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HDAC inhibitor-induced activation of NF-κB prevents apoptotic response of E1A + Ras-transformed cells to proapoptotic stimuli

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ABSTRACT

HDAC inhibitors (HDACIs) are capable of suppressing the cell growth of tumour cells due to the induction of apoptosis and/or cell cycle arrest. This allows of considering HDACIs as promising agents for tumour therapy. The final outcome – apoptotic cell death or cell cycle arrest – depends on the type of tumour and cellular context. In this report, we addressed the issue by analysing effects produced in E1A + Ras-transformed MEF cells by HDAC inhibitors sodium butyrate (NaB), Trichostatin A (TSA) and some others. It has been shown that the HDACIs induced cell cycle arrest in E1A + Ras-transformed cells but not apoptosis. The antiapoptotic effect of HDACIs is likely to be a result of NF-κB-dependent signaling pathway activation. HDACI-induced activation of NF-κB takes place in spite of a deregulated PI3K/Akt pathway in E1A + Ras cells, suggesting an alternative mechanism for the activation of NF-κB based on acetylation. HDACI-dependent activation of NF-κB prevents the induction of apoptosis by cytostatic agent adriamycin and serum deprivation. Accordingly, suppression of NF-κB activity in HDACI-arrested cells by the chemical inhibitor CAPE or RelA-siRNA resulted in the induction of an apoptotic programme. Thus, our findings suggest that the activation of the NF-κB pathway in HDACI-treated E1A + Ras-transformed cells blocks apoptosis and may thereby play a role in triggering the programme of cell cycle arrest and cellular senescence.

1. Introduction

HDAC inhibitors are able to induce G1 or G2 cell cycle arrest, apoptosis and/or differentiation of transformed cells (Marks et al., 2000; Weidle and Grossmann, 2000). They selectively affect the expression of a subset of genes, including those positively or negatively regulating cell cycle progression (Van Lint et al., 1996). However, little is known so far about why HDAC inhibitors induce cell cycle arrest in one cell line and apoptosis in another cell line. Cell growth-arrested tumour cells may eventually undergo cellular senescence thereby suppressing tumor progression, but this does not eliminate tumor cells. Correspondingly, the induction of apoptosis in HDAC inhibitor-arrested cells would allow a substantial decrease in tumour cells survival. Programmed cell death (apoptosis) is determined by the interplay of pro- and antiapoptotic signals in a cell (Dragovich et al., 1998).

The NF-κB transcription factor is a key antiapoptotic factor in a cell due to its ability to activate the expression of target genes responsible for cell survival. The NF-κB by itself is a subject for post-translational modifications such as phosphorylation and acetylation, which represent another level of NF-κB-pathway regulation (Quivy and Van Lint, 2004; Perkins, 2006). HDAC inhibitors interfere with pro- and antiapoptotic signals owing to the acetylation of histones as well as various non-histone proteins including the p65 subunit of NF-κB. However, whether the acetylation of the p65 finally activates or suppresses NF-κB target gene transcription seems to depend on the acetylation of the specific sites of the NF-κB subunits and the cellular context.

Deregulation of NF-κB activity is directly associated with cellular transformation (Karin et al., 2002). In E1A-expressing cells, E1A regulates the activity of NF-κB through the binding and inactivation of p300, preventing p300 from carrying out acetylation of NF-κB subunits RelA/p65 (Jennings-Gee et al., 2006) and p50 (Deng and Wu, 2003). In addition, the NF-κB transcription factor is activated in response to oncogenic Ras, and this up-regulation occurs largely through stimulation of the transcriptional function of the NF-κB RelA/p65 subunit (Finco et al., 1997). Moreover, NF-κB is required for Ras-mediated overgrowth foci-formation (Finco et al., 1997). In general, the activation of the NF-κB transcription factor provides protection against apoptosis (Liu et al., 1996; Kim et al., 2006a).

We have previously shown that sodium butyrate induces G1/S arrest of the cycle in E1A + Ras-transformed cells but it does not cause apoptotic cell death (Abramova et al., 2006). Moreover,

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long-term treatment with sodium butyrate induced irreversible cell cycle arrest followed by the induction of cellular senescence (Demidenko et al., 2009). There are recent data showing that NF-κB can function both as an antiapoptotic factor and as a positive modulator of cellular senescence. Importantly, it is also involved in the maintenance of genome stability and the regulation of DNA repair (Wang et al., 2009). To address this issue in more detail, we demonstrate here that the lack of apoptotic response in HDACI-treated E1A + Ras cells results from the activation of the antiapoptotic transcription factor NF-κB and its target genes. Activation of the pathway is provided by an accumulation of the acetylated NF-κB in the nuclei, resulting from an increase in the p65 subunit acetylation and the dissociation of NF-κB from its cytoplasmic inhibitor IκBα. Correspondingly, suppression of NF-κB activity in HDACI-arrested cells by a specific inhibitor of NF-κB activity CAPE or by RelA-siRNA led to a dramatic induction of the apoptotic programme. We suggest that HDACIs, due to the activation of NF-κB and suppression of apoptosis, may play a substantial role in triggering the programme of irreversible cell cycle arrest and cellular senescence in E1A + Ras-transformed cells.

2. Materials and methods

2.1. Cell culture and transfection

MEF E1A + Ras cells were established by Ca-phosphate transfection of E1A Ad5 and activated cHa-Ras oncogenes, as described earlier (Pospelova et al., 1999). For stable plasmid integration, E1A + Ras cells were co-transfected with a luciferase reporter under promoter of NF-κB-responsive element (Promega) or pGL3basic-luc (Promega) and a selective pBABE-puro construct bearing the puromycin resistance gene. The cells were transfected using Lipofectamine-2000 (Invitrogen) as recommended by the manufacturer. For the luciferase assay, cell extracts were prepared, and luciferase activity was determined according to a protocol supplied with the dual luciferase kit (Promega). Each experimental point was carried out in triplicate, and each experiment was performed not less than three times. NaB (Sigma) was added to the culture medium at concentration of 4 mM for 24–72 h, and TSA (Sigma), SAHA (Cayman Chemical Co.), MS-275 (Calbiochem) and Valproic acid (Sigma) at concentrations of 10 μM, 2.5 μM, 1 μM and 2 mM, respectively. The NF-κB and PI3K inhibitors CAPE (Caffeic Acid Phenethyl Ester) and LY 294002 (both from Calbiochem) were added at a concentration of 25 and 10 μM, respectively. Primary MEF cells, minimally transformed 3T3 cells (a gift of Dr. Peter Angel) and NIH 3T3 cells, as well as the human colon cancer cell lines HCT-116 and HT-29, were used to check the effect of HDAC inhibitors on NF-κB activity.

2.2. siRNA-mediated knockdown

Transfections have been carried out in 12-well plates (Corning) in the presence of Lipofectamine-2000 according to the manufacturer's protocol (Invitrogen). siRNA for RelA, or control siRNA, without or together with a reporter (3*kB-luc) were dissolved in OPTI-MEM (Invitrogen) at a ratio of 100 pmol/0.8 μg plasmid DNA/well, and mixed with Lipofectamine-2000 (4 μl) before transfection. The sequence of used siRNA (Sintol) was: RelA-siRNA target: 5′ GCATGCGATTCCGCTATAAAT 3′ siRNA scramble (control): 5′-GGACATATCGTTACACTAGCT-3′.

2.3. RT-PCR

The total cellular RNA was isolated with Trizol (Invitrogen). The RT step was performed with 2 μg of RNA and 1 μg of random hexaprimers. The PCR step was performed in the presence of 100 ng of primers to the cDNA of mouse genes (*vimentin:* 5′-TCAGCTCACCAACGACAAGGC-3′/5′-TTCTTGGCAGCCACGCTTTC-3′, *MMP-2:* 5′-GAT GGCTTCCTCTGGTGCTC-3′/5′-CACCCACAGTGGACATAGCG-3′, *MMP-9:* 5′-GTCATCCA GTTTGGTGTCGC-3′/5′-TGGTGCAGGCAGAGTAGGAG-3′, *XIAP:* 5′-CAGGCCACCTGAGA CTCATG-3′/5′-TTCTGACCAGGCACGATCAC-3′, *Bcl* X_L*:* 5′-AGCTGGTGGTCGACTTTCTC-3′/5′-AAGAGTGAGCCCAGCAGAAC-3′, *gapdh:* 5′-TGTGATGGGTGTGAACCACG-3′/5′-CCAGTGAGCTTCCCGTTCAG-3′). PCRs proceeded for 25–32 cycles: a denaturation step at 95 °C for 1 min, an annealing step (58 °C for *MMP-2, MMP-9, Bcl* X_L and *gapdh*; 55 °C for *XIAP*; 56.7 °C for *vimentin*) for 1 min, and an elongation step at 72 °C for 2 min. PCR products were resolved by electrophoresis in 2% agarose gel. The mouse *gapdh* gene coding for glyceraldehyde-3-phosphate dehydrogenase enzyme was selected as an internal control in a PCR assay. Other used primers are given in Supplementary Data.

2.4. Cell cycle analysis

Cells were washed and permeabilized for 30 min with 0.01% saponin. Then about 10^5 cells were washed with PBS and incubated with 40 μg/ml propidium iodide and 0.1 mg/ml RNase A for 15 min at 37 °C prior to analysis with an ATC300 cytometer (Brucker).

2.5. Cell extracts, immunoblots, and immunoprecipitations

For immunoblotting and immunoprecipitation, cells were lysed in a buffer containing 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM EGTA, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride and protease inhibitors (10 μg/ml of leupeptin, pepstatin and aprotinin) (Bulavin et al., 1999). Proteins were separated by electrophoresis in 10% polyacrylamide gel in the presence of 0.1% SDS, transferred onto a membrane (Immobilon P), and probed with appropriate antibodies. Broad molecular mass coloured markers were used as molecular weight standards (Bio-Labs). For immunoprecipitation, IP-complexes were collected with Protein A-Sepharose beads. As primary antibodies, we used rabbit antibodies to caspase-3 (both uncleaved and cleaved forms), p65 NF-κB subunit, IκBα, and acetylated lysines, and mouse antibodies to GAPDH. 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 (ECL, Amersham Biosciences).

2.6. Nuclear extracts and electrophoretic mobility shift assay (EMSA)

For nuclear extract preparation, cells were lysed in a buffer containing 100 mM HEPES-KOH pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.6% Nonidet P-40, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM EGTA, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (10 μg/ml of leupeptin, pepstatin, and aprotinin). Nuclear fraction was precipitated by centrifugation and lysed in a buffer containing 20 mM HEPES-KOH pH 7.9, 0.6 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and protease inhibitors. The reaction mixture for DNA binding included 5 mM HEPES pH 7.9, 1 mM dithiothreitol, 2.5 mM EDTA, 2.5 mM $MgCl_2$, 15% glycerol, 2 mM spermidine and 5 μg of nuclear extracts. The mixtures were incubated at room temperature for 20 min, and then labelled oligonucleotide was added (30,000 cpm/ng) and incubation was continued for additional 20 min. The NF-κB binding site was used as probe for retardation (italic): 5′AGT TGA *GGG GAC TTT CCC* AGG C 3′. Oligonucleotides were labelled by polynucleotide kinase in the presence of [γ-^{32}P]-ATP. Specific and nonspecific oligonucleotides were added in competition experiments at 10–100-fold molar excesses over the labelled probe. DNA–protein complexes were separated by elec-