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Histone deacetylase inhibitor sodium butyrate enhances cellular radiosensitivity by inhibiting both DNA nonhomologous end joining and homologous recombination

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ABSTRACT

HDAC inhibitors have been proposed as radiosensitizers in cancer therapy. Their application would permit the use of lower radiation doses and would reduce the adverse effects of the treatment. However, the molecular mechanisms of their action remain unclear. In the present article, we have studied the radiosensitizing effect of sodium butyrate on HeLa cells. FACS analysis showed that it did not abrogate the γ-radiation imposed G2 cell cycle arrest. The dynamics of γ-H2AX foci disappearance in the presence and in the absence of butyrate, however, demonstrated that butyrate inhibited DSB repair. In an attempt to clarify which one of the two major DSBs repair pathways was affected, we synchronized HeLa cells in G1 phase and after γ-irradiation followed the repair of the DSBs by agarose gel electrophoresis. Since HR is not operational during G1 phase, by this approach we determined the rates of NHEJ only. The results showed that NHEJ decreased in the presence of butyrate. In another set of experiments, we followed the dynamics of disappearance of RAD51 foci in the presence and in the absence of butyrate after γ-radiation of HeLa cells. Since RAD51 takes part in HR only, this experiment allows the effect of butyrate on DSB repair by homologous recombination to be assessed. It showed that HR was also obstructed by butyrate. These results were confirmed by host cell reactivation assays in which the repair of plasmids containing a single DSB by NHEJ or HR was monitored. We suggest that after a DSB is formed, HDACs deacetylated core histones in the vicinity of the breaks in order to compact the chromatin structure and prevent the broken DNA ends from moving apart from each other, thus ensuring effective repair.

1. Introduction

The agents used in cancer therapy are genotoxic, i.e., they interact with DNA inflicting different types of lesions that hinder DNA replication and trigger apoptosis. One of the most potent and universal anticancer agents is γ-radiation which introduces DSBs in DNA. The rationale in applying such agents is that cancer cells are actively proliferating, while normal cells are not, and for this reason cancer cells should be predominantly killed. Unfortunately, many types of normal cells are also actively proliferating and are consequently killed by the therapeutic agent. For this reason, anticancer drugs have severe side effects and it is not possible to treat the patients with therapeutic doses high enough to eliminate all cancer cells. In an attempt to circumvent this problem, enhancers of the anticancer drugs have been developed and tested. The enhancers are typically epigenetic agents that do not directly interact with DNA but, when applied simultaneously with the anticancer agents, increase their killing effect. By their mechanism of action, the potential enhancers fall in several categories. First, they can abrogate the cell cycle checkpoints that arrest cells at discrete phases of the cell cycle until the damage is repaired. Checkpoint pathways represent complex signaling cascades in which the phosphatidylinositol-3-kinases family members ATM and ATR phosphorylate many DNA repair and cell cycle control proteins to achieve cell cycle arrest. For this reason, protein kinase inhibitors are considered as enhancers that can override the cell cycle checkpoints [1]. Second, there are agents that can serve as transcription activators and increase the expression of proapoptotic molecules such as caspases and p21. Such agents shift the balance between apoptosis and survival towards apoptosis, thus increasing the killing effects of the anticancer drugs [2]. Finally, agents that block repair pathways eventually lead to cell death and are also potential enhancers of anticancer drugs [3–5].

Abbreviations: DSB, double strand break; FACS, fluorescence activated cell sorting; HAT, histone acetyltransferase; HDAC, histone deacetylase; HR, homologous recombination; NHEJ, nonhomologous end joining.

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Many compounds have been tested as enhancers for anticancer drugs. The most promising are histone deacetylase (HDAC) inhibitors, some of which have already undergone clinical trials as supplementary agents in chemo- and radio-therapy [6,7]. Sodium butyrate is one such inhibitor. It is the sodium salt of the butyric acid, a four carbon fatty acid that is a natural metabolite in many organisms, including bacteria populating the gastrointestinal tract. Roles for butyrate have been established in a number of epigenetically controlled activities such as cell differentiation, proliferation, motility, induction of cell cycle arrest, apoptosis [8], and even in memory formation [9]. However, neither the mechanisms by which butyrate induces cellular differentiation and suppresses growth, nor the mechanisms through which it regulates the decision between apoptosis and survival are known in detail [10]. Microarray assays of global gene expression profiles have shown that the expression of over 450 genes are significantly regulated by butyrate in bovine kidney epithelial cells. Most genes were down-regulated, but over 30 were up-regulated [11]. Among the down-regulated genes were genes crucial for initiation of DNA synthesis, including the MCM and Orc proteins which are essential for the assembly of the prereplication complex. CDC2/Cdk1 and related cyclins were also down-regulated. On the other hand, genes related to apoptosis were up-regulated. In another assay over 10,000 genes were responsive to butyrate regulation in human epithelial cells [12]. Butyrate exerts modulatory effects on nuclear proteins and DNA through inhibition of histone deacetylation and phosphorylation, and hypermethylation of cytosine residues. Of these various effects, maintaining the acetylation of core histones, which is achieved by inhibition of HDACs, is the best characterized [7]. This posttranslational modification has been correlated with the processes of chromatin assembly and transcription [13]; actively transcribed genes are associated with hyperacetylated core histones, while repressed genes are associated with hypoacetylated histones [14]. In addition, core histones associated with DNA replication origins are hypoacetylated when the origins are inactive, but undergo hyperacetylation before their firing [15,16]. Acetylation of core histones occurs at specific lysine residues in the NH_2-terminal tails, which leads to relaxation of the compact chromatin structure and allows transcriptional activators to access DNA [17]. The state of histone acetylation is controlled by the opposing activities of two distinct families of enzymes; histone acetyltransferases (HATs) and HDACs. Core histone acetylation and deacetylation are also connected with checkpoint activation and repression [18]. However, recent reports have suggested that the relation of chromatin function and histone acetylation could be more complex than the simple scheme in which acetylation means activity and deacetylation means inactivity. It has been shown that acetylation turnover, which can be very rapid, rather than acetylation status may be important [17,19]. This could explain the results of microarray assays in which butyrate treatment, which causes global and permanent histone acetylation actually brings about repression of most of the genes assayed. In the present paper we examine the mechanism by which butyrate sensitizes HeLa cells to γ-irradiation. We find that butyrate inhibits both NHEJ and HR and suggest that core histone deacetylation and compaction of the chromatin structure prevent the broken DNA ends from moving away from each other.

2. Materials and methods

2.1. Cell treatment

HeLa cells (obtained from the American Type Culture Collection) were cultured in monolayer in D-MEM with 10% foetal bovine serum supplemented with antibiotics in 5% CO_2 atmosphere. To obtain cells synchronized in early G1 phase of the cell cycle, cells were cultured in the presence of 0.2 μg/ml nocodazole for 12 h and were vigorously shaken to detach the mitotic cells. These cells were cultured in fresh medium for 2 h to attach and enter G1 phase [16]. Irradiation was carried out with ^{137}Cs γ-source at a rate of 2 Gy/min. For treatment with butyrate, a 1 M stock solution of Na-butyrate was prepared by titration of butyric acid (analytical grade, Sigma) with 12 M NaOH to pH 7.5. The cell cycle distribution was determined by fluorescence activated cell sorting (FACS) analysis. Cells were washed with phosphate buffered saline (PBS), pH 7.4, fixed in 70% ethanol, treated with 20 μg/mL RNase A at 37 °C for 30 min and stained with 20 μg/mL propidium iodide at room temperature for 90 min. 2×10^4 cells/sample were analyzed with a Becton Dickinson (Facscalibur) cell sorter, using CellQuest software (Becton Dickinson).

2.2. Gel electrophoresis

For electrophoresis of DNA, cells were embedded in 1% low melting agarose blocks. The blocks were treated with lysis buffer containing 1% SDS, 1 M NaCl, 50 mM EDTA, 50 mM Tris–HCl, pH 7.8, and 0.1 mg/mL Proteinase K at 37 °C for 24 h. The blocks were washed 3 times for 1 h each time with 50 mM Tris–HCl, 50 mM EDTA, pH 7.8 at 4 °C and then treated with 0.1 mg/mL RNase A at 37 °C for 2 h. Electrophoresis was carried out in 0.5% agarose gel in Tris/acetate buffer at 2 V/cm overnight and DNA was stained with ethidium bromide.

2.3. γ-H2AX and RAD51 foci visualization

HeLa cells were grown and treated on coverslips. At the specified times the medium was removed and cells were fixed in 2% formaldehyde for 7 min at room temperature. The cells were permeabilized with 0.3% Triton X-100 for 5 min, blocked in 3% bovine serum albumin in PBS for 1 h and then treated with anti-γ-H2AX (Abcam), or anti-RAD51 antibody (Abcam) followed by secondary antibodies conjugated with dyes. The slides were observed with an AxioVert 200 M fluorescent microscope (Carl Zeiss, Hamburg, Germany) and the pictures were taken with AxioCam HR camera (Carl Zeiss).

2.4. Host cell reactivation assays

To measure NHEJ and HR repair activities separately, we employed host cell reactivation assays in which reporter plasmids were specifically constructed to measure either NHEJ or HR [20]. The NHEJ reporter cassette contains a GFP gene with an engineered 3 kb intron from the Pem1 gene. The Pem1 intron contains an adenoviral exon flanked by recognition sequences for HindIII and I-SceI endonucleases for induction of DSBs. The I-SceI sites are in inverted orientation. I-SceI has a nonpalindromic recognition sequence and for this reason the two inverted sites generate incompatible DNA ends that can be linked by NHEJ but not by HR. The intact NHEJ cassette is GFP negative as the adenoviral exon disrupts the GFP ORF. Upon induction of DSB by I-SceI, the adenoviral exon is removed and NHEJ restores the function of the GFP gene.

The HR cassette is also based on GFP-Pem1. The first exon of the GFP-Pem1 contains a 22 bp deletion combined with insertion of 3 restriction sites - I-SceI/HindIII/I-SceI. The deletion ensures that GFP cannot be reconstituted by an NHEJ event. The two I-SceI sites are in inverted orientation, so that I-SceI digestion leaves incompatible ends. The first copy of GFP-Pem1 is followed by a promoterless/ATGless first exon and intron of GFP-Pem1. The intact construct is GFP negative. Upon induction of a DSB by I-SceI, functional GFP is reconstituted by gene conversion between the