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A Mechanistic Study of δ-Aminolevulinic Acid-Based Photodynamic Therapy for Cutaneous Leishmaniasis

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TO THE EDITOR

Photodynamic therapy (PDT) is based on the concept that a certain photoactivatable compound, called a photosensitizer, can be excited by light of the appropriate wavelength to generate cytotoxic singlet oxygen and free radicals (Hasan *et al.*, 2006). Although earlier reports of PDT involved microbiological applications (Raab, 1900), clinically PDT has been developed most extensively for oncologic and ophthalmologic applications. Recent clinical outcomes of PDT using δ-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX), termed ALA-PDT, in patients with cutaneous leishmaniasis (CL) have been promising (Enk *et al.*, 2003; Gardlo *et al.*, 2003, 2004; Asilian and Davami, 2006), but far from being curative.

ALA by itself is not a photosensitizer, but is processed to PpIX in the heme biosynthetic pathway where it serves as a biological precursor of PpIX. It is known that *Leishmania amazonensis* parasites are deficient in seven out of eight enzymes in the heme biosynthetic pathway (Sah *et al.*, 2002). Although *Leishmania major* (*L. major*) have not been studied for this enzyme deficiency, as they require extracorporeal supplementation with PpIX or hemin as a growth factor *in vitro* (Steiger and Steiger, 1976; Chang and Chang, 1985), it is reasonable to assume that they may also be deficient in these enzymes, and that they cannot produce PpIX from ALA. Therefore, the reported efficacy of ALA-PDT for CL is somewhat intriguing.

Encouraged by clinical reports, and with a view to increasing the efficacy of ALA-PDT for CL, we performed a series of *in vitro* and *in vivo* experiments to unravel the underlying mechanisms associated with this clinical response. In an attempt to better understand the basis of ALA-PDT for CL, we identified several key questions that are addressed here: first of all, how does ALA-PDT work for CL? At the cellular level, is ALA-PpIX produced by *Leishmania* or by host cells, or by both? Do *Leishmania* parasites take up ALA-PpIX from host cells?

We studied a series of cellular events in *Leishmania* parasites and in infected immortalized macrophages (J774.2) during ALA-PDT. The amount of PpIX in the stationary phase of a

Abbreviations: ALA, δ-aminolevulinic acid; ALA-PDT, photodynamic therapy with 5-aminolevulinic acid-induced protoporphyrin IX; ALA-PpIX, 5-aminolevulinic acid-induced protoporphyrin IX CL, cutaneous leishmaniasis; PDT, photodynamic therapy; PpIX, protoporphyrin IX

metacyclic parasite ($3.00 \times 10^{-17} \pm 0.2 \times 10^{-17}$ moles/cell) was 2.54-fold greater than the amount of PpIX, which could be accumulated by an amastigote ($7.6 \times 10^{-17} \pm 0.5 \times 10^{-17}$ moles/cell). Amastigotes are different from promastigotes in morphological, biochemical, molecular, and antigenic phenotype, as they assume the ability to grow at the mammalian body temperature and to infect the host's cells (Schuster and Sullivan, 2002). Amastigotes are physiologically more active than promastigotes and are likely to actively take up more PpIX (Figure 1a and b). In spite of this observation, the number of PpIX molecules that one amastigote could accumulate ($4.6 \times 10^{7} \pm 0.3 \times 10^{7}$ molecules/cell) was still much lower than the number of ALA-PpIX molecules required for killing one metacyclic parasite (2.3×10^{10} molecules/cell). ALA-PpIX localized distinctively in amastigotes in infected J774.2 cells at 4 hours co-incubation with ALA, and furthermore, the amastigotes in J774.2 cells were not eliminated by ALA-PDT with light doses up to 50 J/cm^2 (data not shown). These results would suggest that a good clinical outcome *in vivo* is unlikely to involve the direct killing of *Leishmania* by ALA-PDT.

In contrast to the *in vitro* results, which failed to demonstrate a parasiticidal effect (Figure 1c and d), in *in vivo* experiments in a murine CL model (all animal procedures were performed according to protocols approved by the Massachusetts General Hospital Subcommittee on Research Animal Care), we observed a reduction of the parasite load after ALA-PDT (Figure 1e). Based on the fact that the uptake of PpIX by the *Leishmania* was not enough to kill the parasites, and that *in vivo* ALA-PDT does reduce the parasite load, it can be assumed that an alternative indirect pathway is operational.

It is known that there are several other mechanisms involved in tissue damage by PDT (Luksiene, 2003). An initial destruction of the vascular system and the intracellular matrix is followed by hypoxia, which results in cell death. We speculated that the vascular damage would have less effect in PDT for CL as compared to PDT against tumors with their abundant vascularity. In our *in vivo* study, all of the ALA-PDT treated ears demonstrated a wedge-shaped necrosis in the peripheral treatment area (Figure 1f, arrows). As the PDT was performed centrally to the lesion, the appearance of a circular necrosis at the site of irradiation could be expected. Anatomically, the blood supply of the murine ear is composed of one major branched blood vessel extending from base to edge of the ear. Considering this, the observed wedge-shaped distortion is consistent with a lack of blood supply

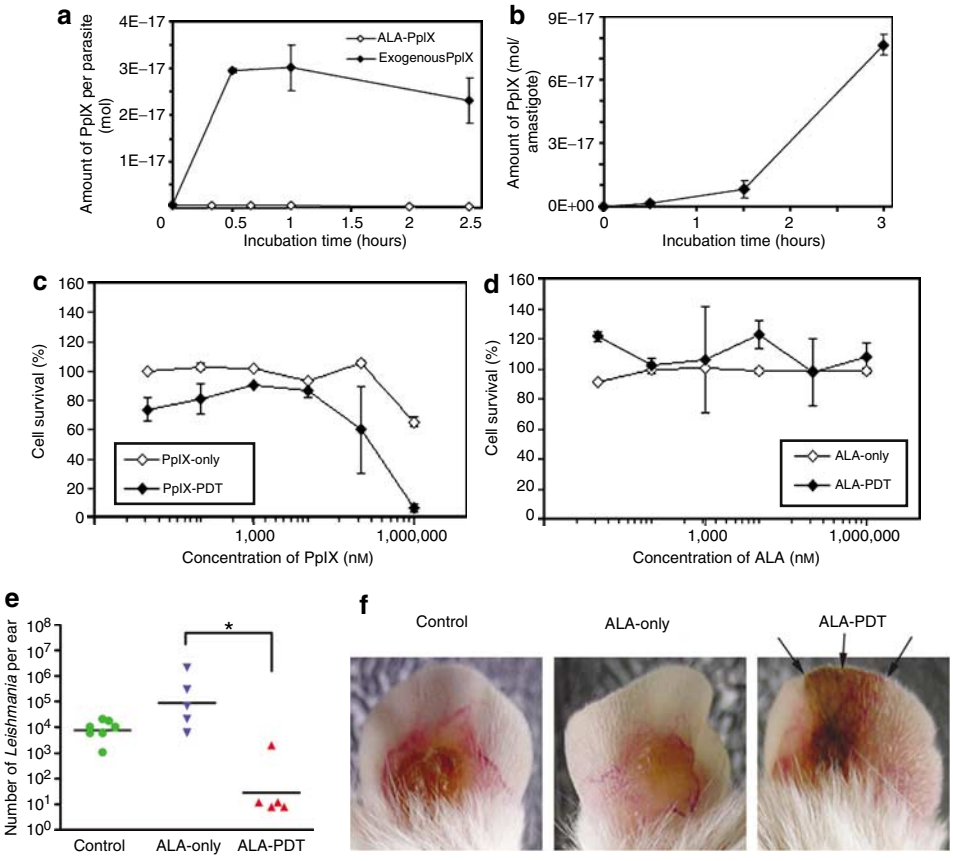

Figure 1. ***In vitro* and *in vivo* efficacies of ALA-PDT against *Leishmania*.** (**a**) The PpIX level in a parasite after co-incubation with exogenous PpIX or ALA (0.1 μM). (**b**) Quantification of PpIX in an amastigote. (**c**–**d**) Exogenous PpIX mediated phototoxicity at high concentrations, whereas ALA did not. *L. major* parasites were co-incubated with PpIX (**c**) or ALA (**d**) and subsequently irradiated at 10 J/cm^2. The viability of parasites was determined as the percentage of 3-[4,5]dimethylthiazol-2,5-diphenyltetrazolium bromide conversion activity. (**e**) Intralesional quantification of *L. major* parasites by standard dilution (Belkaid *et al.*, 1998). *$P<0.05$ (**f**) Topical ALA-PDT on CL lesions 3 weeks after *L. major* infection (635 nM diode-laser at 50 J/cm^2).

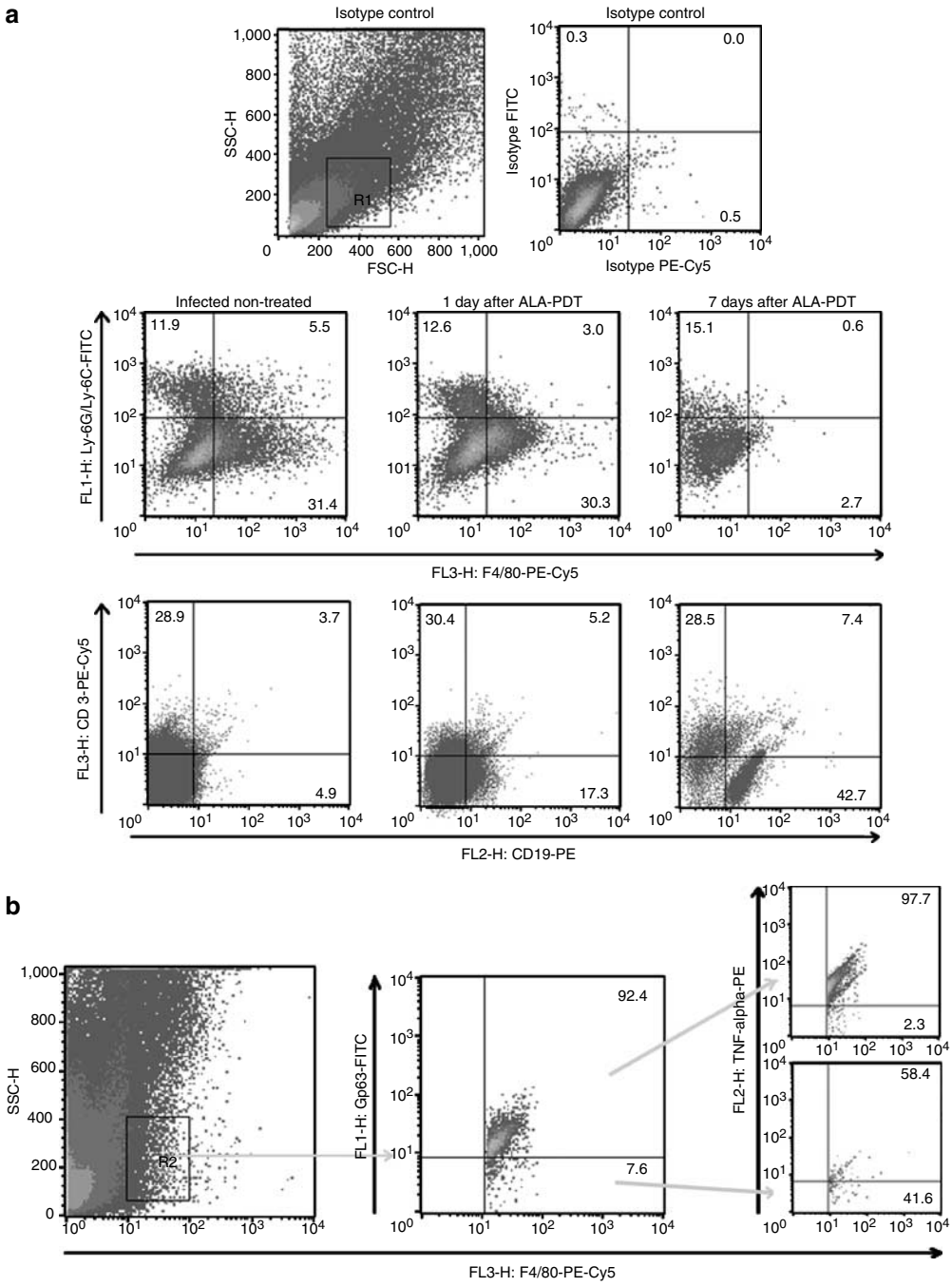

Figure 2. Phenotypic profile of cells in ears with CL lesions after ALA-PDT. (**a**) A representative dot plot (top left chart) used for cell gating (R1). Dot plots for neutrophil (Ly-6G/Ly-6C), macrophage (F4/80), T lymphocyte (CD3), and B lymphocyte (CD19) expression are shown for infected untreated mice, treated mice 1 day, and 7 days after ALA-PDT. (**b**) A representative dot plot displays SS and F4/80$^+$ cells used for cell gating (R2). A dot plot for F4/80 and *L. major* surface glycoprotein (gp63-FITC) expression is shown in the middle. Tumor necrosis factor-α expression was determined in infected (gp63-positive cells) and uninfected (gp63-negative cells) F4/80$^+$ cells and presented as two charts on the right side of the picture.

resulting from vascular obstruction after treatment.

On the other hand, the necrosis may be the result of unspecific destruction of the particular type of cells in the murine CL lesion. In order to determine the phototoxic effects of ALA-PDT on the cellular components of CL lesions, we estimated the percentage of neutrophils (Ly-6G/Ly-6C), macrophages (F4/80), T- (CD3) and B- (CD19) cells in the ears of non-treated infected mice, treated infected mice at 1 day after ALA-PDT, and treated infected mice 7 days after ALA-PDT. We observed that among neutrophils, T- and B cells, the percentage of macrophages was dramatically decreased 7 days after ALA-PDT (Figure 2a). This result suggests that macrophages are the most sensitive to ALA-PDT and would be the main target

of this regimen. However, almost all remaining macrophages (2.7%) were infected (92.4%), providing a potential source of future reactivation of the infection (Figure 2b). The vast majority of those infected macrophages produced tumor necrosis factor-α (97.7%), compared to non-infected F4/80-positive cells, of which only 58.4% produced tumor necrosis factor-α. Thus, one treatment is not sufficient for the complete eradication of *L. major*-infected cells.

The findings from this study suggest that the clinical outcome observed with ALA-PDT is likely the result of unspecific tissue destruction accompanied by a depopulation of macrophages rather than any direct killing of parasites. Other approaches, such as those studying new photosensitizers, or PDT targeted specifically against *Leishmania*, may also be worth exploring.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Nuclear c-Myc: A Molecular Marker for Early Stage Pemphigus Vulgaris

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TO THE EDITOR

Pemphigus vulgaris (PV) is the most common variant of the human autoimmune blistering disorders in the pemphigus complex and accounts for over 80% of all cases (Greenberg, 1994). Although generally considered as disease of middle-aged adults, PV has also been reported in children and neonates (Bjarnason and Flosadottir, 1999). In order to rapidly institute appropriate therapeutic measures and thus prevent the serious morbidity that results from extensive loss of epidermal cohesion, it is of importance to diagnose PV in its very initial phase.

PV is diagnosed by virtue of its clinical and histological presentation, the latter being examined on routine paraffin-embedded biopsies. Characteristically PV presents with fragile blisters and subsequent erosions, matched by suprabasal cleft formation and the presence of acantholytic cells in light microscopy. In early stages, diagnosis may be missed in the presence of nonspecific, pre-bullous inflammatory skin lesions. Upon clinical suspicion of PV, the demonstration of intraepidermal IgG deposits and circulating anti-keratinocyte membrane antibodies by direct (DIF) and indirect (IIF) immunofluorescence, respectively, was mandatory to prove the autoimmune origin of the disease until recently. DIF is carried out on native tissue from the patient as described by Beutner and Jordon (1964) requiring a second biopsy in case the first biopsy was solely processed for routine histology. A commercially

Abbreviations: BP, bullos pemphigoid; DIF and IIF, direct and indirect immunofluorescence analysis; Dsg3, desmoglein 3; PF, pemphigus foliaceus; PV, pemphigus vulgaris; SD, seborrheic dermatitis; TEN, toxic epidermal necrolysis