Biochemical Pharmacology 83 (2012) 1643–1654

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

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

Mitochondrial superoxide mediates doxorubicin-induced keratinocyte apoptosis through oxidative modification of ERK and Bcl-2 ubiquitination

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ARTICLE INFO

Article history:
Received 20 January 2012
Received in revised form 13 March 2012
Accepted 15 March 2012
Available online 24 March 2012

Keywords:
Apoptosis
Doxorubicin
Keratinocytes
Reactive oxygen species
ERK
Bcl-2

ABSTRACT

Massive apoptosis of keratinocytes has been implicated in the pathogenesis of chemotherapy-induced skin toxicities, but the underlying mechanisms of action are not well understood. The present study investigated the apoptotic effect of doxorubicin (DOX) on HaCaT keratinocytes and determined the underlying mechanisms. Treatment of the cells with DOX induced reactive oxygen species (ROS) generation and a concomitant increase in apoptotic cell death through the mitochondrial death pathway independent of p53. Electron spin resonance and flow cytometry studies showed that superoxide is the primary oxidative species induced by DOX and responsible for the death inducing effect. Ectopic expression of mitochondrial superoxide scavenging enzyme (MnSOD) or treatment with MnSOD mimetic (MnTBAP) inhibited DOX-induced superoxide generation and apoptosis. The mechanism by which superoxide mediates the apoptotic effect of DOX was shown to involve downregulation of Bcl-2 through ubiquitin–proteasomal degradation. Superoxide induces dephosphorylation of Bcl-2 through MAP kinase ERK1/2 inactivation, which promotes ubiquitination of Bcl-2. We also provide evidence for the oxidative modification of ERK1/2 through cysteine sulfenic acid formation. These findings indicate a novel pathway for redox regulation of apoptosis regulatory proteins, which could be important in the understanding of chemotherapy-induced toxicities and development of preventive treatment strategies which are currently lacking.

1. Introduction

Doxorubicin (DOX) is the mainstay chemotherapeutic agent against various hematological malignancies and solid tumors, including breast cancer, lung cancer and sarcoma, but with limited cumulative doses [1,2]. Numerous side effects have been reported in patients undergoing DOX treatment, including skin toxicities such as alopecia and skin ulcerations, myelosuppression and myopathy [1,3–5]. Alopecia or massive hair loss is arguably the most feared and most common side effect of chemotherapy. In addition, occupational cutaneous exposure to DOX has been reported [6,7]. Accumulating evidence indicate that uncontrolled apoptosis plays a key role in skin toxicities of DOX [8,9]. Apoptosis is a tightly regulated process and is executed along two major pathways (extrinsic and intrinsic) [10,11]. The extrinsic pathway involves death receptor activation and induction of death signaling complex leading to apoptosis, while the intrinsic pathway involves the release of cytochrome *c* from the mitochondria which binds to caspase-activating proteins such as Apaf-1 and activates the caspase cascade [12]. Bcl-2 is a key regulator of the intrinsic pathway by interfering with the release of cytochrome *c* or its binding to Apaf-1 through its interaction with Bax [13,14]. Thus, downregulation of Bcl-2 by DOX provides a key mechanism of apoptosis induction by the agent.

DOX has been shown to induce reactive oxygen species (ROS) generation in various cell types, which has been linked to its action [15–17]. Mitochondria are the major source of ROS generation and are involved in apoptotic cell death induced by various stimuli

Abbreviations: DOX, doxorubicin; ROS, reactive oxygen species; zVAD-fmk, benzyloxy-carbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone; zIETD-fmk, benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone; zLEHD-fmk, benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone; control, CNTL; MnTBAP, Mn(III)tetrakis(4-benzoic acid) porphyrin chloride; CAT, cell permeable catalase, catalase-polyethylene glycol; DMTU, dimethylthiourea; MnSOD, manganese superoxide dismutase; H_2DCF-DA, dihydrodichlorofluorescein diacetate; DHE, dihydroethidium; ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LAC, lactacystin; CMA, concanamycin A; Bcl-2-Ub, Bcl-2 ubiquitination; Cys-SOH, cysteine sulfenic acid.

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doi:10.1016/j.bcp.2012.03.010

[18,19]. The role of ROS in Bcl-2 regulation has been reported to occur primarily through ubiquitin–proteasomal degradation [20,21], but the underlying mechanism and specific ROS involved have not been demonstrated. Dephosphorylation of Bcl-2 is an essential step for Bcl-2 ubiquitination [21,22]. One of the major phosphorylation–dephosphorylation signaling cascades is mitogen-activated protein (MAP) kinases, including p44/p42 extracellular signal-related kinases (ERK1/2), c-Jun-N-terminal protein kinase (JNK), and p38 kinase [23,24]. We hypothesized that one or more of these kinases are responsible for the dephosphorylation and ubiquitination of Bcl-2 in response to DOX treatment.

The overall objective of this study was to identify the specific ROS involved and determine the underlying mechanisms of DOX-induced skin toxicities. We identified mitochondrial superoxide as a key mediator of DOX-induced apoptosis and discovered an oxidative protein modification process via cysteine sulfenic acids as a potential regulator of ERK signaling and DOX-induced apoptosis. This novel finding could have a major implication in the regulation of redox-sensitive proteins under oxidative stress conditions. Human keratinocytes were tested in this study since they are the primary target for DOX-induced toxicities. HaCaT keratinocytes, although exhibited alterations in their life-span (immortalizion), were used as they express typical epidermal phenotype [25–27], of which making them common in vitro models to aid the mechanistic studies of skin and hair follicle [28,29].

2. Materials and methods

2.1. Cell culture

Human keratinocyte HaCaT cells were obtained from Cell Lines Service (Heidelberg, Germany) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml of penicillin and 100 μg/ml of streptomycin in 5% CO_2 at 37 °C.

2.2. Reagents

Doxorubicin, Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) and concanamycin A were obtained from EMD Biosciences (La Jolla, CA). *N*-acetyl cysteine (NAC), cell permeable catalase (catalase-polyethylene glycol; CAT), dimethylthiourea (DMTU), lactacystin, and antibody for ubiquitin were obtained from Sigma Chemical (St. Louis, MO). Hoechst 33342, di-hydrodichlorofluorescein diacetate (H_2DCF-DA), dihydroethidium (DHE), and Mitosox™ were obtained from Molecular Probes (Eugene, OR). Caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethylketone (zVAD-fmk), caspase-8 inhibitor benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (zIETD-fmk), and caspase-9 inhibitor benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone (zLEHD-fmk) were from Alexis Biochemicals (San Diego, CA). Antibody for Bcl-2, phospho-Bcl-2 (serine 87), and protein G-conjugated agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for Bax, MAP kinases and phospho-MAP kinases ERK1/2, SAPK/JNK, p38, β-actin, and peroxidase-conjugated secondary antibodies were from Cell Signaling Technology (Boston, MA). PD98059 (MAP kinase ERK1/2 inhibitor), SP600165 (c-Jun N-terminal kinase inhibitor), and SB203580 (p38 MAP kinase inhibitor) were from Calbiochem (La Jolla, CA). Antibody for cysteine sulfenic acid was from Millipore (Billerica, MA).

2.3. Plasmids and transfection

Manganese superoxide dismutase (MnSOD) plasmid was generously provided by Dr. X. Shi (University of Kentucky, School of Medicine, Lexington, KY). Authenticity of the plasmid construct was verified by DNA sequencing. HaCaT cells were transfected with MnSOD or pcDNA3 control plasmid by nucleofection using Amexa Biosystems Nucleofector (Cologne, Germany), according to the manufacturer's instructions. Cells were suspended in 100 μl of nucleofection solution with 2 μg of plasmid and nucleofected using the device program U020. The cells were resuspended in 500 μl of complete medium and seeded in 6-mm cell culture dish, after which they were allowed to recover for 72 h before each experiment. Transfection efficiency was determined by using a GFP reporter plasmid and was found to be approximately 85%.

2.4. Apoptosis assays

Apoptosis was determined by Hoechst 33342 and caspase activation assays. In the Hoechst assay, cells were incubated with 10 μg/ml of Hoechst 33342 for 30 min and analyzed for apoptosis by scoring the percentage of cells having intensely condensed chromatin and/or fragmented nuclei by fluorescence microscopy (Leica Microsystems, Bannockburn, IL). Approximately 1000 nuclei from ten random fields were analyzed for each sample. The apoptotic index was calculated as the percentage of cells with apoptotic nuclei over total number of cells.

2.5. Caspase activity assays

Caspase activity was determined by using APO LOGIX carboxyfluorescein caspase detection kit (Cell Technology, Minneapolis, MN), according to the manufacturer's instructions. After specific treatments, cells were incubated with 10 μl of 30× FAM-DEVD-fmk, FAM-LETD-fmk, or FAM-LEHD-fmk for 2 h in dark for caspase-3, -8, or -9 activity determinations, respectively. The cells were washed with 1× working dilution wash buffer, which was supplied with the kit. The fluorescence signals were measured using a fluorescence microplate reader (FLUOstar, BMG Labtech, Durham, NC) at the excitation and emission wavelengths of 488 and 520 nm, respectively. Caspase activity was expressed as the ratio of fluorescence signal from the treated and control samples.

2.6. ROS detection

ROS generation was measured by flow cytometry and by electron spin resonance (ESR). The former utilizes dichlorodihydrofluorescein diacetate (H_2DCF-DA) as a general oxidative probe and dihydroethidium (DHE) as a superoxide anion probe, whereas the latter uses 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trapper. For flow cytometric measurements, cells were incubated with the probe (10 μM) for 30 min at 37 °C, after which they were washed and resuspended in phosphate buffered saline (PBS) and immediately analyzed for dichlorofluorescein (DCF) intensity using a 485-nm excitation beam and 538-nm band-pass filter, or dihydroethidium (DHE) intensity using a 488-nm excitation beam and a 610-nm band-pass filter (FACSort, Becton Dickinson, Rutherford, NJ). Mean fluorescence intensity was quantified by CellQuest software (Becton Dickinson) analysis of the recorded histograms. For ESR measurements, cells were incubated with the spin trapper DMPO (10 mM) for 10 min at 37 °C in the presence or absence of specific ROS scavengers to aid characterization of the generated free radicals. The ESR signals were measured using a Bruker EMX spectrometer (Bruker Instruments, Billerica, MA) and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate and 1,1-diphenyl-2-picrylhydrazyl as reference