Mitochondrial translocation of p53 and mitochondrial membrane potential ($\Delta \Psi_m$) dissipation are early events in staurosporine-induced apoptosis of wild type and mutated p53 epithelial cells

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The mitochondrial localization of p53 is an important event in p53-dependent apoptosis. Some p53 mutants defective for transcription also facilitate apoptosis through changes of the mitochondria. Here, apoptosis of HeLa and CaSki cells (p53wt), C33A and HaCat cells (p53mt) and SaOs-2 cells (p53 deficient) was induced by 300 nM staurosporine. We showed that wild-type p53, as well as p53 mutants, were transiently located to the mitochondria with changes in the mitochondrial membrane potential ($\Delta \Psi_m$). However, in C33A cells harboring a p53 mutated on its DNA binding domain, $\Delta \Psi_m$ collapse and Sub-G1 DNA content were reduced compared to p53 wt cells, whereas no significant difference was observed in HaCat cells with a p53 mutated on UV hot spots. In addition, inhibition of the mitochondrial permeability transition pores by cyclosporine A significantly reduced the $\Delta \Psi_m$ loss and the sub-G1 DNA content in p53 positive cells. These results indicate that $\Delta \Psi_m$ collapse is an early and necessary event, which plays an important role in apoptosis of immortal mammalian cells.

Keywords: apoptosis; depolarization; mitochondria; p53.

Abbreviations: ST, staurosporine; CsA, cyclosporine A; MPT, mitochondrial permeability transition; MPTP, mitochondrial permeability transition pore; $\Delta \Psi_m$, mitochondrial membrane potential; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide translocase; AIF, apoptosis inducing factor; APAF-1, apoptosis protease activating factor; NAO, nonyl acridine orange; BSA, bovine serum albumin; PBS, phosphate-buffered saline; wt, wild-type; mt, mutated; HPV, human papillomavirus.

Introduction

Mitochondria play a pivotal role in respiratory and oxidative functions of eukaryotic cells, as well as in apoptotic death. To date, two main apoptotic cascades have been described. Firstly, extrinsic stimuli can induce trimerization of cell membrane-bound death receptors (such as Fas, TRAIL). Then, the death domain can attach adapter molecules, which in turn recruit initiator procaspase 8 inducing its activation. Effector caspases (like caspase 3) are subsequently cleaved which results in DNA fragmentation and death. Caspase 8 can also truncate Bid into tBid which mediates apoptotic mitochondrial dysfunctions after binding to cardiolipin. A second pathway also leads to mitochondrial dysfunction in response to intrinsic stimuli (such as DNA damage, reactive oxygen species). Indeed, mitochondria undergo permeabilization by opening of different mitochondrial membrane pores according to the death stimuli (intrinsic or extrinsic). This results in loss of mitochondrial membrane potential ($\Delta \Psi_m$) and release of apoptogenic factors, like cytochrome c, Smac/Diablo, AIF. Once in the cytosol, cytochrome c associates with procaspases, APAF-1 and ATP to form apopotosomes, which then cleave downstream caspases. However, it has been demonstrated that release of cytochrome c and subsequently caspase activation may occur before any detectable loss of $\Delta \Psi_m$, while release of AIF is strictly dependent on $\Delta \Psi_m$ (for review see10). The nature of pores implicated in the mitochondrial permeability transition (MPT) is not yet fully defined, and several models of pore are involved in regulated MPT. The mitochondrial permeability transition pores (MPTP) are constituted by an outer membrane voltage-dependent anion channel (VDAC) and an inner membrane adenine nucleotide translocase (ANT). Mitochondrial benzo diazepine receptor in the outer membrane, cyclophilin D and cardiolipin in the mitochondrial matrix complete the MPTP. These channels can be inhibited with numerous compounds, like cyclosporine A (CsA) that targets cyclophilin D and rescues cells from apoptosis in some systems. On the other hand, members of the
Bcl-2 superfamily control the mitochondrial membrane permeability by at least two mechanisms. First, pro-apoptotic proteins, like Bax, cause an increase in permeability by formation of conducting channels (for review see18).19 Thus, Bax channels, Bax/Bid pores and Bax/VDAC channels have been distinguished so far and cytochrome c can be shuttled through these channels.1,20–22 Second, anti-apoptotic proteins like Bcl-2, Bcl-xL, which insert into the OMM, prevent an increase of permeability by directly closing the VDAC or by forming heterodimers with pro-apoptotic Bcl-2 family proteins.19,23

Other proteins such as p53 can also reside, at least transiently, in the mitochondria (for review see24). Thus, Marchenko et al.25 have shown that a fraction of p53 localizes rapidly to mitochondria in response to death signals which precedes loss of ΔΨm and cytochrome c release. More recently, Mihara et al.4 have shown that p53 can interact, at the mitochondrial level, with Bcl-2 and Bcl-xL and p53 binds to Bcl-xL by its DNA binding domain. Tumor-derived p53 mutants severely impair in forming Bcl-xL complexes and reduce cell sensitivity to apoptotic stimulus.4

We have previously reported that staurosporine (ST) can induce apoptosis of human cervical carcinoma-derived cells, whether they are human papillomavirus (HPV) positive and p53 wild type (HeLa and CaSkI cells) or whether they are HPV negative and p53 mutated (C33A).26 We next demonstrated in HPV positive cells that ST inhibited expression of MDM2 and E6 viral gene (that do not anymore favour p53 degradation), leading to increased levels of p53. Additionally, the expression of p53-targeted genes, p21waf1 and Bax, was increased while Bcl-2 and Bcl-xL expression was lowered. Moreover, western-blotting performed on sub-cellular fractions showed a transient mitochondrial localization of p53, preceding cytochrome c release, caspase activation and DNA fragmentation. Finally, characteristic morphological signs confirmed the apoptosis execution.27

Given the evidence for a role of p53 in programmed cell death with sometimes dissipation of mitochondrial ΔΨm, we explored by immunocytochemistry the temporal and spatial distribution of p53 in p53wt HeLa and CaSkI cells and in p53mt C33A and HaCat cells upon ST exposure. Thereafter, we investigated the possible connection between p53 and changes in MPT.

Materials and methods

Cell lines

Five human cell lines, collected from ATCC (Rockville, MD), were used in this study. HeLa and CaSkI cells are HPV positive cells derived from human cervical carcinomas. Their p53 status is of wild type (p53wt). C33A cells, also derived from human cervical carcinoma, are HPV negative and their p53 is not competent for trans-activation because of mutation on DNA binding domain (amino acid 273). The epidermal immortalized HaCat cells present a mutated p53 on UV hot spots but not on the DNA binding domain. SaOs-2 cells, derived from osteosarcoma, are deficient in p53 and used as control. HeLa and CaSkI cells were respectively cultured in RPMI (Bio Whittaker Europe, Verviers, France) and EMEM (Bio Whittaker) supplemented with 5% (v/v) fetal bovine serum (Bio Whittaker) and 2mM L-glutamine (Bio Whittaker). C33A cells were cultured in the same medium as Hela cells supplemented with 5% (v/v) sodium pyruvate (Sigma, St Louis, MO). HaCat and SaOs-2 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (Bio Whittaker) and 4mM L-glutamine (Bio Whittaker). All cells were incubated at 37°C under a humidified atmosphere of 95% air and 5% CO2(v/v). They were routinely monitored and found to be free of mycoplasma infection.

Cell treatments

Apoptosis induction. Staurosporine (ST) (Sigma) was dissolved in dimethyl sulfoxide. To induce apoptosis, cells were exposed to 300 nM ST for 15, 30 min, 1, 2, 3, 6 and 12 h to measure ΔΨm variations and for 3, 6, 12 and 24 h to quantify the percentage of cells that present depolarized mitochondria and/or fragmented DNA. For immunocytochemistry studies, cells were treated during 15, 30 min, 1, 2, 4, 6, 8, 10 and 12 h. All experiments were performed at least three times.

Inhibition of mitochondrial membrane depolarization. CsA (Novartis International AG, Basel, Switzerland) was diluted in DMSO. The five cell lines were cultured with ST (300 nM) plus CsA (10 µM) for 24 h and then analyzed for mitochondrial depolarization and sub G1 DNA content. The different cell lines treated for 24 h with only CsA were used as controls.

Immunocytochemistry studies

Cells were plated onto glass coverslips and grown until 60% of confluence. As indicated in experiments, cells were rinsed twice with PBS before fixation and permeabilization with cold acetone/methanol (1:1) for 3 min. Cells were then washed 3 times with cold PBS and incubated for 20 min at room temperature (RT) in PBS-BSA 1%. Fixed cells were incubated for 1 h at RT with mouse primary anti-human p53 antibody (1:200; p53 DO-7, PharMingen, San Diego, CA) diluted in PBS-BSA 0.1%-Triton

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