Biochimica et Biophysica Acta 1823 (2012) 1343–1352

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

zVAD-fmk upregulates caspase-9 cleavage and activity in etoposide-induced cell death of mouse embryonic fibroblasts

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ARTICLE INFO

Article history:
Received 26 December 2011
Received in revised form 4 May 2012
Accepted 10 May 2012
Available online 18 May 2012

Keywords:
Caspase-9
zVAD-fmk
p53-dependent cell death
Mouse embryonic fibroblast

ABSTRACT

Caspases are key effectors of programmed cell death. Down- and up-regulation of their activity are involved in different pathologies. In most cells, zVAD-fmk prevents apoptosis. However, unexpected effects of zVAD-fmk have been characterized in different laboratories, cell models and cell death processes. We have previously shown that zVAD-fmk accelerates p53-dependent apoptosis in rat embryonic fibroblasts. In this study, we pursued our investigations on zVAD-fmk effects and focused our study at the mitochondrial level in mouse embryonic fibroblasts (MEFs). In both primary and immortalized (by AgT or 3T9 protocol) MEFs, zVAD-fmk increased etoposide-induced loss of $\Delta\Psi m$. This increase correlated with an increase of the number of apoptotic cells in primary and 3T9 MEFs, but did not in AgT MEFs. In both types of immortalized MEFs, zVAD-fmk regulated neither p53 levels nor transcriptional activities, suggesting that zVAD-fmk acts downstream of p53. In MEFs, zVAD-fmk increased p53-dependent loss of $\Delta\Psi m$, cytochrome c release and caspase-9 activity. Indeed, zVAD-fmk inhibited effector caspases (caspases-3, -6, -7) as expected but increased caspase-9 cleavage and activity in etoposide-treated MEFs. Q-VD-OPh, another caspase inhibitor, also increased both loss of $\Delta\Psi m$ and caspase-9 cleavage in etoposide-treated MEFs. Invalidation of *bax* and *bak* suppressed p53-dependent cell death and zVAD-fmk regulation of this process. Invalidation of *caspase-9* did not inhibit mitochondrial membrane depolarization but suppressed zVAD-fmk amplification of this process. Altogether, our data suggest that caspase-9 activity is upregulated by zVAD-fmk and is involved in an amplification loop of etoposide-induced cell death at the mitochondrial level in MEFs.

1. Introduction

Programmed cell death (PCD) is a physiological process required for embryogenesis, metamorphosis, tissue homeostasis and elimination of cells that are potentially detrimental to the organism. Misregulation of PCD has been implicated in many pathologies, such as cancers, neurodegenerative and autoimmune diseases [1,2]. Different types of PCD have been described including apoptosis, necrosis and autophagy, which diverge on criteria such as the initiating death signals, morphological alterations, mitochondrial events, protease and/or nuclease activations, as well as functional and immunological aspects [3,4]. We focused our study on mitochondrial-dependent cell death induced by the tumor suppressor p53. In response to stresses such as DNA damages, oncogene activation or hypoxia, the transcription factor p53 induces the intrinsic pathway of apoptosis [5,6] by trans-activating genes encoding proteins involved in the induction of apoptosis, such as proapoptotic Bcl-2 family members (Bax, Puma or noxa) [7,8]. These proteins as well as p53 itself [9] can induce mitochondrial modifications, such as mitochondrial internal membrane depolarization and outer membrane permeabilization. These modifications induce the release of apoptogenic proteins, including cytochrome c and Smac/DIABLO, from the mitochondrial inter-membrane space to the cytosol. Cytochrome c release results in the formation of the apoptosome, a large complex composed of cytochrome c, ATP/dATP, Apaf-1 and procaspase-9. The formation of this apoptosome requires the interaction of Apaf-1 and procaspase-9 *via* their respective CARD domain, and activates the initiator caspase-9, which in turn activates effector caspases (caspase-3, -6 and -7) [10,11]. Smac/DIABLO cooperates to the regulation of the apoptotic process by neutralizing the caspase-3, -7, -9 inhibitor XIAP in the cytosol [12,13]. In some cell types, p53 also regulates the extrinsic apoptotic pathway by controlling the expression of genes coding for death receptor family members. This pathway is activated by death receptors able to cross-activate the intrinsic pathway *via* the caspase-8-dependent cleavage of Bid, a proapoptotic Bcl-2 family member [14].

A molecular hallmark of apoptosis is the activation of caspases, cysteinyl proteases that execute cell death through the cleavage of a broad

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doi:10.1016/j.bbamcr.2012.05.013

spectrum of cellular protein targets after an Asp residue [11,15–17]. Apoptotic caspases can be classified as initiator (caspase-2, -8, -9, -10) or effector caspases (caspase-3, -6, -7). The classical apoptotic pathways require a sequential activation of initiator caspases (requiring adapter proteins) and effector caspases (cleaved by activated initiator or effector caspases). Most of the studies on both the function and the regulation of caspases have been performed using pharmacological inhibitors [18]. Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk) is the most used cell permeable broad-caspase inhibitor. In most of the cells, zVAD-fmk prevents the apoptotic process. However, we have previously shown that zVAD-fmk increases p53-dependent cell death in rat embryonic fibroblasts [19–21]. In REtsAFs, a rat embryonic fibroblast cell line immortalized with a temperature-sensitive mutant (tsA58) of the simian virus 40 large tumor antigen (AgT), we have shown that p53-dependent cell death in the presence of zVAD-fmk diverges from the classical pathway. This cell death process induced in the presence of zVAD-fmk could not be inhibited by Bcl-2 overexpression, in contrast to the classical pathway [19,21]. Furthermore, we have shown that zVAD-fmk could modify p53-transcriptional activities in these cells. Indeed, p53-dependent trans-activation was decreased while p53-dependent trans-repression was increased in the presence of zVAD-fmk as shown by the study of p53 target gene expression and microarray analysis [20]. Different laboratories presented evidences that zVAD-fmk can induce switches between apoptotic, necrotic and autophagic cell deaths [22,23] and that zVAD-fmk cannot prevent all types of caspase-dependent apoptosis [19–21,24,25]. These unexpected effects of zVAD-fmk were detected on different cell death pathways, in different cell types and after different cell death stimuli. Altogether, these results suggest that the inability of zVAD-fmk to prevent some apoptotic cell deaths or to induce a switch in the cell death process might be due to the fact that this inhibitor does not inhibit all caspases to the same extent. To test this hypothesis and to progress in the comprehension of zVAD-fmk effect on p53-dependent cell death, we pursued our investigations on the zVAD-fmk effect in mouse embryonic fibroblasts (MEFs), in primary cultures and in immortalized cell lines issued from wild type, Bax$^{-/-}$ Bak$^{-/-}$ and caspase-9$^{-/-}$ mice. In the present study, we show that: (i) zVAD-fmk accelerates mitochondrial membrane depolarization and cytochrome c release occurring during etoposide-induced cell death; (ii) although zVAD-fmk classically inhibited effector caspases, it did not inhibit the initiator caspase-9 but in contrast it induced the accumulation of the active form of this caspase; and (iii) caspase-9 induces a mitochondrial amplification loop during etoposide-induced cell death in MEFs.

2. Materials and methods

2.1. Cell culture and drugs

Primary cultures of mouse embryonic fibroblasts (primary MEFs) were a generous gift from Alice Jouneau (INRA, Jouy-en-Josas, France). Established cell lines from wild type (3T9 MEFs) and Bax$^{-/-}$ Bak$^{-/-}$ (DKO 3T9 MEFs) mouse embryonic fibroblasts were a generous gift from Peter Daniel (Clinical and Molecular Oncology, University Medical Center Charité, Berlin, Germany). SV40 Antigen T immortalized MEFs from wild type (AgT MEFs) and Caspase-9$^{-/-}$ (C9KO AgT MEFs) mice were a generous gift from Richard Flavell (Yale School of Medicine, Department of Immunology, New Haven, USA). MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% (3T9, AgT, C9KO AgT and primary MEFs, HeLa cells) or 15% (DKO 3T9 MEFs) fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 1% Glutamax at 37 °C in 5% CO_2 humidified atmosphere. Etoposide (50 μg/ml, Sigma), which is an inhibitor of Topoisomerase II and a DNA damage-inducing drug, was used to induce p53-dependent cell death. zVAD-fmk (50 μM, Z-Val-Ala-DL-Asp-fluromethylketone, Bachem) and Q-VD-OPh (20 μM, Quinolyl-Val-Asp-OPh, Biovision) were used as caspase inhibitors.

2.2. Flow cytometry analysis of cell death

Cells were plated in 12-well plates at a density of 7.10^4 cells/ml. At 70% confluence, different treatments (etoposide, zVAD-fmk and/or Q-VD-OPh) were performed. Both attached and floating cells were collected and analyzed by flow cytometry after $DiOC_6(3)$ and propidium iodide (PI) staining as previously described [26]. Three parameters were examined: the $DiOC_6(3)$ staining (representative of the mitochondrial membrane potential, $\Delta\Psi m$), cell size (representative of the condensation of the cells), and the PI staining (representative of primary or secondary necrotic cells). In this study, we focused our attention on: cells with low $\Delta\Psi m$ which correspond to cells with low $DiOC_6(3)$ and low PI staining cells; and, apoptotic cells which correspond to cells with small cell size, low $DiOC_6(3)$ and low PI staining cells. MitoTracker® Red CMXRos was used to analyze the mitochondrial membrane potential of transfected cells. Cells were incubated with 150 nM MitoTracker® Red CMXRos for 30 min at 37 °C before flow cytometry analysis.

2.3. ROS production analysis by flow cytometry

3T9 MEFs cells and HeLa cells were plated in 12-well plates at a density of 7.10^4 cells/ml. At 70% confluence, etoposide and/or zVAD-fmk treatments were performed and reactive oxygen species (ROS) production was evaluated by flow cytometry after DCFH-DA staining as previously described (Dumay et al., 2006).

2.4. RT-PCR assay

At 70% confluence, 3T9 MEFs were incubated with or without etoposide +/− zVAD-fmk. After 16 h of treatment, total RNA was isolated using the guanidium isothiocyanate method. RT-PCR was performed to examine the levels of p53, mdm2, p21 and noxa mRNAs as previously described [26]. RT-PCR of 18S rRNA was used as a control.

2.5. Cytosol fraction preparation

At 70% confluence, 3T9 MEFs were incubated with etoposide +/− zVAD-fmk for different treatment times (0, 4, 8, 12, 16 and 19 h). Attached and floating cells were collected and centrifuged 5 min at 200 *g*. Pellets were resuspended in 200 μL of Lysis buffer (10 mM Hepes–KOH pH 7.4, 0.1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM protease inhibitor cocktail AEBSF (Roche)). After 30 min incubation at 4 °C, cell disruption was completed by passing the cells through a 0.4×20 mm needle 10 times. 1/3 of the extract was conserved as the total extract, 2/3 of the extract was centrifuged for 5 min at 4 °C at 52 *g* to eliminate nuclei. The supernatant was further centrifuged 30 min at 4 °C at 7000 *g* to eliminate mitochondria and reticulum. The supernatant corresponded to the cytosol fraction and was analyzed by Western blot.

2.6. Western blot analysis

At 70% confluence, MEFs were incubated with or without etoposide, zVAD-fmk and/or Q-VD-OPh. After different treatment times, cells were harvested, lysed and frozen at −20 °C. Proteins (10–30 μg), from total cell extracts or from cytosolic extracts, were separated in NuPAGE 4–12% Bis–Tris polyacrylamide gels according to the manufacturer's instructions (Invitrogen) and transferred onto a PVDF membrane (Millipore). The primary antibodies used were anti-p53 (Pab 122, gift from E. May, IRSC, Villejuif, France), anti-phospho-p53 (Ser-15, Cell Signaling), anti-actin (Sigma), anti-cytochrome c (BD Pharmigen), anti-VDAC (gift from C. Brenner, UVSQ, Versailles, France), anti-lamin A/C (Cell Signaling), anti-enolase (gift from N. Lamande, College de France, Paris), anti-caspase-9 (5B4, Abcam), anti-cleaved caspase-3 (Asp175, Cell Signaling), anti-cleaved caspase-6 (Asp162, Cell Signaling), and anti-tubulin (MAS078, Sera-Lab). Secondary antibodies (peroxidase-