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Class I histone deacetylases regulate p53/NF-κB crosstalk in cancer cells

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ABSTRACT

The transcription factors NF-κB and p53 as well as their crosstalk determine the fate of tumor cells upon therapeutic interventions. Replicative stress and cytokines promote signaling cascades that lead to the co-regulation of p53 and NF-κB. Consequently, nuclear p53/NF-κB signaling complexes activate NF-κB-dependent survival genes. The 18 histone deacetylases (HDACs) are epigenetic modulators that fall into four classes (I-IV). Inhibitors of histone deacetylases (HDACi) become increasingly appreciated as anti-cancer agents. Based on their effects on p53 and NF-κB, we addressed whether clinically relevant HDACi affect the NF-κB/p53 crosstalk. The chemotherapeutics hydroxyurea, etoposide, and fludarabine halt cell cycle progression, induce DNA damage, and lead to DNA fragmentation. These agents co-induce p53 and NF-κB-dependent gene expression in cell lines from breast and colon cancer and in primary chronic lymphatic leukemia (CLL) cells. Using specific HDACi, we find that the class I subgroup of HDACs, but not the class IIb deacetylase HDAC6, are required for the hydroxyurea-induced crosstalk between p53 and NF-κB. HDACi decrease the basal and stress-induced expression of p53 and block NF-κB-regulated gene expression. We further show that class I HDACi induce senescence in pancreatic cancer cells with mutant p53.

1. Introduction

The NF-κB family consists of five transcription factors, namely p65/Rel-A, Rel-B, c-Rel, NF-κB1/p50, and NF-κB2/p52 [1,2]. These proteins control immunological functions, cell proliferation, transformation, and drug sensitivity [1,2]. Depending on the upstream signals, the activation of NF-κB occurs via classical or alternative pathways [3]. In addition, replicative stress and DNA damage engage atypical pathways of NF-κB activation [1,4].

Simultaneously to NF-κB activation, replicative stress and DNA damage induce the phosphorylation and acetylation of the tumor suppressor p53 [5]. These posttranslational modifications (PTMs) stabilize p53 and they induce its capacity to activate genes regulating cell cycle progression and cell death [5–7]. The growth-restricting activities of p53 explain why tumors express mutated p53 very frequently. Moreover, mutant p53 acquires new oncogenic, gain-of-function qualities, including the positive regulation of pro-tumorigenic functions of NF-κB [4,8].

In addition to individual functions of NF-κB and p53, there is an intense, context-dependent crosstalk between them [1,4]. The stress- and cytokine-dependent control of several NF-κB target genes relies on chromatin-bound p53/NF-κB p65 complexes in vivo [9,10]. Oren and colleagues confirmed our finding that mutant p53 activates NF-κB and they showed that this pathway controls chronic inflammatory responses and colorectal tumorigenesis [11]. Furthermore, Murphy and colleagues verified the interaction between p53 and NF-κB p65 and revealed that a p53 polymorphic isoform with a proline residue at position 72 (p53-P72, $p53^{P72}$) interacted more avidly with p65 than p53 carrying

Abbreviations: BCL, B cell lymphoma; CLL, chronic lymphatic leukemia; DSB, DNA double strand break; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; MS-275, entinostat; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B cells; PDAC, pancreatic ductal adenocarcinoma; PTM, posttranslational modification; VPA, valproic acid.

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an arginine residue (p53-R72, $p53^{R72}$) [12]. The observation that mice with the $p53^{P72}$ variant show increased inflammatory responses [12] illustrates that p53-p65 interactions affect cancer cell growth as well as immunological functions. The finding that mutant p53 controls NF-κB, cell proliferation, and survival in other systems [4] stresses the importance of the interaction between these transcription factors.

HDACs are epigenetic modifiers that affect NF-κB and p53 at several levels [4,5,13]. HDACs fall into four classes (I, IIa/b, III, IV). Cancer-relevant functions have most frequently been reported for the subgroup of class I HDACs (HDAC1,-2,-3,-8) and for HDAC6 [13–16]. Accordingly, an increasing number of HDACi undergo clinical testing; these agents specifically block zinc-dependent HDACs [13,14]. Of note, the FDA has recently approved four HDACi for the treatment of cancer [17,18].

It is unclear how the attenuation of wild-type p53 by HDACi affects tumor cells under replicative stress, a scenario in which wild-type p53 positively regulates NF-κB-dependent anti-apoptotic genes [4,9]. Therefore, we addressed further details on the replicative stress-induced NF-κB/p53 crosstalk. We investigated whether the HDACi entinostat (MS-275), valproic acid (VPA), and Marbostat-100 affected wild-type and mutant p53 as well as NF-κB-dependent gene expression. As the ability of HDACi to decrease mutant p53 helps to eliminate cancers that depend on this tumor driver [19], we also analyzed if this beneficial activity of HDACi is associated with a modulation of the p53/NF-κB crosstalk. We report that HDACi against class I HDACs are necessary for the expression of mutant p53 and the activity of NF-κB in cancer cells. Thus, HDACi can modulate a biologically relevant crosstalk between p53 and NF-κB to shift the therapeutic outcome to a favorable response.

2. Materials and methods

2.1. Reagents

Drugs, chemicals, and buffers are described in [9,20]. Marbostat-100 is patented as *Mahboobi*, *Siavosh*; *Sellmer*, *Andreas*; *Pongratz*, *Herwig*; *Leonhardt*, *Michel*; *Krämer*, *Oliver*; *Böhmer*, *Frank-Dietmar*; *Kelter*, *Gerhard*; *Preparation of fused heterocyclic compounds as HDAC6 inhibitors and their uses*; *PCT Int. Appl. (2016)*, *WO 2016020369* A1.

2.2. Cells, transfections, luciferase, and MTT assay

For details on cells and assays see [9,20] and Supplemental Table 1; cells were regularly tested to exclude mycoplasma contamination. MCF7 cells were plated at densities of 5×10^4/mL and transfected with Lipofectamine (Invitrogen). Peripheral blood samples were obtained from patients with CLL after informed consent in accordance with the Declaration of Helsinki. Sample collection was approved by the local ethics committee of the University Hospital of Heidelberg as part of the tumor bank of the National Centre for Tumor Diseases, Heidelberg, Germany. Cases matched standard diagnostic criteria for CLL. Both patients had wild-type TP53.

2.3. Flow cytometry analyses

Cell cycle profiles were measured as stated in [9]. Intracellular staining of p53, Survivin, and BCL-XL was done according to the manufacturers' protocols with the antibodies Survivin (D-8) Alexa Fluor® 647, Santa Cruz sc-17779 AF647; BCL-XL Antibody (H-5) PE, sc-8392 PE; and TP53-FITC, BD Biosciences.

2.4. Preparation of cell lysates, immunoblotting, ABCD assays, and microscopy

For lysate preparations, Western blots, ABCD assays, and co-immunoprecipitations see [9,20,21]. Proteins were detected with Western blots generated with X-ray films (grey backgrounds) or with the Odyssey Infrared Imaging System (Licor) using IRDye® 680RD- or IRDye® 800CW-coupled secondary antibodies (white backgrounds). Antibodies were from Santa Cruz Biotechnology: p65, sc8008; p50, sc7178; p53, sc263/6243/fl-393X; STAT1, sc346/417; β-Actin, sc47778; XAF1, sc-398,012; Sigma: β-Actin, A2066/5060, acetylated Tubulin T7451, α-Tubulin T5168; Pharmingen: BCL-XL, 66461A; Novus: Survivin, 500-201L; p52, 05–361, Calbiochem: p53, OP03; Abcam: α-Tubulin, ab 176,560; Novocastra: p53 (CM5); Enzo: HSP90 ADI-SPA 830 (ac88); BD: XIAP, BD-610716; Cell Signaling: p-p65 Ser-536, #3036; Bethyl Laboratories anti-p-Ser317-CHK1 (A300-163A). For ac-*H3*/ac-H4 antibodies see [22]. Western blots were probed for housekeeping genes to ensure equal sample loading. Microscopy techniques are described in [9].

2.5. Senescence associate β-galactosidase (SA-β-gal) staining and analysis of XAF1 promoter methylation

These methods are described as Supplemental information.

3. Results

3.1. NF-κB and p53 target gene induction upon replicative stress and DNA damage

Hydroxyurea potently arrests cells in S phase via the inhibition of ribonucleoside diphosphate reductase, which provides dNTPs for DNA replication [23–25]. Etoposide inhibits topoisomerase II and thereby causes DNA double strand breaks (DSBs) [26]. After 24 h, hydroxyurea arrested HCT116 colon cancer cells in S phase and etoposide caused their accumulation in G2/M phase (Fig. 1A). After 48 h, both drugs caused cell death, which we measured as an occurrence of cells with fragmented DNA by flow cytometry (Fig. 1B).

Concomitantly with these cellular alterations, hydroxyurea and etoposide induced p53 and its direct target gene BAX. Consistent with the literature [9,10,27], hydroxyurea and etoposide evoked an accumulation of the NF-κB targets Survivin and BCL-XL (Fig. 1C).

However, the accumulation of p53 and Survivin as detected by Western blot (Fig. 1C), does not necessarily reflect a co-induction in the same cells. Therefore, we used flow cytometry to assess the putative co-expression of p53 with BCL-XL and Survivin. We stained p53 and one of these proteins with antibodies coupled to fluorescent dyes. This approach verified that hydroxyurea and etoposide caused a joint accumulation of p53 with BCL-XL and Survivin in HCT116 cell populations (Fig. 1D and data not shown).

The cytokine-induced phosphorylation of p65 at serine 536 is linked to its nuclear translocation [28]. Therefore, we analyzed whether hydroxyurea-induced replicative stress also triggers this PTM. However, we did not observe serine 536 phosphorylation of p65 in hydroxyurea-treated HCT116 cells (Supplemental Fig. S1). We consistently observed no alteration in the levels of I-κB proteins, which retain NF-κB subunits in the cytoplasm (data not shown).

These data illustrate the co-regulation of p53/NF-κB in response to various conditions evoking replicative stress and DNA damage. These data agree with our previous findings illustrating that p53 is a permissive factor for NF-κB-dependent gene expression [4,9,10,13].

3.2. Co-regulation of p53 and NF-κB in CLL cells

To test for a chemotherapy-induced p53/NF-κB crosstalk in primary cancer cells, we exposed fresh CLL cells to the DNA synthesis inhibitor fludarabine, which is a standard drug against this incurable disease [29]. We also chose this malignancy because fludarabine induces p53 signaling in CLL cells [30,31] and since such cells depend on p65 for their survival [32]. As expected [30,31], fludarabine potently induced apoptosis in CLL cells (Fig. 2A).