Strong and prolonged induction of c-jun and c-fos proto-oncogenes by photodynamic therapy

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Summary Photodynamic therapy (PDT) is currently under investigation in phase II and III clinical studies for the treatment of tumours in superficial localisations. Thus far, the underlying mechanisms of PDT regarding cellular responses and gene regulation are poorly understood. Photochemically generated singlet oxygen (¹O₂) is mainly responsible for cytotoxicity induced by PDT. If targeted cells are not disintegrated, photo-oxidative stress leads to transcription and translation of various stress response and cytokine genes. Tumour necrosis factor (TNF) α, interleukin (IL) 1 and IL-6 are strongly induced by photodynamic treatment, supporting inflammatory action and immunological anti-tumour responses. To investigate the first steps of gene activation, this study focused on the proto-oncogenes c-jun and c-fos, both coding for the transcription factor activator protein 1 (AP-1), which was found to mediate IL-6 gene expression. We here determine the effects of photodynamic treatment on transcriptional regulation and DNA binding of transcription factor AP-1 in order to understand the modulation of subsequent regulatory steps. Photodynamic treatment of epithelial HeLa cells was performed by incubation with Photofrin and illumination with 630 nm laser light in vitro. Expression of the c-jun and c-fos genes was determined by way of Northern blot analysis, and DNA-binding activity of the transcription factor AP-1 was evaluated by electrophoretic mobility shift assay (EMSA). Photofrin-mediated photosensitisation of HeLa cells resulted in a rapid and dose-dependent induction of both genes but preferential expression of c-jun. Compared with the transient expression of c-jun and c-fos by phorbol ester stimulation, photodynamic treatment led to a prolonged activation pattern of both immediate early genes. Furthermore, mRNA stability studies revealed an increased half-life of c-jun and c-fos transcripts resulting from photosensitisation. Although mRNA accumulation after PDT was stronger and more prolonged compared with phorbol ester stimulation, with regard to AP-1 DNA-binding activity, phorbol ester was more efficient. Surprisingly, in addition to the activation of AP-1 DNA-binding via PDT, photodynamic treatment can decrease AP-1 DNA-binding of other strong inducers, such as the protein kinase C-mediated pathway of phorbol esters and the antioxidant pyrrolidone carboxylate (PDTC). This may explain the induction of c-jun and c-fos expression by PDT, with prolonged kinetics and mRNA stabilisation as compared with activation by phorbol esters. Interestingly, this observation is not coincident with an overinduction of AP-1 DNA-binding, hence suggesting that post-translational modifications are dominant regulatory mechanisms after PDT that tightly control AP-1 activity in the nucleus thus limiting the risk of deregulated oncogene expression.

Keywords: photodynamic therapy; photosensitisation; oxidative stress; proto-oncogene

Photodynamic therapy (PDT) is a novel therapeutic approach consisting of topical or systemic application of a photosensitising agent and activation by subsequent illumination with tissue-penetrating visible light. The combination of drug uptake in malignant tissue and selective light delivery provides an effective tumour therapy with efficient cytotoxicity and minimal damage to normal tissue (Pass, 1993).

Oxidative stress caused by singlet oxygen (¹O₂) is responsible for the effects induced by PDT (Weichshaupt et al., 1976). Photosensitisation activates expression of a variety of stress-response genes coding for heat shock proteins, glucose-regulated proteins and haem oxygenase (Gomer et al., 1991; Ryter et al., 1991). The exact functions of these stress proteins are still unclear; however, they might participate in a protective reaction to oxidative stress. Evans et al. (1990) demonstrated the dose-dependent production of murine tumour necrosis factor (TNF) in supernatants obtained from PDT-treated macrophages in vitro. Furthermore, interleukin (IL) 6 (Kick et al., 1995) and IL-1α/β (G Kick et al., manuscript submitted) protein synthesis induced by PDT in epithelial cell lines was recently discovered. These cytokines have the ability to up-regulate anti-tumour responses and may be good candidates to mediate the local inflammatory response. In studying molecular mechanisms of PDT-induced IL-6 expression at the level of gene regulation, electrophoretic mobility shift assays (EMSAs) revealed increased AP-1 DNA-binding activity at the distal AP-1 element of the IL-6 promoter (Kick et al., 1995).

To elucidate further the underlying regulation, the present study analysed the effect of PDT on the AP-1 transcription factor genes c-jun and c-fos, which are known as proto-oncogenes. Both genes are members of a multigene family coding for transcription factors (Curran and Franz, 1988; Ryder et al., 1989). By interaction of their leucine zipper domain, Jun and Fos proteins form dimeric complexes (Sassone-Corsi et al., 1988; Landschulz et al., 1988; Kouzarides and Ziff, 1988; Gentz et al., 1989). This mode of dimerisation is a characteristic feature of the bZIP family, which includes Jun-, Fos-, activating transcription factor (ATF), cAMP-responsive element-binding (CREB) proteins and others that can bind to the AP-1 DNA motifs and closely related sequence elements (Ivashkiv et al., 1990; Hai and Curran, 1991; Kerppola and Curran, 1993; Hsu et al., 1994; Kataoka et al., 1995), and are regulated by phosphorylation and redox changes (Abate et al., 1990; Boyle et al., 1991; Hunter and Karin, 1992; Dérijard et al., 1994; Kallunki et al., 1994; Kyriakis et al., 1994). AP-1 DNA-binding is considered to be a crucial event in the transcriptional regulation of a variety of genes associated with growth, differentiation, cellular stress and tumorigenesis (Angel and Karin, 1991; Morgan and Curran, 1991; Holbrook and Fornace, 1991; Saed et al., 1995).

Studying the regulation of this ubiquitous transcription
factor in response to singlet oxygen was a further reason for the present investigation, as in vitro photosensitisation by Photofrin is a system that generates this reactive oxygen intermediate (Gollnick, 1968; Weishaupt et al., 1976).

Moreover, proto-oncogene expression could be a relevant aspect of cancer therapy. Recent studies describe c-jun and c-fos expression as associated with programmed cell death (Smeyne et al., 1993; Manome et al., 1993; Kim and Beck, 1994), multidrug-resistant phenotype (Scanlon et al., 1994), and deregulated expression has been discussed as a prerequisite of their transforming potential (Millet et al., 1984; Bos et al., 1990; Lee et al., 1988; van den Berg et al., 1993).

Materials and methods
Cell line and culture conditions
The human epithelial carcinoma cell line HeLa was grown in adherent culture in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat complement-inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin (all purchased from Gibco-BRL, Eggenstein, Germany). For the experiments, HeLa cells were removed by trypsinisation, then washed with phosphate-buffered saline (PBS) and transferred to 5 cm Petri dishes containing medium with only 0.5% FCS. Cells were cultured in a humidified atmosphere at 37°C and 5% carbon dioxide.

Reagents
Pyrrolidine dithiocarbamate (PDTC) was dissolved in PBS as a 1 M stock solution. N-acetyl-L-cysteine (NAC) was dissolved in water and adjusted to pH 7.4 by the addition of sodium hydroxide. Both solutions were prepared immediately before use. PDTC, NAC, 12-O-tetradecanoylphorbol-13-acetate (TPA), cycloheximide (CHX) and actinomycin D (act-D) were purchased from Sigma (Deisenhofen, Germany). Photofrin (Cyanamid Lederle, Wolfratshausen, Germany) was aliquoted at 2.5 mg ml⁻¹ stock solution and stored at −20°C.

Photodynamic treatment
Photofrin, a haematoporphyrin derivative preparation, was used as a photosensitiser. HeLa cells were incubated with Photofrin 1 h before illumination, appropriate for an almost complete Photofrin uptake (Leung et al., 1994). Light application on the monolayers was performed via a 600 µm optical fibre by an argon-pumped dye laser (Aesclupal Meditech, Heroldseberg, Germany) at a wavelength of 630 nm. This light application system was modified to maintain a homogeneous power density throughout the diameter of the plate. Power density and homogeneity of illumination were continuously controlled by a calibrated power meter (Coherent, Frankfurt am Main, Germany). At a constant power density of 40 mW cm⁻² up to 400 s, thermal effects upon illumination could be excluded. As determined 24 h after PDT by the trypan blue exclusion method, viability of cells was higher than 80% at all concentrations of Photofrin used up to 2 µg ml⁻¹ and treated by a light dose of 4 J cm⁻² as well as under co-treatment.

RNA isolation and Northern blot analysis
Northern blot analyses were performed for detection of specific c-jun and c-fos transcripts. Hybridisation with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was carried out for relative quantification of mRNA levels. Total cellular RNA was isolated by the guanidine isothiocyanate method and acid phenol extraction (Chomczynski and Sacchi, 1987). Cellular RNA (20 µg) was size-fractionated by electrophoresis using 1.5% denaturing formaldehyde-agarose gels, then transferred to Hybond N+ nylon membranes (Amersham Buchler, Braunschweig, Germany) by capillary blotting. Filters were sequentially hybridised with probes encompassing 1.1 kb of the mouse c-jun gene, 1.4 kb of the mouse c-fos gene and 210 bp of the human GAPDH gene; all probes were radiolabelled with [α-32P]dATP (Hartmann Analytic, Braunschweig, Germany) by random hexamer priming (Feinberg and Vogelstein, 1983). Three × 10⁶ c.p.m. of labelled probe was used per ml of hybridisation solution [1% bovine serum albumin (BSA), 1 mM disodium EDTA, 250 mM disodium hydrogen phosphate, 7% sodium dodecyl sulphate (SDS); all purchased from Sigma]. Hybridisation was performed at 65°C for 12 h in glass tubes by continuous rotation. Membranes were washed twice at 65°C for 10 min each in a wash buffer (1 mM disodium EDTA, 250 mM disodium hydrogen phosphate, 1% SDS; all purchased from Sigma) to remove unspecifically bound nucleotides. Using amplifying screens, blots were exposed to Kodak X-Omat AR films (Linhardt, Munich, Germany) at −80°C for up to 2 days.

Preparation of cell extracts
Whole cell extracts were prepared from 5 × 10⁶ cells. Monolayer cultures were washed twice with ice-cold PBS and scraped from the plates with a rubber spatula. Haryestated cells were centrifuged for 10 min at 500 g at 4°C. Following a single wash with ice-cold PBS, cleared cells were resuspended in 50 µl of lysis buffer [20 mM Heps potassium hydroxide (KOH), pH 7.5, 350 mM potassium chloride, 1 mM magnesium chloride, 0.5 mM EDTA, 0.1 mM EGTA, 5 mM dithiothreitol (DTT), 10 µg ml⁻¹ leupeptin, 10 µg ml⁻¹ aprotinin, 0.5% (v/v) of a saturated phenylmethylsulphonyl fluoride (PMSF) solution in ethanol, 20% (v/v) glycerol and 1% (v/v) Nonidet P 40]. After a 15 min incubation on ice, the lysate was centrifuged in a precooled microfuge for 20 min at 14 000 r.p.m. The resulting supernatant was diluted with one volume of buffer H [20 mM Heps/potassium hydroxide pH 7.9, 0.2 mM EDTA and 20% (v/v) glycerol] to reduce salt concentration (all reagents purchased from Sigma). For all samples, the amount of protein was determined by a Bradford microassay procedure (BioRad, Munich, Germany).

Electrophoretic mobility shift assay
In order to detect the DNA-binding activity of transcription factor AP-1, we used double-stranded (ds) DNA oligonucleotides that encompassed the palindromic AP-1 consensus sequence 5'-TTCCGGCTGACTCATCAAGCG-3' (purchased from Promega, Heidelberg, Germany). Oligonucleotides were labelled via T4 polynucleotide kinase and [γ-32P]ATP (Hartmann Analytic, Braunschweig, Germany) phosphorylating free 5'-hydroxy groups. DNA protein-binding reactions were carried out in a 20 µl reaction volume containing 1–3 µl of whole cell extracts (3 µg of protein). DNA-binding was initiated by the addition of protein extracts to a mix containing 1–2.5 µg of poly (dl-D), 2 µg BSA as a carrier, 2 µl of a 10 × binding buffer [100 mM Tris-HCl, pH 7.5, 500 mM sodium chloride, 10 mM EDTA, 50% (v/v) glycerol, 2 mM DTT, 2 µl 1% (v/v) Nonidet P 40 (Sigma), and approximately 10 000 c.p.m. of a γ-32P-labelled dsDNA probe with a specific activity not less than 3000 Ci mmol⁻¹. After 30 min at room temperature, the samples were loaded on a 4% non-denaturing polyacrylamide gel in 0.5 × Tris–borate-EDTA buffer. Electrophoresis was performed in a vertical EMSA system (AGS, Heidelberg, Germany) at 15 V cm⁻¹ for 1.5 h. Gels were dried at 80°C for 30 min and then exposed for autoradiography.

Results
Our previous study identified the DNA binding of transcription factor AP-1 as an important regulatory event
of PDT-induced IL-6 gene expression (Kick et al., 1995). The present investigation proposed to elucidate the regulation of the AP-1 transcription factor genes c-jun and c-fos by photodynamic treatment. The same cell line and photosensitisation conditions were tested that were previously proven to be efficient in the activation of IL-6 gene expression and AP-1 DNA-binding.

Thus, for the first set of experiments, HeLa cells (5 x 10⁶) were incubated with Photofrin at a concentration of 1 μg ml⁻¹ for 1 h, then illuminated with monochromatic (630 nm) light at a dose of 4 J cm⁻². Total RNA was harvested at different points in time following irradiation. The response to phorbol ester TPA (100 ng ml⁻¹) that induces comparable levels of IL-6 mRNA and protein (Kick et al., 1995) and c-jun and c-fos via the protein kinase C pathway was determined as a reference. Thirty minutes after photodynamic treatment, mRNA levels of c-jun were already significantly increased, reaching a plateau of maximum levels after 1 to 4 h (Figure 1). Up-regulation of c-jun mRNA by PDT was much stronger than by TPA stimulation. In contrast TPA treatment led to a stronger, but more transient induction of c-fos mRNA expression. Surprisingly, activation of both proto-oncogenes was clearly prolonged following photodynamic treatment and was still detectable after 8 h. Accumulation of c-jun and c-fos mRNA was dose-dependent and led to maximum amounts of transcripts at the photosensitiser concentration and illumination conditions used above for kinetics studies. Specific mRNA signals could not be detected in untreated cells or following treatment with either Photofrin or laser light alone (data not shown).

In order to determine whether the up-regulation of c-jun and c-fos mRNA levels is caused by induction of transcription, HeLa cells were incubated for 2 h with the transcriptional inhibitor actinomycin D (10 μg ml⁻¹) before PDT. As shown in Figure 2, PDT could not induce c-jun or c-fos mRNA significantly in cells without associated transcriptional activity (lane 3). To define whether the stability of c-jun and c-fos mRNA is altered by PDT, HeLa cells were treated with TPA or PDT, then incubated with actinomycin D (act-D) 1 h after activation to stop further transcription. Cells were harvested immediately after the addition of actinomycin D (Figure 2, lanes 4 and 9) and 30 min, 1 h, 2 h and 4 h thereafter. In TPA-treated cells, c-jun and c-fos mRNA decreased, then disappeared 1 h after adding actinomycin D (Figure 2, lanes 4 to 8). By comparison, following PDT both transcripts showed signals up to 4 h after the addition of actinomycin D (Figure 2, lanes 9 to 13). This result demonstrates that PDT-mediated induction of c-jun and c-fos mRNA is regulated by the activation of transcription and a prolongation of the mRNA half-life.

To study further whether PDT-induced c-jun and c-fos mRNA expression is dependent on de novo protein synthesis, HeLa cells were treated with PDT in both the presence and absence of cycloheximide, an inhibitor of translation. At concentrations that efficiently inhibit protein synthesis, cycloheximide led to a superinduction of c-jun and c-fos mRNA (Figure 3, lane 4) with about the same high amounts as upon stimulation by the combination of PDT and cycloheximide (lane 8). Thus, conclusions concerning the necessity of de novo protein synthesis could not be drawn from this experiment. However, the early initiation of c-jun and c-fos mRNA induction by PDT within 30 min indirectly indicates that de novo protein synthesis of transcription factors is not involved in the initial step.

Since photodynamic action is caused by oxidative stress, we were prompted to explore the influence of antioxidants on PDT-mediated mRNA uptake. Particularly pyrrolidine dithiocarbamate (PDTC) and N-acetyl-L-cysteine (NAC) were tested in multiple studies for efficient radical scavenging at non-cytotoxic concentrations (Schreck et al., 1992; Aruoma et al., 1989; Burgunder et al., 1989). In our in vitro system, PDTC (100 μM) led to induction of c-jun and c-fos mRNA expression (Figure 3, lane 2). This finding concurs with a previous study that found AP-1 active under both oxidant and antioxidant conditions (Meyer et al., 1993). After exposure to NAC (30 mM), only very weak signals were detectable (Figure 3, lane 3), but both antioxidants preferentially induced c-fos transcripts. Interestingly, c-jun mRNA induction upon PDT was reduced by PDTC and NAC, and for the latter c-jun was selectively suppressed (lanes 5 to 7). Thus, it appeared that c-jun expression is mainly caused by the oxidative potential of PDT and could be regarded as an oxidative stress-responsive member of the AP-1 transcription factor family. However, c-fos appeared to be much more strongly activated at the mRNA level under antioxidant conditions.

In order to analyse if the strong and prolonged expression of c-jun and c-fos mRNA by PDT is paralleled by an increased AP-1 DNA-binding activity, protein extracts were prepared at various points in time after photodynamic treatment. Again, TPA stimulation was used as a control. Nuclear extracts were incubated with a 32P-labelled dsDNA oligonucleotide encompassing the AP-1 consensus sequence. For a comparison at the level of AP-1-binding activity, mobility shift assays were carried out with equal amounts of extracted proteins. An increased AP-1 DNA-binding activity could be detected as early as 15 min following PDT or phorbol ester stimulation (data not shown), and strongest activity was observed after 4 h (Figure 4). At that point in time, AP-1 protein was barely detectable in extracts of untreated HeLa cells (Figure 4, lane 1), but the binding was

Figure 1 Kinetics of c-fos and c-jun mRNA induction in HeLa cells by PDT in comparison with stimulation by TPA. Cells (5 x 10⁶) were treated with 1 μg ml⁻¹ Photofrin and illuminated with monochromatic light of 630 nm (4 J cm⁻²) or incubated with 100 ng ml⁻¹ TPA for mitogenic stimulation. The HeLa cells were harvested at the indicated points in time (in h) after treatment. C-jun and c-fos mRNA was analysed by hybridisation with specific cDNA probes. For relative quantification of mRNA levels, hybridisation with a GAPDH-specific probe was performed after complete stripping of the probes used previously. An autoradiogram is shown.
photodynamic resulted in independently reduced if (Figure 2). Despite photodynamic treatment (lanes 1–3) and mRNA stabilisation (lanes 4–13). Total RNA from HeLa cells was isolated 1 h after PDT with 1 μg ml⁻¹ Photofrin and 4 J cm⁻² (lanes 2 and 3). Incubation with actinomycin D (act-D; 10 μg ml⁻¹) 2 h before photosensitisation (lane 3), and of actinomycin D (act-D; 10 μg ml⁻¹) 1 h after treatment by TPA (100 ng ml⁻¹; lanes 4–8) or PDT (lanes 9–13) was performed. Total RNA was isolated immediately (0), 0.5, 1, 2 and 4 h after the addition of actinomycin D. The results for the specific mRNA signals of c-jun, c-fos and GAPDH as quantitative control are depicted.

Figure 2 C-jun and c-fos mRNA accumulation by PDT owing to transcriptional activation (lanes 1–3) and mRNA stabilisation (lanes 4–13). Total RNA from HeLa cells was isolated 1 h after PDT with 1 μg ml⁻¹ Photofrin and 4 J cm⁻² (lanes 2 and 3). Incubation with actinomycin D (act-D; 10 μg ml⁻¹) 2 h before photosensitisation (lane 3), and of actinomycin D (act-D; 10 μg ml⁻¹) 1 h after treatment by TPA (100 ng ml⁻¹; lanes 4–8) or PDT (lanes 9–13) was performed. Total RNA was isolated immediately (0), 0.5, 1, 2 and 4 h after the addition of actinomycin D. The results for the specific mRNA signals of c-jun, c-fos and GAPDH as quantitative control are depicted.

The antioxidant PDT was shown to activate transcription factor AP-1 at the transcriptional and post-translational level (Meyer et al., 1993). Accordingly, we detected an increased AP-1 DNA-binding activity following antioxidant treatment (Figure 4, lane 6). Interestingly, the strong AP-1 activity upon PDT addition was markedly reduced if antioxidant treatment was combined with photodynamic treatment (Figure 4, lanes 6 and 7) at conditions that otherwise independently led to AP-1 activation (lane 2).

The DNA-binding activity induced by TPA was also reduced if phorbol ester stimulation was combined with photodynamic treatment. The addition of TPA 15 min after (Figure 4, lane 4) and 15 min before (lane 5) photodynamic treatment resulted in a reduced binding activity as compared with TPA stimulation alone (lane 3).

Discussion

In this study, photodynamic treatment of human epithelial HeLa cells led to rapid and strong induction of c-jun and c-fos mRNA expression. Both genes were induced by similar kinetics, but c-jun was much more responsive to PDT. Striking differences were apparent when kinetics of c-jun and c-fos gene induction by PDT and TPA were compared. The prolonged induction of both nuclear oncoproteins by photodynamic treatment was in sharp contrast to their transient activation by the phorbol ester TPA. These results suggest a specific regulatory modus of immediate early genes by Photofrin-mediated photosensitisation, predominantly generating singlet oxygen. Other reactive oxygen intermediates, such as superoxide anions (Crawford et al., 1988; Shibahama

Figure 3 Effects of antioxidant treatment and inhibition of protein synthesis on the induction of c-jun and c-fos mRNA expression by PDT in HeLa cells. For antioxidant treatment, PDT was applied at a concentration of 100 μM and NAC at a concentration of 30 mM. Inhibition of protein synthesis was induced by cycloheximide (10 μg ml⁻¹). For co-incubation experiments, PDT and NAC were added 1.5 h and cycloheximide 0.5 h before light application. Total cellular RNA of HeLa cells was isolated 1 h after PDT (1 μg ml⁻¹ Photofrin; 4 J cm⁻²) and analysed as described in Figure 1.

Figure 4 Induction of AP-1 DNA-binding in HeLa cells. For electrophoretic mobility shift assay (EMSA), a radioactive labelled dsDNA oligonucleotide comprising the AP-1 canonical consensus sequence motif was incubated with equal amounts of protein extracts from HeLa cells. Cells (5 × 10⁶) were treated by PDT (1 μg ml⁻¹ Photofrin; light: 4 J cm⁻² and 630 nm), TPA (100 ng ml⁻¹), PDTC (100 μM) or kept in medium without further treatment (Co). Co-stimulation with TPA was performed 15 min after (PDT+TPA) or before (TPA+PDT) photodynamic treatment. PDT was added 1.5 h before illumination. The fluorogram of a native gel is shown. The position of the protein DNA complex containing AP-1 is indicated with a filled arrowhead, whereas the free radiolabelled oligonucleotide is marked with an open triangle. Exposure of the autoradiogram was performed for 15 h at −80°C with an intensifier screen.
Regulation of c-jun and c-fos expression after photodynamic treatment alone occurred at both the transcriptional and post-transcriptional levels. Our mRNA stability studies clearly demonstrated increased half-life of induced c-jun and c-fos transcripts after PDT. Thus, mRNA stabilisation of proto-oncogenes by singlet oxygen might account for the strong and prolonged induction of c-jun and c-fos. Degradation of mRNA is described as an important step of c-jun and c-fos gene regulation, and is responsible for rapid regulation between synthesis and activation. The mRNA depends on two instability domains, the first located within the 3' untranslated region of the mRNA and consisting of an AU-rich sequence element (Shyu et al., 1989; Chen and Shyu, 1994). The second domain is positioned in the transcribed sequence and referred to as the coding region determinant of mRNA instability of the c-fos gene (Kabnick and Housman, 1988; Chen et al., 1992). At the same time, AT-rich sequences of the untranslated region of c-jun may be involved in the post-transcriptional regulation of the c-jun gene (Hattori et al., 1988). Oxidative stress resulting from photosensitisation might interfere with protein behaviour at the described destabilising sites, with unispecific mRNA-degrading activities or transcriptional elongation blocks (Plet et al., 1995).

Regarding the fundamental importance of AP-1 as a mediator of cell transformation by phorbol ester-mediated phorbol ester, it seems likely that aberrant regulation of any component involved might have dramatic consequences, such as eventual loss of growth control and neoplastic transformation. Is deregulated c-jun and c-fos mRNA expression a prerequisite for neoplastic transformation by photodynamic therapy? Although there is evidence for a causal relationship between mRNA stability and AP-1-mediated transformation, potential oncogenic activation of c-jun or c-fos requires both deregulated expression and structural changes. To activate the transforming potential of c-fos, a 67 bp element in the 3' non-coding region which leads to mRNA destabilisation must be deleted (Miller et al., 1984; Lee et al., 1988; Meijlink et al., 1985; Rahmsdorf et al., 1987). Tumour induction by high-level c-fos expression has been observed in an animal model: c-fos transgenic mice were constructed under the control of the metallothionein promoter and the FBJ 3' LTR, which was necessary to stabilise the exogenous fos mRNA (Rüther et al., 1989).

Furthermore, it appears insufficient to evaluate the risk of carcinogenesis by AP-1 at the level of mRNA expression, since there are several post-translational regulatory mechanisms and, moreover, synergistic or antagonistic interactions between AP-1 family members and possibly other transcription factors.

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In the case of photodynamic treatment, post-translational regulation also appears to be a powerful mechanism in controlling AP-1 activity. Although mRNA accumulation by PDT was stronger and even prolonged in comparison with phorbol ester stimulation, on the level of protein DNA-binding, photodynamic treatment was much less efficient. Apparently, the binding capacity of AP-1 is determined by regulatory mechanisms other than transcriptional activation of the nuclear factor genes. According to previous reports, AP-1 was shown to be induced under both pro-oxidant and anti-oxidant conditions (Abate et al., 1990; Xanthoudakis et al., 1992). Stronger DNA-binding was caused by the antioxidant state (Meyer et al., 1993; Schenk et al., 1994; Li and Jaiswal, 1994). Superoxide anions and hydroperoxide were found to be weak inducers of AP-1 binding (Amstad et al., 1992; Devary et al., 1991). Reversible oxidation of a single, conserved cysteine residue located in the DNA-binding domain of AP-1 proteins modulates their DNA-binding activity (Abate et al., 1990). Oxidation of these cysteine sites inhibits the DNA-binding activity of AP-1 (Abate et al., 1990; Bannister et al., 1991; Frame et al., 1991). Ref-1 (redox factor 1), a DNA repair protein, was found to reactivate in vitro oxidised AP-1 by reducing this cysteine residue (Xanthoudakis et al., 1992). Singlet oxygen-mediated redox regulation of AP-1 binding could therefore explain the discrepancy between strong mRNA induction and weak DNA-binding of AP-1 after PDT. Furthermore, oxidation of cysteine residues might be responsible for the down-regulation of AP-1 DNA-binding induced by the antioxidant PDT during concomitant photodynamic treatment. As phorbol ester-mediated AP-1 DNA activation is caused by dephosphorylation (Boyle et al., 1991; Papavassiliou et al., 1992) and PKC seems not to be involved in PDT-mediated regulation of AP-1 in HeLa cells (Krick et al., 1995), redox regulation might be regarded as the dominant pathway of AP-1 DNA-binding modulated upon PDT. This argument is strengthened by the fact that activation of AP-1 upon TPA was suppressed by PDT both before and after the addition of the phorbol ester. However, there is growing evidence for AP-1 regulating cytokine gene expression in response to PDT.

This research has identified Photofrin-mediated photosensitisation as a potent inducer of c-jun and c-fos mRNA expression but a comparatively weak activator of AP-1 DNA-binding. Mechanisms of PDT-mediated AP-1 regulation differ from mechanisms of activation by other reactive oxygen intermediates and phorbol esters, thus suggesting a unique regulatory pathway. It seems unlikely that deregulated mRNA expression could enhance the potential risk of PDT-induced carcinogenesis, since AP-1 DNA-binding is tightly controlled.

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