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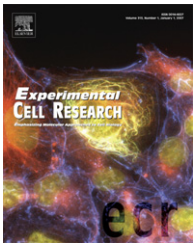

Research Article

The activation of beta-catenin by Wnt signaling mediates the effects of histone deacetylase inhibitors

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ABSTRACT

Most colorectal carcinomas (CRCs) exhibit constitutively active Wnt signaling. We have reported that (a) the histone deacetylase inhibitor (HDACi)[2] sodium butyrate (NaB) modulates the canonical Wnt transcriptional activity of CRC cells *in vitro* and (b) a linear relationship exists between the increase in Wnt transcriptional activity and the levels of apoptosis in ten CRC cell lines treated with NaB. Herein we report that structurally different HDACis modulate Wnt signaling in CRC cells and a mechanism involved in this action is an increase in beta-catenin that is dephosphorylated at Ser-37 and Thr-41 residues. The increase of active (Ser-37 and Thr-41 dephosphorylated) beta-catenin in CRC cells treated with HDACis is initiated at the ligand level and the inhibition of this increase suppresses Wnt signaling and lowers the levels of apoptosis. CRC cells that develop resistance to the apoptotic effects of HDACis exhibit lower levels of active beta-catenin compared to apoptosis-sensitive parental cells and this resistance is reversed by increasing the levels of active beta-catenin. Results from comparative studies between HDACi-resistant and HDACi-sensitive cells suggest that non-histone targets of HDACis mediate the effects on Wnt signaling and apoptosis.

Introduction

Inhibitors of histone deacetylases (HDACis) are promising anticancer agents that preferentially induce growth arrest, differentiation, and apoptosis in malignant, but not normal, cells (reviewed in [1–5]). Several HDACis are currently in clinical trials, and, recently, the U.S. Food and Drug Administration gave approval for the HDACi vorinostat (SAHA) to be used in the treatment of cutaneous T-cell lymphoma. Thus, knowledge of how these agents express their antineoplastic properties is important. The major activity of HDACis is believed to involve inhibition of histone deacetylases, resulting in modified chromatin assembly and altered gene expression [1–5]; however, an increasing body of evidence suggests

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Abbreviations: APC, adenomatous polyposis coli; CRC, colorectal carcinoma; Dkk1, Dickkopf-1; EGFP, enhanced green fluorescent protein; GSK-3beta, glycogen synthase-kinase 3beta; BCT, beta-catenin-Tcf; HDACi, histone deacetylase inhibitors; LRP, low-density lipoprotein receptor-related protein; NaB, sodium butyrate; OA, okadaic acid; PBS, phosphate buffered saline; sFRP, secreted Frizzled related protein; SAHA, suberoylanilide hydroxamic acid; Tcf/Lef, T-cell factor/lymphocyte enhancer factor; TOP/FOP, ratio of luciferase reporter activity of pTOPFLASH to that of pFOPFLASH; TSA, trichostatin A

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that non-histone proteins are essential mediators of HDACi function [6].

We have established that HDACis such as sodium butyrate (NaB) and trichostatin A (TSA) modulate Wnt transcriptional activity in human colorectal carcinoma (CRC) cells [7,8]. Canonical Wnt transcriptional activity is induced by the binding of Wnt ligands to cell surface receptors, resulting in inhibition of glycogen synthase kinase-3 beta (GSK-3 beta) activity [9–12]. When active, GSK-3 beta, in complex with adenomatous polyposis coli (APC) and Axin, promotes the phosphorylation and degradation of beta-catenin [13–15]; however, when GSK-3 beta activity is inhibited, dephosphorylated beta-catenin accumulates and interacts with Tcf/Lef DNA binding proteins [16–20]. Beta-catenin-Tcf (BCT) transcriptional complexes are detected by their ability to drive transcription from Tcf/Lef site-containing promoter constructs [19,20].

The constitutive activation of canonical Wnt signaling due to mutations in *APC* [21–23] and *beta-catenin* [20] is believed to promote cell proliferation and tumorigenesis in the colon. However, we and several other research groups have reported that relatively high levels of Wnt signaling result in apoptosis [24–29]. Our findings indicate that hyper-activation of canonical Wnt transcriptional activity induces apoptosis since (a) there is a linear relationship between the fold induction of Wnt transcriptional activity and the degree of apoptosis in ten human CRC cell lines exposed to NaB, (b) cells with suppressed induction of Wnt activity exhibit a decrease in apoptosis in the presence of NaB, and (c) cell fractions with high Wnt activity have a higher ratio of apoptotic to live cells than cell fractions with low levels of Wnt activity [29]. We have also established that the increase in canonical Wnt activity precedes the apoptotic event since (a) the inhibition of apoptosis by a general caspase inhibitor does not abrogate the increase in Wnt activity (unpublished data), and (b) flow cytometry-sorted cells with high Wnt activity exhibit high levels of both live and apoptotic cells; however, if apoptosis was a prerequisite for induction of Wnt activity, all cells with high Wnt activity should have been apoptotic [29]. Based upon our results and the findings of others [24–29], we hypothesize that the relative levels of Wnt signaling determine whether cells proliferate or commit to undergo apoptosis. These observations and the findings that Wnt signaling is modulated by HDACis suggest that the reason HDACis induce reversible growth arrest or apoptosis in different cell types is at least partially determined by the levels of induced Wnt signaling. Thus, HDACis influence the physiology of cells that do not carry Wnt activating mutations to a lesser extent; however, in cells with a deregulated Wnt pathway, HDACis induce higher levels of Wnt which lead to apoptosis.

In the present investigation, we have primarily focused on the effects of NaB in CRC cells, since butyrate is a natural fermentation product of dietary fiber in the colon [30] and the preventive role of dietary fiber against CRCs has been convincingly demonstrated in the most recent completed clinical studies [31,32]. We have, however, also evaluated the effects of other HDACis, two of which are in clinical trial, on the modulation of Wnt activity and apoptosis in CRC cells. We report herein that structurally different HDACis also modulate Wnt signaling in CRC cells and a mechanism involved in this effect is an increase in Ser-37/Thr-41-dephosphorylated beta-catenin initiated at the ligand level. Inhibition of the increase in active beta-catenin levels suppresses the induction of Wnt signaling and the induction of apoptosis by these HDACis. In addition, CRC cells resistant to the apoptotic effects of HDACis exhibit lower levels of Ser-37/Thr-41 dephosphorylated beta-catenin compared to apoptosis-sensitive parental cells; this resistance can be reversed by increasing the levels of active beta-catenin. The findings suggest that non-histone targets of HDACis likely mediate the effects of these agents on Wnt signaling and apoptosis.

Materials and methods

Cells, plasmids, transfections, luciferase assays, and clonal growth assays

Human CRC cell lines and human transformed embryonic kidney 293 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in alpha-MEM with 10% fetal bovine serum. Transfections were performed with Lipofectamine 2000 (Life Technologies, Rockville, MD) or GenePorter (Gene Therapy Systems, San Diego, CA) as reported previously [8,29]. The vector pRSV-TK (Promega Corp., Madison, WI) was used for normalization of transfection efficiency. The following vectors were provided by various researchers: mouse Dickkopf1 (Dkk1) and LRP5 (Dr. D. Wu, Yale University School of Medicine, New Haven), secreted Frizzled-related proteins (sFRP) 1, 2, 4, and 5 (Dr. H. Suzuki, Sapporo Medical University, Japan), pTOPFLASH (TOP) and pFOPFLASH (FOP) and Tcf1 expression vector (Dr. Hans Clevers, Netherlands Institute for Developmental Biology, Utrecht, Netherlands), small T antigen (Dr. E. Sontag, University of Texas Southwestern Medical Center, Dallas, Texas). Tcf4 expression vector was from Upstate Biotechnology (Lake Placid, NY). Luciferase assays were performed using a Turner Luminometer and a Dual Luciferase kit (Promega, Madison, WI). Treatment with NaB (Sigma, St. Louis, MO) was performed at 5 mM, with Trichostatin A (Alexis Biochemicals, Carlsbad, CA) at 1 μM, with SAHA (BioVision Research Products, CA) at 10 μM, with MS-275 (Alexis Biochemicals) at 10 μM, and with LiCl (Sigma) at 20 mM. Okadaic acid (Sigma) was used at 20 nM final concentration and was added to cells 15 min prior to exposure to NaB.

Transfections with EGFP-TOP and EGFP-FOP were performed with cells plated at 2×10^6 per well in 12-well dishes 24 h before transfection with 2 μg of DNA and Lipofectamine 2000. At 5 h, cells from each well were washed, trypsinized, and aliquoted into 6 wells of 24-well dishes. At 24 h after transfection, cells were treated with NaB, okadaic acid (OA), or the combination of these two agents. In cotreatment experiments, cells were preincubated with OA for 15 min before the addition of NaB. Cells were harvested 24 h later and subjected to flow cytometry as described [29]. Transfections with inhibitors of Wnt activity were carried out with GenePorter in 24-well dishes with 0.4 μg or 1 μg of Dkk1, sFRP, dnLRP5, or empty expression construct and 0.1 μg or 0.4 μg of luciferase