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Rapamycin enhances lytic replication of Epstein-Barr virus in gastric carcinoma cells by increasing the transcriptional activities of immediate-early lytic promoters

Man Wang[a,*], Wei Wu[a], Yinfeng Zhang[a], Guoliang Yao[b], Bianli Gu[c]

[a] *Institute for Translational Medicine, Medical College of Qingdao University, Qingdao, 266021, China*
[b] *Department of General Surgery, The First Affiliated Hospital of Henan University of Science and Technology, Luoyang, 471003, China*
[c] *Henan Key Laboratory of Cancer Epigenetics, Cancer Hospital, The First Affiliated Hospital, College of Clinical Medicine, Medical College of Henan University of Science and Technology, Luoyang, 471003, China*

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ABSTRACT

Epstein-Barr virus (EBV), a human herpesvirus, is linked to both epithelial and lymphoid malignancies. Induction of EBV reactivation is a potential therapeutic strategy for EBV-associated tumors. In this study, we assessed the effects of rapamycin on EBV reactivation in gastric carcinoma cells. We found that rapamycin upregulated expression of EBV lytic proteins and increased the viral proliferation triggered by the EBV lytic inducer sodium butyrate. Reverse transcription-qPCR, luciferase activity assays, chromatin immunoprecipitation and western blotting were employed to explore the mechanism by which rapamycin promotes EBV reactivation. Our results showed that rapamycin treatment resulted in increased mRNA levels of EBV immediate-early genes. Rapamycin also enhanced the transcriptional activities of the EBV immediate-early lytic promoters Zp and Rp by strengthening Sp1 binding. Repression of the cellular ataxia telangiectasia-mutated/p53 pathway by siRNA-mediated knockdown of the ataxia telangiectasia-mutated gene significantly abrogated virus reactivation by rapamycin/sodium butyrate treatment, indicating that the ataxia telangiectasia-mutated/p53 pathway is involved in rapamycin-promoted EBV reactivation. Taken together, these findings demonstrate that rapamycin might have the potential to enhance the effectiveness of oncolytic viral therapies developed for EBV-associated malignancies.

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous human pathogen that persists as a life-long, latent infection in 95% of the world's population (Liu et al., 2016). EBV infection is closely linked to a wide range of human malignancies, such as nasopharyngeal carcinoma (NPC), gastric carcinoma (GC), Burkitt's lymphoma (BL) and Hodgkin's lymphoma (HL) (Ali et al., 2015). During primary infection, EBV infects cells in a lytic form and then enters into a latent state in infected cells (Cohen, 2000). Lytic EBV infection can be activated by chemical stimuli, including sodium butyrate (SB), 12-*O*-tetradecanoyl-phorbol-1,3-acetate (TPA), anti-immunoglobulin (anti-Ig), and transforming growth factor-β (TGF-β) (Zauner et al., 2010). EBV reactivation can lead to expression of immediate-early (IE), early (E) and late (L) proteins (Ersing et al., 2017). The IE proteins (Zta and Rta) switch EBV from a stage of latency to the lytic cycle by initiating an expression cascade of other lytic proteins (Jha et al., 2016). Zta, a member of the basic region leucine zipper (bZIP) transcription factor family, transactivates lytic promoters by interacting with Zta response elements (ZREs) (Kalla et al., 2010; Schelcher et al., 2007). Zta also serves as an important factor for viral DNA replication (Rennekamp et al., 2010). Similarly, Rta activates EBV lytic gene expression by binding to Rta response elements (RREs) (Heilmann et al., 2012).

Due to the high prevalence of EBV infection, there is an urgent need to develop effective therapies for EBV-associated malignancies. Accumulating studies have proven that EBV persists as a latent infection in host cells and that latent EBV infection is implicated in the etiology of EBV-associated cancers (Bollard, 2013; Grywalska and Rolinski, 2015; Tang et al., 2014). In these cancers, EBV is present exclusively in tumor cells and not in normal cells, suggesting that virus-targeted therapy might be utilized to treat EBV-associated malignancies (Wildeman et al., 2012). Previous studies have shown that switching the latent virus to the lytic phase directly kills EBV-positive tumor cells (Du et al., 2016; Shyamasundar et al., 2016; Wang et al., 2004).

* Corresponding author.
E-mail address: wangman@qdu.edu.cn (M. Wang).

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Moreover, activating EBV lytic replication renders EBV-infected cells more susceptible to the cytotoxic effects of antiviral drugs (Sides et al., 2013). Therefore, virus-targeted therapy might constitute a novel therapeutic approach for EBV-associated tumors.

Rapamycin, a specific inhibitor of mechanistic target of rapamycin (mTOR), possesses anti-tumor activity and is a potential chemotherapeutic drug against EBV-associated tumors (Furukawa et al., 2013; Kawada et al., 2014). For example, rapamycin suppresses expression of Kaposi's sarcoma-associated herpesvirus (KSHV) lytic proteins and significantly reduces viral production (Nichols et al., 2011). Moreover, rapamycin is reported to markedly increase the replication and spread of herpes simplex virus (HSV) (Fu et al., 2011). In addition, Kosowicz et al. (Kosowicz et al., 2017) found that some B-cell receptor (BCR) signaling inhibitors, such as ibrutinib, idelalisib and dasatinib, can suppress BCR-mediated EBV reactivation. The authors also confirmed that immunosuppressant drugs such as cyclosporine and tacrolimus but not rapamycin inhibit BCR-driven EBV reactivation. Moreover, mTORC2 is implicated in BCR-driven virus reactivation, whereas FKBP12 binding alone is not sufficient to inhibit EBV reactivation. Further investigation indicated that BCR signaling induces EBV reactivation not only in tumor cell lines but also in naturally infected patient B cells, a process that can be blocked by BCR signaling inhibitors. Another study showed that rapamycin-mediated suppression of mTOR alters EBV reactivation in a cell-type specific manner (Adamson et al., 2014). Rapamycin treatment of EBV-positive B cells inhibits EBV reactivation, suggesting that EBV reactivation is dependent upon the mTOR pathway in EBV-positive B cells. Conversely, rapamycin promotes virus reactivation by increasing the levels of the viral immediate-early or early proteins in EBV-positive epithelial cell lines. However, the mechanisms underlying rapamycin-promoted EBV reactivation remain unclear. In the present study, we investigated whether rapamycin affects sodium butyrate (SB)-induced EBV reactivation in the human gastric carcinoma cell line SNU-719, which is naturally infected with EBV (Oh et al., 2004). We found that rapamycin significantly increased the expression levels of EBV lytic proteins and resulted in elevated levels of intracellular and extracellular EBV DNA. We further explored the mechanism by which rapamycin stimulates EBV reactivation, revealing that rapamycin could increase the promoter activities of *Zta* (Zp) and *Rta* (Rp). Moreover, rapamycin improved the binding of the transcription factor Sp1 to Zp and Rp, thus promoting the initiation of EBV lytic replication. Finally, our results implicated the cellular ataxia telangiectasia-mutated (ATM)/p53 pathway, which is required for EBV reactivation, in rapamycin-regulated EBV reactivation. These findings provide an alternative strategy for effective virus-targeted therapies for EBV-associated malignancies.

2. Materials and methods

2.1. Cell lines

SNU-719, AGS-EBV and GT38 are EBV-positive gastric carcinoma cell lines. MKN-74 and AGS are EBV-negative gastric carcinoma cell lines. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37 °C in a humidified CO_2 incubator.

2.2. Cell viability assay

Cell viability was estimated using Cell Counting Kit-8 (CCK-8; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Cells (5×10^4 cells/ml) were seeded in 96-well plates. After incubation for 24 h, the cells were treated with dimethyl sulfoxide (DMSO), rapamycin (5 nM), sodium butyrate (SB, 3 mM) or rapamycin/SB for 72 h. CCK-8 solution (10 μl) was then added to each well for 3 h, after which the absorbance at 450 nm (OD450) was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.3. Cell treatment

Cells were pretreated with rapamycin (5 nM) for 24 h. SB (3 mM) was then added to the cultures to induce EBV lytic replication. After incubation for 12 h or 24 h, the cell lysates were collected and analyzed for expression of viral or cellular proteins by western blotting.

2.4. Western blotting

Equal amounts of protein (40 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore Co., MA, USA). The membranes were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline/0.1% Tween-20 (TBST) and then incubated with primary antibodies overnight at 4 °C. The following antibodies were used for western blotting: anti-Zta (Argene, Verniolle, France), anti-Rta (Argene), anti-TK (Abcam, Cambridge, UK), anti-EAD (Millipore, Temecula, CA, USA), anti-DNase (Biorbyt, San Francisco, CA, USA), anti-Sp1 (Cell Signaling Technology, Beverly, MA, USA), anti-ATM (Cell Signaling Technology), anti-ATM-pS1981 (Cell Signaling Technology), anti-p53-pS15 (Cell Signaling Technology), anti-p53 (Cell Signaling Technology), anti-Sp1-pS101 (Active Motif, Carlsbad, CA), and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA). The blots were then incubated with secondary antibodies and developed using enhanced chemiluminescence (ECL).

2.5. Quantification of viral DNA

SNU-719, AGS-EBV and GT38 cells (1×10^6 cells/well) were exposed to rapamycin (5 nM) for 24 h and then incubated with SB (3 mM) for 48 h. Intracellular viral genomic DNA was isolated from cell pellets using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA). The cell culture medium was collected at 5 days post-treatment and passed through 0.45-μm filters. The supernatants were digested with DNase I at 37 °C for 1 h, and the reaction was stopped by EDTA at 70 °C for 10 min followed by proteinase K digestion. The released viral DNA in the supernatants was extracted as previously described (Tsai et al., 2011a) and quantitated using real-time PCR. The EBV *BALF5* gene was amplified with the following primers: forward, 5′-CGGAGTT GTTATCAAAGAGGC-3′, and reverse, 5′-CGAGAAAGACGGAGAT GGC-3′.

2.6. Reverse transcription-qPCR (RT-qPCR)

Total RNA was extracted from SNU-719 cells using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Real-time PCR was conducted using SYBR green PCR Master Mix with a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The real-time PCR primers used in this study were as follows: *Zta* forward, 5′-AATGCCGGGCCAAGTTTAAGCAAC-3′, and reverse, 5′-TTGGGCACA TCTGCTTCAACAGGA-3′; *Rta* forward, 5′-GAGCGATGAGAGACCCATA TTC-3′, and reverse, 5′-GAACATACCTTCCCGGCTATC-3′; internal reference *GAPDH* forward, 5′-GGAAGGTGAAGGTCGGAGTCAACGG-3′, and reverse, 5′-CTCGCTCCTGGAAGATGGTGATGGG-3′. The results were normalized to *GAPDH* expression. All of the samples were analyzed in triplicate.

2.7. Luciferase reporter assay

Zp and Rp reporter plasmids were constructed as previously described (Wu et al., 2013). SNU-719 and MKN-74 cells (1×10^6 cells/well) were seeded in 24-well plates for 24 h and then transfected with the Zp or Rp plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 4 h of transfection, rapamycin (5 nM) was added for 24 h as a pre-treatment. The cells were then exposed to SB (3 mM) for 24 h. Luciferase reporter activity was measured using a luminescence reader

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