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PDT-induced HSP70 externalization up-regulates NO production via TLR2 signal pathway in macrophages

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ABSTRACT

We studied the molecular mechanism underlying PDT-induced apoptosis-dependent macrophage activation, particularly through NO production. We demonstrate that NO production is initially induced by HSP70 on the apoptotic cell surface, and is further enhanced by macrophage phagocytosis. Additionally, we found that apoptotic cells, through TLR2, could activate PI3K, and this could be either dependent or independent of the activation of MyD88. These results reveal a novel pathway linking innate immune signalling to apoptotic cells and point at HSP70 as an important antitumor immunostimulant. They also indicate that PDT-induced apoptosis has an important role in macrophage innate immunity.

Structured summary of protein interactions:
p85α and MyD88 physically interact by fluorescent resonance energy transfer (View interaction)
TLR2 and p85α physically interact by fluorescent resonance energy transfer (View interaction).

1. Introduction

Considerable data support the idea that apoptotic cells (ACs) participate in immune response, but its immunological mechanism is still unclear [1,2]. Although engulfment of ACs has traditionally been regarded as being immunologically suppressive, recent studies have suggested that infected ACs are a critical component of the innate immune signals [3].

The ideal cancer treatment modalities should not only cause tumor regression and eradication, but also induce a systemic antitumor immunity. Photodynamic therapy (PDT) is a relatively new modality for the clinical treatment of cancer. In addition, PDT has shown antitumor effects in regulating the host immune systems [4–7]. Since macrophages exist in most tumor sites and are the most abundant infiltrating cells in tumors, macrophage-targeted PDT has been applied in the selective killing of cells involved in inflammation and tumor [8]. Macrophages are classified as M1 and M2. M1 macrophages promote anti-tumour immunity while M2 macrophages derail it [9]. Mounting evidence shows a more complex progress of macrophage activation during PDT, which performs distinct immunological functions and different physiologies on surrounding cells and tissues [10,11]. Studies have demonstrated that activated macrophages not only can distinguish tumor cells from normal host cells, but also are capable of reducing tumor cell growth and achieving tumor cytotoxicity without the aid of specific antibodies [12]. In addition, accumulating evidence shows that the apoptotic rather than necrotic tumor cells induce a potent immune response [13]. We also demonstrated that TLR2 initiated such signalling cascades in response to PDT-induced AC [14]. It is known that the released endogenous molecules of necrotic cells are recognized as danger signals. Recent evidence has also shown that PDT-induced AC is associated with danger signals that can activate innate immune cells [15].

Since macrophages produce a large volume of ROS (reactive oxygen species) through respiratory burst, nitric oxide (NO) release has been a sign of respiratory burst. The high-output of NO and the over-expressed inducible nitric oxide synthase (iNOS) are considered as "activated macrophage marker". Macrophages can kill tumor cells by releasing high levels of NO and related reactive nitrogen species such as nitroxyl and peroxynitrite, after up-regulation of expression of the iNOS gene [16]. Importantly, as an agent of inflammation and cell-mediated immunity, iNOS-derived NO also plays an important role in host anti-tumor immunities [17]. Since the NO production

Abbreviations: AC, apoptotic cell; CFP, cyan fluorescent protein; CM, conditioned medium; Cyt B, cytochalasin B; DN MyD88, dominant negative myeloid differentiation factor-88; DN TLR2, dominant negative TLR2; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; HSPs, heat shock proteins; iNOS, inducible nitric oxide synthase; NC, necrotic cells; NF-κB, nuclear factor κB; NO, nitric oxide; PDT, photodynamic therapy; shRNA, short hairpin RNA; TLR, toll like receptor; YFP, yellow fluorescent protein

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is mainly derived from M1 macrophages, it is important to determine in the future whether M1/M2 macrophage ratio has significant implications after PDT-treated AC stimulation.

We have previously reported that cytoplasmic heat shock protein 70 (HSP70) could translocate onto the outer surface of ACs after PDT treatment [18]. In the current study, we investigated the mechanism of macrophage activation induced by PDT-treated AC, particularly through NO production. Moreover, the effects of HSP70 translocation onto tumor cell surface and the molecular mechanisms by which macrophages activation is induced by PDT-treated AC, lead to the consequent cellular responses, were also studied.

2. Materials and methods

2.1. Chemicals and plasmids

We used antibodies against iNOS, β-actin (Santa Cruz), pan-Cadherin (Abcam), HSP70 and isotype-matched control anti-HSP70 antibody (Sigma–Aldrich).

The plasmid of CFP-TLR2 was kindly supplied by Dr. Robert W. Finberg (University of Massachusetts Medical School). GFP-MyD88 was kindly supplied by Dr. Hermann Wagner (RWTH Aachen University). Dominant negative MyD88 was a gift from Dr. Ken-ichi Tanamoto (Musashino University). The dominant-negative PI3K construct Δp85α (encompasses a deletion mutant bovine p85 that lacks a binding site for the p110 catalytic subunit of PI3K) was kindly provided by Dr. Geoffrey M. Cooper of Boston University. YFP-p85α was kindly supplied by Dr. Georges Bismuth of University Paris Descartes. YFP-HSP70 was kindly supplied by Dr. Richard I. Morimoto of Northwestern University. HSP70 short hairpin RNA (shRNA) and non-target shRNA were provided by Dr. Tolkovsky and were used as previously described [19]. pNF-κB-Luc was kindly provided by Dr. X. Shen (Institute of Biophysics, Chinese Academy of Sciences). iNOS-Luc was kindly provided by Dr. David A. Geller (University of Pittsburgh School of Medicine). Dominant negative TLR2 was purchased from InvivoGen. pRL-TK was purchased from Promega.

2.2. Cell culture

Murine mammary tumor line EMT6 was maintained in RPMI 1640 medium (GIBCO), murine macrophage-like cell line RAW264.7 was maintained in Dulbecco's modified Eagle's medium (GIBCO), with 15% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in 5% CO_2 at 37 °C in a humidified incubator.

Mouse peritoneal macrophages were obtained as previously described [14].

2.3. Photodynamic therapy treatment of tumor cells

ACs were generated by PDT with a Photofrin (Sinclair Pharmaceuticals) dose of 10 μg/ml and a light dose of 5 J/cm^2 (10 mW/cm^2), as previously described [14]. Briefly, cells were administrated with 10 μg/ml Photofrin, then incubated in a dark, humidified atmosphere of 5% CO_2 at 37 °C for 12 h, rinsed with PBS, and exposed to light of 5 J/cm^2 (10 mW/cm^2), which resulted in more than 90% apoptotic cells. To generate ACs with overexpression HSP70 (HSP70-AC), EMT6 cells were transfected with YFP-HSP70 and G418-resistant cells were collected for PDT treatment. To generate necrotic cells (NCs), tumor cells were heated to 41 °C for 5 min and then placed in −20 °C for 30 min. The process was repeated three times. In all experiments, the ratio of apoptotic or necrotic cells to macrophages was kept at 5:1. Unless stated otherwise, ACs in the figures are PDT-induced ACs.

The AC conditioned medium (CM) was obtained by incubating 2.5×10^6 ACs in 1 ml of medium. After 2 h, cells were centrifuged for 10 min at 1000*g*. The supernatant was removed and filtered through a 0.2-μm cellulose syringe filter. The filtrate was taken as CM. When using CM, full medium was removed and replaced with CM.

2.4. Transient transfection and luciferase activity

Transient transfection of cells was performed using FuGENE HD transfection reagent (Roche) following the manufacturer's instructions. For pNF-κB or iNOS reporter luciferase activity assay, pRL-TK was used as an internal control of transfection efficiency. The ratio of luciferase activity to pRL-TK activity in each sample served as a measure of normalized luciferase activity. Luciferase assays were performed by using Dual Luciferase Reporter Gene Assay Kit (Beyotime Biotech. Institute) according to the manufacturer's instructions and results were expressed as the ratio of luciferase to pRL-TK (mean ± S.E.M.).

2.5. Nitric oxide measurement

NO production in live macrophages was detected with the fluorescent probe DAF-FM DA (Invitrogen) by confocal microscopy at excitation/emission maxima of 495/515 nm. Once inside the cells, it is deacetylate by intracellular esterases to become DAF-FM. DAF-FM is essentially non-fluorescent until it reacts with NO. The fluorescence emission intensities of the DAF-FM-stained macrophages (1×10^5/well) were measured by a 96-well plate reader.

Extracellular NO production secretions by macrophages under different treatments in the supernatants were monitored by Griess assay (Promega) according to the manufacturer's instructions.

2.6. iNOS activity assay

We measured iNOS activity by using the Nitric Oxide Synthase Assay Kit (Beyotime). Macrophage-like cells were preincubated with ACs (ratio 1:5) or with CM for 12 h. Then cells were washed twice with PBS so that only the adherent macrophages were retained for iNOS activity assay. The test was performed according to the manufacturer's instructions.

2.7. Laser confocal scanning microscopy (LCSM) and fluorescence resonance energy transfer (FRET) analysis

FRET was used to detect the interaction between GFP-MyD88/CFP-TLR2 and YFP-p85α. Fluorescent emissions from CFP, GFP, YFP, and DAF-FM were measured with LCSM (Zeiss, Jena, Germany), using different excitation wavelengths and detection filters as previously described [19,20].

2.8. Western blot analysis

Cells were lysed with lysis buffer (50 mM Tris–HCl [pH8.0], 150 mM NaCl, 1% TritonX-100, 100 μg/ml PMSF and Protease Inhibitor Cocktail Set I) for 45 min on ice; the fractionation of membranes was prepared using a plasma membrane protein extraction kit according to the instructions of the manufacturer (BioVision, Mountain View, CA). After centrifugation, the expressions of iNOS, HSP70 and β-actin in resulting lysates were analyzed by western blotting [19].

2.9. Statistics

Data are representative of at least three independent experiments and are expressed as mean ± S.E.M. Significant differences