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N-methyl-N′-nitro-N-nitrosoguanidine induces and cooperates with 12-O-tetradecanoylphorbol-1,3-acetate/sodium butyrate to enhance Epstein-Barr virus reactivation and genome instability in nasopharyngeal carcinoma cells☆

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ABSTRACT

Seroepidemiological studies implicate a correlation between Epstein-Barr virus (EBV) reactivation and the development of nasopharyngeal carcinoma (NPC). Moreover, N-nitroso compounds are known chemical carcinogens in preserved foodstuffs and cigarettes and have been implicated as risk factors contributing to the development of NPC. Here, NPC cell lines latently infected with EBV, NA and HA, and the corresponding EBV-negative NPC cell lines, NPC-TW01 and HONE-1, were used as the model system in this study. We demonstrate that the reactivation of EBV increases with increasing concentrations of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). MNNG at a single non-toxic concentration (0.1 μg/ml) did not induce discernible reactivation of EBV, but repeated treatment with this concentration of MNNG significantly induced viral reactivation. Furthermore, low dose MNNG (0.1 μg/ml) had a synergistic effect with 12-O-tetradecanoylphorbol-1,3-acetate (TPA)/sodium butyrate (SB) (10 ng/ml and 0.75 mM, respectively) on EBV reactivation. Through promoter activity assay, MNNG was found to enhance the transcriptional activity of Rta on Rta and Zta promoters. Using siZta to block EBV reactivation, the concomitant induction of genome instability was diminished indicating that reactivation is critical for enhancing genome instability. Co-treatment with TPA/SB and MNNG markedly increased the levels of γH2AX and ROS formation in NPC cells, which may be responsible for the increase of genome instability. Our findings offer a possible mechanism by which N-nitroso compounds induce reactivation of EBV and contribute to malignant progression by enhancing genome instability in NPC cells.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a common cancer in the southern part of China, Taiwan and southeastern Asia. Genetic, environmental and microbial factors have been incriminated in the carcinogenesis of NPC [1,2]. Epstein-Barr virus (EBV) is a member of human herpesvirus. Retrospective, prospective and cross-sectional sero-epidemiological studies strongly support the notion that EBV plays an etiological role in the carcinogenesis of NPC [3–6].

Ingestion of Cantonese-style salted fish has been linked to NPC [7]. Case–control studies have demonstrated that consumption of salted fish increases the risk for NPC [8,9]. It was also found that volatile N-nitroso compounds are present in foods from NPC high

Abbreviations: EBV, Epstein-Barr virus; NPC, Nasopharyngeal carcinoma; SB, Sodium butyrate; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; TPA, 12-O-tetradecanoylphorbol-1,3-acetate.

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risk areas and may be an etiological factor for NPC [10,11]. The preserved food samples from NPC high risk areas were also found to contain EBV inducers and mutagens, as well as N-nitroso compounds [12]. These results suggest that N-nitroso compounds may contribute to the carcinogenesis of NPC, however, the underlying mechanism remains unclear [13].

Genome instability is a hallmark of cancers and considered to be a cause of carcinogenesis [14,15]. Chromosomal abnormalities have been demonstrated in NPC tissues [16–20]. Taking chromosomal 3p LOH as a marker, it was found that NPC patients have the highest incidence of genome instability, followed by residents in NPC high risk areas, low risk areas, then areas with very few cases of NPC [21]. This observation suggested that genome instability is closely associated with the development of NPC. Because both chemicals and virus have been demonstrated to be co-carcinogens in the development of cancer [22], it would be critically important to examine the interplays between EBV and N-nitroso compounds and their effects on the genome instability of NPC.

Recently, we have demonstrated that recurrent reactivations of EBV by 12-O-tetradecanoylphorbol-1,3-acetate (TPA) and sodium butyrate (SB) could enhance the genome instability, invasiveness and tumorigenicity of NPC cells [23]. This result supports the notion that chemical reactivation of EBV may contribute to the carcinogenesis of NPC [24]. Because N-nitroso compounds were implicated in the carcinogenesis of NPC, we determined to study whether they reactivate EBV and induce genome instability in NPC cells. N-nitrosamide such as N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) can methylate all oxygen and most nitrogen atoms in DNA directly, making MNNG as a good model chemical to elucidate the interplays between EBV and N-nitroso compounds. Moreover, previous reports have also indicated that MNNG could enhance transformation of human leukocytes caused by EBV [25], and induce the reactivation of EBV in lymphocytes [26]. However, those studies were carried out in B lymphocytes and do not explain any effect on the carcinogenesis of NPC.

In this study, we demonstrate that the reactivation of EBV in NPC cells was increased with increasing concentrations of MNNG. Furthermore, low dose MNNG (0.1 μg/ml) had a synergistic effect with TPA/SB on EBV reactivation. Taken together, these data provide evidence for the cooperation between chemical carcinogens and EBV reactivation in the enhancement of genome instability and may contribute to carcinogenesis of NPC.

2. Materials and methods

2.1. Cell lines

NPC-TW01 (TW) and HONE-1 cells are EBV-negative nasopharyngeal carcinoma cell lines derived from nasopharyngeal tumors from Chinese patients [27,28]. NA and HA cells are EBV-positive NPC cells derived from NPC-TW01 and HONE-1 cells, respectively [29]. NPC-TW01 and NA cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with streptomycin, penicillin and 10% fetal bovine serum (HyClone, Waltham, MA) at 37 °C with 5% CO_2. HONE-1 and HA cells were cultured in RPMI 1640 medium (HyClone) supplemented with streptomycin, penicillin and 10% fetal bovine serum at 37 °C with 5% CO_2. G418 (400 μg/ml, Ameresco, Solon, OH) was added to the medium of NA and HA cells to maintain the EBV genome in the cells [29].

2.2. Cytotoxicity

Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and treated with MNNG (Sigma–Aldrich) at various concentrations for 48 h. After treatment, the relative survival rate of cells was measured by standard MTT assay as described previously [30].

2.3. Antibodies

Monoclonal antibodies including 4F10 (anti-Zta) [31], 467 (anti-Rta) and 88A9 (anti-EA-D) [32] were used for detection of EBV proteins. The anti-β-actin antibody AC-15 was purchased from Sigma. FITC-conjugated mouse antibody to γH2AX was purchased from Upstate.

2.4. Western blot analysis

Cells were lysed in lysis buffer (3% sodium dodecyl sulfate, 1.6 M urea, 4% β-mercaptoethanol). Western blot analysis was carried out as described previously [23].

2.5. Detection of EBV-reactivation in NPC cells after chemical treatment

The cells were treated with (1) MNNG at various concentrations for 48 h, (2) 0.1 μg/ml MNNG once daily for 5 days, (3) TPA/SB for 72 h or (4) TPA/SB for 72 h in combination with 0.1 μg/ml MNNG for the final 48 h. In order to reveal the synergistic effect of MNNG and TPA/SB, a sub-optimal concentration of TPA/SB (10 ng/ml and 0.75 mM, respectively) was used throughout this experiment, as compared to 40 ng/ml TPA and 3 mM SB for optimal induction of EBV [29]. Expression of the lytic EBV proteins, BRLF1 (Rta), BZLF1 (Zta) and BMRF1 (EA-D) were detected as EBV-reactivation markers and cellular β-actin was detected as an internal control.

2.6. Promoter activity assay

To assay the Rta or Zta promoter activity, 0.5 μg Rp or Zp-firefly luciferase reporter plasmid (pRp-Luc or pZp-Luc) and 0.1 μg renilla luciferase reporter plasmid (pWP1, Promega) as a control were co-transfected into NPC-TW01 cells. Twenty-four hours post-transfection, the cells were treated with (1) MNNG at various concentrations for 48 h, (2) TPA/SB for 72 h or (3) TPA/SB for 72 h in combination with 0.1 μg/ml MNNG for the final 48 h. The cells were harvested and subjected to the luciferase assay with a Dual-Glo assay kit (Promega). The firefly luciferase activity of each sample was normalized to the renilla luciferase activity. The activity of the cell treated with solvent was taken as the control and set at 1.0. The fold of relative promoter activity was calculated by dividing that of the drug-treated transfectants by that of solvent control transfectants. To determine the transactivational function of Rta or Zta, 0.5 μg Rta protein expressing plasmid (pRts15) or 0.5 μg Zta protein expressing plasmid (pCMV-Zta) was added into the promoter activity assay system, respectively.

2.7. Micronucleus formation assay

To determine whether the genome instability is a result of EBV reactivation in EBV-positive NPC cells by chemicals treatment, we evaluated the occurrence of micronuclei (MN), which arise from acentric chromatids and have been recognized as a marker for genome instability [33]. The cells were seeded onto glass slides at a density of 2×10^5 cells/well in 6-well plates for 24 h and then treated with MNNG and TPA/SB as described in Section 2.5. Micronucleus formation was evaluated as described previously. At least 1000 cells were examined for each experiment [23].