International Immunopharmacology 28 (2015) 44–51

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Low dose photodynamic-therapy induce immune escape of tumor cells in a HIF-1α dependent manner through PI3K/Akt pathway

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ARTICLE INFO

Article history:
Received 2 February 2015
Received in revised form 4 May 2015
Accepted 18 May 2015
Available online 29 May 2015

Keywords:
Photodynamic therapy
Lewis lung carcinoma
Immune escape
HIF-1α
PI3K/Akt pathway

ABSTRACT

As a regulatory approved antitumor therapy, photodynamic therapy (PDT) shows poor effect in clinical application. The current study aimed at investigating the mechanism through which low-dose PDT affects the immune escape ability of Lewis lung carcinoma (LLC) cells.

Our data show that low-dose PDT could increase HIF-1α expression through the activation of the PI3K/Akt pathway. Our results also show that low-dose PDT treatment can increase tumor growth rate in C57BL/6 mice, reducing the survival of tumor-bearing mice. Furthermore, low-dose PDT could increase regulatory T cells (Tregs) number, decrease the cytotoxic T lymphocytes (CTLs) number, and induce CTLs apoptosis in co-culture system *in vitro*. The immunosuppression caused by low dose PDT could be partially abolished by knocking down the hypoxia-inducible factor 1α (HIF-1α) and blocking the phosphatidylinositol 3-kinase (PI3K).

Our results suggest that low dose PDT may enhance immune escape of LLC cells in a HIF-1α dependent manner through PI3K/Akt pathway. The present study thus offers a new insight on elucidating the mechanism whereby low dose PDT induces tumor progression and therapy-resistance, providing a novel approach for cancer immunotherapy.

1. Introduction

Photodynamic therapy (PDT) is a regulatory approved antitumor therapy [1]. It possesses the ability to destroy tumor mass by triggering a photochemical reactions after local or systemic administration of a photosensitizer. The exposure of the treated area to light generates singlet oxygen ($^1O_2$) or reactive oxygen species (ROS) [2]. The antitumor effects of PDT involve multiple and interacting mechanisms including direct tumor cell killing, damage to the microvasculature of the tumor bed, and tumor specific reactions [3,4]. However, the recurrence of the tumor and the immune-resistance of the tumor cells, that represent the main problem of the anticancer therapies, are also observed after PDT treatment [5]. It is assumed that PDT can affect the tumor microenvironment by the dysregulation of cytoprotective molecules [5]. This dysregulation leads to a malignant phenotype characterized by an enhanced angiogenesis, cell proliferation, and cell invasion, thus promoting tumor progression or recurrence [6]. Therefore, specific attention to microenvironment should be paid, focusing on these cytoprotective molecules that may be involved in the tumor recurrence and therapy-resistance, to obtain an improvement of the clinical outcomes of PDT.

The cytoprotective molecules involved in PDT-induced tumor recurrence and therapy-resistance, include hypoxia-inducible factor 1α (HIF-1α), cyclooxygenase-2 (COX2), vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), the anti-apoptotic protein survivin, and heat shock proteins (HSPs) [6]. Among them, HIF-1α plays an important role participating in the regulation of the immune responses in the tumor microenvironment [7,8]. Hypoxia is the result of the tumor microenvironment's insufficient blood supply required to support proliferating tumor cells. In response to hypoxia, the cells counterbalance the low level of oxygen by changing the transcription of several genes. Since HIF-1α plays a key role in the hypoxia stress, it regulates the cellular response to hypoxia, through which tumor cells acquire enhanced malignant properties and become less sensitive to antitumor therapies [9].

Accumulating evidence indicates that phosphatidylinositol 3-kinase (PI3K)/Akt pathway is involved in HIF-1α expression induced by hypoxia [10]. In addition, this pathway contributes to maintaining the malignant phenotype of tumor cells [11–14]. Moreover, HIF-1α expression through PI3K/Akt pathway is associated with the tumor microenvironment [8]. However, their relationship in regulating the ability of tumors to escape immune surveillance remains unclear.

Tumor cells possess indeed the ability to evade immune system-mediated destruction, thus surviving from the attack of the adaptive

Abbreviations: PDT, photodynamic therapy; LLC, Lewis lung carcinoma; ROS, reactive oxygen species; HIF-1α, hypoxia-inducible factor 1α; CTL, cytotoxic T lymphocytes; Hyp, hypericin; μM, μmol/L; nM, nmol/L.

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http://dx.doi.org/10.1016/j.intimp.2015.05.025
1567-5769/

immunity. Previous reports showed that this ability of tumor cells controls the balance between tumor dormancy versus progression [15]. Immune escape involves a variety of mechanisms including hypoxia response, and may play a critical role in clinical outcomes [15]. CD4 + CD25 + Foxp3 + Regulatory T cells (Tregs) contribute to the immune tolerance of tumors by inhibiting most types of immune responses [16,17]. Cytotoxic T lymphocytes (CTL, IFN-γ producing CD8 + T) are responsible for deepening the host defense against malignancies *in vivo* [18]. By examining Treg and CTL population, we could evaluate the immune escape status of tumor cells.

In the present study, we investigated whether HIF-1α through PI3K/Akt pathway is involved in the mechanism whereby low dose PDT enhances the immune escape ability of LLC cells. Our data showed that low dose PDT increased the expression of HIF-1α through PI3K/Akt pathway, increased Treg cell population, suppressed the CTLs population, and enhanced the resistance of LLC cells to CTL-mediated killing. Moreover, knockdown of HIF-1α as well as blockage of PI3K resulted in the restoration of tumor cell lysis upon low dose PDT treatment.

2. Material and method

2.1. Antibodies and reagents

Hypericin (Hyp) was purchased from Yuanye bio-technology Co., Ltd (Shanghai, China) and dissolved in dimethyl sulphoxide (DMSO) to obtain a 1 mM stock solution. LY294002 (a highly selective PI3K-inhibitor) and reduced L-glutathione (GSH) were purchased from Sigma. Anti-HIF-1α antibody was purchased from Epitomics Inc. (California, USA). Anti-Akt, anti-p-Akt antibodies, and secondary antibodies conjugated with FITC and HRP were purchased from ProteinTech Group, Inc. (Atlanta, USA). Recombinant murine IL-2 was from Peprotech Inc (Rocky Hill, USA). FITC conjugated-antibodies against CD4 and CD8, APC conjugated-antibodies against Foxp3, PE conjugated-antibodies against CD25 and IFN-γ, and appropriate isotype control antibodies were all purchased from eBioscience, Inc. (California, USA).

2.2. Mice

Six week old male C57BL/6 mice were purchased from Shanghai Laboratory Animal Resource Center (Shanghai, China). The mice were divided into 3 groups (of 7 mice each): control group (untreated control), low dose PDT group, and high dose PDT group.

1×10^6 LLC cells were suspended in 200 μL PBS, and then injected subcutaneously into the lower left flanks of the C57BL/6 mice. When the tumor volume was more than 50 mm^3, the tumor-bearing mice were subjected to PDT treatment.

2.3. PDT treatment both in vivo and in vitro

The tumor-bearing mice were treated with a high dose (5 mg/kg) or a low dose (2.5 mg/kg) Hyp via tail vein injection and incubated for 4 h. This incubation time was chosen according to preliminary metabolic experiments (data not shown). Mice were anesthetized with phenobarbital sodium after 4 h. The rest of mouse body was protected by aluminum foil. The fur was removed from the treated region prior to irradiation. The 600-micron bare fiber was used to perform irradiation on the tumor area of the mice. The light was delivered at a dose of 14 J/cm^2 (low dose PDT group), and 60 J/cm^2 (high dose PDT group). The *in vivo* PDT treatment was performed every five days for a total of three times.

In vitro PDT treatment was performed as described previously [19]. LLC cells treated with PDT were harvested at the appropriated post-PDT time points, and used for the subsequent experiments.

2.4. Immunohistochemistry

Tumors were harvested from the mice belonging to the three groups (control; low dose PDT; high-dose PDT). The tumor tissues were fixed in formalin and embedded by paraffin. Next, the paraffin-embedded tissues were cut into 4 μm sections and mounted on glass slides. Immunohistochemistry was performed as described previously [20,21] to detect HIF-1α expression. Briefly, the sections were deparaffinized and rehydrated, followed by a treatment with 0.3% H_2O_2/methanol solution to block endogenous peroxidase activity, and incubated with blocking buffer (BSA 5% in 0.3% Triton X-100). Sections were incubated with anti-HIF-1α antibody at a dilution of 1:200 overnight at 4 °C and 3,3-diaminobenzidine tetra-hydrochloride (DAB) Peroxidase Substrate kit (Beyotime Biotech, Haimen, China) was used to detect the antigen, according to the manufacturer's instructions. Sections were further stained with hematoxylin to locate of the nucleus. Slides were observed using Nikon Ti-S microscope, and images were collected by NIS-Elements D3.2 software. The intensity of HIF-1α positive signals was evaluated using the Image-Pro Plus 6.0 software.

2.5. The knockdown of HIF-1α and the blockage of PI3K/Akt pathway

LLC cells were seeded into 6-well plate, and incubated overnight to allow the attachment of the cells. Cells were then transfected with Silencer Select siRNA for mouse HIF-1α (Ambion Inc.) using lipofectamine 2000 according to the manufacturer's instructions. 4 h after transfection, cell medium was removed. Cells were incubated with 125 nM Hyp and 50 μM LY294002 (a specific PI3K inhibitor) for 16 h and then subjected to irradiation with light at low dose (1.85 J/cm^2). Cells without any pretreatment before low dose PDT were used as control group. Cells were harvested 8 h post-PDT, and used for subsequent experiments.

2.6. Western blot

For kinetic analysis of HIF-1α levels, cells were harvested at 0, 4, 8, 12, 24 h post PDT. For analysis of the expression of PI3K/Akt pathway and HIF-1α protein, cells were harvested at 8 h post-PDT. Next, the cell lysates were resolved by SDS/PAGE gel, and western blot was performed as previously described [22]. Briefly, the harvested cells were lysed using protein lysis buffer (Beyotime, Beijing, China). Cell lysates were centrifuged at 14,000 rpm for 15 min at 4 °C, and the supernatants were collected. After determination of total protein concentrations by using BCA assay, approximately 50 mg of proteins was loaded onto 7.5% to 15% SDS/PAGE gels. The gels were transferred to PVDF (polyvinylidene fluoride) membrane (Millipore). The PVDF membranes were then blocked with blocking buffer (5% non-fat dry milk, in TBST) for 2 h at room temperature, incubated with primary antibodies at 4 °C overnight, and with the appropriated alkaline-phosphatase (AP)-conjugated secondary antibody for 1 h. The membranes were washed and the bands were detected by color–substrate solution (5 mL AP buffer, 16.5 mL BCIP, 33 mL NBT) at room temperature for 10 to 20 min. β-actin was used as a loading control.

2.7. Measurement of ROS production

Cells were subjected to low dose PDT treatment. The ROS generation in Hyp-PDT treated LLC cells was detected by using a reactive oxygen species assay kit (Beyond time, China). At the indicated post-PDT time points, cells were incubated with DCFH-DA and the fluorescence intensity was measured according to the manufacturer's instruction.

2.8. Immunofluorescence

Following low dose PDT treatment, cells were fixed with 0.25% paraformaldehyde in PBS, and permeabilized with 0.2% Trixton X-100.