Mechanism of enhanced responses after combination photodynamic therapy (cPDT) in carcinoma cells involves C/EBP-mediated transcriptional upregulation of the coproporphyrinogen oxidase (CPO) gene

Sanjay Anand¹,², Tayyaba Hasan³ and Edward V Maytin¹,²,³
Departments of ¹Biomedical Engineering and ²Dermatology, Cleveland Clinic, Cleveland, OH and ³Wellman Center for Photomedicine, Harvard Medical School, Boston, MA, U.S.A.

ABSTRACT
Photodynamic therapy (PDT) with aminolevulinate (ALA) is widely accepted as an effective treatment for superficial carcinomas and pre-cancers. However, PDT is still suboptimal for deeper tumors, mainly due to inadequate ALA penetration and subsequent conversion to PpIX. We are interested in improving the effectiveness of photodynamic therapy (PDT) for deep tumors, using a combination approach (cPDT) in which target protoporphyrin (PpIX) levels are significantly enhanced by differentiation caused by giving Vitamin D or methotrexate (MTX) for 3 days prior to ALA-PDT. In LNCaP and MEL cells, a strong correlation between inducible differentiation and expression of C/EBP transcription factors, as well as between differentiation and mRNA levels of CPO (a key heme-synthetic enzyme), indicates the possibility of CPO transcriptional regulation by the C/EBPs. Sequence analysis of the first 1300 base pairs of the murine CPO upstream region revealed 15 consensus C/EBP binding sites. Electrophoretic Mobility Shift Assays (EMSA) proved that these sites form specific complexes that have strong, moderate or weak affinities for C/EBPs. However, in the context of the full-length CPO promoter, inactivation of any type of site (strong or weak) reduced CPO promoter activity (luciferase assay) to nearly the same extent, suggesting cooperative interactions. A comparative analysis of murine and human CPO promoters revealed possible protein-protein interactions between C/EBPs and several neighboring transcription factors such as NFkB, Sp1, AP-1, CBP/p300 and CREB (an enhanceosome complex). Overall, these results confirm that C/EBP’s are important for CPO expression via complex mechanisms which upregulate PpIX and enhance the outcome of cPDT.

Keywords: Photodynamic therapy, skin cancer, differentiation, aminolevulinic acid, protoporphyrin IX, coproporphyrinogen oxidase, gene transcription, transcription factor, tumor model.

1. INTRODUCTION

Photodynamic therapy of cancers is a non-surgical and non-scarring treatment modality. PDT is a combination treatment that utilizes a photosensitizer and light in the presence of oxygen to kill tumor cells. Aminolevulinic acid-mediated photodynamic therapy (ALA-PDT) is an emerging binary treatment of non-melanoma skin cancers (NMSCs) that involves the administration of a pro-drug or precursor photosensitizer, i.e. ALA, which selectively accumulates in tumors to form the actual photosensitizer (the protoporphyrin IX, PpIX), followed by local irradiation with visible light to induce cell death. The advantage of ALA-PDT is two-fold; (i) preferential accumulation of the photosensitizer within the tumor cells, and (ii) selective irradiation of the target tissue with light ¹.
In dermatology, because of higher recurrence rates as compared to standard surgical excision, ALA-PDT in its current form remains suboptimal for the treatment of deeper tumors such as nodular BCC and SCC. Therefore, new approaches are needed to improve the clinical response of tumors to ALA-PDT. The outcome of ALA-PDT relies upon efficient conversion of the ALA to PpIX, and subsequent interactions between the photosensitizer, visible light and reactive oxygen species generated inside the tumor. Tumor destruction occurs through apoptosis, necrosis, autophagy or immune-mediated mechanisms, depending on the tumor cell type and the type of the photosensitizer used. Three determinants of PDT i.e. photosensitizer, light and oxygen, either alone or in combination, can contribute to the efficacy of the therapy. Insufficient penetration of photosensitizer into deep tumors, inadequate penetration of light, or hypoxia in tumors that limits the generation of reactive oxygen species, can severely limit the outcome of therapy. However, even when these three factors are optimized, a fourth factor equally important for the success of ALA-PDT is the ability of target tissue to convert precursor photosensitizer (ALA) into photosensitizer (PpIX) within the tumor. This fourth factor is the focus of clinical and translational studies in our lab 1;2;3.

In our laboratory we developed a new approach of combining Differentiation Therapy (DT) with ALA-PDT, also called Combination PDT (cPDT), to improve the biological response and susceptibility of tumors to the therapy 1. The basis of cPDT is the fact that cancer cells often bypass normal physiological pathways of growth arrest, differentiation and apoptosis, and thus can circumvent cell death. To counteract these abnormalities, certain differentiation promoting agents such as methotrexate (MTX) and vitamin D (vit D) when given prior to ALA-PDT make cells susceptible to cell killing through accumulation of higher levels of PpIX 4-9. The conversion of ALA to PpIX requires sequential action of eight enzymes from heme-biosynthetic pathway. Coproporphyrinogen oxidase (CPO) which acts upstream of PpIX formation, is of particular interest because it could be a rate limiting step for efficient production of PpIX required for cell killing during the PDT. Interestingly, many pharmacological agents (MTX, vit D and androgens) that improve PpIX accumulation and the therapeutic response to ALA-PDT also involve the upregulation of CPO 5;6;8;9.

The CCAAT Enhancer Binding Proteins (C/EBPs) are leucine zipper transcription factors that regulate a variety of physiological processes such as development, proliferation, differentiation, metabolism and cell death 10-12. More recently, abnormal expression of C/EBPα and C/EBPβ has been implicated in the pathophysiology of breast, lung, myeloid and skin cancers. Differentiation promoting agents such as MTX, vit D, androgens or calcium have been shown to exert their effect through upregulation of C/EBPs in different model systems. In this study we asked whether C/EBPs could be involved in the upregulation of CPO during differentiation combination PDT regimens. The results show that C/EBPs upregulate CPO expression and this leads to higher PpIX levels. This study provides evidence that C/EBPs could constitute a missing link between several differentiation promoting agents, CPO expression and PpIX levels suggesting that C/EBPs could be a central mechanism for cPDT.

2. METHODS

2.1 Cell culture, pretreatment and PpIX analysis

Murine erythroleukemia (MEL) cells, LNCaP prostate carcinoma cells, A431, and COS-7 cells were obtained from ATCC and cultured as recommended by the supplier. MEL cells in 25 cm² flasks were incubated with or without dimethyl sulfoxide (DMSO, 1% final concentration). After 24 h, medium with 5-aminolevulinic acid (ALA; 1 mM final) was added for 4 hours. Cells were centrifuged and processed for RT-PCR, western blot, or PpIX analysis (spectrofluorimetry). For PpIX measurements, cell pellet were lysed in 1 ml Solvable (Perkin Elmer) and centrifuged at 10,000 x G at 4 °C. PpIX content of 100 μl aliquots was measured in triplicate in 96 well plates (Corning) using a SpectraMAX Gemini-XS spectrofluorimeter ( Molecular Devices) at 395 nm excitation and 635 nm emission wavelengths, respectively. PpIX levels were expressed as fluorescence units (FUs) per mg of total protein.

Lymph node carcinoma of prostate (LNCaP) cells were plated on plastic cover slips in 35 mm dishes (50,000 cells/dish). At 24 h, the medium was changed to fresh medium containing 10⁻¹²⁻¹⁰⁻⁶ M vit D3 or vehicle (ethanol) alone.
After 72 h of incubation, medium containing 1 mM ALA was added for 4 h prior to PpIX analysis. PpIX-specific fluorescence in living cells was measured on a confocal laser-scanning microscope and digital images analyzed by image processing as described. PpIX levels were expressed as arbitrary fluorescence units per cell.

2.2 C/EBP expression and PpIX analysis by confocal microscopy

LNCaP cells were plated on 22 X 22 mm, No. 1 plastic cover slips in a 35 mm dish (50,000 cells/dish). At 24 h cells were transiently transfected with C/EBP expression vectors (10 to 250 ng), or with pcDNA3.1 empty vector, using FuGENE 6 (Roche Diagnostics, Germany) as per the manufacturer's guidelines. At 24 h post transfection, fresh medium containing 1 mM ALA was applied for 4 hours, then PpIX levels analyzed and quantified as described.

2.3 RNA and protein analysis

To detect semiquantitative changes in CPO mRNA levels in MEL cells, the following oligonucleotide (oligos) pairs were used. CPO: sense, 5'-AGGATGCTGTCATTCAC-3'; CPO: antisense, 5'-GGGGAGTCAAGATCGTCAAA-3'; GAPDH: sense, 5'ACACAGTCGTGACATAC-3'; GAPDH: antisense, 5'-TCCACCACCTGTGCTGTA-3' (IDT DNA technology). RNA (2 µg) extracted from cells using Trizol reagent (Invitrogen) were used for first strand synthesis using gene-specific primers (antisense oligos for the genes mentioned above, 2 pM each). The primers were employed in reactions with SuperScript III H-reverse transcriptase (Invitrogen) as described by the manufacturer. For PCR, amplification, cycles were: 94 °C 2 min, 94 °C 45 s, 60 °C 45 s, 72 °C 45 s X 25-28 cycles, and 72 °C for 2 min. Amplification products were analyzed on 1.5% agarose gels along with size markers.

Western analyses were performed as described. The source and dilution of antisera used were as follows: C/EBPβ and GAPDH (Santa Cruz, 1:5000); CPO (custom made, 1:5000); HRP conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 1:20,000).

2.4 Generation of subcutaneous tumors, vit D pretreatment and PpIX analysis by confocal microscopy

Nude mice received intradermal injections of 2 x 10^6 A431 cells (a human SCC line originally obtained from a cutaneous squamous carcinoma) in each flank. On day 6, visible nodules were observed. For the differentiation therapy, tumors were preconditioned with Vit D (5 µg/kg, intraperitoneal, daily for 3 days) in half of the animals; the other half received sham PBS injections. On day 9 of the protocol, ALA was administered in PBS (200 mg/kg, intraperitoneal) for 4 h. Tumors were harvested, processed and analyzed by H&E and immunohistochemistry, western blotting and PpIX analysis. For PpIX analysis, cryosections (10 µ) of tumors embedded in OCT were placed on glass slides, briefly air dried, coverslipped with Vectashield, and viewed on the Leica confocal microscope, using excitation at 635 nm and image collection in the red channel (emission 670-780 nm).

2.5 Functional analysis by Electrophoretic mobility shift assay (EMSA) and Luciferase reporter assay

Complementary oligonucleotides (~ 43 nt) spanning either one or two C/EBP motifs from the CPO promoter, along with corresponding mutated C/EBP motifs were synthesized by Integrated DNA Technologies. Duplex oligonucleotides were annealed, labeled with [α-32P]dCTP, incubated with nuclear extracts, and the DNA-protein complexes resolved on polyacrylamide gels as described. For supershift experiments, 1 µl of antibody was added to the incubation mixture for 15 min prior to addition of labeled oligonucleotides. For competition experiments, a 10 or 100 molar excess of unlabeled oligonucleotides was added to the mixture.
Nuclear extracts from COS-7 cells overexpressing individual C/EBP isoforms (α, β, δ and ξ) were prepared as described. Expression of the C/EBP proteins in nuclear extracts was verified by western blotting.

Luciferase expression vector pSKLuc containing CPO promoter sequences (pSKLuc-946) was constructed by cloning a 946 bp fragment of the promoter at NotI and XhoI sites. Three of the C/EBP motifs were mutated individually, using the site-specific mutagenesis by overlap extension technique. PCR-amplified products were cloned in pCRII-TOPO vector and sequenced to verify the mutation. Depending on their orientation in pCRII-TOPO, inserts carrying the C/EBP site mutations were cloned in pSKLuc vector as following; C7m at XhoI and KpnI; C6del, C6m, and C4m at SpeI and XhoI sites. LNCaP cells were cultured in 6-well plates (100,000 cells/well) for 24 h, then transfected with 1 μg of lerp expression vectors (pSKLuc or pSKLuc-946) along with 10 to 250 ng of expression plasmid for various C/EBP isoforms (α, β and δ) and 1 μg of pSV-galactosidase expression plasmid (internal control), using FuGENE 6 as per manufacturers guidelines. Cells were harvested 24 h post-transfection; light output was measured using a luciferase assay kit and normalized to the activity of beta-galactosidase.

3. RESULTS & DISCUSSION

3.1 Differentiation-induced C/EBPs regulate CPO expression in cancer cells

MEL cells, a well-established model to study the regulation of heme pathways and also used for cloning the CPO gene, were incubated with 1% DMSO to induce differentiation. The differentiation treatment of MEL cells resulted in upregulation of C/EBPβ and concurrently, increased CPO mRNA and protein levels (Fig. 1a & b). Increased levels of CPO resulted in higher levels of PpIX in MEL cells in response to ALA incubation (Fig. 1c).

Because our previous studies involving differentiation agents and PDT were carried out in cells of epithelial origin, we asked whether C/EBPβ overexpression or differentiation pretreatment in LNCaP cells (an epithelial cell line from human prostate carcinoma) would result in higher levels of PpIX. As seen in Figure 1d, forced expression of
C/EBPβ in LNCaP cells resulted in increased PpIX levels indicating a link between C/EBPs and the heme pathway. Similarly, induction of differentiation in LNCaP cells by vit D pretreatment resulted in higher levels of PpIX (Fig. 1e).

### 3.2 Differentiation induced C/EBPβ expression and PpIX production in A431 subcutaneous tumor model

Subcutaneous tumors created by injection of A431 human SCCs were pretreated for 3 days with vit D or vehicle alone. The differentiation pretreatment clearly upregulated the nuclear expression of C/EBPβ in the vit D treated tumors (Fig. 2, top panel). The differentiation status of vit D treated tumors was verified by increased levels of E-cadherin localized to membrane junctions (Fig. 2 middle panel). The vit D treatment of A431 tumors resulted in increased levels of PpIX when analyzed by confocal microscopy (Fig. 2, lower panel). The increase in PpIX levels was the result of increased CPO protein (data not shown)9. These results support our hypothesis that elevated C/EBPβ upregulates CPO expression, leading to increased PpIX synthesis after vit D pretreatment in vivo.

**Figure 2** Vit D pretreatment induces differentiation, C/EBPβ expression and PpIX levels in vivo. A431 tumors histologically analyzed for expression of C/EBPβ (upper panel), E-cadherin (middle panel) and PpIX levels by confocal microscopy (lower panel). Tumors shown on left and right were treated with vehicle (ethanol) or Vit D, respectively, followed by 4h of ALA. See methods for details.

### 3.3 Identification and characterization of C/EBP binding sites in murine CPO (mCPO) promoter region

To explore the link between increased C/EBPs and CPO expression resulting in elevated PpIX levels, we analyzed the upstream promoter sequence of the mCPO gene by Transfac pattern recognition software. On the mCPO promoter, 946 base pairs (bp) of nucleotide sequence upstream from the transcription start site contained 17 sites with nucleotides homologous to C/EBP consensus binding sites 13; 14. Starting from the 3' transcription start site, these sites were numbered 1-17 (Fig. 3a). To test the binding of these sites to C/EBP transcription factors, we tested these sites on Electrophoretic Mobility Shift Assay (EMSA) using nuclear extracts from COS-7 cells overexpressing each of the C/EBP isoforms. Expression of each isoform was confirmed by western blot analysis. The relative ability of each site to bind C/EBPs was determined as a function of nucleotide sequence and as a function of a particular C/EBP isoform. For example, an EMSA gel showing the binding of C/EBPδ to each site under identical experimental conditions is shown in Figure 3b. Based on their binding affinities, as seen in Figure 3b, these sites were classified into four categories: strong (3 sites), moderate (6 sites), weak (6 sites) and non-binding (2 sites). One may predict that the binding hierarchy observed among the binding sites may translate functionally under in vivo conditions, making strong affinity sites more important than weaker ones. To test this possibility, three C/EBP binding sites with different binding affinities (strong, moderate or weak) were randomly chosen for mutagenesis experiments. A full length promoter-Luc reporter construct
containing 946 bp of wildtype mCPO promoter showed robust activity and was further induced when cotransfected with a C/EBPβ expression vector (Fig. 3c). However, when C/EBP sites were mutated, all constructs showed a similar decrease in their activity irrespective of their binding strengths (Fig. 3c). Since the mutation of individual sites, independent of binding strength, completely abolished the transcriptional activation of the reporter, we analyzed the full promoter sequence for cooperative interactions among C/EBP sites and other transcription factors known to interact with C/EBPs.

Cooperative interactions between multiple C/EBP sites have been described for several gene promoters including the keratin 10 (k10) promoter by our group. Careful scanning of nucleotide sequences on mCPO promoter revealed binding sites of several transcription factors such as NF-κB, Sp1, AP-1, CREB and CBP/p300, all known to interact with C/EBPs (Fig. 4). These observations lead us to interpret our data in the context of an “enhanceosome”.

Enhanceosomes are higher order complexes of DNA and proteins, which through their assembly promote the recruitment of RNA polymerase machinery to the transcription start site. In this situation, as observed in Figure 3c, deletion or mutation of a single site is sufficient to inactivate the transcriptional complex. The complexity of molecular interactions among the transcription factors present on the mCPO gene promoter makes it an interesting gene to study, and in particular its response to signaling pathways crucial for regulation of heme-biosynthetic pathways.

4. CONCLUSIONS

In clinical studies up to now, ALA-PDT has remained suboptimal to treat skin cancers due to current limitations including insufficient penetration of the drug, inability to deliver light to deep targets, or the hypoxic nature of the tumor. However, even when these factors are optimized, the outcome of PDT relies on the state of the target tissue for efficient conversion of the drug to photosensitizer. In our laboratory we have developed a new approach of combining differentiation pretreatment with ALA-PDT, which we named cPDT. We have found that the use of differentiation pretreatment enhances PpIX accumulation and distribution in deep tumors, which results in increased cell killing. In this study we showed that the differentiation promoting agents (MTX and vit D) not only improved PpIX accumulation, but also induced C/EBP transcription factors, and that the concurrent induction of CPO expression appears to involve C/EBP.
consensus binding sites present on CPO gene promoter. Differentiation-induced transcriptional regulation of CPO by C/EBPs is proposed as the mechanism underlying the upregulation of CPO, PpIX levels and cell killing that is observed during a cPDT regimen. Based on these results, C/EBP mediated cellular differentiation may serve as a central mechanism for new approaches to provide enhanced efficacy of ALA-based combination treatment for cancer, through upregulation the of CPO gene.

Figure 4 Location of C/EBP binding sites and consensus-binding sites for other important transcription factors in the upstream regulatory region of mCPO gene. The promoter sequence showing transcription and translation start site and C/EBP binding sites (rectangles) is shown. Locations of other important transcription factor binding motifs, identified by Transfac search database are shown by other symbols. See the key for details.

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6. REFERENCES


