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Enrichment of stem-like cell population comprises transformation ability of Epstein–Barr virus latent membrane protein 2A for non-transformed cells

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ABSTRACT

Epstein–Barr virus (EBV) is a representative human oncogenic virus that causes malignancies of various cell lineages. LMP2A, an EBV-encoded latent membrane protein, is expressed in EBV-associated malignancies of various cell lineages. LMP2A caused visible tumor formation transplanted in nude mice when transferred to immortalized non-transformed fibroblasts, NIH3T3. LMP2A-expressing cells showed higher ability of colony formation in soft agar than empty vector-transfected control cells, although the expression of LMP2A did not cause focus transformation in low serum concentrations. LMP2A expression increased the size of Hoechst 33,342 dye excreting side population (SP), in which cancer-initiating cells or cancer stem-like cells were enriched. SP increase by LMP2A was also responsible for colony formation in soft agar. The LMP2A-mediated SP increase depended on the activations of Stat3, MEK/ERK, and PI3K pathways, and on upregulation of *HMGA2*. Enrichment of SP, stem-like cells, by LMP2A promoted the transformation capability of LMP2A from non-transformed cells. The enrichment of stem-like cell population by a virus-encoded factor might explain the oncogenic functions of oncogenic viruses.

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1. Introduction

Human oncogenic viruses cause various malignancies after a long latent period in a limited population of virus carriers. A few infected cells evade the immune system and await an opportunity to grow. Alternatively, they modify the microenvironment to expand within the body. Epstein–Barr virus (EBV) is a representative human oncogenic virus that causes malignancies of various cell lineages, including Burkitt lymphoma, nasopharyngeal and gastric carcinoma, and some mesenchymal tumors (Fukayama et al., 2001, 1994, 2008; Fukayama and Ushiku, 2011; Takada, 2000; Young and Rickinson, 2004). EBV maintains a latent type of infection in malignant cells, with characteristic patterns of the expression of latent genes such as EBNA1, 2 and 3 s and latent membrane protein (LMP) 1 and 2A. The gene expression patterns are classified as Latency I (EBNA1, LMP2A), Latency II (EBNA1, LMP1, LMP2A), and Latency III (EBNA1, EBNA2, EBNA3s, LMP1, LMP2A) according to the type of malignant cell and the status of host immunocompetence. One common viral protein, EBNA1, resides in host nuclei, where it maintains viral DNA in an episomal form, although oncogenic functions of EBNA1 have not been identified conclusively. LMP2A is also expressed constantly in EBV-associated malignancies, suggesting that this protein might be a driving force in tumorigenesis. LMP2A is a 12-pass transmembrane protein that includes a 119-amino-acid cytoplasmic domain at its N-terminus. This domain includes motifs that associate with signaling molecules in the cytoplasm. The outer membrane domain of LMP2A has no function as a receptor (Longnecker, 1994). LMP2A is therefore regarded as a constitutive signal activator that is independent of exogenous stimulation (Longnecker, 1994; Young and Rickinson, 2004) involving the MAPK, PI3K/Akt, NF-κB, and Wnt pathways (Caldwell et al., 1998; Fukayama et al., 2008; Fukuda and Longnecker, 2007; Hino et al., 2009; Morrison and Raab-Traub, 2005; Siler and Raab-Traub, 2008; Stewart et al., 2004). However, most such studies have been performed using established cancer cells. Few researchers have examined the role of LMP2A in the dynamic process of transformation.

To ascertain the role of LMP2A in viral oncogenesis, we used an immortalized non-transformed cell, NIH3T3. Because it is not established from cancer cells, the analysis of its transformation process might clarify the oncogenic processes of EBV, especially the earliest phases of transformation toward the neoplastic cells (McCoy et al., 1983). In the present study, we specifically examined stem cell-like cells. One characteristic is their ability to excrete Hoechst 33,342 dye. These cells are detected as side populations (SP) using fluorescence-activated cell sorting (FACS) analysis.

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Cancer-initiating cells have been identified within the SP fraction of cancer cells. They are responsible for tumor initiation, metastasis and recurrence (Dean et al., 2005; Hadnagy et al., 2006; Hirschmann-Jax et al., 2004; Patrawala et al., 2005; Zhou et al., 2007, 2001). Consequently, it is our aim to address whether and how oncogenic viruses affect stem-like cells during the transformation process and how they promote oncogenesis.

2. Materials and methods

2.1. Expression vector constructions and cell lines

LMP2A expressing vector was constructed using the *LMP2A* cDNA (gift from Dr. Paul J. Farrell, Tumor Virology, Division of Investigative Science, Imperial College, London, United Kingdom) with a DNA fragment coding FLAG/HA tag, which was inserted into a retrovirus vector, pMx-IRES Puro vector (pMx-IP vector, gifted from Prof. Toshio Kitamura, The Institute of Medical Science, The University of Tokyo) (Kitamura, 1998; Nakatani and Ogryzko, 2003). The pMx-IP empty vector or pMx-IP FLAG/HA-LMP2A vector was transfected into PLATA packaging cells (from Dr. Toshio Kitamura) using a FuGene6 transfection kit (Promega Corp., Madison, WI, USA). Then, NIH3T3 cells were infected using the supernatants of PLATA packaging cells. The retrovirus-infected cells were selected with puromycin (Merck Millipore, Darmstadt, Germany) at 2 μg/ml for more than 3 days, until the control cells without transfection had died completely. They were maintained as bulk clusters to avoid compounding effects from insertional mutagenesis.

2.2. Transplantation of the cells in nude mice

NIH3T3 cells expressing pMx-IP empty vector or pMx-IP FLAG/HA-LMP2A vector (or the deletion mutants, Δ21–36, Δ21–64) were collected and resuspended in 1×10^6 cells in 100 μl HBSS (Life Technologies Inc., Carlsbad, CA, USA). These cells were then injected subcutaneously into 8-week-old female nude mice (Charles River Japan Inc., Kanagawa, Japan), following institutional and national regulations. Tumor formation was confirmed using histopathological analysis.

2.3. Reverse transcription-PCR

Reverse transcription (RT)-PCR was performed as described in an earlier report (Hino et al., 2009). We used the following primer pairs: LMP2A, 5′-GGGGCAGTGGAAATAGAACA-3′ (F) and 5′-GCCAATGAGGAAAATCAGGA-3′ (R); GAPDH, 5′-GAAGGTGAAGGTCGGAGTC-3′ (F) and 5′-GAAGATGGTGATGGGATTC-3′(R).

2.4. Real time PCR analysis

Total RNA was prepared using ISOGEN II (Nippon Gene Co. Ltd., Toyama, Japan) from cells. Total RNA (0.5 μg) was used for the subsequent synthesis of cDNA with the ReverTra Ace qPCR RT Kit (Toyobo Co. Ltd., Osaka, Japan) as recommended by the manufacturer. The mRNA levels were measured using an ECO Real-Time PCR system (Illumina Inc., San Diego, CA, USA) and KAPA SYBR Fast qPCR Kit (Kapa Biosystems Inc., Woburn, MA, USA) with the following primer pairs: LMP2A, 5′-ATGACTCATCTCAACACATA-3′ (F) and 5′-CATGTTAGGCAAATTGCAAA-3′ (R); GAPDH (same sequences as RT-PCR). All PCRs were performed in a 10 μL volume, using 48-well PCR plates (Illumina Inc.). The cycling conditions were 95 °C for 1 min (enzyme activation) followed by 45 cycles of 95 °C for 5 s, 58 °C for 30 s. After amplification, the samples were heated slowly from 55 °C to 95 °C with continuous reading of fluorescence to obtain a melting curve. The relative mRNA level was calculated using the arithmetic formula, where is the difference between the threshold cycle of a given target cDNA and that of an endogenous reference cDNA.

2.5. Focus formation assay and soft agar colony formation assay

Focus formation assay was performed according to procedures described by Tsuei et al. (2004). In total, 2×10^4 cells were seeded into a six-well plate with DMEM containing 0.5% fetal bovine serum. After 2 weeks, cells were observed for counting.

The colony formation capability in soft agar was assayed in 4×10^4 cells per well in 0.33% agar with 2 ml DMEM and 10% fetal bovine serum, which was overlaid on the basal layer of 0.5% agar consisting of 1.5 ml × 1 DMEM (diluted with 5× DMEM; Nitta Gelatin Inc., Osaka, Japan) and 10% fetal bovine serum per well in a six-well tissue culture plate. To evaluate the effect of signal pathways, IS3 295: Stat3 inhibitor (National Cancer Institute, USA) (Turkson et al., 2005), U0126: MEK inhibitor (Merck Millipore), or LY294002: PI3K inhibitor (Cell Signaling Technology Inc., Beverly, MA, USA) was added to both the lower layer and upper layer. At 2 weeks after seeding the cells, we counted the cell colonies which included more than 10 cells under a microscope.

2.6. Side population analysis

The cultured cells were suspended at 1×10^6 cells/ml in HBSS (Life Technologies Inc.) with 5.0 μg/ml Hoechst 33,342 (Sigma–Aldrich Corp., St. Louis, MO, USA) with or without verapamil (150 μM) for 30 min at 37 °C. Then cells were resuspended in 1 μg/ml propidium iodide (PI) (Sigma–Aldrich Corp.) containing HBSS. The cells were sorted with blue (488 nm) and UV (355 nm) lasers using FACS Vantage SE (Becton-Dickinson, Franklin Lakes, NJ, USA), and the living cells with Hoechst Blue (422/444 nm fluorescence) and Hoechst Red (585/542 nm). Side population (SP) cells were defined as Hoechst Blue^{low} and Hoechst Red^{low} populations, and as non-SP as Hoechst Blue^{high} and Hoechst Red^{high} populations. The SP cells (1×10^3 cells), after cell sorting, were subject to colony formation assay, as described above.

To evaluate the effects of signal pathways, the cells were seeded in a culture flask. Inhibitors were added the next day. After incubating the cells for 6 days with or without kinase inhibitors, the SP fractions of the cells were analyzed as described above.

2.7. Phosphorylated kinase antibody array

Phosphorylated kinase antibody array was performed using Human Phospho-Kinase Antibody Array (ARY003; R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Phosphorylated kinase levels were detected by chemiluminescence. Then the signal intensity was normalized by the actin expression level from the same protein lysates.

2.8. Western blot analysis

Western blot analysis was performed as described previously (Hino et al., 2009). Whole cell lysates were prepared with high salt RIPA buffer (1% Triton X-100, 0.1% SDS, 500 mM NaCl, 50 mM Tris–HCl [pH 7.4], 10 mM $MgCl_2$, 0.5% sodium deoxycholate) (Watanabe et al., 2009) with Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). The extracts were subjected to sodium sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with the antibodies against HA (H9658, Sigma–Aldrich Corp.), HMGI-C (sc-23,684, Santa Cruz biotechnology Inc., Santa Cruz, CA), Akt1/2 (sc-1619, Santa Cruz biotechnology Inc.), Phospho-Akt (Ser473) (#9271, Cell Signaling Technology Inc.), Stat3 (#9132, Cell Signaling Technology Inc.), Phospho-Stat3 (Tyr705) (#9131, Cell Signaling Technology Inc.), p44/42 MAPK

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