1999).

2. The regulation of MAPK

Figure 2. Schematic overview of MAPK modules.

MAPK kinase kinases (MAPKKK) activate MAPK kinases (MAPKK) by phosphorylation. The activated MAPKKs then further phosphorylate and activate MAPKs. The activation of MAPKs results in cell proliferation, differentiation and/or apoptosis.


Stimulus
Growth factors
Cellular stresses
MAPKKK
Raf
MEKK MLK
MAPKK
MER1/2
MEKK4/7
MEKK3/6
MAPK
ERK1/2
JNK1
p38
Response
Proliferation
Differentiation
Apoptosis,

Activation of MAPKs

MAPK activation requires dual phosphorylation on Thr and Tyr residues with the motif of Thr-Xaa-Tyr (Cobb and Goldsmith 1995; Kyriakis and Avruch 1996). The best-characterized sequential protein kinase pathway that leads to the activation of MAPKs is the growth factor-induced ERK pathway. The ligands of growth factor to receptor lead to the activation of receptor tyrosine kinase, which in turn activates ras. The activated ras further binds to the raf family of proteins, mainly raf-1, and brings it to the plasma membrane where raf-1 is activated (Jelinek et al. 1996). Once activated, raf-1 activates its downstream target, MEK1 and MEK2 by phosphorylation (Ahn et al. 1991; Seger et al. 1992). MEK1 and MEK2 are the direct upstream activators of ERK1 and ERK2 (Crews et al. 1992; Zheng and Guan 1993b; Zheng and Guan 1993a). The activation of the ERK cascade is thought to occur mainly in response to mitogenic stimuli, and is involved in cell proliferation, differentiation and survival (for review see Lewis et al. 1998). However, cellular stresses including heat shock (Ng and Bogoyevitch 2000), DNA damaging agents (Cui et al. 2000) and reactive oxygen species (Wang and Bonner 2000) also can activate ERK1/2 pathway. In some cell types, the activation of the ERK pathway can be raf-1 or ras independent (Ng and Bogoyevitch 2000; Garcia et al. 2001).

The JNK and p38 pathways are activated by a variety of environmental stresses such as osmotic shock, UV radiation, heat shock and reactive oxygen species, and play an important role in stress-induced apoptosis (for review sees Lewis et al. 1998). The direct upstream activators of JNK are SEK1/MKK4 and MKK7 (Sanchez et al. 1994; Derijard et al. 1995; Lawler et al. 1998; Tournier et al. 1997) although *in vitro* SEK1/MKK4 also phosphorylates and activates p38 (Derijard et al. 1995). SEK1/MKK4 and MKK7 do not appear to have any preference for the different JNK isoforms. MKK3 and MKK6 are direct upstream activators of p38. However, they differentially activate p38 kinases. MKK3 only activates p38α and p38γ, while MKK6 activates p38α, p38β and p38γ (Enslen et al. 1998). In addition several other kinases have been demonstrated to be able to activate these MKKs (for review see Davis 2000). It includes MKK kinase1 (MEKK1) and mixed lineage kinases (MLKs). The former is an activator of MKK7 and MKK4/SEK1 (Yao et al. 1997; Yan et al. 1994; Cuenda and Dorow 1998), whereas the later is activator of MKK 6 and MKK4/SEK1 (Rana et al. 1996; Tibbles et al. 1996).

B. Negative regulation of MAPKs

MAPK pathways are activated by a series of

phosphorylation events which can be inactivated by phosphatases. Several specific phosphatases that target different MAPK have been identified, including dual-specificity phosphatases (MKPs) (for review see Lewis et al. 1998). MKPs are dual specificity phosphatases that inactivate MAPKs by dephosphorylation of both Thr and Tyr residues within their signature sequence. At least nine distinct MKP family members have been cloned. Most of them are the products of immediate early genes, and are under tight transcriptional control (Keyse 1998; Bokemeyer et al. 1996; Cook et al. 1997). MAPK phosphatase 1 (MKP-1) shows selectivity for ERK1/2 *in vitro* (Franklin and Kraft 1995; Sun et al. 1993b). MKP-1 has been reported to block growth factor-induced ERK activation and suppress cell proliferation (Brondello et al. 1995; Sun et al. 1993a). MKP-1 expression can be induced by mitogens (Brondello et al. 1999) and also by a variety of stress stimuli including oxidative stress (Guyton et al. 1996; Metzler et al. 1998; Bokemeyer et al. 1998; Kojima et al. 2000). MKP-1 is also a target of ERK1/2. ERK1/2 stabilizes MKP-1 by phosphorylation (Brondello et al. 1999). Thus, there is a negative feed back loop of regulation between ERK1/2 and MKP-1. In addition, MKP-1 has been reported to inactivate JNK and p38 in some cases (Sanchez-Perez et al. 2000; Hirsch and Stork 1997; Franklin and Kraft 1997).

3. General functions of MAPK

A. Gene expression

One of the most explored functions of MAPK signaling modules is the regulation of gene expression in response to extracellular stimuli (for review see Lewis et al. 1998). Many downstream target genes of MAPK have been identified. JNKs phosphorylate jun proteins and enhance their ability to activate transcription (Kallunki et al. 1996). Most MAPKs phosphorylate Ets transcription factors. Ets is involved in the induction of c-fos which heterodimerizes with jun proteins to form the activation protein 1 (AP-1) complex (for review see Lewis et al. 1998; Kallunki et al. 1996). Some of the substrates may be regulated by more than one MAPK pathway. For instance, c-jun can also be phosphorylated by ERK1/2 (Chou et al. 1992). This suggests that different stimuli may result in the same effect via different pathways. In addition to regulating transcription factors, MAPKs also regulate gene expression through post-transcriptional or post-translational mechanisms. JNK, for example, phosphorylates p53, and thereby, stabilizes its protein (Fuchs et al. 1998).

B. Cell proliferation

A number of studies indicate the role for ERK1/2 in

cell proliferation (for review see Lewis et al. 1998). A correlation between the activation of ERK1/2 and DNA synthesis or cell growth induced by mitogenic stimuli has been shown in many cell types. This suggests that the ERK pathway is necessary for cell proliferation (Liang and Chen 2001; Mizuno and Nishida 2001; Ravenhall et al. 2000). Blocking ERK1/2 activation using PD98059, an inhibitor of MEK, prevents or attenuates mitogen-induced DNA synthesis in human cultured airway smooth muscle cells (Ravenhall et al. 2000). Constitutive expression of active mutant MKK1 increases cell growth (Brunet et al. 1994; Seger et al. 1994). The mechanism by which ERK1/2 stimulates cell proliferation may be through the regulation of cyclin D1 expression, as evidenced by the report that the activation of ERK1/2 is required for the induction of cyclin D1 in primary bovine airway smooth muscle cells (Ramakrishnan et al. 1998), as well as in Chinese hamster lung cells (Lavoie et al. 1996). The JNK and p38 pathways have also been shown to be involved in cell proliferation in some cell types. For example, the activation of JNK is required for epidermal growth factor-induced lung cancer cell growth (Bost et al. 1997) and in vascular smooth muscle cells, treatment with SD203580, an inhibitor of p38, results in the inhibition of thrombin-induced DNA synthesis (Kanda et al. 2001). Furthermore, hypoxia-induced cell

proliferation is ablated by the treatment of either antisense oligonucleotides of JNK1 or the p38 inhibitor SD202190 (Das et al. 2001).

C. Cell survival

MAPKs play a crucial role in controlling cell survival. In most cases, activation of ERK has been linked to cell survival while JNK and p38 are involved in the induction of apoptosis (Xia et al. 1995a). Inhibition of ERK1/2 activation by PD98059 results in enhanced cisplatin cytotoxicity in ovarian carcinoma cell lines (Persons et al. 1999), and increases H_2O_2- induced apoptosis in cardiac myocytes (Aikawa et al. 1997) and hyperoxia-induced apoptosis in alveolar epithelial cells (Buckley et al. 1999). Moreover, accumulated evidence demonstrates that ionizing radiation-induced ERK activation is able to protect cells from radiation-induced cell death (Abbott and Holt 1999; Hagan et al. 2000; Dent et al. 1999). However, the role of JNK and p38 in cell survival appears to be highly cell type and context dependent. Expression of dominant negative form of JNK1 or treatment with a p38 inhibitor SB202190 increased the viability and prevented cadmium-induced apoptosis in lung carcinoma cells (Chuang et al. 2000). In mouse fibroblasts, expression of a constitutively active mutant of MEKK1, an

upstream activator of JNK, induced apoptosis (Johnson et al. 1996). Similarly, expression of the constitutively active mutant of MKK3 (an upstream activator of p38) in PC12 cells induced apoptosis (Xia et al. 1995b). In contrast, TNF-α receptor-mediated JNK activation was not linked to apoptosis (Liu et al. 1996b). A study using human eosinophils shows that p38 is required for TNF-α mediated cell survival (Tsukahara et al. 1999). The activation of JNK increased T98G glioblastoma cell survival after treatment of cisplatin (Potapova et al. 1997) and expression of the dominant negative mutant of c-jun decreased the viability after the treatment of cisplatin. These results suggest that JNK and p38 also mediate a survival signal under specific circumstances.

4. MAPKs and cell response to cancer therapy

Several studies have shown a role for MAPKs in the cell response to cancer therapy. As mentioned previously, blocking ERK1/2 activation by PD98059 resulted in enhanced cisplatin cytotoxicity in ovarian carcinoma cell lines (Persons et al. 1999), whereas increased JNK activity was necessary for cisplatin-induced apoptosis. Cells derived from c-jun knock out mice are more resistant to cisplatin-induced cell death compared to normal cells (Sanchez-Perez and Perona

1999). Blocking the p38 pathway with SB20219 inhibits Pc-4-PDT-induced apoptosis in murine leukemic lymphoblasts (Xue et al. 1999). Similarly, ERK activity has been reported to protect cells from ionizing radiation-induced cell death (Hagan et al. 2000). JNK activation has also been suggested to play a critical role in ionizing radiation induced apoptosis (for review see Verheij et al. 1998). In contrast, cells derived from c-jun knock out mice show no change in cell survival following adriamycin treatment (Sanchez-Perez and Perona 1999). Expression of a dominant negative mutant c-jun inhibits DNA repair and thereby decreases cell viability following cisplatin treatment in several tumor cell lines (Potapova et al. 1997). Moreover, the activation of JNK and p38 has been shown to protect HeLa cells from apoptosis induced by hypericin-mediated PDT (Assefa et al. 1999). Therefore, the role of MAPK in cell response to cancer therapy seems to depend upon the cell type and/or the type of stimuli.

V. Proposed study

Although PDT is a promising cancer treatment, the cell sensitivity to PDT varies between different cell types as reviewed above. There are many mechanisms that could contribute to the cellular response to PDT. However, the mechanism of cell sensitivity to PDT is far from clear.

Increasing cell sensitivity to PDT has the potential to increase the effectiveness of clinical treatment. Therefore, a better understanding of the mechanism(s) involved in the cellular sensitivity to PDT may lead to novel strategies to increase the tumor killing effects of PDT. p53 and MAPKs have both been shown to play a role in the cellular response to chemotherapy and radiation therapy. However, the exact details concerning the involvement of p53 and MAPKs in the cellular response to PDT are far from clear. The goals of this study focus on the potential roles of p53 and MAPK pathways in the cell sensitivity to Photofrin-mediated PDT.

In the present study, normal human skin fibroblast (NHF) strains and immortalized Li-Fraumeni syndrome (LFS) skin fibroblast cells were used as cell models. Immortalized LFS cells are derived from individuals with an inherited germ-line mutation in one allele of p53 gene (Malkin 1994). The genetic mutation in LFS is thought to be specific for p53 (Varley et al. 1997). Immortalization of LFS skin fibroblasts involves loss of the wild-type p53 allele such that the established LFS line expresses only mutant p53. Thus LFS cells have been used as a model system to examine the role of p53 in apoptosis, DNA repair, and the sensitivity of cells to a variety of DNA damage agents (McKay et al. 1997; Ford and Hanawalt 1995). The work presented in this thesis shows that LFS cells have an

increased resistance to Photofrin-mediated PDT compared to NHF cells.

The sensitivity of NHF and LFS cells to Photofrin-mediated PDT was determined using a clonogenic survival assay. It was found that LFS exhibited a G_2/M arrest and were more resistant to Photofrin-mediated PDT than NHF cells. Reintroduction of wild type of p53 by recombinant adenovirus infection increased LFS cell sensitivity to PDT. These results suggest that the p53 mutation in LFS cells contributes to the greater resistance of LFS cells to Photofrin-mediated PDT.

The activation of MAPK pathways in LFS and NHF cells was also studied. The activation of MAPKs including ERK1/2, JNK1 and p38 was observed in both cell types following Photofrin-mediated PDT. NHF cells exhibited a transient activation of MAPKs while the activation of MAPKs in LFS cells was prolonged. Blocking the prolonged ERK1/2 activation increased the sensitivity of LFS cells to PDT. However, blocking p38 activity had no effect on LFS cell survival after PDT. These data suggest that sustained activation of ERK1/2 protects cell from Photofrin-induced phototoxicity, whereas P38 activation alone does not appear to play an important role in cell sensitivity to PDT. The actual role of JNK1 activation remains to be further investigated.

Work presented in this thesis also shows that PDT

increased MKP-1 expression in both cell types. The expression of MKP-1 was inversely correlated with the kinetics of PDT-induced ERK1/2 inactivation. In addition, it was observed that PDT reduced the expression of raf-1, suggesting that PDT-induced ERK activity is raf-1 independent and regulated, in part at least, by MKP-1.

Since p53 plays a role in cell response to Photofrin-mediated PDT, we have also examined its potential roles in the second generation photosensitizer, Nile Blue A (NBA)-mediated PDT. We found that NHF cells showed extreme dark toxicity to NBA compared to LFS cells and some human tumor cells. In addition, we were unable to detect significant phototoxicity of Nile Blue A in the human cells tested using NBA drug alone in concentrations that have relatively low toxicity for normal human fibroblasts. It is possible that the high cytotoxicity of NBA in normal human skin fibroblasts may cause side effects when using Nile Blue A as photosensitizer in the clinic. However, to what extent the extreme dark toxicity of NHF cells to the drug NBA would influence the outcome of NBA-mediated PDT in the treatment of human tumors remains to be investigated.

Chapter Two

Materials and Methods

Materials

Cell lines and fibroblast strains

The normal human fibroblast (NHF) strains were obtained from the National Institute of General Medical Sciences Repository (Camden, NJ). The immortalized Li-Fraumeni syndrome (LFS) cell lines, LFS087 and LFS041 were obtained from Dr M. A. Tainsky, M. D. Anderson Cancer Center, Houston, TX. HeLa cells (C1/A5) stably expressing FLAG-tagged dominant negative p38 mutant (p38 (AGF)) (Taher et al. 1999) and parental HeLa cells (A5) were obtained from Dr. K. Valerie, Medical College of Virginia, Richmond, VA. The 293 cell line was a human embryonic kidney cell line transformed with the left end of Adenovirus 5 (Ad5). This cell line expresses early region 1 of Ad5 therefore permitting propagation and titration of E1 deleted recombinant Ad constructions (Hitt et al. 1997). These cells were provided by Dr. F. L. Graham, McMaster University, Hamilton, Ontario.

Recombinant adenovirus constructs

Replication defective viruses expressing various gene products were obtained from several investigators. Recombinant expressing lacZ (Ad5HCMVsp1lacZ) or wild-type p53 (Ad5p53wt)

under the control of the human cytomegalovirus (HCMV) immediate early promoter was provided by Dr. F. L. Graham, McMaster University, Hamilton, Ontario. The recombinant adenovirus expressing a dominant negative mutant of p38 (Ad5p38(AGF)) was obtained from Dr. K. Valerie, Medical College of Virginia, Richmond, VA.

Reagents

Photofrin was a gift from QLT Inc. (Vancouver, BC). The RNeasy mini kit and reagents used for RT-PCR were obtained from Qiagen Inc. (Missisauga, ON). The Random primer labeling kits were purchased from Life Technology (Burlington, ON). The MEK1/2 inhibitor, PD989059, and antibodies to phophorylated ERK1/2 and p38 or total ERK1/2 and p38 were purchased from New England Biolabs, Inc. (Beverly, MA). HRP conjugated secondary antibodies and antibodies to Raf-1, actin or MKP-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-human JNK1 antibody and protein-G sepharose were purchased from PharMingen (San Diego, CA.). Chemical reagents were purchased from either GibcoBRL or Sigma (St. Louis, Missouri, USA).

Methods

Cell cultures:

All cells were grown in monolayer culture in alpha-minimal essential medium (α-MEM medium) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Gibco BRL) and were cultured in a humidified atmosphere at 37 ^{0}C and 5% CO_2.

Virus propagation and titration:

293 cells were grown to near confluence in 150 mm dishes. The cells were infected with the virus of interest at a multiplicity of infection (MOI) of 2-4 plaque forming units (PFU)/cell. Following a 45-minute period, cells were re-fed with 26 ml of complete growth medium. When most cells were round up, usually three to five days following infection, cells were harvested by scraping and pelleted by centrifugation at 1000 rpm for 5 minute. Cells were resuspended in 100 µl of α-MEM containing 10% of sterilized glycerol per dish. The resuspended cells then were subjected to three cycles of freezing and thawing to release virus from cells. Virus was stored at -20 ^{0}C.

To titre virus, the virus stock was serially (between

10^6 to 10^9) diluted in serum-free α-MEM medium. Nearly confluent 293 cells were infected with each dilution in triplicate for 45 minute using 6 well plate. Thereafter, cells were overlaid with 8 ml of complete α-MEM medium. The overlay was removed when plaques formed (usually 5 days after infection). The monolayers were stained with 0.5 % of methylene blue in 70 % ethanol. The titre of virus stock was calculated from the number of plaques formed at each dilution.

Photodynamic treatment:

Cells were incubated with Photofrin at the concentrations indicated in individual experiments for 18 hours and followed by replacement with fresh culture medium before exposure to red light. Light exposure to cell monolayers was performed as described previously (DiProspero et al. 1997). The light source was a light box illuminated by a parallel series of fluorescent tubes (Phillips type TL83) and the light was filtered with red acetate filters (Roscolux, No. 19, Rosco, CA). The energy fluence rate was indicated in individual experiments at a wavelength of 630 nM. The light dose used was as indicated in individual expriments. In the experiment where PD98059 was used, cells were incubated with variou concentrations of PD98059 (dissolved in DMSO; final

concentration in medium, <=0.15%) for 2 hours prior to exposure to red light.

Clonogenic assay:

Exponentially growing cells were plated at low density (100-150 cells/well for LFS and HeLa cells, 500 cells /dish for NHF cells) and incubated for 4 hours to allow cells to adhere before treatment with PDT as described above. Following PDT, cells were allowed to grow for 5-7 days for LFS and HeLa cells or 12-14 days for NHF cells. Colonies were stained with a solution containing 0.5% methylene blue in 70% ethanol. Colonies greater then 30 cells were counted. The surviving fraction was calculated as the percentage of the PDT-treated samples compared to the non-PDT-treated samples that were treated with drug alone (without light exposure) or without light and drug.

Photofrin uptake and protein content:

Cells were seeded at 7.5 x 10^5/100 mm dish for 3-4 hours allowing cells to adhere and then treated with Photofrin at various concentrations. Eighteen hours late, the medium containing Photofrin was removed. The cells were trypsinized, washed with phosphate-buffered saline (PBS) and subsequently resuspended in 1 ml of PBS for Photofrin uptake analysis. The

Photofrin concentrations of cells were measured by flow cytometry using an excitation wavelength of 488 nm and emission measurement of 575 nm. The cellular concentration of Photofrin was expressed as the average Photofrin fluorescence per cell. In order to determine the protein content per cell, cells were seeded at 7.5 x 10^5/100 mm dish. Twenty-four hours later cells were trypsinized, washed with PBS and counted using a hemocytometer. Following low-speed centrifugation, cells were lysed with lysis buffer containing 50 mM of Tris (PH 8.0), 150 nM of NaCl, 0.5% NP-40, 2 mM of EDTA, 100 mM of NaF, 10 mM of sodium orthovanadate and a protease inhibitor cocktail for 30 min on ice. Insoluble material was removed from the cell lysate by centrifugation at 14,000 g for 15 min. The protein concentration of the cellular extracts was determined using a BCA protein detection kit (Pierce Inc.) or BioRed protein assay (Munich, Germany) according the protocol provided by the manufacturer.

Expression of Ad5p38(AGF) or wild-type p53:

Cells were seeded in 6 well plates at 2.5 x 10^5 cells per well overnight and subsequently infected with Ad5p38 (AGF) or Ad5p53wt in 500 µl serum-free α-MEM medium at the MOI indicated in individual experiment for 45 minutes. Cells infected with Ad5HCMVsp1lacZ were used as control. At 4-6

hours postinfection, infected cells were seeded at 100 cells/well for LFS cells, 500 cells/dish for NHF cells and assayed for clonogenic survival as described above. To detect the expression of Ad5p38(AGF) or Ad5p53wt after infection, infected and mock-infected cells were harvested before exposure to red light and lysed with a lysis buffer as described above. Equal amounts of protein (100 μg) from infected or mock infected cells were inmmunoprecipated using protein-G sepharose together with 2 μg of anti-FLAG antibodies (for Adp38(AGF)) or anti-p53wt antibodies overnight at 4^0C with rotation. The immunoprecipate was analyzed by western blotting with anti-p38 antibodies or anti p53 antibodies as described below.

Preparation of c-jun protein:

E. coli expressing GST-fusion protein of c-jun (GST-Jun) was obtained from Dr. M. Rudnicki, McMaster, Hamilton, Ontario. Over expression of the proteins was induced by 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hours. The cells were then pelleted and lysed by sonication in PBS containing protease inhibitor. The fusion proteins were isolated with glutathione-Sepharose beads (Amersham Pharmacia Biotech, England) for 2 hours at 4 ^{0}C with rotation and eluted

from beads by incubation with 5 mM reduced glutathione Sepharose. The quantity and purity of the fusion proteins were estimated on 12% SDS-polyacrylamide gels using a standard curve of bovine serum albumen (BSA).

In Vitro Kinase Assay for JNK1 Activity:

Cells treated with or without PDT were harvested by scraping at the indicated times and subjected to protein extraction as described above. The cell lysates (100 to 150 μg proteins) were immunoprecipitated with anti-human JNK1 antibody and protein G sepharose overnight. The immunoprecipitates were washed three times with lysis buffer containing the same components as described above and three times with kinase buffer containing 1X HEPES, 20 mM of MgCl, 10 mM of β-Glycero-phosphate, 2 mg/ml of p-Nitrophenyl phosphate, 10 nM of sodium orthovanadate and 2 mM of DTT. The kinase assays were performed in a final volume of 30 μl of kinase buffer with 5 μCi of [γ-^{32}P]ATP and 2 μg GST-c-Jun (1-79), a substrate of JNK1 prepared as described above. Reactions were conducted for 20 min at 30 ^{0}C. Thereafter, samples were denatured, resolved on 12% SDS-PAGE, transferred on to a nitrocellular membrane and autoradiographed. The phosphorylation of c-jun was quantitated using PhosphorImage

analysis. Following autoradiography, the nitrocellulose membrane was blocked with 5% skim milk in tris-buffered saline with 1% tween-20 (TBST), probed with anti-human JNK1 primary antibody and then incubated with HRP conjugated secondary antibody. The total JNK1 was detected using enhanced chemiluminescence detection procedure according to the manufacturer's recommendations (Amersham Pharmacia Biotech, England) and quantified by using PhosphorImage analysis. The JNK1 activity was presented as a ratio of the amount of phosphorylation of c-jun to total JNK1 and normalized to that obtained for untreated cells.

Flow Cytometry:

Cells with or without PDT treatment were collected at various times after PDT treatment by trypsinization and washed twice with PBS. Cells were then resuspended in a solution containing 0.1 % Triton X-100, 66 units/ml RNase and 5 mg % propidium iodide (PI) and incubated for 30 minute. A total of 2 X 10^4 cells was analyzed for DNA-PI fluorescence using flow cytometry at an excitation wavelength of 488 nm. Resulting DNA distributions were analyzed for the proportion of cells in apoptosis and in G_0/G_1, S and G_2/M phases of the cell cycle. Data were analyzed by an M-cycle Software program.

Staining for apoptosis:

Cells from the same cultures as used for flow cytometry were resuspended in Hoechst Stain solution containing 11 μg/ml Heochst 33258, 0.7 % NP-40 and 4.7 % formaldehyde and incubated for 10 minute. Stained cells were then examined under a fluorescence microscope. Ultraviolet light was used for excitation. Cells with a condensed chromatin pattern were considered apoptotic and the percentage of apoptotic cells (in three fields of 100 cells) were determined.

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 manufactory using RNeasy mini kit. 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, England). The cDNA was synthesized by RT-PCR using primers that were designed according to the sequence of MKP-1 or GAPDH gene. cDNA probes were labeled with [α^{32}p]dCTP using a random primer labeling kit according the procedure described by the

manufacturer. Membranes were hybridized with the cDNA probes of MKP-1 overnight at 42^{0}C in a solution containing 5X SSC, 50% formamide, 1X Denhardt's reagent, and 1% SDS. After the membrane was washed 3X with 1X 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 re-probed with a cDNA probe of GAPDH, which was used to normalize for differences in loading and transferring among the samples.

Western Blot Analysis:

Cellular extracts (15-20 μg protein) from PDT treatment cells were separated on 12% SDS-polyacrylamide gels and electroblotted onto a nitrocellular membrane. The membrane was then blocked with 5% skim milk in a tris-buffered saline with 1% tween-20 (TBST) for 1 hour at room temperature, incubated at 4 ^{0}C overnight with corresponding anti-bodies in the same buffer as the block buffer. Specific antibody-labeled proteins were detected by using HRP conjugated secondary antibodies and the ECL plus Western blotting detection system (Amersham Pharmacia Biotech, England). Thereafter, blots were stripped and re-probed with antibodies to actin or total ERK1/2, or total p38 as indicated in individual experiment. The data were quantified using PhosphorImager analysis and

normalized with actin. The activity of p38 or ERK1/2 was presented as the ratio of the amount of phosphorylated p38 or ERK1/2 to total p38 or ERK1/2 and normalized to that obtained for untreated cells.

Chapter three

The role the p53 tumor suppressor in the response of human cells to Photofrin-mediated photodynamic therapy

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The Role of the p53 Tumor Suppressor in the Response of Human Cells to Photofrin-mediated Photodynamic Therapy

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ABSTRACT

Although there is evidence that the p53 tumor suppressor plays a role in the response of some human cells to chemotherapy and radiation therapy, its role in the response of human cells to photodynamic therapy (PDT) is less clear. In order to examine the role of p53 in cellular sensitivity to PDT, we have examined the clonogenic survival of normal human fibroblasts that express wild-type p53 and immortalized Li–Fraumeni syndrome (LFS) cells that express only mutant p53, following Photofrin-mediated PDT. The LFS cells were found to be more resistant to PDT compared to normal human fibroblasts. The D_{37} (LFS cells)/D_{37} (normal human fibroblasts) was 2.8 ± 0.3 for seven independent experiments. Although the uptake of Photofrin per cell was 1.6 ± 0.1-fold greater in normal human fibroblast cells compared to that in LFS cells over the range of Photofrin concentrations employed, PDT treatment at equivalent cellular Photofrin levels also demonstrated an increased resistance for LFS cells compared to normal human fibroblasts. Furthermore, adenovirus-mediated transfer and expression of wild-type p53 in LFS cells resulted in an increased sensitivity to PDT but no change in the uptake of Photofrin per cell. These results suggest a role for p53 in the response of human cells to PDT. Although normal human fibroblasts displayed increased levels of p53 following PDT, we did not detect apoptosis or any marked alteration in the cell cycle of GM38 cells, despite a marked loss of cell viability. In contrast, LFS cells exhibited a prolonged accumulation of cells in G_2 phase and underwent apoptosis following PDT at equivalent Photofrin levels. The number of apoptotic LFS cells increased with time after PDT and correlated with the loss of cell viability. A p53-independent induction of apoptosis appears to be an important mechanism contributing to loss of clonogenic survival after PDT in LFS cells, whereas the induction of apoptosis does not appear to be an important mechanism leading to loss of cell survival in the more sensitive normal human fibroblasts following PDT at equivalent cellular Photofrin levels.

INTRODUCTION

Photodynamic therapy (PDT)† with photosensitizing drugs activated by visible light has been applied in many clinics throughout the world and shows considerable promise for solid tumors, including those from lung, bladder, breast, esophagus, brain and colon (1). 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 (2–6). Photodynamic therapy kills tumor cells *via* apoptosis and/or necrosis both *in vivo* and *in vitro*. The particular mode of cell death in response to PDT depends on experimental conditions, such as the dose of PDT and the subcellular localization of photosensitizer (7). The sensitivity of cells to PDT varies between different cell types, even between very closely related cell lines (8), and there are many different mechanisms that could contribute to the cellular sensitivity to PDT (9). Increasing the sensitivity of tumor cells to the lethal effects of PDT has the potential to improve significantly the cure rate of cancers. Therefore, there is considerable interest in the response to PDT at the molecular level that could provide an understanding of the determinants of intrinsic cell photosensitivity and lead to the design of new biological approaches for increasing the sensitivity of tumors to PDT.

Studies have provided evidence that the protein product of the p53 tumor suppressor gene may be critical in mediating certain cellular responses to DNA damage in mammalian cells including cell cycle regulation, DNA repair and apoptotis (10,11). Several laboratories have examined the influence of cellular p53 status on the response of cells to various DNA-damaging therapeutic agents, with differing results. Loss of p53 or p53 mutations has been reported to increase the resistance of some tumor cells to chemotherapy

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†*Abbreviations:* HCMV, human cytomegalovirus; HPD, hematoporphyrin derivative; HPV-16, human papillomavirus type 16; IR, ionizing radiation; LFS, Li–Fraumeni syndrome; α-MEM, alpha-minimal essential medium; PBS, phosphate-buffered saline; PDT, photodynamic therapy; PFU, plaque-forming units; PI, propidium iodide; RIF, radiation-induced fibrosarcoma; SnET2, tin ethyl etiopurpurin; TBST, Tris-buffered saline with Tween-20.

and radiation therapy, both *in vitro* and *in vivo* (12,13), and reintroduction of wild-type of p53 into some types of tumor cells with mutant p53 has been reported to result in an increased chemosensitivity and/or radiosensitivity (14,15). In contrast, disruption of wild-type p53 increases the sensitivity of human breast and colon carcinoma cell lines to cisplatin but does not alter their sensitivity to ionizing radiation, adriamycin or etoposide (16). Other reports show that the presence of wild-type p53 is not a determinant for cisplatin cytotoxicity in human ovarian tumor cell lines (17) and that abrogation of p53 function does not enhance the resistance of several different human tumor cell lines to ionizing radiation (18).

The effect of p53 status on the sensitivity of cells to PDT has not been as extensively studied. The SV40-transformed IMR-90 cells in which p53 function is abrogated were more sensitive to hematoporphyrin derivative (HPD)-mediated PDT compared to their untransformed counterparts (19). In contrast, human promyelocyte leukemia HL60 cells expressing wild-type p53 were found to be more sensitive to cell killing by Photofrin or tin ethyl etiopurpurin (SnET2)-mediated PDT compared to HL60 cells in which p53 is not expressed or is inactive (20), and a similar result has been reported for a human colon carcinoma cell line (21). However, selective abrogation of p53 expression with human papillomavirus type 16 (HPV-16) E6 did not alter the clonogenic survival of colon or breast carcinoma cells exposed to Photofrin-mediated PDT (22). The differing effects of p53 status on cellular sensitivity to PDT in these studies may result from confounding effects of additional genetic alterations present in the tumor cells that can modulate p53-dependent pathways or otherwise modulate PDT sensitivity. In the present study, we have examined the cellular sensitivity to Photofrin-mediated PDT and PDT-induced cell cycle alterations and apoptosis in normal human skin fibroblasts that express wild-type p53 and in immortalized Li–Fraumeni syndrome (LFS) cells that express only mutant p53. Individuals with LFS inherit a germ-line mutation in one allele of the p53 gene and exhibit an increased risk for developing a variety of neoplasia at early ages, associated with loss of the remaining wild-type p53 gene (23). The genetic mutation in LFS is thought to be specific for p53 (24), and LFS cells have been used as a model system to examine the role of p53 in apoptosis, DNA repair and the sensitivity to various DNA damaging agents (25,26). We show that the LFS cells were more resistant to Photofrin-mediated PDT, took up less Photofrin per cell and showed evidence of apoptosis and a G_2–M arrest following PDT. In contrast, although p53 levels were increased, we found no evidence of cell cycle arrest or apoptosis after PDT treatment of normal human fibroblasts at equivalent cellular Photofrin levels, despite a substantially greater loss of cell viability compared to that in LFS cells. In order to test directly the effects of p53 expression on cell sensitivity to PDT we used adenovirus-mediated transfer and expression of wild-type p53 in LFS cells. We show that expression of wild-type p53 in LFS cells resulted in a significant increase in sensitivity to PDT but no change in the uptake of Photofrin per cell.

MATERIALS AND METHODS

Cells. The normal human fibroblast strains GM38A and GM37F were obtained from the National Institute of General Medical Sciences Repository (Camden, NJ). The LFS087 and LFS041 cells express only mutant p53 and were obtained by spontaneous transformation of MDAH087 (087wt/mut) and MDAH041 (041wt/mut) fibroblasts, respectively, that are heterozygous for single-base mutations in p53 at codons 248 and 184, respectively (27). The LFS cells were obtained from Dr. M. A. Tainsky, M. D. Anderson Cancer Center, Houston, TX. Human 293 cells were obtained from Dr. F. L. Graham, McMaster University, Hamilton, Ontario, Canada. All cell cultures were grown as monolayer in alpha-minimal essential media (α-MEM) supplemented with fetal bovine serum (10%), penicillin (100 mg/mL), streptomycin (100 mg/mL) and amphotericin (250 mg/mL) (Gibco BRL). Under these growth conditions the doubling times for the normal human fibroblast strains and the LFS cell lines were about 60 and 25 h, respectively.

Viruses. Recombinant adenoviruses Ad5HCMVsp1*lacZ* and Ad5p53wt were obtained from Dr. F. L. Graham, McMaster University, Hamilton, Ontario, Canada. The Ad5p53wt is a nonreplicating recombinant adenovirus expressing wild-type p53 under control of the human cytomegalovirus (HCMV) immediate early promoter (28). The Ad5HCMVsp1*lacZ* is also a nonreplicating recombinant adenovirus expressing β-galactosidase under control of the HCMV immediate early promoter and was used as a control (29). The viral constructs express p53 or β-galactosidase, respectively, in most human cell types without replication of the virus. Viruses were replicated and titered in plaque-forming units (PFU)/mL on human 293 cells (30).

Photosensitizer and light source. The photosensitizer, Photofrin, was a gift from QLT Phototherapeutics, Inc., Vancouver, BC, Canada. For the PDT treatments, tissue culture petri dishes containing cell monolayers that had been incubated with or without Photofrin were exposed to red light as described previously (31). The light source was a 21" × 32.75" light box illuminated by a parallel series of 12 fluorescent tubes (Philips type TL83), enclosed on top with a sheet of clear Plexiglas and filtered with red acetate filters (Roscolux, No. 19, Rosco, CA) that gave a wideband illumination above 585 nm. The energy fluence rate was 0.34 W m^{-2} in the wavelength band of 640 nm as measured using a Jobin-Yvon model CP-200 spectrometer and a Princeton Instruments charge-coupled device. Exposure for 5 min resulted in an incident energy fluence of 10^2 J m^{-2} in the wavelength band of 640 nm.

Clonogenic survival. Exponentially growing cells were plated at low density (100 cells/well for LFS cells, 500 cells/dish for the normal human fibroblast strains) and incubated in the dark with growth medium containing an appropriate concentration of Photofrin for 18 h. The drug-containing medium was then replaced with fresh drug-free growth medium, and the cell monolayer was exposed to red light for 5 min as described above. Irradiated cells were allowed to grow for 5–7 (LFS cells) or 12–14 days (normal human fibroblasts). Colonies were stained with methylene blue (0.5% methylene in 70% ethanol), and colonies greater than 30 cells were counted. The surviving fraction was calculated as the relative plating efficiency of the PDT-treated compared to the non-PDT-treated cultures. The absolute plating efficiency for untreated cells was 11.5 ± 3.2% (mean ± standard error of eight independent experiments) for the GM38A normal human fibroblast strain, 5.6% (one experiment) for the GM37F normal strain, 63.7 ± 8.4% (mean ± standard error of seven independent experiments) for the LFS087 cell line and 77.4% (mean of two experiments) for the LFS041 cell line. After the addition of photosensitizer to the cells, all procedures were carried out in minimal ambient lighting until the addition of the stain.

Expression of wild-type p53 in LFS cells. Cells were seeded in 6 well plates at 2.5 × 10^5 per well and 16 h later infected or mock infected with Ad5HCMVsp1*lacZ* or Ad5p53wt suspended in 500 μL of serum-free α-MEM at 20 PFU/cell. At 4–6 h postinfection, infected or mock-infected cells were seeded to low density and assayed for clonogenic survival as described above. The absolute plating efficiencies in these experiments for untreated LFS cells were 51.5 ± 19.5% (mean ± standard error of two experiments) for noninfected cells, 43.0 ± 7.0% (mean ± standard error of four experiments) for Ad5p53wt-infected cells and 39.1 ± 6.9% (mean ± standard error of four experiments) for Ad5HCMVsp1*lacZ*-infected cells. The absolute plating efficiencies for untreated GM38A cells was 14.4 ± 1.4% (mean ± standard error of two experiments) for noninfected cells, 12.9 ± 1.8% (mean ± standard error of three

experiments) for Ad5p53wt-infected cells and 12.9 ± 1.3% (mean ± standard error of three experiments) for Ad5HCMVsp1*lacZ*-infected cells. To detect the expression of wild-type p53 in LFS cells, infected and mock-infected cells were harvested after drug treatment but before exposure to red light and lysed with lysis buffer (50 m*M* Tris, pH 8.0, 150 n*M* NaCl, 0.5% NP-40, 2 m*M* EDTA, 100 m*M* NaF, 10 m*M* Na pyrophosphate, 1 m*M* Na_3VO_4 [sodium orthovanadate] together with a protease inhibitor cocktail [Boehringer Manheim, Germany]). The cell lysate was then immunoprecipated using protein-A agarose together with anti-p53 wild-type antibody, Ab-5 (CalBiochem, CA) that recognizes wild-type p53 only, for 16 h and the immunoprecipitate was analyzed by western blot as described below.

Photofrin uptake and protein content. Cells were seeded at 7.5 × 10^5/100 mm dish for 3–4 h allowing cells to adhere and then exposed to Photofrin at various concentrations. Eighteen hours later, the medium containing Photofrin was removed. The cells were then trypsinized, washed with phosphate-buffered saline (PBS) and subsequently resuspended in 1 mL PBS for Photofrin uptake analysis. The Photofrin concentrations of cells were measured by flow cytometry using an excitation wavelength of 488 nm and emission measurements at 575 nm (32). The cellular concentration of Photofrin was expressed as the average Photofrin fluorescence per cell. In order to determine the protein content per cell, cells were seeded at 7.5 × 10^5/100 mm dish. Twenty-four hours later cells were trypsinized, washed with PBS and counted using a hemocytometer. Following low-speed centrifugation, cells were treated with lysis buffer for 30 min on ice. The lysate was cleared by centrifugation at 13 000 *g* for 15 min at 4°C and the protein concentration was determined using a Bio-Rad protein assay or a BCA Protein Assay Reagent Kit (Pierce Inc.).

Immunoblot analysis. Expression of p53 in the GM38A cell line was assessed by western blot analysis. Cells were treated with Photofrin at various concentrations for 18 h and then exposed to red light for 5 min to give a total exposure of 10^2 J/m^2. Twenty-four hours after light treatment both detached and adherent cells were resuspended in lysis buffer for 30 min on ice. The lysate was cleared by centrifugation at 13 000 *g* for 15 min at 4°C and the protein concentration was determined using a Bio-Rad protein assay. Fifteen microgram protein samples were resolved over 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 5% skim milk in Tris-buffered saline with Tween-20 (TBST), washed and incubated with p53 specific mouse IgG_{2a} (Ab-3, Oncogene Science) for 2 h followed by incubation with anti-mouse secondary antibodies. Specific antibody-labeled proteins were detected using an ECL chemiluminescence detection system. The data were analyzed using IQ software and the relative amount of p53 protein was determined by comparison with the amount of actin in order to control for differences in loading. The antibody against actin used was polyclonal IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Flow cytometry. Treated and control cells were collected at various times after PDT treatment by trypsinization and washed twice with PBS. Cells were then resuspend in a solution containing 0.1% Triton X-100, 66 units/mL RNase and 5 mg% propidium iodide (PI) and incubated for 30 min. A total of 2 × 10^4 cells were analyzed for DNA–PI fluorescence using flow cytometry at an excitation wavelength of 488 nm. Resulting DNA distributions were analyzed for the proportion of cells in apoptosis and in G_0/G_1, S and G_2–M phases of the cell cycle. Data were analyzed by an M-CYCLE Software program.

Staining for apoptosis. Cells from the same cultures as used for flow cytometry were resuspended in Hocchst stain solution containing 11 μg/mL Hoechst 33258, 0.7% NP-40 and 4.7% formaldehyde and incubated for 10 min. Stained cells were then examined under a florescence microscope. Ultraviolet light was used for excitation. Cells with a condensed chromatin pattern were considered apoptotic and the percentage of apoptotic cells (in three fields of 100 cells) were determined.

RESULTS

p53 status and photosensitivity

Clonogenic survival assays demonstrated that LFS cells expressing only mutant p53 were significantly more resistant

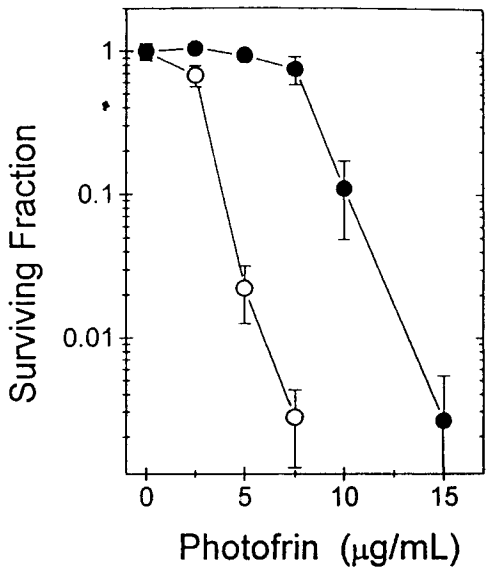

Figure 1. Clonogenic survival curves for the normal fibroblast strain GM38A (○) and LFS087 cells (●) following Photofrin-mediated PDT treatment. Cells were incubated with Photofrin for 18 h and subsequently exposed to 10^2 J/m^2 of red light. Data points represent the mean ± standard error of colony counts for three determinations from a typical experiment. The plating efficiency for untreated cells was 12.1 ± 0.68% for the GM38A normal human fibroblast strain and 85.3 ± 6.1% for the LFS087 cells.

to the cytotoxic effects of Photofrin-mediated PDT compared to the normal human fibroblast strains. Results of a representative experiment using LFS087 and GM38A are shown in Fig. 1. Clonogenic survival curves were fitted to the equation $Ln(SF) = -(\alpha X + \beta X^2 + \gamma)$ that was then used to determine the D_{37} for each cell line. Mean D_{37} values obtained from a number of independent experiments were 3.93 + 0.42 μg/mL (mean + standard error for seven experiments) for GM38A, 1.9 μg/mL for GM 37 (1 experiment), 9.23 + 0.74 μg/mL (mean + standard error for six experiments) and 8.85 + 2.56 μg/mL (mean + standard error for three experiments) for LFS041. In experiments that included a normal fibroblast strain and an LFS cell line, the ratio of D_{37} for LFS cells compared to the D_{37} for a normal fibroblast strain was also calculated. The mean value for this ratio ± standard error for seven independent experiments was 2.8 ± 0.3.

Because drug uptake is a potential factor influencing cellular sensitivity to PDT, we examined the uptake of Photofrin in GM38A and LFS087 cells. Comparisons of the Photofrin uptake per cell between GM38A and LFS087 cells are shown in Fig. 2. It can be seen that the Photofrin fluorescence per cell was significantly higher in GM38 cells compared to LFS087 cells. Over the range of Photofrin concentrations employed, the uptake of Photofrin per cell was 1.57-± 0.11-fold greater in the normal human fibroblast strain compared to that in the LFS cells. Because the differential Photofrin uptake may result from differences in cell size, we also measured the protein content per cell and found that the protein content of GM38A cells was not significantly different from that of LFS087 cells. The mean ± standard error obtained for five independent experiments gave values of 430 ± 38 and 360 ± 54 μg/10^6 cells for GM38A and

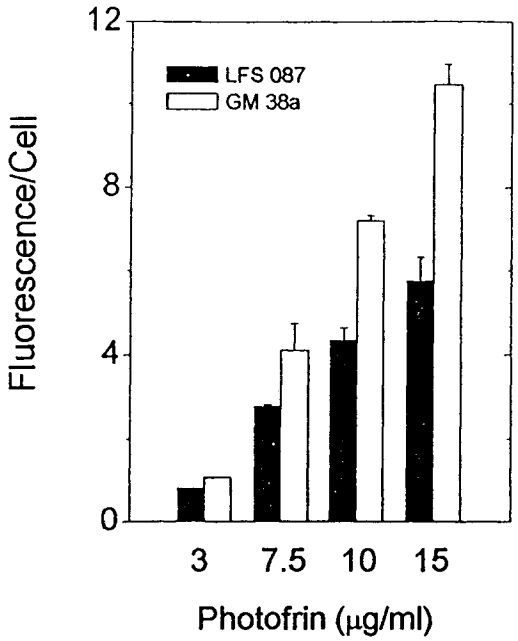

Figure 2. Photofrin uptake in GM38A and LFS087 cells. Average Photofrin uptake (expressed as Photofrin fluorescence per cell) measured following 18 h incubation in the presence of the drug was determined by flow cytometry using an excitation wavelength of 488 nm and emission measurements at 575 nm. Two to three independent experiments were conducted with each cell line and the data points represent the mean ± standard error. The mean ± standard error for the ratio of Photofrin uptake per cell in GM38A cells compared to that in LFS087 cells was 1.33, 1.49 ± 0.23, 1.66 ± 0.12 and 1.81 ± 0.20 at Photofrin concentrations of 3 μg/mL, 7.5 μg/mL, 10 μg/mL and 15 μg/mL, respectively.

LFS087 cells, respectively (the difference of the means by an independent *t*-test gave $P = 0.32$). Comparison of clonogenic survival after PDT at an equivalent cellular Photofrin level (10 μg/mL for LFS cells and 7.5 μg/mL for GM38A cells, from Fig. 2) also demonstrated a substantial reduction in survival for GM38A compared to LFS cells (Fig. 1).

In order to examine further the effects of p53 on cell sensitivity to Photofrin-mediated PDT, we determined the sensitivity to PDT of cells infected with Ad5p53wt, a recombinant adenovirus construct expressing wild-type p53. A similar recombinant adenovirus construct, Ad5HCMVsp1*lacZ*, expressing β-galactosidase was used as a control. Clonogenic survival following Photofrin-mediated PDT was assessed in normal human fibroblasts and LFS cells infected at a multiplicity of 20 PFU per cell with recombinant adenovirus constructs expressing either wild-type p53 or β-galactosidase. A western blot using anti-p53 antisera that recognizes wild-type p53 only showed that expression of wild-type p53 occurred in LFS cells infected with Ad5p53wt. The level of wild-type p53 expression in the Ad3p53wt-infected LFS cells was similar to that in nontreated and noninfected GM38A cells (data not shown). Also the uptake of Photofrin per cell was not significantly affected by infection of the LFS cells with recombinant adenovirus (data not shown).

Results of a typical experiment comparing the sensitivity of LFS cells infected with either Ad5p53wt or Ad5-HCMVsp1*lacZ* are shown in Fig. 3A. It can be seen that there was a reduced survival for Adp53wt-infected cells

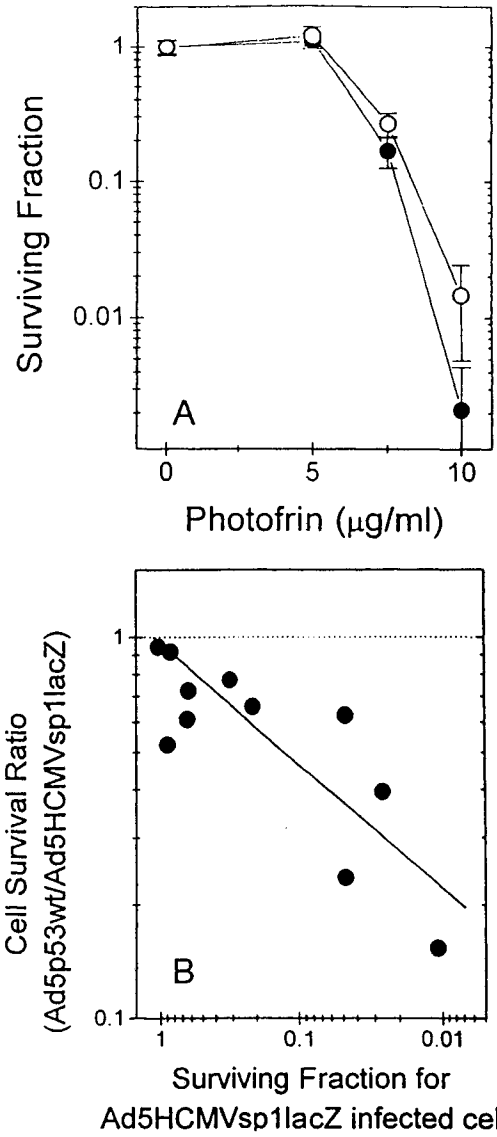

Figure 3. Expression of wild-type p53 increases the sensitivity of LFS087 cells to Photofrin-mediated PDT. (A) The LFS087 cells were infected with either Ad5p53wt (●) or Ad5HCMVsp1*lacZ* (○) at 20 PFU/cell and subsequently assayed for clonogenic survival following Photofrin-mediated PDT. Data points represent the mean ± standard error of colony counts for three determinations from a typical experiment. The plating efficiencies for untreated LFS cells was 69.7 ± 0.3% for noninfected cells, 59.7 ± 2.6% for Adp53wt-infected cells and 58.3 ± 3.4% for Ad5HCMVsp1*lacZ*-infected cells. (B) Clonogenic survival of cells infected with Ad5p53wt relative to that for cells infected with Ad5HCMVsp1*lacZ* plotted as a function of survival for cells infected with Ad5HCMVsp1*lacZ*. Shows pooled results for three experiments.

compared to Ad5HCMVsp1*lacZ*-infected cells at all the Photofrin concentrations employed. A reduction in survival for Ad5p53wt-infected compared to Ad5HCMVsp1*lacZ*-infected LFS cells was also observed at all Photofrin doses tested in two other experiments. A paired *t*-test on the pooled data for survival values in Ad5p53wt-infected compared to Ad5HCMVsp1*lacZ*-infected LFS cells showed a significant difference ($P = 0.018$, $n = 11$). In contrast, a paired *t*-test on the survival values in noninfected compared to Ad5HCMVsp1*lacZ*-infected LFS cells showed no significant

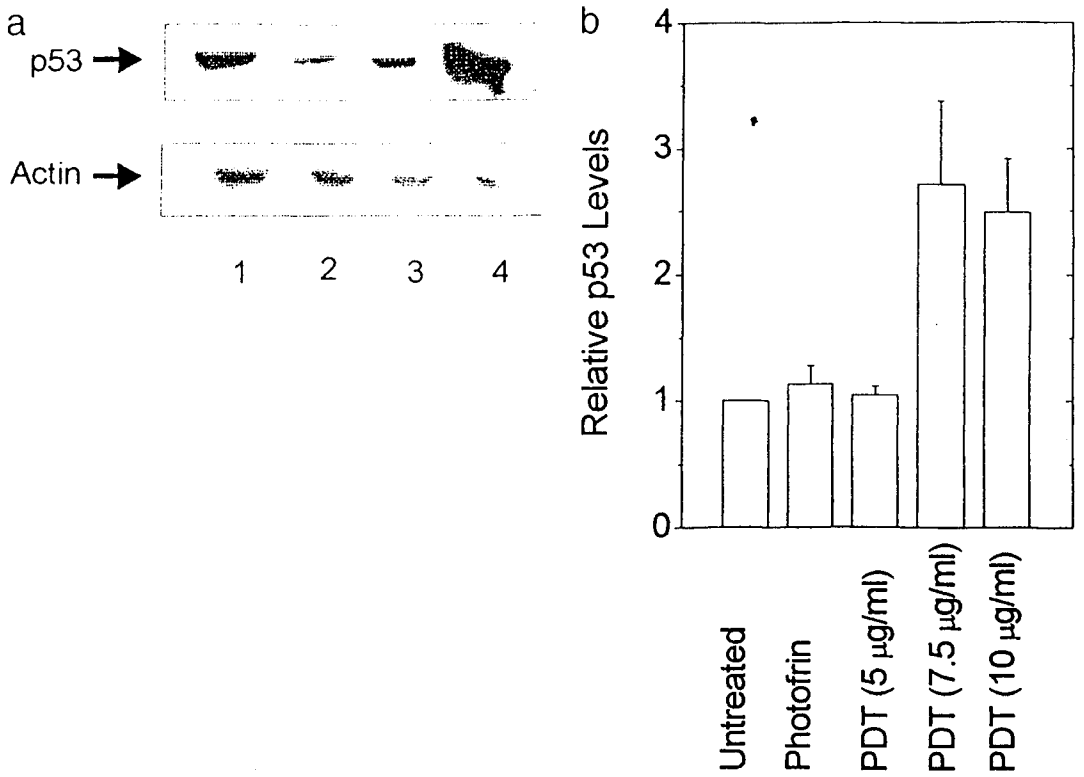

Figure 4. Induction of cellular p53 in GM38A cells following Photofrin-mediated PDT. Cells were treated with Photofrin for 18 h prior to light exposure. At 24 h after PDT, the p53 levels were monitored by immunoblotting. (Left) Shows western blot using anti-p53 antisera. Lane 1, untreated GM38 cells; lane 2, cells treated with drug only (7.5 μg/mL Photofrin); lane 3, cells treated with PDT using 5 μg/mL Photofrin plus light; lane 4, cells treated with PDT using 7.5 μg/mL Photofrin plus light. A western blot using anti-actin antisera is also shown for comparison purposes. (Right) The amount of p53 protein detected was normalized to the amount of actin present and expressed as the relative amount of p53 compared to that in the untreated controls. The drug alone treatment, shown in column 2, is for a combination of data obtained at 7.5 and 10 μg/mL Photofrin. Values for cellular p53 levels are the mean of at least three independent experiments ± standard error.

difference (P = 0.17, n = 8). In order to display the pooled data from these experiments, the ratio of cell survival in Ad5p53wt-infected compared to that in Ad5HCMVsp1*lacZ*-infected LFS cells was plotted as a function of survival for cells infected with Ad5HCMVsp1*lacZ* (Fig. 3B). Linear regression analysis of this plot showed that there was a significant correlation between the ratio of cell survival in Ad5p53wt-infected compared to that in Ad5HCMVsp1*lacZ*-infected LFS cells and survival for cells infected with Ad5HCMVsp1*lacZ* (P = 0.0025, n = 11). In contrast, no significant correlation was found between the ratio of cell survival in noninfected compared to that in Ad5HCMVsp1*lacZ*-infected LFS cells and survival for cells infected with Ad5HCMVsp1*lacZ* (P = 0.62, n = 8) (data not shown). In addition, a paired t-test on the survival values for Ad5p53wt-infected compared to Ad5HCMVsp1*lacZ*-infected GM38A cells (P = 0.14, n = 9) as well as the survival values for noninfected compared to Ad5HCMVsp1*lacZ*-infected GM38A cells (P = 0.56, n = 7) showed no significant difference. These results indicate that adenovirus-mediated expression of wild-type p53 led to a significant increase in sensitivity to Photofrin-mediated PDT in LFS cells but not in normal human fibroblasts.

p53 induction by PDT

A variety of different treatments, including UV light or ionizing radiation (33,34), can induce p53. The induced response appears to be cell-type dependent (10). In addition, p53 has been proposed to mediate a more general stress response to suboptimal growth conditions. Recently, Photofrin-mediated PDT has been reported to induce cellular accumulation of p53 in two different tumor cell lines that exhibit a wild-type p53 phenotype (22). In the current study, we have examined the cellular p53 levels following Photofrin-mediated PDT in normal human fibroblasts. We found that Photofrin alone did not change the cellular p53 level. However, increased accumulation of p53 was observed when cells were treated with PDT at Photofrin concentrations of 7.5 μg/mL and 10 μg/mL. Figure 4 shows results for p53 induction at 24 h after PDT treatment of cells. In other experiments the induction of p53 was noted as early as 6 h following PDT (data not shown).

P53 status and cell cycle arrest

The p53 protein is an important modulator of the cell cycle and may be involved in the determination of photosensitivity. It is possible that the difference in sensitivity to PDT of normal and LFS cells results from differential effects of PDT on the cell cycle. In particular, the p53 protein has been shown to mediate a p21-dependent G_1 arrest in cells exposed to ionizing radiation and certain other DNA-damaging agents (35). It was therefore considered of interest to examine the cell cycle changes in GM38A and LFS cells fol-

Table 1. Effects of photofrin-mediated PDT on the cell cycle*

Hours post-PDT	G_1/G_0			S			G_2/M		
	Untreated	Photofrin	PDT	Untreated	Photofrin	PDT	Untreated	Photofrin	PDT
GM38A cells									
0	87.4 ± 7.4	81.9 ± 14.2	87.0 ± 8.9	13.1 ± 5.9	16.5 ± 14.3	9.2 ± 7.2	3.6 ± 1.9	1.6 ± 0.29	3.8 ± 1.8
4	74.5 ± 3.2	76.8 ± 14.8	87.1 ± 10.2	21.6 ± 0.4	21.4 ± 13.1	8.1 ± 6.6	3.7 ± 2.7	1.8 ± 1.7	4.7 ± 1.8
12	56.6 ± 10.6	46.8 ± 12.6	61.3 ± 7.2	32.9 ± 6.7	33.5 ± 7.9	35.9 ± 4.5	10.5 ± 5.7	19.4 ± 6.5	1.6 ± 1.6
24	68.4 ± 5.8	64.3 ± 4.5	61.3 ± 7.2	24.7 ± 6.2	25.5 ± 3.9	35.2 ± 8.5	6.0 ± 1.3	10 ± 2.3	3.5 ± 2.5
36	66.0 ± 7.6	68.4 ± 4.6	59.1 ± 9.6	29.4 ± 7.1	28.4 ± 6.7	32.6 ± 7.9	4.5 ± 1.0	3.2 ± 2.1	8.3 ± 1.8
LFS087 cells									
0	34.7 ± 4.2	24.0 ± 9.3	25.8 ± 9.0	37.7 ± 5.3	43.1 ± 7.0	44.9 ± 5.0	27.5 ± 8.8	32.9 ± 6.0	29.3 ± 6.9
4	43.3 ± 18	46.8 ± 1.5	33.7 ± 8.9	48.6 ± 24	34.1 ± 12.3	37.4 ± 3.9	8.2 ± 6.4	19.2 ± 12.8	29.0 ± 12.8
12	39.3 ± 8.4	38.4 ± 6.3	25.3 ± 6.5	47.1 ± 5.3	53.0 ± 3.5	22.1 ± 6.5	13.6 ± 4.9	8.7 ± 2.8	52.7 ± 0
24	58.8 ± 4.4	54.1 ± 3.8	42.9 ± 2.3	34.4 ± 5.9	35.9 ± 3.3	35.5 ± 2.1	6.8 ± 2.9	10.1 ± 2.8	21.6 ± 3.1
36	62.3 ± 1.3	58.9 ± 3.3	27.7 ± 9.0	33.1 ± 1.6	33.7 ± 4.9	36.3 ± 5.5	4.7 ± 1.0	7.1 ± 2.8	35.9 ± 4.9

*Values represent the percentage of cells in each phase of the cell cycle. Data are the means ± SE from three independent experiments.

lowing PDT. Perturbations of the cell cycle following PDT were analyzed using flow cytometry at drug concentrations that resulted in equivalent cellular photofrin levels for LFS087 (10 μg/mL Photofrin) and GM38 (7.5 μg/mL Photofrin) cells. No significant G_1 arrest or other marked accumulation of cells in any phase of the cell cycle was detected in GM38A cells after PDT (Table 1). A recent report also finds no evidence for G_1 arrest or other significant changes to the cell cycle in normal human fibroblasts following moderate exposure to UV (36). However, a significant and prolonged G_2–M cell cycle arrest was observed in LFS cells subjected to PDT (Table 1, Figs. 5 and 6). The PDT-induced G_2–M phase cell cycle arrest in LFS cells was sustained for at least 36 h. Because the LFS cells express only mutant p53, these results suggest that the marked G_2–M arrest in LFS cells results from a p53-independent mechanism.

P53 status and cell death

After PDT, both apoptosis and necrosis of tumor cells have been observed *in vivo* (9). Apoptosis is a prominent form of cell death in response to PDT for many, but not all, cells in culture (37). In order to evaluate the relationships between apoptosis, sensitivity to PDT and p53 status, the apoptosis and cell viability for normal human fibroblasts and LFS cells were compared using drug concentrations that resulted in equivalent cellular Photofrin levels (10 μg/mL and 7.5 μg/mL Photofrin for LFS087 and GM38A cells, respectively). Apoptotic cells were assessed by both morphological analysis using Hoechst stain and flow cytometry analysis and results using the two different methods were comparable. Cell viability was assessed by trypan blue exclusion that is thought to reflect primarily the integrity of the cell membrane. Evaluation of cell viability using trypan blue exclusion rather than clonogenic survival allowed coincident measurements of both apoptosis and cell viability as a function of time after PDT treatment. As shown in Fig. 7, no significant amount of apoptosis was detected in GM38A cells over the course of the experiment in spite of an immediate and substantial loss of cell viability. It is possible that p53 is

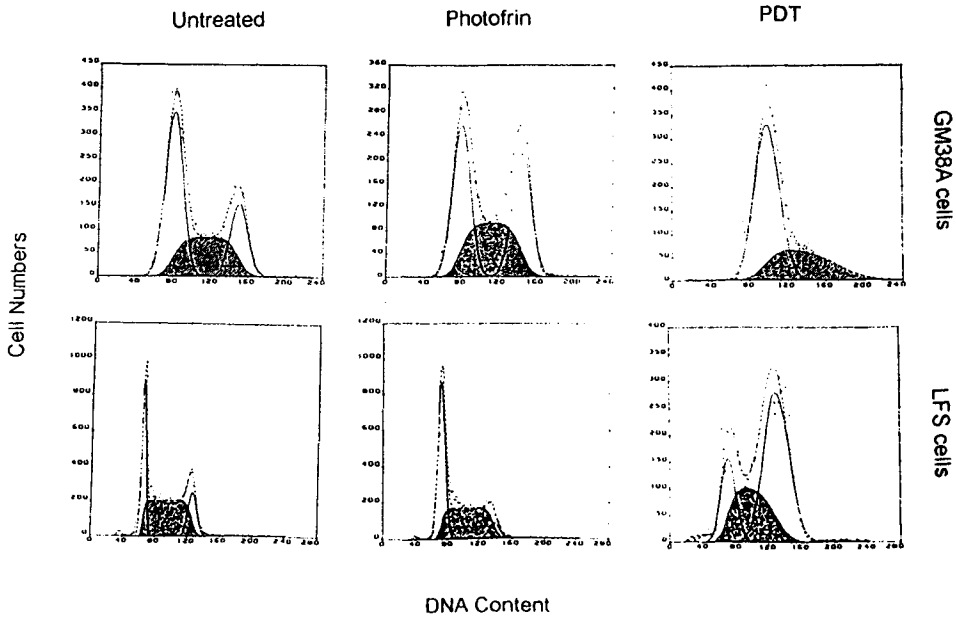

Figure 5. The PDT-induced effects on the cell cycle in GM38A and LFS087 cells. The LFS cells were treated with 10 μg/mL Photofrin and GM38 cells treated with 7.5 μg/mL Photofrin for 18 h and subsequently exposed to 10^2 J/m^2 of red light. Thirty-six hours later cells were analyzed for DNA–PI fluorescence using flow cytometry at an excitation wavelength of 488 nm, and the resulting DNA distributions were analyzed using an M-cycle Software program. The S cell populations are highlighted by the hatched profiles, while the G_1 and G_2/M cell populations are shown by the profiles at left and right of the S cell population, respectively.

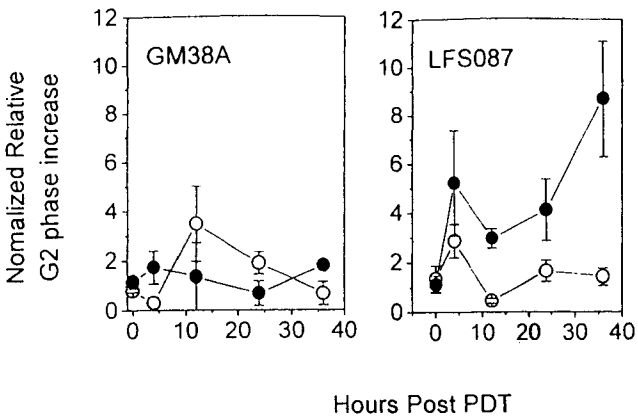

Figure 6. The PDT-induced G_2-phase arrest in LFS087 cells. Cells were treated with Photofrin for 18 h and subsequently exposed to 10^2 J/m^2 of red light as indicated in Fig. 5. At various times after treatment, cells were analyzed for DNA–PI fluorescence using flow cytometry at an excitation wavelength of 488 nm. Resulting DNA distributions were analyzed using an M-cycle Software program. Shows the normalized percentage increase (Photofrin alone or PDT compared to untreated) in G_2/M with time following treatment for (○) drug alone and (●) PDT. (Left) GM38 cells using 7.5 μg/mL Photofrin and (right) LFS cells using 10 μg/mL Photofrin.

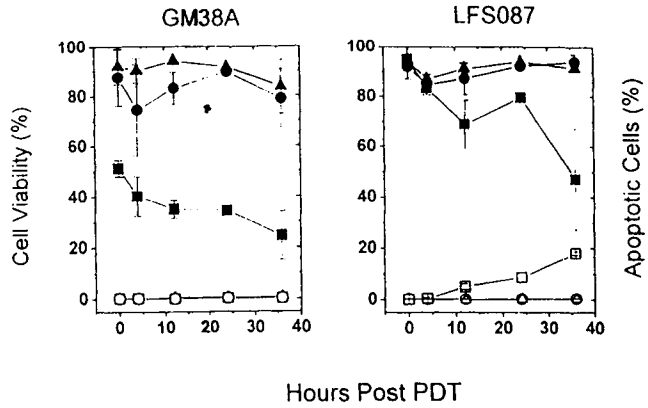

Figure 7. Cell viability and apoptosis in GM38A (left) and LFS087 (right) cells after Photofrin-mediated PDT. Cells were treated without Photofrin or light (▲), with Photofrin in the absence of light (●) or with Photofrin followed by red light exposure (PDT) (■). Photofrin concentrations used were 7.5 μg/mL for GM38A and 10 μg/mL for LFS cells. Cells were harvested at 0, 12, 24 and 36 h after PDT treatment and subsequently scored for viability (closed symbols) and apoptosis (open symbols) using trypan blue exclusion and Hoechst stain, respectively.

protective against the induction of apoptosis in normal human fibroblasts after this level of exposure to Photofrin-mediated PDT by a mechanism that does not involve activation of a G_1 checkpoint. In contrast, LFS cells exhibited apoptotic changes including condensed chromatin and apoptotic bodies. The percentage of LFS cells showing apoptosis increased with time following PDT throughout the course of the experiment and correlated with cell viability. These results indicate that the increased resistance of LFS cells compared to normal human fibroblasts following PDT does not result from an absence of p53-mediated apoptosis in LFS cells. In contrast, it appears that a p53-independent apoptotic pathway plays a major role in cell killing for LFS cells following Photofrin-mediated PDT.

DISCUSSION

In this report, we show that LFS cells expressing only mutant p53 are more resistant to Photofrin-mediated PDT compared to normal human fibroblasts that express wild-type p53. The D_{37} value for the clonogenic survival curves of the LFS cells was about three-fold greater than that for the normal human fibroblasts. Furthermore, we found that transient expression of wild-type p53 using a recombinant adenovirus increased the sensitivity of LFS cells to PDT. These results suggest a role for the p53 tumor supressor gene in the cellular sensitivity of some human cells to Photofrin-mediated PDT.

The uptake of Photofrin per cell was about 1.6-fold greater in the PDT-sensitive GM38A cells compared to LFS087 cells, although the protein content of the two cell types was not significantly different. Transfectant HL60 cells expressing wild-type p53 have also been reported to show a similar increased uptake of Photofrin per cell compared to HL60 cells exhibiting mutated p53 or deleted p53 expression and were also more sensitive the PDT compared to the parental p53 null human leukemia HL60 cells (20). In contrast, the LS513 colon carcinoma cell line expressing wild-type p53 showed an increased sensitivity to Photofrin-mediated PDT compared to the LS1034 cell line that expresses a mutated p53, although the drug uptake was similar in the two cell lines (21). In the present work, adenovirus-mediated transient expression of wild-type p53 in LFS cells resulted in an increased sensitivity to PDT but did not effect drug uptake. It is thus not clear whether the difference in drug uptake for LFS compared to normal human fibroblasts reported here is related to the difference in p53 status of the two cell types. It is possible that other factors such as differences in cell morphology and growth rate between transformed LFS cells and nontransformed human fibroblasts may account for differences in drug uptake. Furthermore, because the time of exposure to Photofrin has been reported to affect the mode of cell death in one cell line (38), it appears likely that the drug concentration at specific sites of photodamage within the cell may be a more important determinant of cell sensitivity than simply the uptake of drug per cell or per μg of cellular protein. Thus, although the survival of the normal human fibroblasts was substantially reduced compared to the survival of LFS cells even for similar levels of drug uptake per cell, it is not clear to what extent the difference in sensitivity between normal human fibroblasts and LFS cells is a reflection of p53 status.

Consistent with the results presented here, transfectant HL60 cells expressing wild-type p53 were more sensitive to Photofrin-mediated PDT compared to parental p53 null human leukemia HL60 cells at similar levels of Photofrin uptake (20) and the wild-type p53 expressing LS513 colon carcinoma cell line was significantly more sensitive to Photofrin-mediated PDT compared to the LS1034 cell line that expresses a mutated p53 (21). In contrast, a more recent report indicates that abrogation of p53 function by transfection with the HPV-16 E6 gene does not alter the sensitivity of LS513 human colon and MCF-7 breast carcinoma cells (22).

The p53 accumulates in response to DNA damage caused

by a variety of agents such as ionizing and UV radiation, alkylating agents and nucleases (35). Photodynamic therapy activates many signaling reactions that have been characterized in response to other oxidative stresses or physiological stimuli (7). For example, Photofrin-mediated PDT activates NF-kB in mouse L1210 cells (39) and fos, jun and myc are activated by PDT in mouse radiation-induced fibrosarcoma (RIF) cells (40). However, the information of p53 induction by PDT is limited. We have found that PDT induces p53 in GM38A cells. The induction of p53 is observed as early as 6 h after PDT. In agreement with our data, Fisher *et al.* (22) report that Photofrin-mediated PDT induces the accumulation of p53 in colon carcinoma and breast carcinoma cells that exhibit a wild-type p53 phenotype.

A correlation between apoptosis and the cellular sensitivity of cells to chemotherapy and radiotherapy has been reported in several different cell types (41). The p53 protein has been shown to be important for apoptotic cell death in several cell types and the loss or mutation of p53 can result in an alteration in cellular sensitivity to DNA-damaging agents due to loss of p53-dependent apoptosis (42). Although many types of mammalian cells undergo apoptosis after exposure to PDT, an apoptotic response to PDT is not always observed (20–22,43–46). In the work reported here, we found no correlation between cell death and apoptosis in human normal fibroblasts following exposure to Photofrin-mediated PDT. No apoptosis was detected in GM38A cells although they showed a marked sensitivity to PDT as assayed by either colony formation or trypan blue exclusion. However, LFS cells that were less sensitive to PDT, exhibited apoptosis. The number of apoptotic LFS cells correlated with cell killing and increased as a function of time after PDT treatment. The apoptosis in LFS cells indicates that Photofrin-mediated PDT can cause p53-independent apoptosis as has been reported for HL60 cells (20). Because the trypan blue assay for viability primarily reflects the membrane integrity of the cell, the results presented in Fig. 7 suggest a more rapid loss of membrane integrity in normal human fibroblasts compared to LFS cells following PDT at equivalent Photofrin levels.

In addition to the induction of apoptosis, p53 can block the G_1/S transition in cells exposed to DNA-damaging agents through the accumulation of p21 (47). The p53 also regulates a mitotic spindle checkpoint that prevents DNA synthesis before chromosome segregation (48). Additionally, p53 controls a G_2 checkpoint that may be due to decreasing intracellular levels of cyclin B1 (49). However, the role of p53 in cell cycle control is controversial and varies between cell lines. Abrogation of p53 reduces G_1 arrest induced by Photofrin-mediated PDT in breast carcinoma cells but not in colon carcinoma cells (22) and phthalocyanine-mediated PDT-induced G_1 arrest is found in human epidermoid carcinoma cells (50). We have found no marked alteration of the cell cycle in GM38A cells following Photofrin-mediated PDT. However, there was a prolonged G_2/M arrest after Photofrin-mediated PDT in LFS cells. This difference in the effects of PDT on the cell cycle in GM38A compared to LFS087 cells may result, in part at least, from the different p53 status of these two cell types. Many tumor cells with defective or absent p53, including HeLa cells, have been found to undergo G_2 arrest in response to radiation and other cytotoxic agents (51–53). There are reports of a strong correlation between G_2 arrest and cell survival after ionizing radiation (IR). Prolonged G_2 delay has been shown to increase radioresistance and conversely, treatments that abolish G_2 arrest generally increase IR-induced cell death (54). Cells transformed by SV40 in which there is an abrogation of p53 have a longer G_2 phase and less radiosensitivity than parental cells (55). In MCF-7 cells with disruption of p53 by HPV-16 E6, abrogation of G_2 arrest enhances the cytotoxicity of cisplatin (52). It has been proposed that G_2/M arrest allows cells more time to repair damaged DNA so that chromosomes can be segregated efficiently at mitosis (56). Thus, the prolonged G_2/M arrest induced by PDT in LFS cells could, in part at least, account for their resistance to PDT. This result suggests that disruption of the G_2 checkpoint may be an effective method of increasing the sensitivity of tumor cells to PDT.

In summary, we found that LFS cells expressing only mutant p53 are resistant to Photofrin-mediated PDT and that reintroduction of wild-type p53 increased the sensitivity of LFS cells to PDT. We show also that PDT induces p53 accumulation in normal human fibroblasts at Photofrin concentrations of 7.5 μg/mL and 10 μg/mL and LFS087 cells exhibit a prolonged G_2 arrest after PDT. These results suggest that p53 plays a role in the cellular sensitivity to PDT, and the loss of p53 may result in a pronounced G_2 arrest that contributes to the photoresistance of LFS087 cells.

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Chapter Four

Extreme dark cytotoxicity of Nile Blue A in normal human fibroblasts

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(Accepted to Photochemistry and Photobiology)

ABSTRACT

Early reports using mouse models indicated that Nile Blue A (NBA) is taken up more efficiently by tumour cells than normal tissue and retards tumour growth. NBA also shows both dark toxicity and phototoxicity of human tumour cells in vitro. However, studies on the dark toxicity of NBA and the effects of NBA-mediated photodynamic treatment in normal human cells are lacking. In the current study we have examined the cytotoxicity of NBA, in normal human fibroblasts, spontaneously immortalized Li-Fraumeni Syndrome (LFS) cells and three different human tumour cell lines. The normal human fibroblasts showed extreme dark toxicity to NBA compared to LFS cells and the human tumour cell lines. Treatment with 0.1□g/ml of NBA for one hour reduced the colony formation of normal human fibroblasts by greater than 95%, but had no significant effect on the colony formation of LFS cells. No significant numbers of apoptotic cells were detected in either normal human fibroblasts or LFS cells following this drug concentration indicating the extreme sensitivity of normal human fibroblasts to NBA does not result from p53 dependent apoptosis. Although the drug uptake was higher in normal human fibroblasts compared to LFS cells, the difference in sensitivity between normal human fibroblasts and LFS cells

could not be accounted for by the difference of drug uptake alone. In addition, we could not detect any significant photo-cytotoxic effect of NBA in either normal human fibroblasts or LFS cells for a drug concentration of 0.05 μg/ml at light exposures of up to 6.7 J/cm^2. These data indicate an extreme sensitivity of normal human fibroblasts to NBA and an inability to produce a significant photo-cytotoxic effect on human cells using NBA concentrations that have relatively low toxicity for normal human fibroblasts.

Abbreviations: □-MEM, alpha minimal essential medium; EC_{50}, extracellular drug concentration needed to cause a 50% reduction in colony formation; EtNBS, 5-Ethylamino-9-diethyaminobenzo-[*a*]phenothiazinium choride; HPV-16, human papilloma virus-16; LFS, Li-Fraumeni syndrome; MDR1, multi drug resistance protein; NBA, Nile Blue A; PDT photodynamic therapy; NB2B, Nile blue 2B; PI, propidium iodide; SV40, simian virus 40; SnET2, tin ethyl etiopurpurin;

INTRODUCTION

Photodynamic therapy (PDT) is a cancer treatment which involves the systemic administration to a patient of a photosensitizing drug that localizes in tumour tissue and is activated when subsequently irradiated with light of the appropriate wavelength. Since the photosensitizing drug exerts its major cytotoxic effect after light absorption, the selectivity of tumour cell destruction can be achieved by either increasing its accumulation in the tumour (compared to the surrounding tissue) or increasing the light delivery to the tumour tissue. Photofrin-based PDT has been proven to be effective for the treatment of superficial bladder cancer, lung cancer, cervical cancer and cancers of skin and upper digestive tract in several countries (1).

Although Photofrin is an effective photosensitizer, it can result in a prolonged photosensitivity in numerous normal tissues, such as skin, and has a relatively low absorbance at wavelengths above 600 nm (2). For these reasons, several secondary generation photosensitizers are under investigation, with a view to reducing skin photosensitivity of the patient and increasing photosensitizer light absorbance. Early reports indicated that various benzophenoxazines, such as Nile blue A

(NBA) and Nile blue 2B (NB2B), are taken up more efficiently by tumours than normal tissue and retard tumour growth in the mouse (3-6). Although NBA and NB2B both show some dark toxicity and phototoxicity to human tumour cells *in vitro* (7-10), the phototoxicity is lower than that of some other photosensitizers (7). The relatively low phototoxicity of NBA and NB2B has been attributed to their inability to efficiently form triplet states during irradiation (11) and several more efficient benzophenoxazine photosensitizers which demonstrate substantial phototoxicity both in vitro (8,9,11) and *in vivo* (12,13) have been developed.

Although studies on NBA-mediated PDT indicate both dark toxicity and phototoxicity to human tumour cells *in vitro*, to our knowledge there are no previous reports which describe the effects of NBA-mediated PDT on normal human cells. In the present study, we have examined the dark toxicity of NBA in normal human skin fibroblasts, immortalized Li-Fraumeni syndrome cells and three different human tumour cell lines. Normal human fibroblasts showed extreme dark toxicity to NBA compared to LFS cells and the human tumour cells. In addition, we were unable to produce a significant photo-cytotoxic effect in human cells using a drug concentration that was relatively non-toxic to normal human fibroblasts.

MATERIALS AND METHODS

Cells and photosensitizer. All cell cultures used in this study were grown as monolayers in alpha-minimal essential medium (□-MEM) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotericin. The normal human fibroblasts GM38A and GM9503 were obtained from the National Institute of General Medical Science Repository, Camden NJ, USA). The LFS087 and LFS041 cell lines express only mutant p53 (14) and are the spontaneously immortalised counterparts of the Li-Fraumeni syndrome fibroblast strains MDAH087 and MDAH041 respectively and were obtained from Dr. M.A. Tainsky, M.D. Anderson Cancer Center, Houston, TX, USA. The HT29 human colon adenocarcinoma cell line, the HT1376 human bladder carcinoma cell line and the SK-N-MC human neuroblastoma cell line have mutations in the p53 gene (15-18) and were obtained from The American Type Culture Collection, Rockville, Maryland, USA. The Nile blue A was obtained from Sigma Inc.

Cell treatment. For each clonagenic survival experiment, exponentially growing cells were plated at low density (100

cells/plate for HT29, HT1376, SK-N-MC and LFS cells, 500 cells/plate for normal human fibroblasts) in triplicate for 3-4 hours to allow cells to adhere. Following exposure to various concentration of NBA for 1 hour at 37 ^{0}C, the medium containing NBA was replaced with fresh drug-free medium. Following a 5-7 (LFS, HT29, HT1376, SK-N-MC cells) or 10 (normal fibroblasts) day incubation, cells were fixed, stained with methylene blue and the number of colonies containing >30 cells were counted. Average colony counts together with the standard error were determined from the triplicate plates for each NBA treatment. The surviving fraction was calculated as the relative plating efficiency of the NBA treated versus the non-NBA-treated cultures.

For PDT, cells were treated with NBA at 0.05 μg/ml for 1 hour and exposed to a red light at various fluencies. The light source was a 21" X 32.75" light box illuminated by a parallel series of 12 fluorescent tubes (Phillips type TL83), enclosed on top with a sheet of clear Plexiglass and filtered with red acetate filters (Rosco-lux, No 19, Rosco, CA) that gave a wide band illumination above 585 nm. The energy fluence rate was 22.4 mW/cm^2 at the wavelength of 670 nm as measured using a Jobin-Yvon model CP-200 spectrometer and a Princeton Instruments charge-coupled device. Cell survival was determined using the colony forming assay as described above.

Drug uptake studies. Cells were seeded at 7.5 X 10^5 /dish for 3-4 hours allowing cells to adhere, and subsequently exposed to NBA at various concentrations for 1 hour. The medium containing NBA then was removed. The cells were trypsinized, washed with PBS, and resuspended in 1 ml PBS for NBA uptake determination. Cellular uptake of NBA was measured by flow cytometry using an excitation wavelength of 488 nm and emission at 675 nm. Data from 10^4 cells were collected for each sample and the cellular NBA concentration was expressed as the average fluorescence of NBA per cell.

Detection of apoptosis. Cells were treated with or without NBA as described above. At 24 hours following 1 hour of drug exposure, cells including detached cells were collected using trypsin and washed twice with PBS. For flow cytometry, cells were stained with a solution containing 0.1% Triton X-100, 66 units/ml RNase and 5mg % propidium iodide (PI) for 30 min. A total 2 X 10^4 cells was analysed for DNA-PI fluorescence by flow cytometry at an excitation wavelength of 488 nm. The cell fraction with sub diploid DNA was considered to represent the fraction of apoptotic cells. Cells from the same sample for flow cytometry were fixed and stained for 10 min with 11 μg/ml

Hoechst dye 33258 (Sigma) diluted in PBS. The stained samples were examined under a fluorescence microscope. Cells with condensed chromatin pattern were considered as apoptotic cells. 300 Cells were examined in each of three independent fields of view and the average percentage of apoptotic cells was calculated.

RESULTS

Extreme sensitivity to Nile blue A of normal human fibroblasts compared to LFS cells

We have reported previously that immortalized LFS cells are more resistant than normal human fibroblasts to Photofrin-mediated PDT (19). It was therefore considered of interest to determine if a differential sensitivity of LFS cells and normal human fibroblasts was also present following NBA-mediated PDT. Previous reports of successful NBA-mediated PDT of human tumor cells have employed exposure of cells to drug concentrations ranging from 0.2 to 5 μM over periods of 10 minutes to 1 hour followed by red light exposures ranging from 4.8 to 20 J/m^2 (8-10). In the present study we exposed cells to NBA drug concentrations ranging from 0.05 up to 4 μg/ml (0.068 to 5.44 μM) for 1 hour. We found that normal human fibroblasts showed an extreme dark toxicity to NBA compared to LFS cells. Fig. 1 shows the colony survival following treatment with NBA for two normal human fibroblast strains and two LFS cell lines. It can be seen that exposure of cells to 0.1 μg/ml of NBA for 1 hour resulted in a more than 95% reduction in colony survival for normal human

fibroblasts, whereas this drug concentration had no significant effect on the survival of LFS cells. Similar results were obtained for GM38 and LFS087 cells in two additional independent experiments.

In order to examine whether the difference in drug toxicity of normal human fibroblasts and LFS cells was due to a difference in drug uptake, we compared the drug uptake between GM38A and LFS087 by flow cytometry. It can be seen from Fig. 2 that the NBA-fluorescence per cell was higher in GM38A cells compared to that in LFS087 cells. Using the uptake data from Fig. 2, the colony survival of GM38A and LFS087 cells was plotted as a function of drug uptake per cell in Fig. 3. It can be seen that for the same drug uptake, the normal human fibroblasts still showed an extreme sensitivity to NBA compared to the LFS cells. Thus the mechanism(s) leading to the extreme sensitivity of normal human fibroblasts compared to LFS cells is not due to differences in drug uptake alone.

It has been demonstrated that p53 plays an important role in apoptosis induced by DNA damage (20) and apoptosis is considered to be a critical determinant of tumor cell sensitivity to chemotherapy (21). Since LFS cells express only mutant p53, wild-type p53 dependent apoptosis was a possible mechanism leading to the increased sensitivity of the normal

human fibroblasts to NBA. We therefore examined cells for the presence of apoptosis following treatment with NBA using both flow cytometry and Hoechst staining. At a concentration of 0.1 μg/ml NBA for 1 hour, which killed more than 95% of the cells, we did not detect any significant apoptosis in GM38A cells at 24 or 48 hours following exposure. In addition, no significant apoptosis was observed in LFS087 cells following treatment with 0.1 or 0.4 μg/ml of NBA for 1 hour. The average percentage of apoptotic cells was less than 1% for both normal human fibroblasts and LFS cells following the NBA treatment (data not shown). Thus the sensitivity of normal human fibroblasts to NBA did not result from p53 dependent apoptosis.

We also attempted to examine the sensitivity of normal human fibroblasts and LFS cells to NBA-mediated PDT using drug concentrations that showed relatively low dark toxicity in normal human fibroblasts. However, even at a drug concentration of 0.05 μg/ml (0.068 μM), which resulted in greater than 80% cell killing for normal human fibroblasts, exposure of LFS087 and GM38A cells to red light at fluencies of up to 6.7 J/cm^2 resulted in no significant light induced decrease in cell survival (data not shown).

Sensitivity of human tumour cells to Nile blue A

We also examined the dark toxicity to NBA in three human tumour cell lines. As shown in Fig. 4, the tumour cell lines HT29, HT1376 and SK-N-MC, like LFS cells, all showed a considerably greater resistance to NBA compared to that of normal human fibroblasts. EC_{50} (extracellular drug concentration needed to cause a 50% reduction in colony formation) values for drug alone (dark toxicity) were: GM38A normal human fibroblasts, 0.02 μg/ml; SK-N-MC cells, 0.40 μg/ml; HT1376 cells, 0.73 μg/ml; LFS087cells, 1.15 μg/ml; HT 29 cells, 1.63 μg/ml.

DISCUSSION

Previous reports of successful NBA-mediated PDT of human tumour cells have employed exposure of cells to drug concentrations ranging from 0.2 to 5 μM over periods of 10 minutes to 1 hour followed by red light exposures ranging from 4.8 to 20 J/cm^2 (8-10). Cellular uptake of NBA in MGH-U1 human bladder tumour cells occurs with a rapid initial uptake followed by a more gradual increase in the intracellular concentration reaching saturation by about 80 minutes, such that the intracellular drug concentration at 10 minutes is at least 50% of that achieved at 60 minutes following the initial exposure of the drug to the cells (9). Thus the intracellular drug concentration following exposure of cells to a given NBA concentration for 10, 30 or 60 minutes would not be expected to differ by more than 2 fold at most.

In the present work we found that normal human fibroblasts showed high dark toxicity to NBA compared to LFS cells and three different human tumour cell lines. An extracellular NBA concentration of 0.05 μg/ml (0.068 μM) for 1 hour resulted in more that 80% lethality for normal human fibroblasts. The EC_{50} dark toxicity values for normal human fibroblasts were about 56 fold less compared to LFS cells and

20, 35 and 80 fold less compared to SK-N-MC, HT1376 and HT29 human tumour cells respectively (Fig. 4). The NBA concentrations reported by others to result in significant tumour cell kill following NBA-mediated PDT are at least two orders of magnitude greater than the EC_{50} value for NBA dark toxicity in normal human fibroblasts (9,10). An EC_{50} of about 4 μM NBA following a light exposure of 20 J/cm^2 and an EC_{50} of more than 10 μM NBA with a light exposure of 4.5 J/cm^2 have been reported for 2008 human ovarian carcinoma cells (10) and MGH-U1 bladder carcinoma cells (9), respectively. Such NBA concentrations would be expected to result in a substantial dark toxicity for normal human fibroblasts.

The p53 protein has been shown to play a role in the sensitivity of cells to radiation therapy (22,23) and chemotherapy (24). Gastrointestinal cancer cells carrying wild-type p53 exhibit higher sensitivity to doxorubicin and 5-fluorouracil than cells carrying mutant p53 (25) and reintroduction of wild-type p53 into ovarian cancer cells (26) and human bladder cancer cells (17) increases their sensitivity to cisplatin. Loss of wild-type p53 has been shown to enhance the activity of the multi drug resistance protein (MDR1) and MDR1 is thought to play a role in the resistance of cells to cytotoxic drugs (27-29). Several, but not all studies suggest a role for p53 in the sensitivity of

cells to PDT (19,30-32).

In the present work we show that two unrelated normal human fibroblast strains expressing wild type p53 have a substantially greater dark toxicity to NBA compared to two unrelated LFS cell lines and three different human tumour cell lines. The LFS cells and the three human tumor cell lines tested all harbour a mutation in the p53 gene (14-18). A relatively low dark toxicity to NBA, compared to that reported here for normal human fibroblasts, has also been reported for other human tumour cells including Hep2 human larynx carcinoma cells (8), MGH-U1 human bladder carcinoma cells (9) and 2008 human ovarian adenocarcinoma cells (10). An NBA dose dependent dark toxicity with an EC_{50} of 17.5 μM following 1 hour exposure to the drug has been reported for 2008 cells and exposures of up to 5 μM NBA for 1 hour had no significant effect on their cell growth (10). Exposure of Hep2 cells to 0.5 μM of NBA alone for 10 minutes resulted in no significant cell toxicity as determined by [^{3}H]leucine incorporation (8). Hep2 cells (33) and MGH-U1 cells (34) both harbour a mutation in the p53 gene and 2008 cells show constitutively high p53 expression levels indicating abnormal p53 expression in these cells (35). Taken together these results suggest that some pathway(s) which is/are altered in these human tumour cells compared to

untransformed human fibroblasts play(s) a role in the dark toxicity of NBA. However, it is not clear whether the differences in NBA sensitivity are due to differences in cellular p53 status or some other alteration, since the cell lines used in the present study were derived from different genetic backgrounds and, in addition, some mutant p53s retain partial function, whereas others do not. Notwithstanding, these results are consistent with an involvement of p53 and/or a p53 dependent pathway (which does not lead to apoptosis induction) in the dark toxicity of NBA in human cells.

Several recently developed novel benzophenoxazines with greater photosensitizing efficacy compared to NBA have been developed (8,9) which have a higher photocytotoxicity to human rectal carcinoma cells compared to primary normal bovine kidney cells (8). Photodynamic therapy employing one of these, 5-ethylamino-9-diethyaminobenzo[*a*]phenothiazinium chloride (EtNBS), shows efficacy against experimental murine tumors (12) and naturally occurring tumours in cats and dogs (13). To date, the dark toxicity of normal human fibroblasts to these novel benzophenoxazines has not been reported.

Since numerous mechanisms contribute to the success of PDT *in vivo*, it is not clear to what extent the extreme dark toxicity of normal human fibroblasts to the drug NBA would influence the outcome of NBA-mediated PDT in the treatment of

human tumours.

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Figure 1. NBA cytotoxicity in GM38A (▲), GM9503 (△), LFS087 (●) and LFS041 (○).

Cells were exposed to NBA at the concentrations indicated for 1 hour. Cell survival was determined by the colony forming assay as described in materials and methods. Data points represent the mean ± standard error for three determinations from a typical experiment. Similar results were obtained for GM38A and LFS087 in two additional experiments.

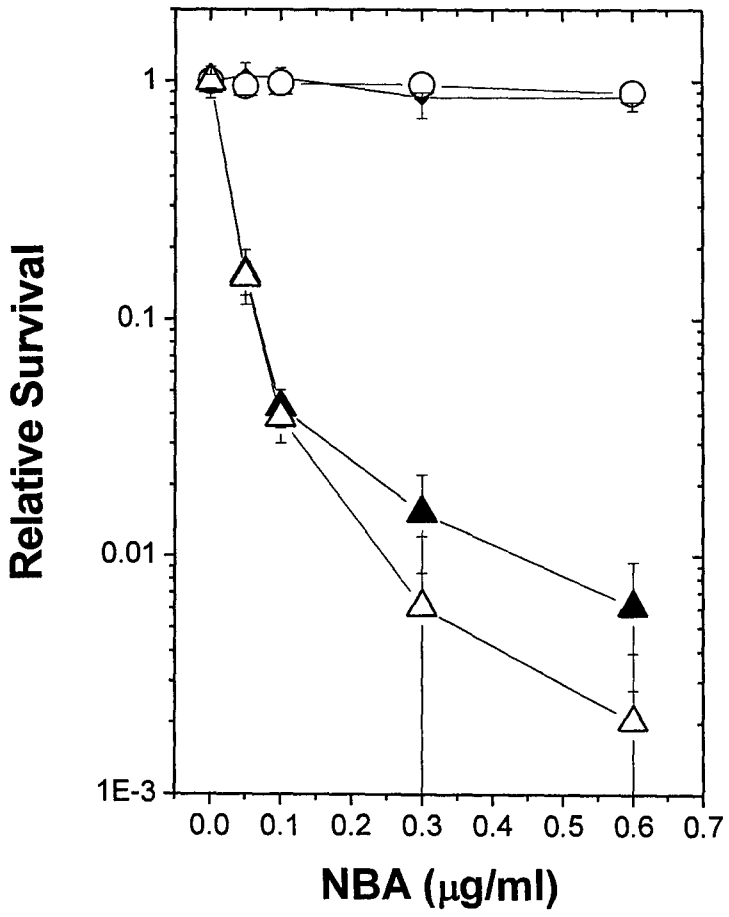

Relative Survival
1
0.1
0.01
1E-3
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
NBA (μg/ml)

Figure 2. Drug uptake in GM38A (○) and LFS087 (●).

Cells were treated with NBA at the concentrations indicated. Cellular NBA concentrations were determined by flow cytometry following 1 hour incubation with NBA at 37 ^{0}C. Data points represent the mean ± standard error of two independent experiments. Error bars are contained within the data points for LFS087 cells.

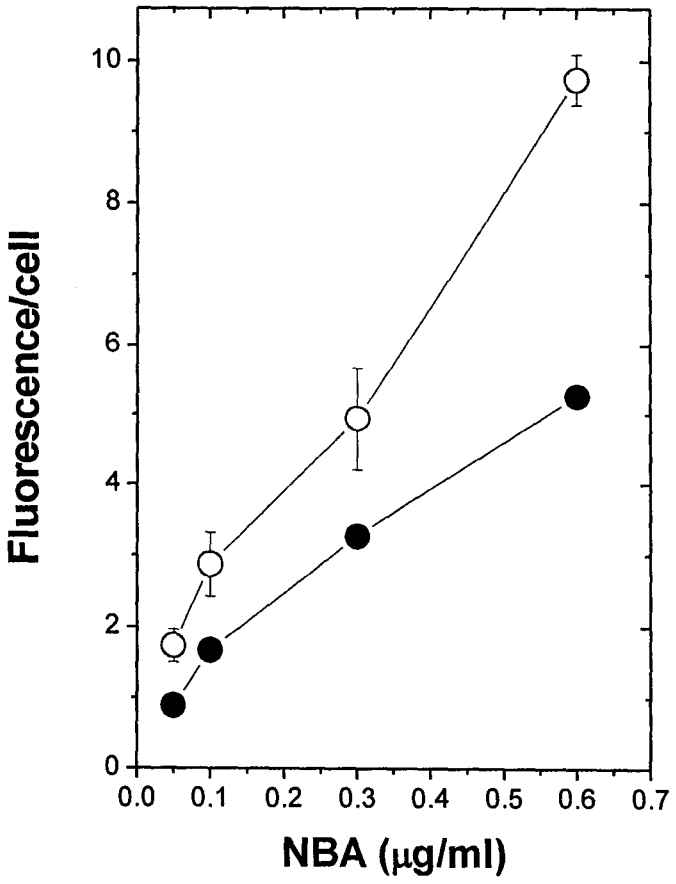

Fluorescence/cell
10
8
6
4
2
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
NBA (μg/ml)

Figure 3. Cell survival versus drug uptake following NBA treatment in GM38A (○) and LFS087 (●).

Using the uptake data from Fig. 2, the colony survival of GM38A and LFS087 cells was plotted as a function of drug uptake per cell. Cell survival was measured using colony-forming ability and drug uptake was determined using flow cytometry as described in materials and methods. Data represent the mean ± standard error of two independent experiments.

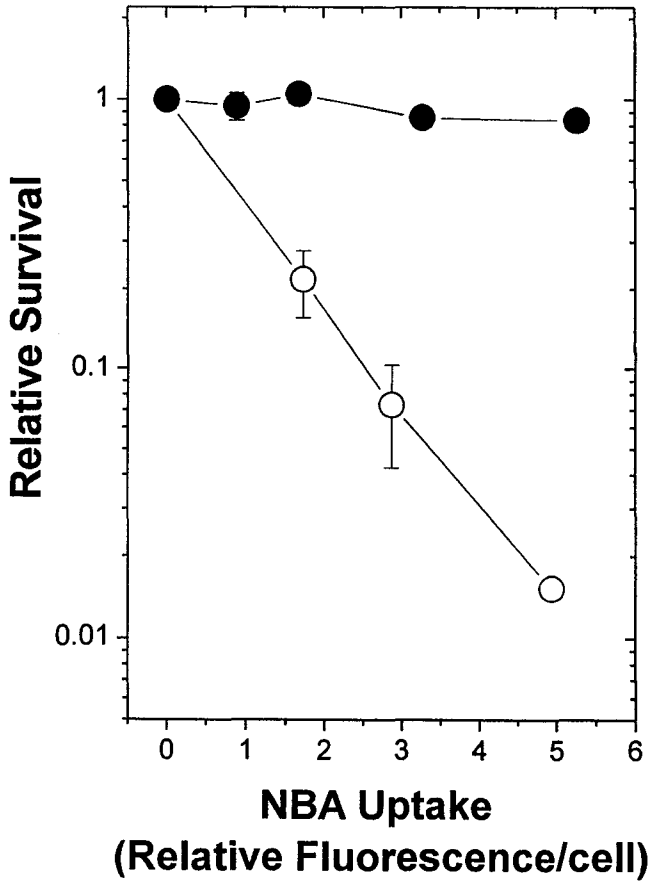
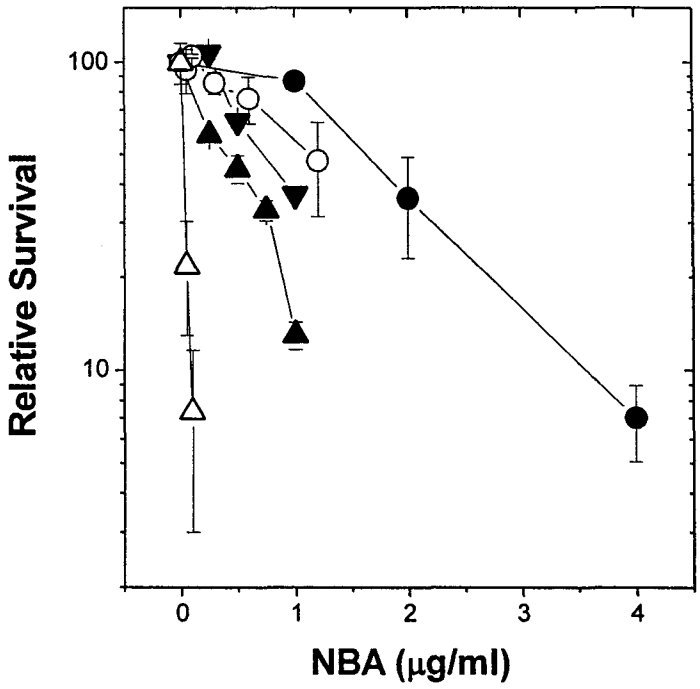

Relative Survival
1
0.1
0.01
0
1
2
3
4
5
6
NBA Uptake
(Relative Fluorescence/cell)

Figure 4. NBA cytotoxicity in HT29 (●), HT1376 (▼) and SK-N-MC (▲) human tumor cells.

Cells were exposed to NBA at the concentrations indicated for 1 hour. Cell survival was determined by the colony forming assay as described in materials and methods. Survival of the normal human fibroblast strain GM38A (△) and the LFS087 (○) cell line are shown for comparison. Data points represent the mean ± standard error of two independent experiments for GM38A and LFS087 cells and three independent experiments for HT29, HT1376 and SK-N-MC cells.


Relative Survival
100
10
0
1
2
3
4
NBA (μg/ml)

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Chapter Five

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

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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 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 (MAPKs), play a crucial role in the cellular response to radiation therapy and chemotherapy. However, the role of ERKs in the cell survival following PDT is less clear. We have reported previously that immortalized Li-Fraumeni syndrome (LFS) skin cells are more resistant to Photofrin-mediated PDT compared to normal human skin fibroblast strains (NHF) at equivalent cellular Photofrin levels. In order to investigate the possible role of the ERK pathway in cellular sensitivity to PDT, we have examined the response of ERK1/2 in both LFS cells (LFS087) and NHF cells (GM38A) following Photofrin-mediated PDT. ERK1/2 activity was induced rapidly in both cell types, as early as 30 min following PDT. In GM38A cells, the PDT-induced ERK1/2 activity

was transient and by 3 h had returned to a level significantly lower than basal levels. In contrast, the induction of ERK1/2 was sustained in LFS087 cells and lasted at least for 11 h. Blocking of the sustained ERK activity with PD98059, an inhibitor of MEK, significantly decreased cell survival of LFS cells following PDT. PDT treatment also induced the expression of MAPK phosphatase, MKP-1, and reduced the level of Raf-1 proteins in both cell types. Both basal and induced levels of MKP-1 were substantially greater in NHF compared to LFS cells and the substantial levels induced in NHF cells correlated with the transient activation of ERK1/2 by PDT in these 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

Photodynamic Therapy (PDT) is a novel treatment that has been applied to both neoplastic and non-neoplastic disease (Levy 1995). 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 presence of molecular oxygen, which leads to cell death through the generation of several chemically reactive molecular species (Pervaiz 2001). 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 (Levy 1995). Photofrin-mediated PDT has been used in many countries throughout the world and shows considerable promise in the treatment of several solid tumors (Levy 1995). Although the molecular mechanisms of tumor regression induced by PDT are not fully understood, there is evidence that the reactive oxygen species (ROS) generated by PDT can lead to apoptosis and/or necrosis of cells and are responsible for tumor destruction (Pervaiz 2001).

ROS, including the ROS induced by PDT, have been shown to be very efficient in triggering a variety of cellular signaling pathways (for a review see Gabbita et al. 2000). Photofrin-mediated PDT induces the expression of early

response genes including c-jun, c-fos and c-Myc (Kick et al. 1996) and several stress proteins (Gomer et al. 1996; Curry and Levy 1993). The former are involved in the regulation of cell proliferation, differentiation and survival while the latter are thought to act as protection signals (Cotto and Morimoto 1999). Photofrin-mediated PDT causes an increase in intracellular second messenger Calcium (Ca^{2+}) by releasing it from mitochondria and the endoplasmic reticulum (ER) (Dellinger et al. 1994). In addition, PDT using various photosensitizers activates NF-kB (Granville et al. 2000) and releases cytochrome c that leads to apoptosis (Chiu et al. 2001). The activation of mitogen-activated protein kinases (MAPKs) by PDT has been reported in several different cell models using several different photosensitizers (Assefa et al. 1999). However, the biological functions associated with PDT-induced activation of MAPKs are somewhat controversial (Xue et al. 1999) 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 ATF2 (Chang and Karin 2001). MAPKs constitute a superfamily of three related kinases that are activated by a diverse array of extracellular stimuli. They include extracellular signal-regulated kinases (ERKs), c-jun NH_2-terminal kinases (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 (Graves et al. 2000). In contrast, JNK and p38 are activated primarily by environmental stresses such as UV light (Tournier et al. 2000), heat shock (Kyriakis et al. 1994) and other cellular stress (Kyriakis et al. 1994) 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 (For a review see Chang and Karin 2001). 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 (Thrane et al. 2001), enhances cell sensitivity to cisplatin treatment (Cui et al. 2000) and increases cytosine arabiniside-induced apoptosis (Anderson and Tolkovsky 1999).

The activities of ERKs are regulated by MAPK phosphatases (MKPs). MKPs are dual specificity phosphatases that inactivate MAPKs by dephosphorylation of both Thr and Tyr residues within their signature sequence (Chang and Karin 2001). MKP-1, one member of this family, is the product of an immediate early gene and shows selectivity for ERK1/2 *in vitro* (Franklin and Kraft 1995; Sun et al. 1993). MKP-1 inactivates growth factor-induced ERK and suppresses cell proliferation (Brondello et al. 1995). Thus MKP-1 plays an important role in the regulation of the dynamic activation of ERKs.

In spite of 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 (Assefa et al. 1999). In contrast, using phthalocyanines Pc 4 as a photosensitizer, Xue et al, (Xue et al. 1999) reported a transient activation of ERK2 in CHO 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 following PDT.

We have previously reported that immortalized Li Fraumeni syndrome (LFS) cells are significantly more resistant

to Photofrin-mediated PDT compared to normal human fibroblasts (NHF). The D_{37} value (Photofrin concentration resulting in 37% colony survival following light exposure) was about 3 fold greater for LFS cells compared to normal human fibroblast (Tong et al. 2000). 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 following 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 hours after PDT. The duration of PDT-induced ERK activity therefore 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 since Photofrin-mediated PDT actually reduced Raf-1 expression. This suggests that the prolonged upregulation of

ERK1/2, through a Raf-1 independent pathway, contribute to the resistance of LFS cells to PDT.

Materials and Methods

Cell lines and culture conditions:

The human normal fibroblast stains GM38A and GM9503 were obtained from the National Institute of General Medical Sciences Repository (Camden, NJ). The immortalized Li-Fraumeni syndrome (LFS) cell lines were obtained from Dr M. A. Tainsky, M. D. Anderson Cancer Center, Houston, TX. All cells were grown in monolayer culture in alpha-minimal essential medium (α-MEM medium) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Gibco BRL) and were cultured in a humidified atmosphere at 37 ^{0}C and 5% CO_2.

Reagents:

Photofrin was a gift from QLT Inc. (Vancouver, BC). The MEK1/2 inhibitor, PD98059, and antibodies to phophorylated ERK1/2 or total ERK1/2 were purchased from New England Biolabs, Inc. (Beverly, MA). HRP conjugated secondary antibodies and antibodies to Raf-1, actin or MKP-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [α-^{32}P]dCTP was purchased from MANDEL scientific company Ltd. (Guelph, ON), and random primer labeling kits from Life Technology (Burlington, ON)

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 (Tong et al. 2000). 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, No. 19, Rosco, CA). The energy fluence rate was 7 mW/cm^2 at a wavelength of 630 nM. In the experiment where PD98059 was used, cells were incubated with various concentrations of PD98059 (dissolved in DMSO; final concentration in medium was less than 0.15%) for 2 h prior to exposure to red light.

Clonogenic assay:

Exponentially growing LFS cells were plated at 100 cells/well and incubated for 4 h to allow cells to adhere before treatment with PDT as described above. Following 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 greater then 30 cells were counted. The

surviving fraction was calculated as the percentage of the PDT-treated samples compared to 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., Missisauga, ON). 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, England). The cDNA was synthesized by RT-PCR using primers that were designed according to the sequence of MKP-1 or GAPDH gene. cDNA probes were labeled with [α^{32}p]dCTP using a random primer labeling kit according the procedure described by the manufacturer. Membranes were hybridized with the cDNA probes of MKP-1 overnight at 42^{0}C in a solution containing 5X SSC, 50% formamide, 1X Denhardt's reagent, and 1% SDS. After the membrane was washed 3X with 1X 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 re-probed with a cDNA probe of GAPDH, which was

used to normalize for differences in loading and transferring among the samples.

Western blotting analysis:

Following PDT, cells were washed twice with ice-cold PBS buffer and lysed in a buffer containing 50 mM of Tris (PH 8.0), 150 nM of NaCl, 0.5% NP-40, 2 mM of EDTA, 100 mM of NaF, 10 mM of sodium orthovanadate and a protease inhibitor cocktail (Boehringer Manheim, Germany) for 30 min on ice. The debris of cells then was pelleted by centrifugation (>10,000 xg) for 15 min and discarded. The protein concentration of the cell lysate was determined using the Bradford microassay procedure (BioRed, Munich, Germany). Equal amounts of protein (15-30 μg) were resolved on 12% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to an ECL membrane (Amersham Pharmacia Biotech, England). The blots were blocked in 5% skim milk in a tris-buffered saline with 1% tween-20 (TBST) for 1 h at room temperature and then incubated at 4 ^{0}C overnight with corresponding anti-bodies in the same buffer as the block buffer. Specific antibody-labeled proteins were detected by using HRP conjugated secondary antibodies and the ECL plus Western blotting detection system (Amersham Pharmacia Biotech, England). Thereafter, blots were stripped and re-probed 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.

In order 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/cm^2, the phosphorylation of ERK1/2 was examined by Western blot analysis using antibodies that only recognize phosphorylated ERK1/2. As showed in Figure 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 to LFS087 cells at later times. For GM38A cells, the phosphorylation of ERK1/2 declined after 30 min. and by three 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 hours. 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 hours following PDT. Photofrin without photo-activation 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 due 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 phosphorylations contribute to the difference in cell sensitivity to PDT between LFS087 and GM38A cells. The decreased sensitivity of LFS087 cells to Photofrin-mediated PDT might due to the prolonged 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 various concentrations for 2 hours before exposure to a red light. We found the PDT-induced phosphorylation of ERK1/2 was reduced by more than 50% when cells were treated with 75 μM of PD98059 (Fig. 2). The inhibition of ERK phosphorylation by PD98059 was observed at 30 min following 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 75 μM PD98059 for 2 hours before exposure to red light. It was found that cells pre-treated with PD98095 showed a significantly reduced viability compared to cells without PD98059 treatment as determined using a clonogenic assay (Fig. 3). The reduction in PDT-induced ERK1/2 phosphorylation and concomitant reduction in cell viability 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 following the initial transient increase after PDT suggested a negative regulation of PDT-induced ERK1/2 occurred in NHF cells a few hours after PDT. MKP-1 has been shown to be a specific inactivator of ERK1/2 by dephosphorylation of ERK1/2 in vitro (Franklin and Kraft 1995;Sun et al. 1993). MKP-1 is a product of immediate early genes and can be rapidly induced by cellular stresses such as UV (Liu et al. 1995) and heat shock (Keyse and Emslie 1992) 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 following 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). The induction of MKP-1 mRNA was observed by 1 h following PDT and lasted at least for 1 hour. 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 significant 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 further 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 as early as 30 min and remained at increased levels for at least 5 hours (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 (Marshall 1994). 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 (Samuels et al. 1993) and dominant negative Raf-1 mutants inhibit growth factor-induced ERK activity in fibroblasts (Chao et al. 1994). In contrast, there are some reports which suggest that the endogenous Raf-1 protein is not an important MEK activator since ERK activity can be induced in cell lacking raf-1 genes (Huser et al. 2001; Mikula et al. 2001). It was therefore 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 following PDT under conditions shown previously to activate ERK1/2. We found PDT significantly reduced the Raf-1 protein by more than 60% compared to control values in LFS 087 cells (Fig. 5). The reduction of Raf-1 protein by PDT was observed as early as 30 min following 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 resulted in the activation of ERK1/2 in either a transient or a sustained manner. The cells with lower sensitivity (LFS087 and LFS041) exhibited a prolonged ERK1/2 activation, while cells with high sensitivity (GM38A and GM9053) showed a transient activation of ERK1/2 after PDT. The MEK inhibitor, PD98059, blocked the sustained activation of ERK1/2 after PDT and reduced cell survival in LFS087 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 were originally associated with the stimulation of growth factors (Derkinderen et al. 1999), there is accumulating evidence suggesting that activation of ERK1/2 can be induced by a variety of cellular stresses including reactive oxygen species (ROS) (Wang and Bonner 2000; Yoshizumi et al. 2000)). ROS have been shown to act as secondary messengers capable of activating a number of signaling pathways (Sundaresan et al.

1995; Briviba et al. 1997; Grether-Beck et al. 1996). 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 NF-κB (Piret et al. 1995), 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 (Assefa et al. 1999; Xue et al. 1999). Photofrin-mediated PDT induces early response genes such as c-fos, c-jun and c-myc (Kick et al. 1996; Luna et al. 1994) that are downstream substrates of ERK1/2 (Seger and Krebs 1995). 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 (Assefa et al. 1999; Xue et al. 1999). This difference might be due to the difference in cell line and/or photosensitizer used.

Activation of ERK is critical not only in cell proliferation and differentiation (Davis 1993; Seger and Krebs 1995), but also as a survival signal (Eastman 1995; Xia et al. 1995). Inhibition of ERK1/2 activation by PD98059 results in enhanced cisplatin cytotoxicity in ovarian carcinoma cell

lines (Persons et al. 1999), as well as increases H_2O_2- induced apoptosis in cardiac myocytes (Aikawa et al. 1997) and hyperoxia-induced apoptosis in alveolar epithelial cells (Buckley et al. 1999). 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 ERKs 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 was reported to depend upon the duration and intensity of ERK activation in a number of cells. For example, sustained activity of ERKs induced by thrombin and bFGF results in cell proliferation in airway smooth muscle while endothelin-1 that induces a transient activation of ERK does not promote cell proliferation (Ravenhall et al. 2000). Sustained ERK activation resulting from constitutively active MEK mutation shows an enhanced resistance to H_2O_2 in NIH 3T3 cells (Guyton et al. 1996). In the same cell model, expression of constitutively active form of ERK2 protects cells from doxorucin-induced cell death (von Gise et al. 2001). 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 (Huang et al. 2001). However, sustained activation of ERK has been also shown to inhibit cell growth in some systems (Kimura et al. 1999). Apparently, the sustained ERK activation induced by Photofrin-mediated PDT protected cells from phototoxicity. Our data further 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 down stream targets. In Madin-Darby canine kidney cells, hepatocyte growth factor induces sustained ERK activation and in turn increases the expression of intergrin 2, while epidermal growth factor which induces a transient activation of ERK only slightly increases the expression of intergrin 2 (Liang and Chen 2001). Similar results are observed in the growth factors-induced expression of serum- and glucocorticoid-inducible kinase (Mizuno and Nishida 2001). 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 second major new finding 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 regulate ERK activity by dephosphorylating ERKs (Keyse and Emslie 1992; Charles et al. 1992). 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 to 1 h after PDT and remained at increased levels for at least 5 h, 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 due to 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 in the MKP-1 expression. MKP-1 mRNA and protein levels have been shown to be regulated by ERK1/2 (Brondello et al. 1997; Li et al. 1999). ERK phosphorylates MKP-1 and in turn stabilizes the MKP-1 protein by reducing its proteolytic degradation (Brondello et al. 1999). 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 to the induction of MKP-1 mRNA. This suggests that the expression of MKP-1 in response to PDT may be regulated by the 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 of this idea, Simon et al. (Cook et al. 1997) have reported that lysophosphatidic acid and FGF-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 ATF/CREB sites, which can respond to Ca^{2+}/cAMP and ERKs (Kwak et al.

1994). Moreover, Photofrin-mediated PDT results in an increase of intracellular Ca^{2+} by release of Ca^{2+} from mitochondria, ER or influx from the 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>>Raf>>MEK mediated ERK activation occurs in response to cellular stresses (Aikawa et al. 1997; Chen et al. 1998). In contrast, the activation of ERKs following heat shock was via a Raf-1- independent route (Ng and Bogoyevitch 2000). It was also reported that normal ERK activation was induced by growth factors in cells in which the Raf-1 gene was knocked out (Huser et al. 2001; Mikula et al. 2001). 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 (Chakravortty et al. 2001), treated with complex I inhibitor of the electron transport chain (Cassarino et al. 2000), and infected with *salmonella* (Jesenberger et al. 2001). These reports suggested that the rapid reduction of Raf-1 results from degradation or cleavage. In the case of PDT-

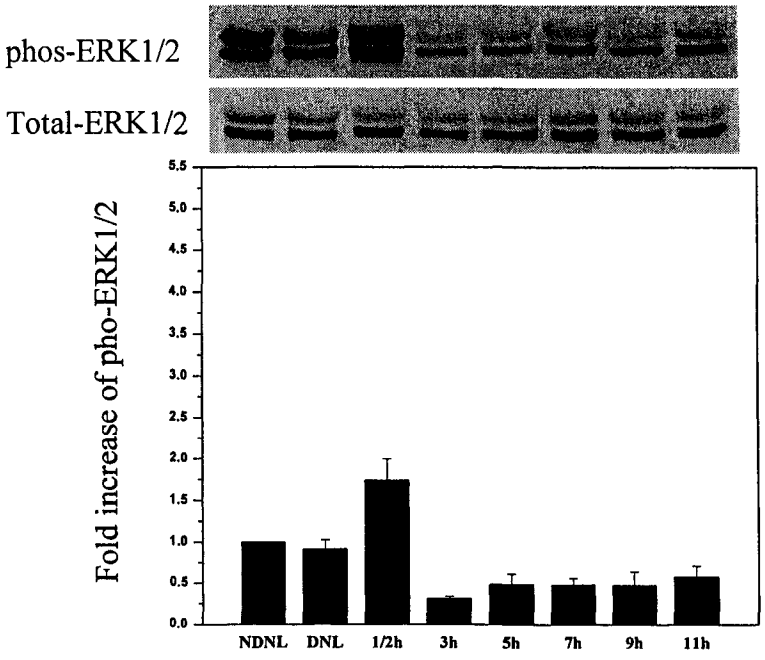
reduced Raf-1, it is possible that PDT-induced ROS resulted in Raf-1 destablization, since the loss of Raf-1 occurred rapidly, in less than 30 min following PDT. Furthermore, PDT-induced ROS could directly damage proteins (Kim et al. 1999) and disturb the electron transport chain. However, the importance of the reduction in Raf-1 in the cellular response to PDT is not clear.

In summary, we have shown 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 following Photofrin-mediated PDT (Fig. 6). In addition, we have demonstrated that MEK is the upstream activator of PDT-induced ERK pathway but Raf-1 is not. The precise nature of the upstream signal of the PDT-induced MEK/ERK pathway will require further investigation.

Figure 1. Activation of ERK1/2 in GM38A (A) and LFS087 cells (B) by Photofrin-mediated PDT.

A representative autoradiograph of a Western Blot (top) from cells treated with Photofrin (10 μg/ml for LFS087 and 7.5 μg/ml for GM38A cells) or untreated with Photofrin for 18 hours before exposure to red light at a light exposure of 270 mJ/cm^2 as described in the materials and methods. (DNL: with Photofrin but without light). Cell lysates were harvested at the indicated times after PDT (1/2h to 11h after PDT). (Bottom) The activity of ERK1/2 was calculated as the ratio of the phosphorylated ERK1/2 to total ERK1/2 and is shown relative to the ERK1/2 activity in the untreated, no drug, and no light (NDNL) control cells. The error bars represent standard error, n = 3.

A.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Total-ERK1/2
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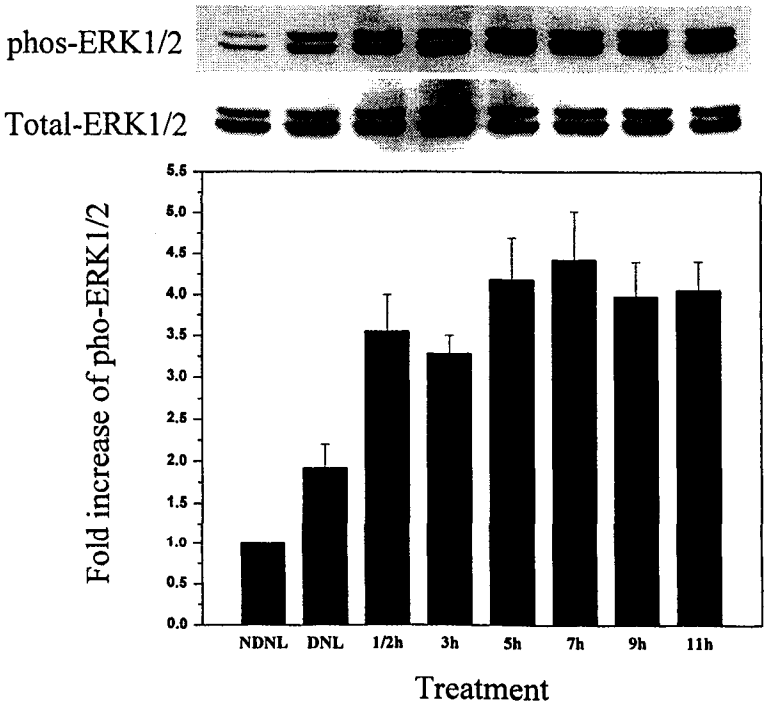
B.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Total-ERK1/2
Fold increase of pho-ERK1/2
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NDNL
DNL
1/2h
3h
5h
7h
9h
11h
Treatment

Figure 2. The effects of PD98059 on the PDT-induced ERK activity in LFS087 cells.

Cells were treated with Photofrin (10 μg/ml) for 18 hours and various concentrations of PD98059 (PD) or DMSO for 2 hours before exposure to 270 mJ/cm^2 of red light. The protein lysate was prepared at the indicated time. (Top) The phosphorylation of ERK1/2 was examined by Western Blot analysis using anti-phosphorylated ERK antibodies and quantified by phosphoImage analysis. (Bottom) The activity of ERK1/2 was calculated as the ratio of the phosphorylated ERK1/2 to total ERK1/2 and is shown relative to the ERK1/2 activity in the untreated, no drug, and no light (NDNL) control cells. DNL, Photofrin only.

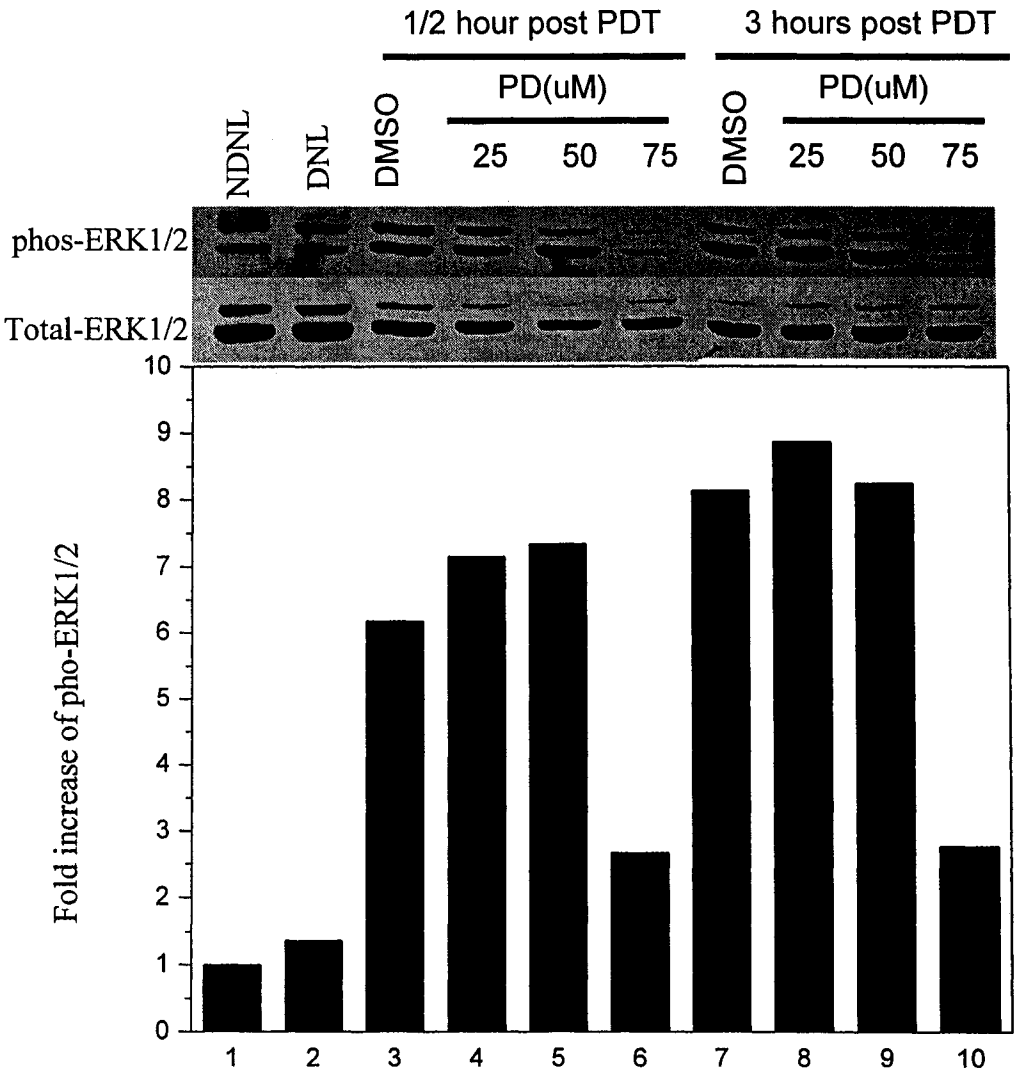

1/2 hour post PDT
3 hours post PDT
PD(uM)
PD(uM)
NDNL
DNL
DMSO
25
50
75
DMSO
25
50
75
phos-ERK1/2
Total-ERK1/2
Fold increase of pho-ERK1/2
0
1
2
3
4
5
6
7
8
9
10
1
2
3
4
5
6
7
8
9
10

Figure 3. The effect of ERK1/2 inhibitor PD98059 on the PDT induced cell death in LFS087 cells.

LFS087 cells were incubated with 10 μg/ml of Photofrin for 18 hours in the presence (■) or absence (▲) of 75 μM PD98059 or DMSO (●) or cells were treated with DMSO (▼) only, for 2 hours before exposure to red light. Results show the mean ± standard error of colony counts for three determinations from a typical experiment. Similar results were observed in three independent experiments. The difference in the survival curves for cells treated with DMSO+PDT (●) compared to cells treated with PD98059 +PDT (■) was significant by chi-square analysis ($p<0.001$).

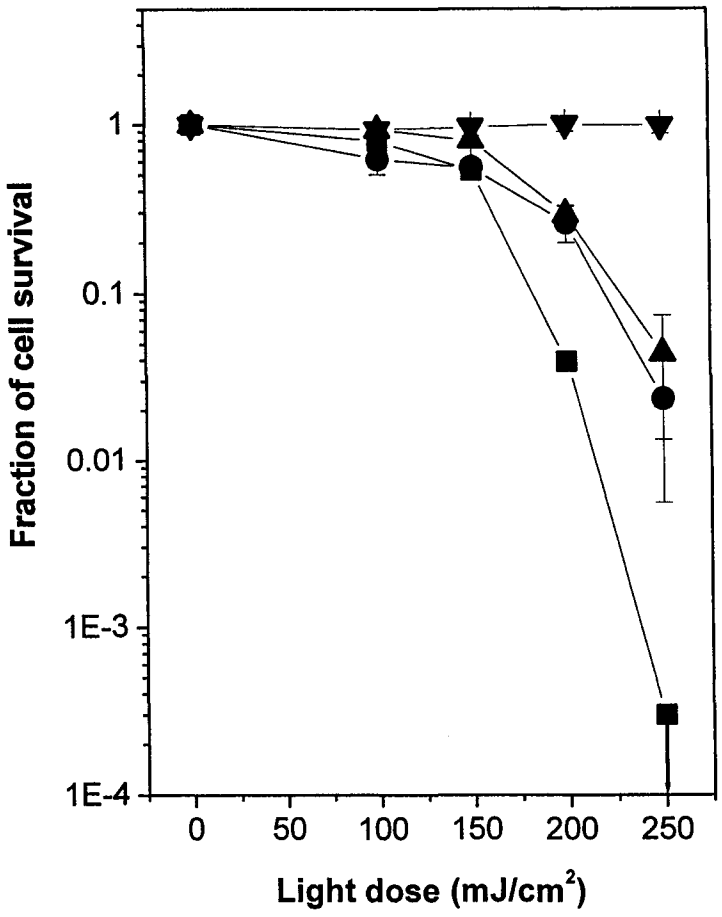
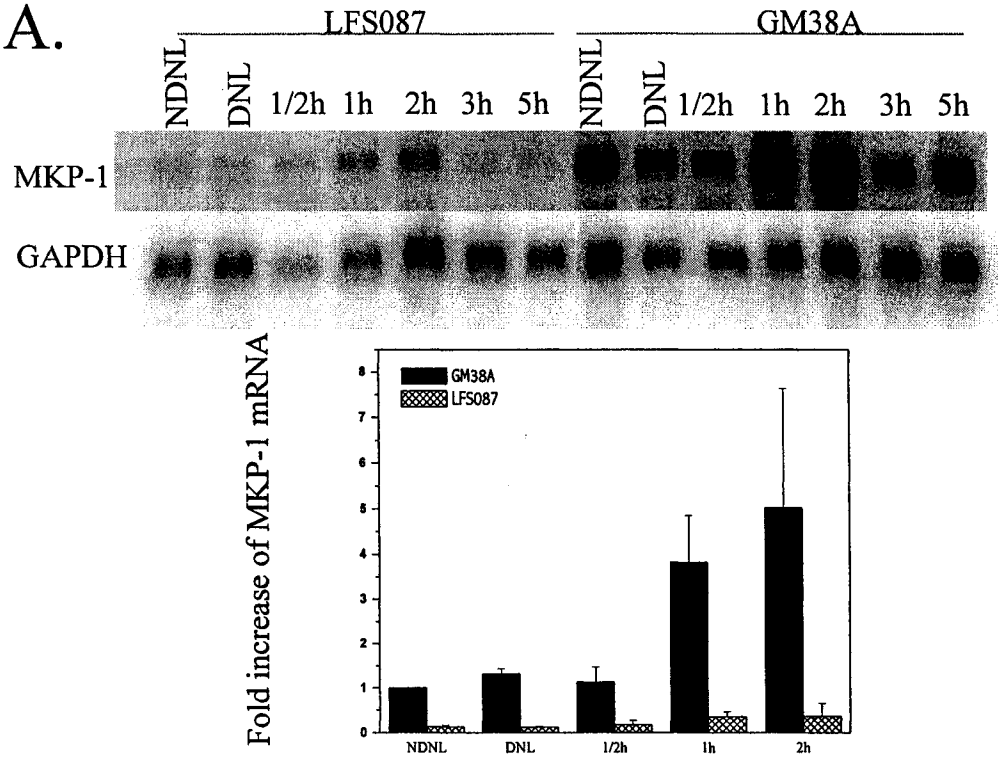

Fraction of cell survival
1
0.1
0.01
1E-3
1E-4
0
50
100
150
200
250
Light dose (mJ/cm²)

Figure 4. Expression of MKP-1 in LFS087 and GM38A cells after Photofrin-mediated PDT.

A. Cells were incubated with or without Photofrin at 10 μg/ml for LFS087 cells or 7.5 μg/ml for GM38A cells for 18 hours and followed by exposure to a red light at 270 mJ/cm^2. (Top) Total RNA was extracted from the cells at the indicated time and then subjected to Northern Blot analysis with an MKP-1 probe that was generated by RT-PCR. (Bottom) Data were normalised using GAPDH and presented as the ratio of treated to untreated samples (NDNL: without light or Photofrin; DNL: Photofrin only; 1/2h to 2 h, hours after PDT). Error bars shows standard error from three independent experiments). B. Whole cell lysates from GM38A cells treated with PDT (7.5 μg/ml of Photofrin for 18 hours before exposure to 270 mJ/cm^2 of red light) were analysed by Western Blotting with anti-MKP-1 antibodies. Data were normalised with actin and expressed as the ratio of treated to untreated controls (NDNL: without Photofrin or light; DNL: Photofrin only; 1/2h to 5h, hours after PDT). The MKP-1 protein in LFS087 cells was undetectable using Western Blot analysis. The bar represents the standard error of the mean of three independent experiments.

A.


LFS087
GM38A
NDNL
DNL
1/2h
1h
2h
3h
5h
MKP-1
GAPDH
Fold increase of MKP-1 mRNA
GM38A
LFS087
NDNL
DNL
1/2h
1h
2h

B.

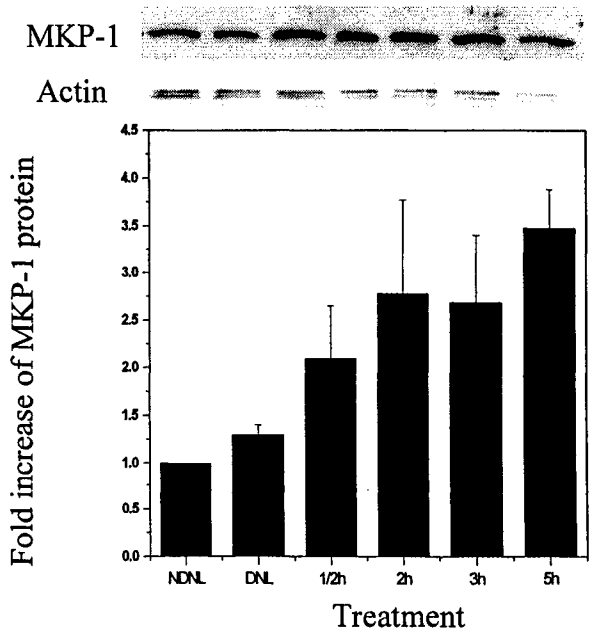

MKP-1
Actin
Fold increase of MKP-1 protein
NDNL
DNL
1/2h
2h
3h
5h
Treatment

Figure 5. Reduction of Raf-1 expression by Photofrin-mediated PDT.

Shows a representative autoradiograph of a Western Blot (top) from LFS087 cells treated with Photofrin-mediated PDT at 10 μg/ml and cells harvested at the indicated times after PDT. The amount of Raf-1 was normalized to the actin band and presented as the amount relative to that in the untreated control (NDNL, without Photofrin or light; DNL, Photofrin only; 1/2h to 5h, hours after PDT). The error bar represents standard error from two to three independent experiments.

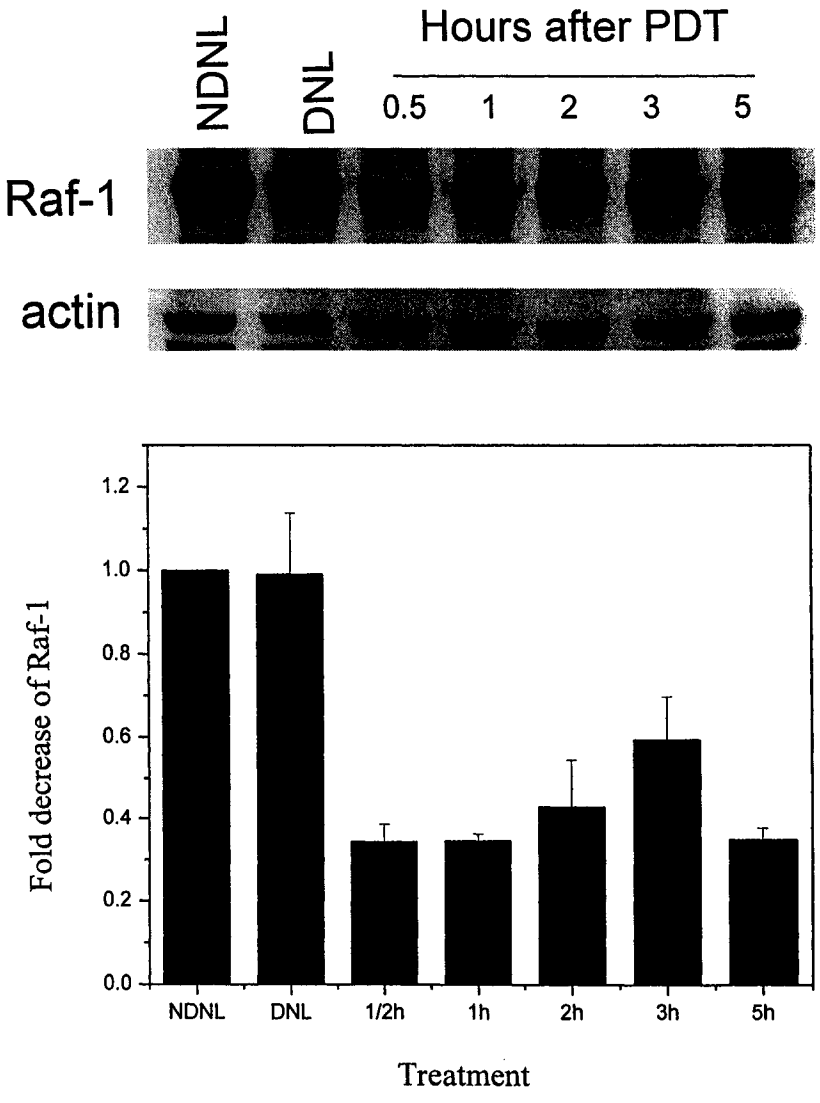
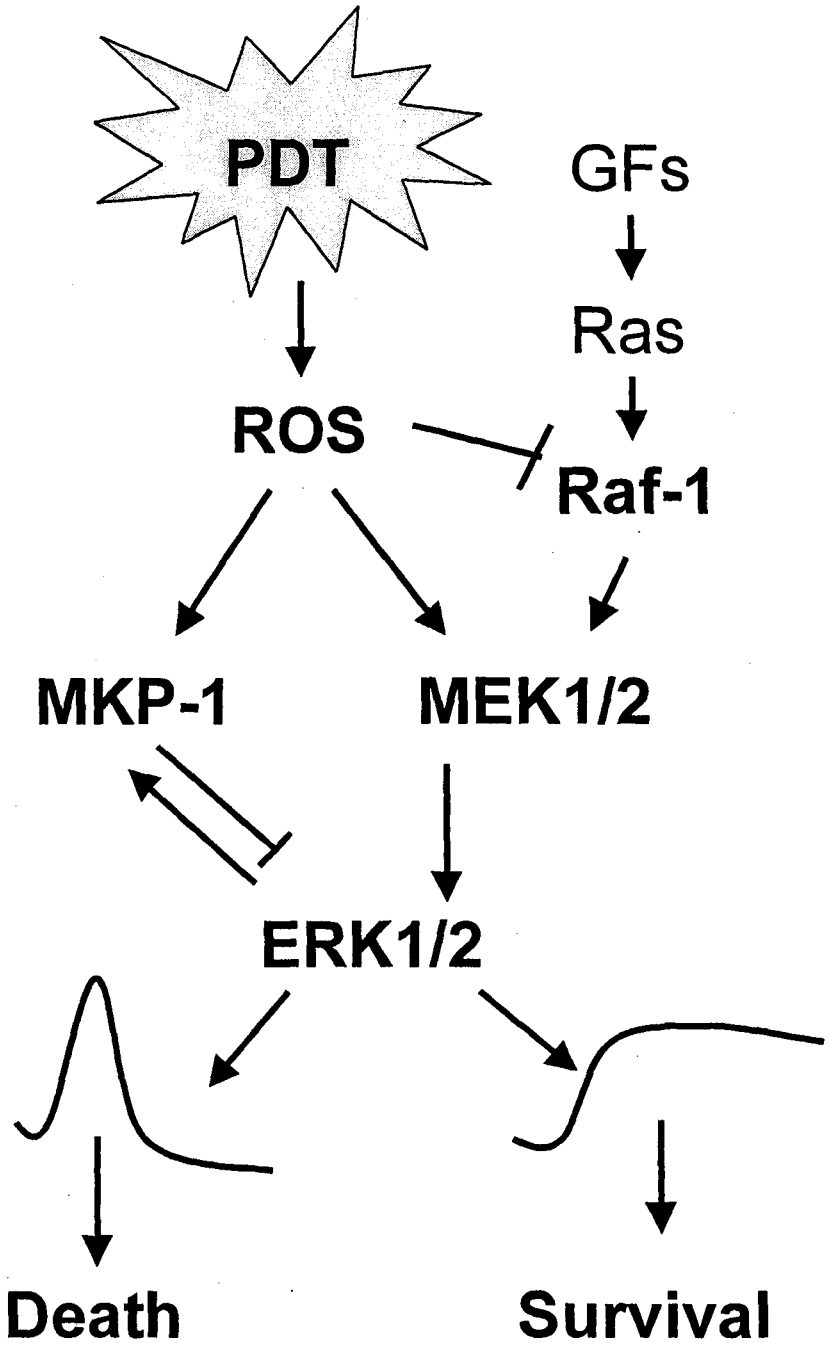

NDNL
DNL
Hours after PDT
0.5
1
2
3
5
Raf-1
actin
Fold decrease of Raf-1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NDNL
DNL
1/2h
1h
2h
3h
5h
Treatment

Figure 6. Model for the possible role of ERK activation in cell 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 while sustained ERK activation supports cell survival.


PDT
GFs
Ras
ROS
Raf-1
MKP-1
MEK1/2
ERK1/2
Death
Survival

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Chapter Six

Activation of the Stress-activated JNK and p38 MAP kinases in human cells by Photofrin-mediated Photodynamic Therapy

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Abstract

Photodynamic therapy is a cancer treatment that eradicates tumor cells via the intracellular generation of reactive oxygen species (ROS). It has been suggested that ROS are very efficient in activating a variety of cellular signaling pathways and recent reports indicate that PDT is able to induce the stress-activated JNK and p38 protein kinases. In the current study we have investigated the activation of JNK and p38 in human cells and their possible role in cellular sensitivity following Photofrin-mediated PDT. We have previously reported that immortalized Li-Fraumeni syndrome (LFS) cells are more resistant to Photofrin-mediated PDT compared to normal human fibroblasts (NHF) at equivalent cellular Photofrin levels. In the current work we report that Photofrin-mediated PDT rapidly increased the activity of JNK1 and p38 in both cell types. However, the activity of JNK1 and p38 induced by PDT was transient in the sensitive NHF cells and returned back to basal levels by 3 h after PDT. In contrast, the resistant LFS cells exhibited a prolonged activation of JNK and p38, which lasted for at least 11 h after PDT. Blocking of the p38 pathway in LFS cells by transient infection with a recombinant adenovirus expressing

a dominant negative mutant of p38 or in HeLa cells by stable transfection with a dominant negative mutant of p38 had no effect on cell survival following PDT. These data suggest that Photofrin-mediated PDT is able to induce the JNK1 and p38 signaling pathways in human cells, but that induction of the p38 pathway alone does not play a major role in the sensitivity of LFS cells to Photofrin-mediated PDT.

Introduction

In mammalian cells, the three major mitogen-activated protein kinases (MAPKs) are the well-defined extracellular signal-regulated kinase (ERK) pathway and the stress activated pathways of the c-jun N-terminal kinase (JNK) and the p38 MAPK. These pathways are central components of the intracellular signaling networks that control many aspects of mammalian cellular physiology, including cell proliferation, differentiation and apoptosis (for review see Lewis et al. 1998). Each of these MAPKs is activated by dual phosphorylation on Thr and Tyr within the motif of Thr-Xaa-Tyr in the catalytic domain (Whitmarsh and Davis 1996; Kyriakis and Avruch 1996; Marshall 1995). This phosphorylation is mediated by a protein cascade, e.g. MAPKKK >>MAPKK >>MAPK (Whitmarsh and Davis 1996; Kyriakis and Avruch 1996; Marshall 1995). Activation of MAPKs results ultimately in the direct or indirect phosphorylation and/or activation of various transcription factors and alterations in gene expression. For instance, c-jun is the downstream substrate of JNK (Klotz et al. 1998a), whereas MAPK-activated protein kinase 2 is the downstream substrate of p38. (Lee et al. 1999).

In contrast to ERK, which is predominantly activated by mitogenic stimuli and is critical for cell proliferation (Kyriakis et al. 1994), JNK and p38 are activated by a variety of environmental stresses such as osmotic shock, UV radiation, heat shock and reactive oxygen species (Kyriakis et al. 1994; Nagata and Todokoro 1999). Accumulated reports suggest a link between JNK/p38 and apoptosis. Expression of a dominant negative form of JNK1 or treatment with the p38 inhibitor SB202190 increases cell viability and prevents apoptosis in human lung carcinoma cells treated with cadmium (Chuang et al. 2000). In mouse fibroblasts, expression of a constitutively active mutant of MEKK1, an upstream activator of JNK, induces apoptosis (Johnson et al. 1996). Similarly, expression of the constitutively active mutant of MKK3 (an upstream activator of p38) in rat PC12 pheochromocytoma cells induces apoptosis (Xia et al. 1995). In contrast, tumor necrosis factor-α (TNF-α) - receptor-mediated JNK activation is not linked to apoptosis (Liu et al. 1996). Studies using human eosinophils showed that p38 is required for TNF-α mediated cell survival (Tsukahara et al. 1999). Furthermore, expression of a dominant negative mutant of c-jun decreases the viability of T98G glioblastoma cells after cisplatin treatment, suggesting that the activation of JNK contributes to cellular resistance to cisplatin (Potapova et al. 1997). Therefore, there is also

evidence that activation of JNK and p38 can protect some cell types from stress stimuli.

Photodynamic therapy (PDT) is a cancer treatment in which a photosensitizing drug is retained by tumor cells and activated by a visible light to create toxic photoproducts including singlet oxygen and other free radicals (Georgakoudi and Foster 1998). Photofrin-mediated PDT is being applied in many clinics throughout the world (Dougherty et al. 1998). In addition, PDT using several photosensitizers other than Photofrin has been introduced in several clinical trials (McCaughan, Jr. 1999). Although PDT is a promising treatment for solid tumors, the precise molecular mechanisms of PDT-induced tumor destruction are not fully understood. There is evidence that both damage to tumor vascular as well as direct tumor cell kill play a role in PDT-induced apoptosis and/or necrosis of the tumor (Dougherty et al. 1998). The differential response to PDT has been studied in several cell types in vitro. Using HPD as a photosensitizer, Foultier et al. (Foultier et al. 1989) report that murine L1210 cells have increased sensitivity compared to normal hematopoietic progenitor cells, but show no difference in uptake of the photosensitizer. Similarly, human smooth muscle cells from atherosclerotic plaques exhibit an increased sensitivity in response to Photofrin-mediated PDT compared to smooth muscle

cells from non-atherosclerotic arteries (Dartsch et al. 1990). There are many mechanisms that could contribute to the cellular response to PDT (Moore et al. 1997). JNK and p38 are induced and protect HeLa cells from apoptosis following Hypericin mediated PDT (Assefa et al. 1999b). In contrast, Xue et al. (Xue et al. 1999b) show that blocking of the p38 cascade by the p38 inhibitor SB202190 abolishes PDT-induced apoptosis in murine LY-R leukemic lymphoblasts but has less effect in CHO cells when using phthalocyanine photosensitizer Pc 4-mediated PDT. However, the activation of JNK and p38 by Photofrin-mediated PDT and the role of these MAPK pathways in the cellular response to Photofrin-mediated PDT have not been reported previously.

We have previously reported that immortalized Li-Fraumeni syndrome (LFS) cells are more resistant to Photofrin-mediated PDT compared to normal human fibroblasts (NHF) at equivalent cellular Photofrin levels (Tong et al. 2000). In the present work we have investigated the activation of JNK and p38 in LFS and NHF cells following Photofrin-mediated PDT. We report that Photofrin-mediated PDT rapidly increased the activity of JNK1 and p38 in both cell types. The activity of JNK1 and p38 induced by PDT was transient in the sensitive NHF cells and returned back to basal levels by 3 h after PDT. In contrast, the resistant LFS cells exhibited a prolonged

activation of JNK and p38, which lasted for at least 11 h after PDT. However, blocking of the p38 pathway in LFS cells had no effect on cell survival suggesting that induction of the p38 pathway alone does not play a major role in the sensitivity of LFS cells to Photofrin-mediated PDT.

Materials and Methods

Cells and cell culture:

The normal human fibroblast strains GM38A and GM9503 were obtained from the National Institute of General Medical Sciences Repository (Camden, NJ). The immortalized Li-Fraumeni syndrome (LFS) cell lines, LFS087 and LFS041 were obtained from Dr M. A. Tainsky, M. D. Anderson Cancer Center, Houston, TX. HeLa cells (C1/A5) stably expressing FLAG-tagged dominant negative p38 mutant (p38(AGF)) and parental HeLa cells (A5) were obtained from Dr. K. Valerie, Medical College of Virginia, Richmond, VA (Taher et al. 1999). All cells were grown in adherent culture in α-MEM medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Gibco BRL) and were cultured in a humidified atmosphere at 37 ^{0}C and 5% CO_2.

Viruses:

The recombinant adenovirus Ad5p38(AGF) was obtained from Dr. K. Valerie, Medical College of Virginia, Richmond, VA., and the recombinant adenovirus Ad5HCMVsp1*lacZ* was obtained from Dr. F. L. Graham, McMaster University, Ontario,

Canada. The Ad5p38(AGF) is a non-replicating recombinant adenovirus expressing a dominant negative mutant of p38. The Ad5HCMVsp1*lacZ* is also a non-replicating adenovirus expressing β-galactosidase and was used as a control. Viruses were replicated and titred in plaque-forming units (PFU)/ml on human 293 cells.

Materials:

Antibodies to phosphorylated p38 or total p38 were purchased from New England Biolabs, Inc. (Beverly, MA). Anti-human JNK1 antibody and protein-G sepharose were purchased from PharMingen (San Diego, CA.). The antibodies to FLAG were purchased from Sigma Inc. (St. Louis, Missouri, USA). Horse radish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The protease inhibitor cocktail was purchased from (Boehringer Manheim, Germany) and the Micro BCA protein detection kit from PIERCE Inc. (Rockford, IL).

PDT treatment and cell lysate preparation:

Cells were treated with Photofrin at an appropriate drug concentration for 18 h before illumination with red light. (10 μg/ml for LFS cells, 7.5 μg/ml for NHF cells) The

light source was a 21" X 32.75" light box illuminated by a parallel series of 12 fluorescent tubes (Phillips type TL83), enclosed on the top with a sheet of clear Plexiglas and filtered with a red acetate filter (Roscolux, No. 19, Rosco, CA) that gave wide band illumination above 585 nm. The energy fluence rate was 0.58 mW/cm^2 or 7 mW/cm^2 at a wavelength of 630 nM as indicated in individual experiments. Samples of treated and control cells were used for either the clonogenic survival assay or for extracting proteins.

For protein extraction, cells were harvested at various times after PDT treatment, washed with ice cold PBS twice and then lysed with a buffer containing 50 mM of Tris (PH 8.0), 150 nM of NaCl, 0.5% NP-40, 2 mM of EDTA, 100 mM of NaF, 10 mM of sodium orthovanadate and a protease inhibitor cocktail for 30 min on ice. Insoluble material was removed from the cell lysate by centrifugation at 14,000 g for 15 min. Portions of the same cellular extracts were used for the JNK1 immune-complex kinase assay and for Western blot analysis of p38 activity. The protein concentration of the cellular extracts was determined using a BCA protein detection kit according the protocol provided by the manufacturer.

Clonogenic survival assay:

Exponentially growing cells were seeded at low density (100-150 cells/plate) and treated with PDT using Photofrin. The PDT-treated cells were allowed to grow for 6-7 days. Cells were fixed and stained with 0.5% methylene blue in 70% methanol and colonies containing greater then 30 cells were counted. The surviving fraction was calculated as the relative plating efficiency of the PDT treated compared to the non-PDT treated (drug only, no light) cultures.

Preparation of c-jun protein:

E. coli expressing GST-fusion protein of c-jun (GST-Jun) was obtained from Dr. M. Rudnicki. Over expression of the proteins was induced by 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hours. The cells were then pelleted and lysed by sonication in PBS containing protease inhibitor. The fusion proteins were isolated with glutathione-Sepharose beads (Amersham Pharmacia Biotech, England) for 2 hours at 4 ^{0}C with rotation and eluted from beads by incubation with 5 mM reduced glutathione Sepharose. The quantity and purity of the fusion proteins were estimated on 12% SDS-polyacrylamide gels using a standard curve of Bovine serum albumin (BSA).

In Vitro Kinase Assay for JNK1 Activity:

The cell lysate (100 to 150 μg of protein) from cells treated with PDT was immunoprecipitated with anti-human JNK1 antibody and protein G sepharose overnight. The immunoprecipitates were washed three times with lysis buffer containing the same components as described above and three times with kinase buffer containing 1X HEPES, 20 mM of MgCl, 10 mM of β-Glycero-phosphate, 2 mg/ml of p-Nitrophenyl phosphate, 10 nM of sodium orthovanadate and 2 mM of DTT. The immune complexes were then incubated in 30 μl of kinase buffer with 5 μCi of [γ^{32}P]ATP and 2 μg GST-c-Jun(1-79). Reactions were conducted for 20 min at 30 ^{0}C. Thereafter, samples were denatured, resolved on 12% SDS-PAGE, transferred on to a nitrocellulose membrane and autoradiographed. The phosphorylation of c-jun was quantitated using PhosphorImage analysis. Following autoradiography, the nitrocellulose membrane was blocked with 5% skim milk in tris-buffered saline with 1% tween-20 (TBST), probed with anti-human JNK1 primary antibody and then incubated with HRP conjugated secondary antibody. The total JNK1 was detected using an enhanced chemiluminescence detection procedure according to the manufacturer's recommendations (Amersham Pharmacia Biotech, England) and quantified by using PhosphorImage analysis. The

JNK1 activity was presented as a ratio of the amount of phosphorylation of c-jun to total JNK1 and normalized to that obtained for untreated cells.

Expression of a dominant negative mutant of p38 in LFS087 cells:

Cells were seeded in 6 well plates at 2.5 x 10^5 cells per well overnight and subsequently infected with Ad5p38 (AGF) in 500 µl serum-free α-MEM medium at 200 PFU/cell for 45 minutes. Cells infected with Ad5HCMVsp1lacZ were used as control. After infection cells were overlayed with fresh growth medium and at 4-6 hours postinfection, infected cells were seeded at 100 cells/well and assayed for clonogenic survival as described above. To detect the expression of the dominant negative mutant of p38 in LFS087 cells, infected and mock-infected cells were harvested before exposure to red light and treated with lysis buffer as described above. Equal amounts of protein (100 µg) from infected or mock infected cells were inmmunoprecipated using protein-G sepharose together with 2 µg of anti-FLAG antibodies overnight at 4^0C with rotation. The immunoprecipate was analyzed by Western blotting with anti-p38 antibodies as described below.

Western Blot Analysis:

Cellular extracts (15-20 μg protein) were separated on 12% SDS-polyacrylamide gels and electroblotted onto a nitrocellulose membrane. The membrane was then blocked with 5% skim milk in TBST for 1 hour at room temperature, probed with primary antibodies in the same solution as blocking buffer over night at 4^0C. Specific antibody-labeled proteins were detected by using HRP conjugated secondary antibodies and the ECL plus Western blotting detection system (Amersham Pharmacia Biotech, England). For the determination of p38 activity, the membranes were stripped and reprobed with anti-p38 antibodies and the amount of p38 was quantified using PhosphorImager analysis. The p38 activity was presented as the ratio of the amount of phosphorylated p38 to total p38 and normalized to that obtained for untreated cells.

Results

The induction of JNK1 activity by Photofrin-mediated PDT:

It has been reported that JNK is activated following exposure of cells to PDT mediated by several different photosensitizers (Assefa et al. 1999a; Klotz et al. 1998a). In order to test whether Photofrin-mediated PDT could induce activation of JNK1, LFS087 and GM38A cells were treated with Photofrin at a drug concentration which resulted in equivalent cellular Photofrin levels for 18 hour before exposure to 270 mJ/cm^2 of red light. The activity of JNK1 was detected in an *in vitro* protein kinase assay using c-jun as a substrate at various times following PDT. As shown in Figure 1, the activity of JNK1 rapidly and dramatically increased in both cell types 30 min after Photofrin-mediated PDT, whereas there was no change of JNK1 activity in cells treated with Photofrin only. The maximum increase of JNK1 activity by PDT was 11.6- and 7.4-fold over the control values in GM38A and LFS087 cells, respectively. Thereafter the induced JNK1 activity gradually returned to basal levels within 3 h in GM38A cells. Whereas in the PDT resistant LFS087 cells, the JNK1 activity was maintained at increased levels for at least 11 h after

PDT. A similar result was observed in GM9503 and LFS041 cells (data not shown).

The activation of p38 by Photofrin-mediated PDT:

Since different members of the MAPK family are usually coordinately regulated (Raingeaud et al. 1995) we also investigated the alteration of p38 activity after Photofrin-mediated PDT. Using Western blot analysis with antibodies specific to phosphorylated p38, we found that p38 phosphorylation was increased as early as 30 min after photofrin-mediated PDT in both LFS087 and GM38A cells (Figure 2). As was found for JNK activation, the phosphorylation of p38 induced by PDT was transient in GM38A cells and by 3 h the phosphorylation of p38 returned to near basal levels. In contrast, LFS087 cells exhibited a sustained phosphorylation of p38. The correlation between the duration of enhanced MAPK activity and the cellular resistance suggested that the JNK1 and/or the p38 pathway may play a role in the sensitivity of cells to Photofrin-mediated PDT.

The effect of the blocking p38 pathway on photosensivity:

The p38 protein kinase pathway has been shown to be involved in cellular survival after Hypericin-mediated or pc

4-mediated PDT treatment (Assefa et al. 1999a; Xue et al. 1999a). According to these reports, the role of p38 in the cellular sensitivity to PDT appears to be cell type and photosensitizer type dependent. In order to assess the possible role of the p38 pathway in the cellular response to Photofrin-mediated PDT, we investigated the effect of blocking the p38 pathway on cell survival after PDT. LFS087 cells were infected with 200 PFU/cell of Ad5p38(AGF), which expresses a FLAG-tagged dominant negative mutant of p38 or Ad5HCMVsp1*lacZ* which served as a control for 24 h before PDT. The expression of this mutant was confirmed by Western blot analysis using anti-FLAG antibodies (Fig. 3). The level of FLAG-tagged p38 protein in cells infected with Ad5p38(AGF) was similar to that in the Hela C1/A5 cells (Taher et al. 1999) that stably express the same FLAG-tagged dominant negative mutant of p38. Cell survival was analyzed in multiple independent experiments by the clonogenic survival assay. As shown in Figure 4, there was no difference in survival following Photofrin-mediated PDT between cells infected with Ad5p38(AGF) and cells infected with Ad5HCMVsp1*lacZ*. To examine further the effect of the p38 MAPK pathway in another human cell type, cell survival after PDT was compared between C1/A5 and parental HeLa cell A5 (Figure 5). It can be seen that no difference in cell survival was observed between C1/A5 and A5 cells following Photofrin-

mediated PDT. These results suggest that the p38 pathway does not play a major role in the sensitivity of LFS or HeLa cells to Photofrin-mediated PDT.

Discussion

In the present study we have examined the ability of Photofrin-mediated PDT to activate the JNK1 and p38 pathways and the effects of the p38 pathway on Photofrin-mediated photocytotoxicity. Both the JNK1 and p38 pathways were activated by Photofrin-mediated PDT. The activation of JNK1 and p38 was transient in NHF cells with high sensitivity while the resistant LFS cells had a prolonged activation of JNK1 and p38 following PDT. Inhibition of p38 by infection of LFS cells with recombinant adenovirus expressing a dominant negative mutant of p38 or stable transfection of HeLa cells with the same dominant negative mutant of p38 did not alter the sensitivity of cells to Photofrin-mediated PDT. These results indicate that like PDT mediated by other photosensitizers, Photofrin-mediated PDT is able to induce the JNK and p38 cascades. However, activation of the p38 pathway appears not to play a major role in the sensitivity of LFS and HeLa cells to PDT.

JNK1 and p38 can be induced by a variety of cellular stresses (Kyriakis et al. 1994). Among the factors implicated in regulating JNK and p38 activity are ROS (Mendelson et al. 1996; Klotz et al. 1997). ROS are induced by PDT and are

considered as one of the main factors leading to tumor eradication in vivo and other cellular responses in vitro following PDT (Matroule and Piette 2000). The activation of JNK1 and p38 by PDT has been reported in a transformed murine keratinocyte cell line using benzoporphyrin derivative (Tao et al. 1996)). These investigators demonstrated that singlet oxygen, one major ROS induced by PDT, is the primary triggering event as evidenced by the finding that ROS scavengers abolish the PDT-induced activation of JNK and p38. Similar results were also reported for cells treated with Rose bengal- (Klotz et al. 1997) or 5-aminolevulinate-mediated PDT (Klotz et al. 1998b). It appears likely that the activity of JNK1 and p38 induced by Photofrin-mediated PDT shown in the present study is also triggered by singlet oxygen. It has been reported that gluthathione S-transferase Pi (GSTp) inhibits JNK1 activity through the formation of GSTp-JNK complexes (Adler et al. 1999). ROS reduce these complexes and increase the activity of JNK1 while ROS scavengers enhance these complexes and reduce JNK1 activity (Adler et al. 1999). The activation of JNK1 by singlet oxygen may therefore arise through the dissociation of gluthathione S-transferase Pi (GSTp) from JNK and be a general cellular response to PDT.

The relevance of the activation of JNK1 associated with Photofrin-mediated PDT to cytotoxicity is unknown.

Several lines of evidence have implicated an involvement of JNK1 in protecting cells from stress stimuli. Blocking of JNK1 activation by the dominant negative mutant of JNK1 significantly reduced the ability of hILP, an anti-apoptosis gene, to protect human 293 cells from interleukin-1β-converting enzyme(ICE)-induced apoptosis (Sanna et al. 1998). CD40-induced B cell survival is accompanied by potent JNK1 activation (Sakata et al. 1995; Berberich et al. 1996). Inhibition of the JNK1 pathway in HeLa cells by transfection with a dominant negative mutant of SEK1, an upstream activator of JNK1, increased apoptosis following Hypericin-mediated PDT (Assefa et al. 1999b). In contrast, the JNK1 pathway is also involved in promoting apoptosis in many cell types (Chuang et al. 2000; Johnson et al. 1996; Xia et al. 1995). These observations demonstrate that the JNK1 pathway can play a role not only in cell survival but also in apoptosis and it has been proposed that it is the duration of JNK1 activation that determines cell fate (Chen and Tan 2000). Transient activation of JNK1 by short exposure to heat shock results in cell differentiation while sustained activation by long exposure causes apoptosis (Nagata and Todokoro 1999). However, several groups have reported that prolonged activation of JNK can also support cell survival (Assefa et al. 1999b; Mazars et al. 2000; Xu et al. 1996; Galley et al. 1997). The discrepancies

among these results may be due to differences in cell type and/or differences in the stress stimulus employed. In the case of Photofrin-mediated JNK activity, the sustained activation of JNK is more likely to support cell survival as the duration of JNK1 activity is correlated with the cellular resistance. In this respect, further study will be required to reveal the precise role of JNK activity in photocytotoxicity.

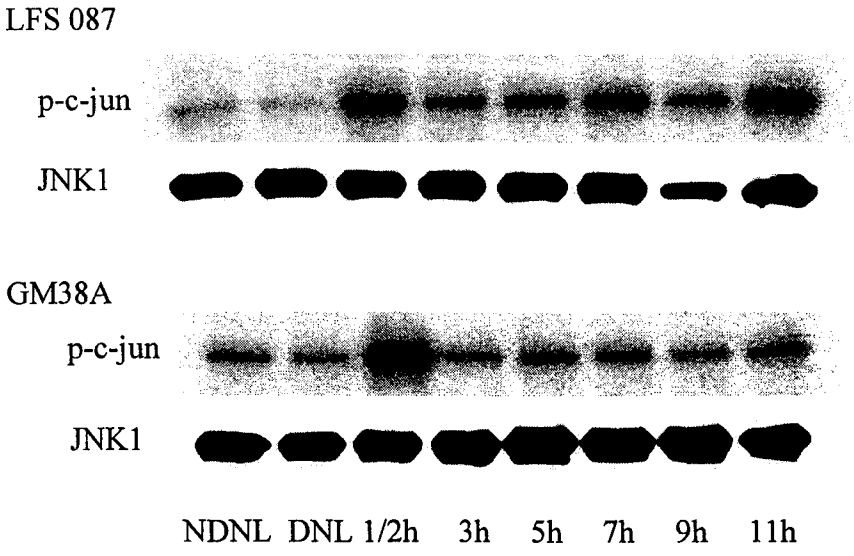
There are differing reports concerning the role of p38 activation in apoptosis, cell growth, and survival. For example, p38 activation is involved in the H_2O_2-induced cell proliferation in Chinese hamster lung fibroblast cells (Kim et al. 2001), and in protecting cells from Hypecirin-induced phototoxicity (Assefa et al. 1999b). In contrast, the activation of p38 participates in ceramide-induced oligdendrocyte apoptosis (Hida et al. 1999) as well as PDT-induced apoptosis with Pc 4 in murine LY-R leukemic lymphoblasts (Xue et al. 1999b). However, the data reported in the current report suggests that the increase in p38 activity in response to Photofrin-mediated PDT is not crucial for cell survival. It is possible that the JNK and/or ERK pathways play a more important role than the p38 pathway in cell sensitivity to Photofrin-mediated PDT. This is supported by our recent finding that Photofrin-mediated PDT caused a prolonged ERK activation in LFS cells and PD 98059, a specific inhibitor of

the ERK pathway, increased cell sensitivity to PDT (Tong et al. 2001,Submitted). Notwithstanding, the data presented here does not exclude a possible role of prolonged p38 activity in PDT induced apoptosis in LFS cells, since Photofrin-mediated PDT leads to apoptosis in a small but significant fraction of LFS cells (Tong et al. 2000). It is possible that the blocking of p38 may not be sufficient to overcome the survival signal resulting from ERK activation in LFS cells. This idea is supported by the finding that expression of MKK1, an upstream activator of ERK, is able to inhibit stress-induced apoptosis which is mediated through activation of p38 and JNK (Nagata and Todokoro 1999). Moreover, the p38-dependent apoptotic signal can be overcome by the activation of ERK (Xia et al. 1995). We suggest that, the balance between survival signals, such as that activated by the ERK pathway, and apoptotic signals such as the p38 and/or JNK pathways, is a crucial determinant of cell fate.

Figure 1. Effect of Photofrin-mediated PDT on the activation of JNK1.

LFS087 and GM38A cells were treated with PDT at equivalent cellular Photofrin levels (10 μg/ml for LFS and 7.5 μg/ml for GM38 cells) followed by exposure to 270 mJ/cm^2 of red light. Cells were harvested at the indicated time points after PDT for the *in vitro* protein kinase assay. (A). JNK1 activity is illustrated by the representative autoradiograph. Total JNK1 was used to normalize the protein loading. (B). Increases in activity (fold activation) of JNK1 were calculated as the ratio of the treated and untreated sample (NDNL) by PhosphorImage analysis (LFS 087: ▲, GM38A: ●). Results represent the mean and standard error of at least three independent experiments. (NDNL, no drug and no light; DNL, drug only; 1.5h to 11h, hours after PDT).

A.


LFS 087
p-c-jun
JNK1
GM38A
p-c-jun
JNK1
NDNL DNL 1/2h 3h 5h 7h 9h 11h

B.

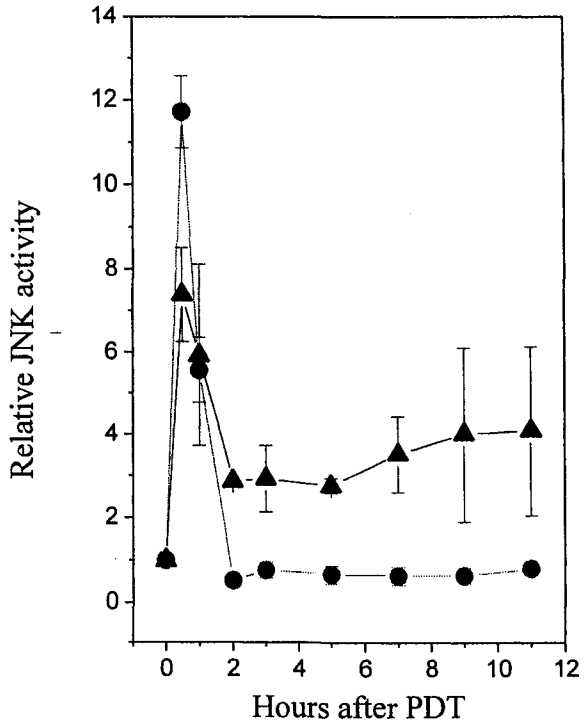
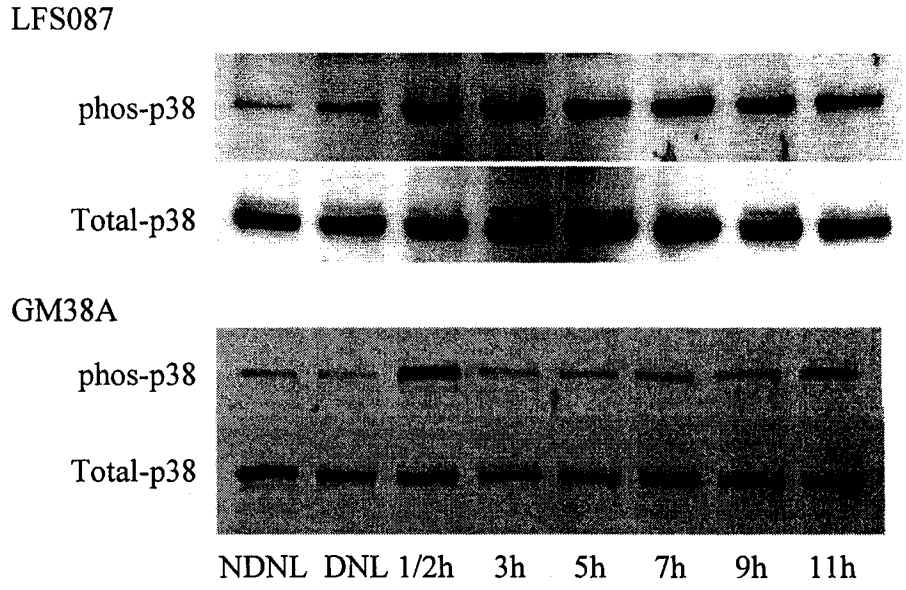

Relative JNK activity
Hours after PDT
0
2
4
6
8
10
12
14

Figure 2. Activation of p38 by Photofrin-mediated PDT in LS087 and GM38A cells.

Cells were treated with PDT as described in the legend of Figure 1. (A). The activity of p38 was detected by Western blot analysis using antibodies that recognize only phosphorylated p38. The total p38 was used to normalize the protein loading. The activity was quantified by PhosphorImage analysis. (B). The histogram shows induction of p38 in LFS 087(■) and GM38A(●) cells treated with PDT normalized to those in untreated controls (NDNL). The error bar represents standard error of three independent experiments. (NDNL, no drug and no light; DNL, drug only; 1.5h to 11h, hours after PDT).

A.


LFS087
phos-p38
Total-p38
GM38A
phos-p38
Total-p38
NDNL DNL 1/2h 3h 5h 7h 9h 11h

B.

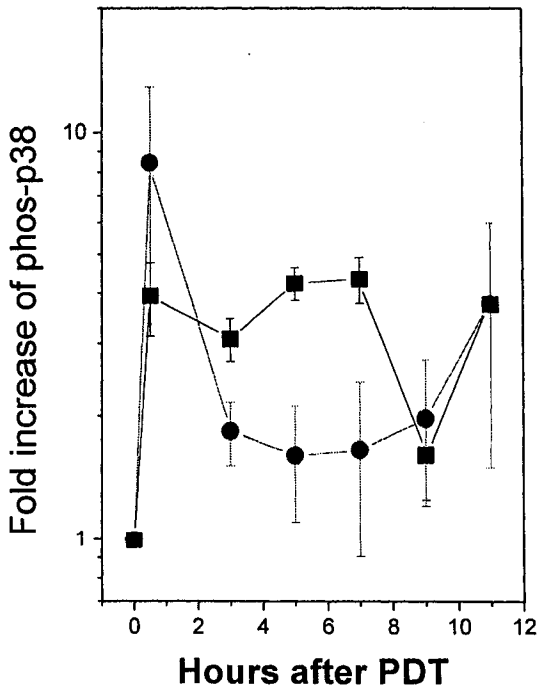

Fold increase of phos-p38
10
1
0 2 4 6 8 10 12
Hours after PDT

Figure 3. The expression of dominant negative p38 protein.

Cell lysates extracted from LFS087 cells that were infected with either Ad5p38(AGF) or Ad5HCMVsp1lacZ at 200 PFU/cell for 24 hours were analyzed by Western blotting as described in Materials and Methods with anti-FLAG antibodies. LFS 087 cells without infection and HeLa cells (A5) were used as negative controls, whereas C1/A5 cells that stably express p38(AGF) were used as a positive control.

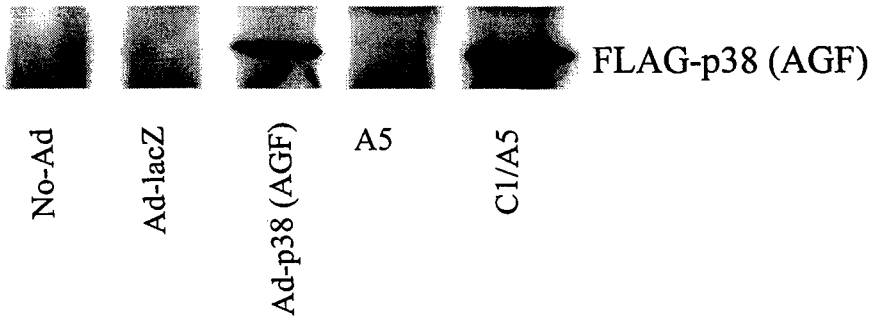

FLAG-p38 (AGF)
No-Ad
Ad-lacZ
Ad-p38 (AGF)
A5
C1/A5

Figure 4. Survival of LFS cells expressing a dominant negative p38 mutant following PDT.

LFS087 cells were infected with or without (■) either Ad5p38(AGF) (●) or Ad5HCMVsp1lacZ (▲) at 200 PFU/cell and subsequently assayed for clonogenic survival following Photofrin-mediated PDT (10 μg/ml of Photofrin) at the indicated red light exposure time. The light energy fluence rate was 0.58 mW/cm^2. Data points represent the mean and standard error of colony counts for three determinations from a typical experiment. Similar results were observed in two other independent experiments. The surviving fraction was calculated as the relative plating efficiency of the PDT treated compared to the non-PDT treated (drug only,no light) cultures.

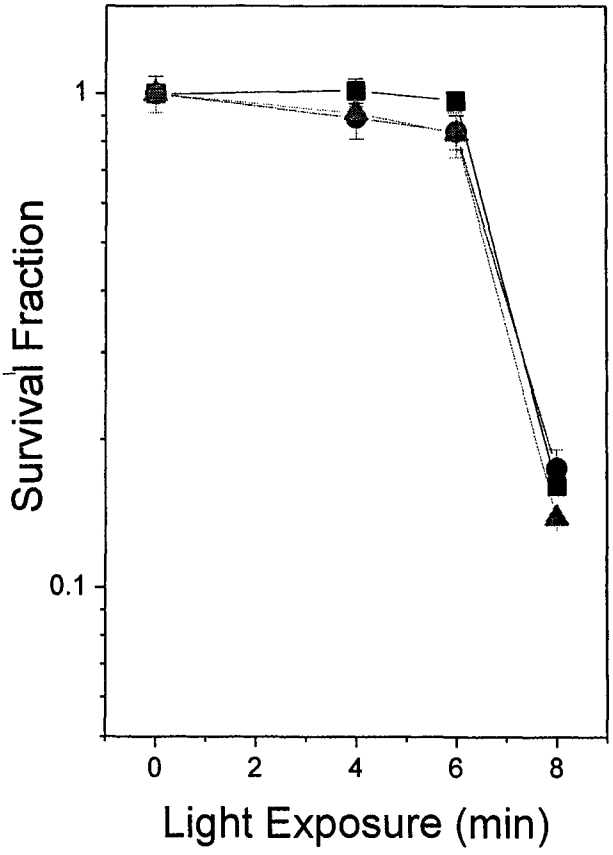
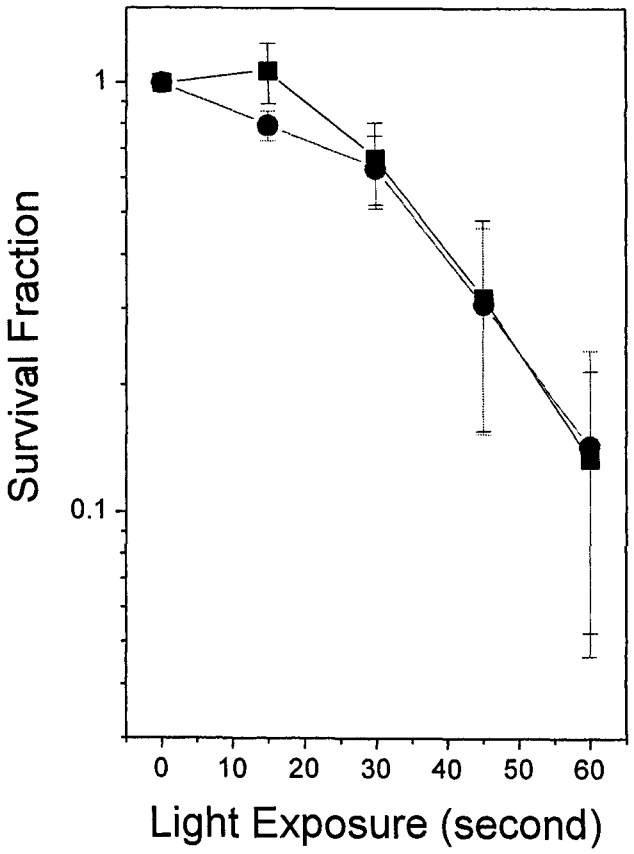

Survival Fraction
1
0.1
0
2
4
6
8
Light Exposure (min)

Figure 5. Survival of HeLa cells expressing a dominant negative p38 mutant following PDT.

Hela cells (C1/A5)(●) stably expressing p38(AGF) and the parental (A1)(■) cell line were treated with 5 μg/ml of Photofrin for 18 hours before exposure to red light for the indicated duration. The light energy fluence rate was 7 mW/cm^2. Cells were assayed by clonogenic survival assay. Results show the mean and standard error of colony survival for three independent experiments each performed in triplicate.


Survival Fraction
1
0.1
0
10
20
30
40
50
60
Light Exposure (second)

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Chapter Seven

Summary and References

Summary

The present studies have addressed the role of p53 and MAPKs in the cellular response to Photofrin-mediated PDT. Using NHF and LFS cells as a model *in vitro* system, we have found LFS cells were more resistant to Photofrin-mediated PDT compared to NHF cells. The treatment of PDT led LFS cells to arrest in the G_2/M phase and display apoptosis while NHF cells did not show marked apoptosis or G_2/M arrest. Reintroduction of wild-type p53 increased the sensitivity of LFS cells to PDT without a change in the uptake of Photofrin. These data demonstrate that the mutation of p53 in LFS cells may result in a pronounced G_2/M arrest that contributes to the resistance of LFS cells to Photofrin-mediated PDT. These results indicate also that PDT is able to cause p53-independent apoptosis.

It has been suggested that the ERK pathway plays a crucial role in cell survival after various stress stimuli (Aikawa et al. 1997; Buckley et al. 1999; Persons et al. 1999). We showed here that Photofrin-mediated PDT caused ERK1/2 activation in either a transient or prolonged manner depending on the cell type. The duration of ERK1/2 activation was inversely correlated with cell sensitivity to PDT. LFS cells with low sensitivity displayed sustained activation,

whereas NHF cells with high sensitivity showed transient activation following PDT. Blocking of the sustained activation of ERK1/2 in LFS cells resulted in a reduction of cell survival after PDT. Thus sustained ERK1/2 activation protects cells from Photofrin-mediated photocytotoxicity.

The data presented here also suggest that MKP-1 participates in the regulation of PDT-induced ERK activity. Photofrin-mediated PDT resulted in a significant increase in the expression of MKP-1 in NHF cells that showed a transient ERK activation. The induction of MKP-1 paralleled the kinetics of ERK1/2 inactivation. Therefore, MKP-1 is a PDT-inducible gene and the increased MKP-1 may be responsible for the transient activation of ERK1/2 in NHF cells. Although LFS cells also showed an increase in MKP-1 transcription after PDT, the overall levels of MKP-1 mRNA were substantially lower than those in NHF cells and the protein levels of MKP-1 were undetectable. This suggests that the prolonged activation of ERK1/2 induced by PDT in LFS cells is due to the low levels of MKP-1 expression in these cells that were below the threshold required for dephosphorylation of ERK1/2.

Previous studies have shown that the activation of ERK1/2 can occur through either Ras-Raf-dependent or a ras-Raf-independent route (Garcia et al. 2001; Lewis et al. 1998). Since a rapid decrease of Raf-1 expression was observed in

response to Photofrin-mediated PDT in both cell types, the data presented here suggest that Photofrin-mediated PDT induced ERK1/2 activity is Raf-independent.

Using an in vitro protein kinase assay or antibody recognizing only phosporylated p38, we have found that Photofrin-mediated PDT rapidly induced JNK1 and p38 activity in both cells types. However, the kinetics of activation were different in LFS compared to NHF cells. NHF cells displayed transient JNK1 and p38 activation while LFS cells exhibited prolonged activation. Blocking the p38 pathway had no effect on cell survival in LFS and HeLa cells following PDT. These results suggest that the p38 pathway alone does not play a major role in cell sensitivity to Photofrin-mediated PDT.

Lastly, we showed here that Nile Blue A was extremely toxic to NHF cell compared to LFS and several tumor cells which express mutant p53. In addition, no significant photocytotoxicity of Nile Blue A was observed in NHF, LFS or the three tumor cell lines tested using NBA concentrations that have relatively low toxicity for NHF. These data suggest caution in the clinical use of Nile Blue A as a photosensitizer for cancer therapy.

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