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HDAC inhibitors induce apoptosis but not cellular senescence in *Gadd*45α-deficient E1A+Ras cells

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ABSTRACT

HDAC inhibitors (HDIs) induce irreversible cell cycle arrest and senescence in E1A+Ras expressing cells. Furthermore, HDIs activate Gadd45α/NF-κB signaling pathway to suppress apoptosis thereby promoting the cell survival. Here, to clarify the role of Gadd45α in realization of the antiapoptotic program, we compared wild-type E1A+Ras cells and the cells with knockout of *gadd45α* gene (Gadd45α−/− cells). As in Gadd45α-expressing E1A+Ras cells, HDIs induce irreversible cell cycle arrest in Gadd45α−/− cells, but the arrested cells do not senesce and eventually die due to activation of the apoptotic death program. These data suggest that the expression of Gadd45α is involved in maintaining the balance of pro- and anti-apoptotic stimuli, while lack or loss of Gadd45 directs the cells to apoptosis after HDIs treatment. Appropriately Gadd45α-deficient cells demonstrate a higher level of pro-apoptotic signals, whereas the anti-apoptotic program is suppressed. The elevated apoptotic background of Gadd45α−/− cells is accompanied by higher levels of Ser15-phosphorylated p53 and p21/Waf1 proteins that additionally commit the cells to HDIs-induced apoptosis. Additionally, loss of Gadd45α protein activates the DDR signaling pathway as demonstrated by nuclear pATM staining, accumulation of γH2AX foci and an increase of single-strand DNA breaks. Thus, in wild-type E1A+Ras cells the p53-dependent expression of Gadd45α is necessary not only for DNA repair and HDI-induced cellular senescence, but also to withstand to apoptosis after DNA damage and stress. Therefore the use of HDIs in combination with agents that block Gadd45α function may have promise for cancer therapy.

1. Introduction

Gadd45 gene family (growth arrest and DNA damage-inducible gene) encodes for the proteins induced by a variety of DNA-damage agents and stress factors. Activation of Gadd45 genes results in growth arrest, DNA repair, cell survival or apoptosis, genomic stability, and modulation of the immune response. The function of Gadd45 is mediated via a complex interplay of physical interactions with other cellular proteins implicated in cell cycle regulation and the response of cells to stress, notably PCNA, p21Waf1, Cdc2/cyclinB1 (Carrier et al., 1999), the MEKK4 and p38 stress response kinases (Liebermann et al., 2011; Tamura et al., 2012). Gadd45 has a tumor-suppressor function because Gadd45-knockout cells can be transformed by a single oncogene such as Ha-Ras (Bulavin et al., 2003). Gadd45α-null mice exhibit significant genomic instability, which is exemplified by aneuploidy, chromosomal aberrations and gene amplification (Hollander et al., 1999). Therefore, Gadd45α appears to be an important player in maintaining the genomic stability. Gadd45α is known to regulate nucleotide and/or base excision DNA repair (NER/BER) (Niehrs and Schafer, 2012). No mutations in *gadd45α* genes were found in various human tumor cell lines (Campomenosi and Hall, 2000). However Gadd45α was identified as a factor in the development of pancreatic cancer as followed from the work where the point mutations in over 13% of tumors from 59 patients with invasive ductal carcinomas of the pancreas have been identified (Yamasawa et al., 2002). Several tumor cell lines have the augmented Gadd45α expression (Carrier et al., 1996). Moreover, the overexpression of

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Gadd45α protein, along with possible p53 loss of function, significantly contributes to poor prognosis, compared with patients with undetectable Gadd45α (Hildesheim and Fornace, 2002).

HDAC inhibitors (HDIs) suppress growth of tumor cells due to induction of cell cycle arrest, senescence or apoptosis. Recent data demonstrate that HDIs can interfere with DNA Damage Response (DDR) thereby sensitizing tumor cells to DNA damaging agents. The final outcome – apoptotic cell death or cell cycle arrest – depends on the type of tumor and cellular context. Rodent primary fibroblasts transformed by *E1A* and *Ras* oncogenes are widely used model of malignant cells to study effects of antitumor agents. Both *Ras* and especially E1A oncogene affect the epigenetic status in the process of transformation, but increased expression of endogenous proto-oncogene is characteristic feature of all tumor cells. Like many tumor cells, E1A+Ras-transformed cells do not cease dividing in response to irradiation and cytostatics. Previously, we have shown that the HDIs are capable of inducing cell cycle arrest and cellular senescence but not apoptosis in E1A+Ras cells. The anti-apoptotic effect of HDIs results from the activation of NF-κB-dependent signaling pathway (Abramova et al., 2010). Many data suggest that NF-κB-mediated cell survival is mostly dependent on functionality of the Gadd45 family members (Zerbini et al., 2004; Tamura et al., 2012). In line with this, inhibition of NF-κB pathway in cancer cells results in Gadd45-dependent induction of apoptosis and inhibition of tumor growth. These findings establish an important role of the Gadd45α family as an essential mediator of cell survival in cancer cells with implications for cancer chemotherapy and novel drug discovery.

Available data allow suggesting that HDAC inhibitors can affect the switching of programs apoptosis and cellular senescence. As *gadd45α* is a HDI-inducible gene, it may also be involved in the regulation of apoptosis and senescence programs. In this paper, we aimed to find out how the knockout of *gadd45α* gene affects the programs of irreversible cell cycle arrest, senescence and apoptosis in HDIs-treated E1A+Ras cells. Here we show that HDIs suppress proliferation of both Gadd45α-expressing and Gadd45α-deficient E1A+Ras-transformed cells evaluated by flow cytometry and cell growth analysis as well as by MTT-test for viability. Similarly to Gadd45α+/+ cells, the Gadd45α−/− transformed cells undergo cell cycle arrest, but they do not demonstrate HDI-dependent cellular senescence exemplified by the absence of SA-betaGal staining. Instead, Gadd45α−/− cells are more sensitive to HDIs treatment as compared with wild-type counterparts due to severe apoptotic cell death. Consistently, HDI-treated Gadd45α−/− cells did not reveal augmentation of NF-κB activity, while demonstrating the elevated levels of caspase-3 activity and transcription of pro-apoptotic genes. Also, they express elevated levels of p53 phosphorylated at Ser15 and p21/Waf1. Hence, the lack of protein Gadd45α sustains the DDR signaling pathway, including nuclear pATM staining, accumulation of γH2AX foci and an increase of single-strand DNA breaks. Altogether, these data confirm that Gadd45α, being a p53-dependent and stress-activated gene, plays an important role in regulation of apoptosis and senescence in E1A+Ras-transformed cells.

2. Materials and methods

2.1. Cell cultures and treatments

E1A+Ras transformed cells were established from primary embryo fibroblasts (Gadd45+/+ wt and Gadd45−/− KO) by Ca-phosphate transfection (Pospelova et al., 1999) and by retroviral infection of E1A Ad5 and activated cHa-Ras oncogenes (Bulavin et al., 2003). Gadd45a-proficient and Gadd45a-deficient MEF cells were infected with pBABE-puro plasmids bearing H-ras and E1A oncogenes and selected in the presence of 2 μg of puromycin/ml. The cells were kindly provided by Dr. Bulavin and Dr. Fornace. In addition, Gadd45+/+ and Gadd45−/− E1A+Ras transformed cells were established by Ca-phosphate transfection of plasmids coding for H-Ras and E1A oncogenes. Wild-type and gadd45−/− E1A+Ras transformed cells obtained by both methods were very similar with respect to the effects produced by HDIs and other agents. Cells were treated with HDIs 4 mM sodium butyrate (NaBut) or 100 nM TSA for 24–72 h, or with genotoxic agents 0.2 μg/ml adriamycin for 40 min or with 12.5 μM etoposide for 40 min (Sigma). The medium was replaced immediately after exposure to genotoxic agents and cells were harvested after 24 h.

2.2. Cell cycle analysis

Cells were washed and permeabilized for 30 min with 0.01% saponin. Then the cells were washed with PBS and incubated with 40 μg/ml propidium iodide, 0.1 mg/ml RNase A for 15 min at 37 °C prior to analysis using FACScan cytometer Bechman Coulter Epicks XL.

2.3. Assessment of mitochondrial membrane potential (Ψ_m)

Cells were loaded with 50 nM tetramethylrhodamine methyl ester (TMRM, Molecular Probes) for 10 min at 37 °C. Alterations of Ψ_m were determined from the fluorescence intensity of TMRM as the transmembrane distribution of this lipophilic, cationic rhodamine derivative is dependent on membrane potential (Scaduto and Grotyohann, 1999). The cells were run immediately for analysis with FACScan cytometer Bechman Coulter Epicks XL, using excitation at 488 nm and detection between 560 and 606 nm.

2.4. Immunofluorescence microscopy

Cells were seeded on coverslips, washed in PBS, fixed in fresh 3.7% paraformaldehyde for 15 min at room temperature, washed in PBS for 10 min and in 0.15 M glycine for 15 min, then permeabilized with 0.2% Triton X-100 for 15 min, and blocked with 3% bovine serum albumin (Sigma) in PBS for 1.5 h. Antibodies were diluted in blocking solution. The coverslips were incubated with primary antibody to pH2AX (Ser139) (Cell Signaling) or pATM (Ser1981) (Calbiochem) overnight at +4 °C and then incubated with secondary Alexa Fluor 488-conjugated goat anti-rabbit antibody or Alexa Fluor 565-conjugated rabbit anti-mouse antibody and 1 mM Topro III (all Invitrogene) for 1 h at room temperature in the dark. Slides were viewed with a Leica DMRE fluorescence microscope.

2.5. Cell extracts, immunoblots, and immunoprecipitations

Cells lysis, immunoblotting and immunoprecipitation were performed as described (Abramova et al., 2010). Proteins were separated by electrophoresis in 8–12.5% polyacrylamide gel in the presence of 0.1% SDS, transferred onto PVDF membrane (Millipore), and probed against caspase-3 (H-277), NF-κB p65 (C-20), IκB-α (C-21), Bax (N-20) (Santa Cruz Biotechnology, Inc.), p21/Waf1 (Ab-6) and p53 (Ab-1) (Calbiochem), phospho-p38 (Thr180/Tyr182), p38, p-p53 (Ser15), pH2AX (Ser139), Akt, GAPDH (14C10) (Cell Signaling). Anti-mouse and anti-rabbit antibodies conjugated with horseradish peroxidase (Sigma) were used as the secondary antibodies. Visualization of membrane-bound proteins was performed by enhanced chemoluminescence (Thermo Sci.).

2.6. Cell fractionation

Cells were washed with PBS, lysed on ice for 20 min in buffer (0.05% digitonin, 250 mM sucrose, 20 mM HEPES pH 7.4,