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Caspase-3 but not caspase-8 is activated by mTHPC-PDT and m-THPC-PDT / Doxorubicin in murine hepatoma MH22 cells

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We have studied activation of two apoptotic enzymes induced in murine hepatoma MH-22A cells *in vitro* following photodynamic treatment (PDT) mediated by *meso*-tetra(3-hydroxyphenyl)-chlorin (mTHPC) alone and in combination with Doxorubicin. Caspase-3, the main effector protease of apoptosis, was activated by mTHPC-PDT alone and in combination with Doxorubicin indicating the apoptotic death of the treated cells. However, no significant activation of initiator caspase-8 was registered, thus excluding the involvement of the caspase-8-dependent apoptosis initiation pathway in mTHPC-photosensitised and mTHPC-photosensitised / Doxorubicin-treated cell death.

Key words: PDT, mTHPC, Doxorubicin, apoptosis, caspase-3, caspase-8

INTRODUCTION

Multimodality therapy has become a conventional treatment for cancer, increasing both local tumor control and patient survival in a number of cancer sites. Photodynamic therapy (PDT), as a novel mode of cancer treatment, is an object of intense investigation aimed at incorporation of PDT into multimodality treatment. PDT is based on the use of photosensitising compounds that localise in neoplastic / hyperplastic tissues and become cytotoxic when exposed to light due to production of reactive oxygen species (ROS) [1, 2]. Oxidative stress evokes many intracellular events including apoptosis, and apoptosis has been recorded in cells subjected to PDT [3].

In this study, we followed the activation of two apoptotic enzymes in murine hepatoma MH-22A cells after a combined treatment with PDT and cytostatic drug. We used *meso*-tetra(3-hydroxyphenyl)chlorin (mTHPC) [4], one of the most potent photosensitisers currently available for clinical use [1], as a photosensitiser and Doxorubicin (Dox), a conventional anticancer drug of anthracyclines group, as a cytostatic drug. Anthracyclines are the most utilised antitumor drugs [5], and their apoptosis-inducing properties involving p53 are well known [6].

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There are two major pathways through which apoptosis is induced; one involves death receptors and is exemplified by Fas-mediated caspase-8 activation, and another is the stress- or mitochondria-mediated caspase-9 activation pathway. Both pathways converge on caspase-3 activation, resulting in nuclear degradation and cellular morphological change [7]. Solid evidence exists to show that during oxidative stress the production of ROS triggers a mechanism which, through the release of cytochrome c from mitochondria and caspase-3 activation, leads to apoptosis [8]. However, mTHPC accumulates in Golgi apparatus predominantly, [9] and it has been shown that Golgi-located pro-apoptotic molecules, including Fas receptor, can translocate from Golgi to the plasma membrane in response to the activation of p53 [10]. These findings rise a question of the involvement of caspase-8-mediated pathway in apoptosis induced by Dox-treatment, which activates p53, and mTHPC, which localises in Golgi membranes.

Here, we present data suggesting that mTHPC-PDT alone and in combination with Dox induces apoptosis without activation of caspase-8.

MATERIALS AND METHODS

Materials

mTHPC (kindly provided by R. Bonnett, Queen Mary, University of London, UK) was dissolved in

ethanol as 1 mg/ml stock solution and stored at –20 °C in the dark. Doxorubicin hydrochloride (Ebewe Arzneimittel, Austria, 2 mg/ml) was stored at 4 °C. All experiments were performed using dilutions of the stock solutions with cell incubation media. Caspase-3 substrate acetyl-aspartyl-glutamyl-valyl-aspartyl-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) and caspase-8 substrate acetyl-isoleucyl-glutamyl-threonyl-aspartyl-7-amino-4-trifluoromethyl coumarin (Ac-IETD-AFC) were from Biomol. Other chemicals were from Sigma.

Cell culture

Culture flasks and plates were Primaria™ from BD Falcon. Foetal calf serum (FCS) was from Gibco BRL. Other tissue culture products were obtained from Sigma. MH-22A cells from murine hepatoma were obtained from the Institute of Cytology, Sankt-Petersburg, Russia. The cells were cultured in monolayer in 25 cm^2 flasks in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FCS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine, at 37 °C in 5% CO_2 atmosphere. Cells were subcultured by dispersal with 0.025% trypsin in 0.02% EDTA and replated at 1:3 dilution twice a week.

Cell treatment

Cell exposure to chemicals and light was carried out in 35 mm Petri dishes. The cells were suspended in DMEM supplemented with FCS at a density of 3.5×10^5 cells/ml in a volume of 2 ml per dish. The cells were incubated with 0.15 μg/ml mTHPC in serum-free DMEM at 37 °C in the dark. After 18 h, the extracellular photosensitiser was removed by rinsing the cell monolayer 3 times with room-temperature DPBS, and DMEM with 10% FCS was added. Cells loaded with mTHPC were exposed to light for 45 s. The light source was a LED array UNIMELA-1 (λ = 660 nm), VU Laser Research Centre, Lithuania, the fluence rate at the cellular level was 10 W/m^2 as measured using an IMO (Russia). In the case of combined treatment, 0.25 μg/ml of doxorubicin was added immediately after light exposure. Cell viability was estimated by the crystal violet assay at 24 h after light exposure as described earlier [11].

Caspase activity

The activity of caspase-3 and caspase-8 was monitored in the control untreated cells and in cells treated at various times post-exposure. Assays were based on the cleavage of caspase-specific fluorogenic peptide substrate. According to manufacturer-recommended procedure, approximately 10^6 cells were dislodged by 0.025% trypsin in 0.02% EDTA and harvested by centrifugation for 10 min at 1000 × g, 4 °C. After centrifugation, the cells were resuspended in 4 °C PBS, counted and collected by a second round of centrifugation. Sample cells were lysed for 5 min in a lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM EDTA) on ice (10^7 cells/ml), the cytosolic extract was cleaned by centrifugation for 10 min at 10 000 × g, 4 °C. 10 μl of cell lysat supernatant was added to 80 μl of measurement buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) and 10 μl of 0.2 mM caspase-specific substrate. Fluorescence of 7-amino-4-trifluoromethyl coumarin (AFC), which is proportional to the caspase-3 or –8 activity in the lysed cell samples, was recorded on an Ascent FL fluoroscan (Labsystems, Finland) at 390 nm for excitation and 510 nm for emission for 60 min at 3 min intervals. Data were plotted as arbitrary fluorescence units (AFU) *versus* time for each sample. The slope of the line, fitted to the linear portion of the data, was obtained using linear regression. Caspase activity was calculated using the equation: activity (pmol/min) = slope (AFU/min) x conversion factor (μM/AFU) × assay vol (μl), where the conversion factor is the 1/slope of the AFC (μM) calibration standard *versus* AFU.

Data analysis

Caspase activity was corrected for the cell count and presented as means ± SE of two independent experiments in duplicate. The statistical significance of differences between curves was assessed by a paired t-test, and the statistical significance of differences between data at one time point were assesed by an unpaired t-test. SigmaPlot 2001 for Windows version 7.101 software was used for the statistical analysis.

RESULTS AND DISCUSSION

Murine hepatoma MH-22A cells were photosensitised with mTHPC, which preferentially localises to cellular membranes [12], especially endoplasmic reticulum and Golgi apparatus [9]. Light-activated photosensitisers generate reactive oxygen species that oxidize various biomolecules in close proximity to the site of localization of the photosensitizer [13]. Therefore, proteins synthesized and processed in these cellular compartments could be modified and / or activated by light exposure. On the other hand, Dox inducing apoptosis through the p53 pathway [6] could evoke translocation of pro-apoptotic molecules in response to the activation of p53 [10]. We attempted to elucidate if the mTHPC-photosensitisation alone or in combination with Dox could initiate the caspase-8-mediated apoptosis pathway.

In this study, cell viability was 51 ± 2% (± SE, n = 4) for cells treated with single PDT , 60 ± 2% for cells treated with single Dox, and 31 ± 2% for

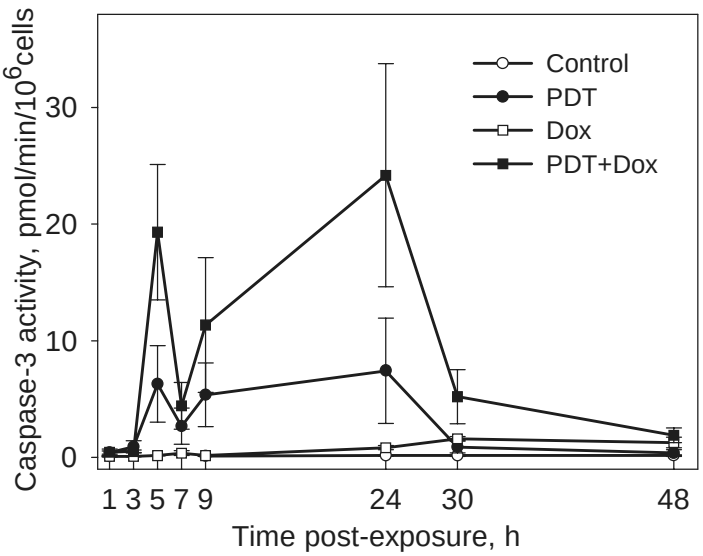

Fig. 1. Activity of caspase-3 in MH-22A cells treated with mTHPC-PDT or/and Doxorubicin. Cells were incubated in the dark in serum-free DMEM containing 0.15 μg/ml m-THPC for 18 h, the medium was replaced with DMEM-containing serum, and cells were exposed to light at λ = 660 nm and fluence 300 J/m^2. After exposure to light, Doxorubicin was added to a final concentration of 0.25 μg/ml. Control, untreated cells; PDT, mTHPC-treated cells exposed to light; Dox, doxorubicin-treated cells: PDT + Dox, mTHPC-treated cells exposed to light and doxorubicin; *bars*, ± SE

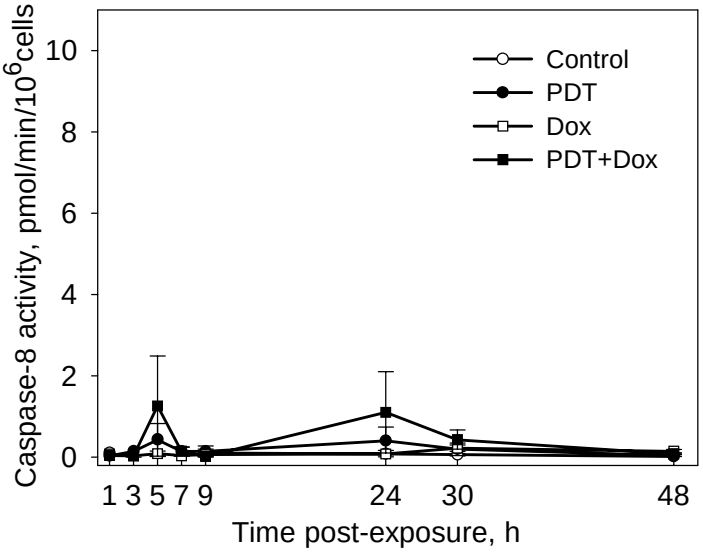

Fig. 2. Activity of caspase-8 in MH-22A cells treated with mTHPC-PDT or/and Doxorubicin. Cells were treated as described in Fig. 1. Control, untreated cells; PDT, mTHPC-treated cells exposed to light; Dox, doxorubicin-treated cells: PDT + Dox, mTHPC-treated cells exposed to light and doxorubicin; *bars*, ± SE

cells treated with PDT/Dox. No manner of caspase-3 activity in untreated control cells was observed.

Cells treated with PDT alone or in combination with Dox expressed the higher caspase-3 activity than untreated control cells with a very similar pattern (Fig. 1). The increase was significant: P = 0.02 in the case of the single PDT, P = 0.01 in the case of PDT/Dox combination for a pairwise comparison of means, and P = 0.05 in the case of comparison between control and combined treatment at a 24 h time-point. Besides the photosensitised cells, a delayed and small-scale, however significant (P = 0.02) increase in caspase-3 activity was recorded in Dox-treated cells. The significant increase in caspase-3 activity indicates the involvement of apoptosis in cell death following mTHPC-PDT and Dox given alone or in combination and the enhancement of apoptosis of m-THPC-photosensitised cells by Dox, and *vice versa* (P = 0.02 for a pairwise comparison between PDT- and PDT/Dox-treated cells, P = 0.01, between Dox- and PDT/Dox-treated cells).

Some momentary and slight increase in caspase-8 activity at 5 h and 24 h post-exposure was observed in cells after combined PDT/Dox treatment (Fig. 2). However, it was not significant: P = 0.27 for a pairwise comparison of means between control cells and cells treated with PDT/Dox, P = 0.45 and 0.42 for a comparison between control and combined treatment at 5 h and 24 h time-points, respectively. The above results lead to the conclusion that our data do not support the involvement of caspase-8-mediated apoptosis initiation pathway in mTHPC-photosensitised and mTHPC-photosensitised / Doxorubicin-treated cell death.

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FOTODINAMINIS IR DOKSORUBICINO KOMBINACIJOS POVEIKIS CASPAZEI-3 IR -8 PELIØ HEPATOMOS MH-22A LÀSTELËSE

S a n t r a u k a

Buvo tirta dviejø apoptozës fermentø aktyvacija peliø hepatomos MH-22A làstelëse po doksorubicino ir/arba *mezo*-tetra(3-hidroksifenil)-chlorino (mTHPC) indukuoto fotodinaminio poveikio (PDT). Gauta, kad svarbiausia apoptozës vyksmo proteazë caspazë-3 buvo aktyvuojama làstelëse tiek po PDT, tiek po PDT kombinacijos su doksorubicinu. Vadinasi, ðios làstelës þuvo apoptozës bûdu, taèiau reikðmingo caspazës-8 iniciacijos aktyvumo nepastebëta. Taigi – nuo caspazës-8 priklausoma apoptozë nebuvo ðiø làsteliø þûties prieþastimi.