Survival Advantage of EBV-Associated Gastric Carcinoma: Survivin Up-regulation by Viral Latent Membrane Protein 2A

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Abstract

EBV-associated gastric carcinoma is a distinct subset of gastric carcinoma infected with EBV, which shows latency I type expression of EBV latent genes (EBNA1, EBER, BARF0, and LMP2A). To clarify the role of EBV in this type of gastric carcinoma, the cell biological characteristics (growth, apoptosis, and migration) were evaluated in gastric carcinoma cell lines (MKN-1, TMK1, MKN-74 and MKN-7) with and without infection of recombinant EBV harboring the neomycin resistance gene. The infection reiterated the latency I type infection, but the only difference observed in EBV-infected gastric carcinoma cell lines was the resistance to serum deprivation–induced apoptosis. Comparative analyses of transcripts of apoptosis-associated genes in MKN-1 and EBV–MKN-1 and subsequent quantitative reverse transcription-PCR analysis showed up-regulation of the cellular survivin gene in EBV-infected gastric carcinoma cell lines. Small interfering RNA–mediated knockdown of survivin increased apoptosis in EBV–MKN-1 to the level of the original MKN-1 cells. Transfection of EBV-latent genes into MKN-1 showed that LMP2A, but not EBNA1, EBER, or BARF0, up-regulated survivin gene expression. LMP2A-mediated survivin up-regulation in gastric carcinoma was inhibited with a nuclear factor–κB (NF-κB) inhibitor, Bay 11-7082. In parallel with these findings in vitro, survivin expression was frequent in carcinoma tissues of gastric carcinoma by immunohistochemistry, and significantly more in EBV-associated gastric carcinoma (12 of 13) than in EBV-negative gastric carcinoma in the advanced stage (P = 0.0307). Thus, EBV uses its latent protein, LMP2A, to activate the NF-κB–survivin pathway to rescue EBV-infected epithelial cells from serum deprivation, and up-regulation of survivin may play a role in the progression of this specific type of gastric carcinoma infected with EBV. [Cancer Res 2008;68(5):1427–35]

Introduction

EBV-associated gastric carcinoma is a distinct subset of gastric carcinoma, accounting for 10% or less of total gastric carcinoma (1, 2). A causal role of EBV in gastric carcinoma has been suggested on the basis of the clonal nature of EBV in neoplastic cells (3), the presence of EBV in all cancer cells, and the absence of EBV in noncancerous mucosa (4); however, the exact role of EBV in the development and progression of this specific type of gastric carcinoma has not been clarified. EBV-associated malignant neoplasms are classified into three types, latency I, II, and III, according to the expression profile of EBV-latent genes (5). EBV-associated gastric carcinoma, as well as Burkitt’s lymphoma, belongs to latency I, in which the expression of viral latent genes is restricted to EBV-determined nuclear antigen 1 (EBNA1), EBV-encoded poly(A) RNAs (EBER), latent membrane protein 2A (LMP2A), and transcripts from the BamHI A region (BARF0; refs. 6–8). Latency II neoplasm includes nasopharyngeal carcinoma and Hodgkin’s lymphoma and is characterized by the expression of LMP1 (9), a transmembrane protein with transforming capacity for rodent fibroblasts. EBNA2 (10), EBER3A, and EBNA3C, essential for immortalizing resting B-lymphocytes, are additionally expressed due to the lack of effective immune mechanisms in patients with latency III neoplasms, such as lymphomas in AIDS or organ transplant patients (11, 12). Because latency I neoplasms arise in nonimmunodeficient patients without the expression of LMP1 or EBNA2, the question has been raised about the role of EBV in these neoplasms. In the case of Burkitt’s lymphoma, however, loss of EBV from the EBV-positive lymphoma cell line, Akata, abolishes colony-forming capacity in soft agar and tumorigenicity in nude mice, demonstrating that EBV contributes to the tumorous growth of latency I–type neoplasms (13, 14).

One crucial problem to investigate this issue in EBV-associated gastric carcinoma is the lack of an ideal experimental model, like the Akata cell line in Burkitt’s lymphoma. It is well known that EBV is hardly maintained in epithelial cells in culture; therefore, to reiterate the role of EBV infection in EBV-associated gastric carcinoma in vitro, we have systematically compared the cell biological characteristics (growth, apoptosis, and migration) of gastric carcinoma cell lines with and without infection of recombinant EBV. In this screening, we observed that resistance to serum deprivation–induced apoptosis is characteristic of EBV-infected gastric carcinoma cell lines. Subsequent analyses showed that up-regulation of the cellular survivin gene, by viral latent protein LMP2A, is responsible for the survival advantage of EBV-infected cells. Survivin is the smallest member of a family of proteins, known as inhibitors of apoptosis protein (IAP; ref. 15), which plays a key role in the regulation of apoptosis and cell division. Using immunohistochemistry, we also showed that survivin expression was significantly high in the advanced stage of EBV-associated gastric carcinoma in vivo.

LMP2A is a viral protein that is expressed as a transmembrane protein in latently infected cells of latency I, II, and III. LMP2A functions to inhibit normal B-cell signal transduction by mimicking an activated B-cell receptor, but simultaneously activates the phosphatidylinositol 3-kinase/Akt pathway for cell survival in latently infected B-cells (16). The expression of LMP2A induces alterations in gene transcription similar to those observed in Reed-Sternberg cells of Hodgkin’s lymphoma. On the other hand, the
function of LMP2A and its modulation of the signal pathway have not yet been fully clarified in epithelial cells, especially stomach epithelial cells. The present study will provide an insight into the mechanisms of the development of a representative latency I epithelial cell malignancy (i.e., EBV-associated gastric carcinoma).

Materials and Methods

Cell lines, culture, and reagents. The gastric carcinoma cell lines used in the study were MKN-7, MKN-74, TMK1, and MKN-1 (17–19), which were originally derived from gastric carcinoma with histologic features of well, moderately, and poorly differentiated adenocarcinoma and adenocarcinoma, respectively. The gastric carcinoma cell lines were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS (MP Biomedicals), penicillin (40 units/ml), and streptomycin (50 μg/ml) at 37°C in a 5% CO2 incubator.

For inhibitor studies, the NF-κB inhibitor Bay 11-7082 (Calbiochem) was used to block the specific signaling pathway.

EBV infection. Each gastric carcinoma cell line was infected with recombinant EBV using the cell-to-cell contact method (20). A Burkitt's lymphoma cell line, Akata, was modified to produce recombinant EBV, in which the neomycin resistance gene (Neo') is inserted into BBLFI (21) and was used as a source of the virus in the present study. Establishment of EBV infection was confirmed by EBER in situ hybridization (ISH), which was applied to gastric carcinoma cells grown on plastic slides after fixation with 10% formalin.

After selection with G418 (700 μg/ml; Sigma), EBV-infected cells were maintained in bulk, but G418 in the medium for EBV-infected gastric carcinoma cell lines was removed 24 h before the experiment. For serum depletion experiments, exponentially growing cells cultured in complete medium were centrifuged, washed twice with serum-free medium, and incubated in RPMI 1640.

Growth assay. The cell growth rate was determined using Cell Counting Kit-8 (Dojindo). Cells were seeded on 96-well plates at 5 × 104/100 μl per well and cultured with 10% FCS. After 0, 24, 48, 72, and 96 h incubation, 10 μl of Cell Counting Kit-8 solution were added to each well and absorbance was measured at 655 nm as a reference.

Terminal deoxynucleotidyl transferase-mediated nick-end labeling assay. Cells were grown on the four-chamber slide well (BD Biosciences) until 70% confluence. After fixing the cells with 10% formalin, terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) reactions were performed using BD ApoAlert DNA Fragmentation Assay Kit (BD Biosciences), according to the manufacturer's instructions. TUNEL labeling index was calculated by counting positive cells per 1,000 under a fluorescence microscope. All experiments were performed in triplicate.

Cell migration assay. The in vitro wound-healing method was used. Cells were grown to confluence in six-well plates and then the monolayer was wounded with a plastic pipette tip. The wound area was photographed under a microscope and >15 h thereafter. The distance that cells had moved into the denuded area was determined using computer-driven image analysis at three distinct points in the wound, and the mean was used for analysis. All experiments were performed in triplicate.

Reverse transcription-PCR of EBV-latent genes. Reverse transcription-PCR (RT-PCR) analysis of EBV latent genes (BABF0, EBER1, EBNA1, EBNA2, LMP1, and LMP2A and LMP2B) was performed in gastric carcinoma cell lines (MKN-1, TMK-1, MKN-74, and MKN-7) with and without EBV infection. Total RNA was extracted from the original and EBV-infected gastric carcinoma cell lines by the acid guanidium/phenol/chloroform method using TRIzol reagent (Invitrogen). Oligonucleotide microarray analysis (GeneChip; Affymetrix) was carried out as described previously (22), according to the instructions from the Affymetrix GeneChip Expression Analysis Technical Manual. In Affymetrix U133plus2.0 array used here, signals from one transcript (= probe set) were measured by 11 probes, which allowed statistical evaluation for signal difference between comparative two objects. The intensities (Table 1) indicate the summed signals from 11 probes and are calculated as described in the Web site. Briefly, to assess the significance of the intensity, the one-sided Wilcoxon’s signed rank test was used where the signals from almost 11 probes needed to be different. When there was little difference in the intensities from several probes among all 11 probes for a particular gene, the gene expression difference between these two samples were regarded as “not statistically significant,” even if the summed signals seemed to be different.

Quantitative RT-PCR. Total RNA (3 μg) was reverse transcribed using the SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen). PCR amplification was carried out using Platinum SYBR Green qPCR SuperMix DUG (Invitrogen). The following primers were used for PCR: survivin primer (forward) 5'-GGGCGGCGGGCTTTCAG-3' (reverse), 5'-CCTTCCAGAAGGAGGCGCATGT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'-GAAGGTGAAGGTCGGAGTC-3’; reverse 5’-GAAGATGGTGATGGATTTC-3’. Analysis was performed in triplicate. To ensure the mRNA stability during the serum deprivation, agarose gel electrophoresis (1%) was performed using each sample of total RNA (3 μg) at 0, 24, 48, 72, and 94 h after serum deprivation.

Western blot analysis. Cells were washed briefly in PBS, centrifuged, and lysed in a lysis buffer consisting of 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EDTA, 1%NP40, 50 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonily fluoride, and protein inhibitor cocktail (Sigma). For normalization of gel loading, protein extracts were assayed with the ABC method (Pierce Laboratories), and, typically, 20 μg of the protein per lane were loaded. The following primary antibodies were used: antisurvivin (mouse monoclonal, clone: 60.11, Novus Biologicals; dilution 1:100), and anti-cIAP1 and anti-cIAP2 (rabbit polyclonal, R&D Systems; 1:1000), phosphorylated Akt (rabbit polyclonal, Cell Signaling Technology; 1:50), Akt (rabbit polyclonal, Santa Cruz Biotechnology; 1:2000), P65 antibody (mouse polyclonal, Santa Cruz Biotechnology; 1:200), and anti-Histone H1 antibody (mouse monoclonal, clone: AE-4, Santa Cruz Biotechnology; 1:100). The filters were incubated with anti-rabbit or anti-mouse antibodies. The antigen was then detected using enhanced chemiluminescence Western blotting detection reagents (Amersham) according to the manufacturer’s instructions.

Small interfering RNAs. Small interfering RNA (siRNA) sequences directed to survivin (SVV-A: 5’-GCGAACGACAAAUUGCGAAC-3’, SVV-B: 5’-GAAAGUGCCGCGGUACAUdTdT-3’, and psiG3L3B, a control vector expressing siRNA against the firefly luciferase transcript (5’-GGUGCCUG CGUGGUGCAAGdTdT-3’) were designed by siDirect® (RNAi Co., Ltd.) and manufactured by Proligo LLC. MKN-1 with or without EBV infection were transfected by transfection reagent RNAiFect (Qiagen) according to the manufacturer’s instructions. Protein was collected at 72 h posttransfection under serum-deprived conditions.

Plasmids and transfection. Each EBV latent gene, EBNA1, BABF0, EBERs, or LMP2A, was cloned into pCDNA3 containing FLAG-tag. MKN-1 was transfected with each expression vector by lipofection. The expression of each gene was confirmed by RT-PCR and immunohistochemical staining

EBNA1, EBNA2, LMP1, and LMP2B, and the Akata cell line was used for LMP2A.

Oligonucleotide microarray analysis. One representative gastric carcinoma cell line (MKN-1) and MKN-1 with EBV infection (EBV-MKN-1), incubated for 96 h under 0% FCS condition, were subjected to oligonucleotide microarray analysis. After the total RNA was isolated by the acid guanidium/phenol/chloroform method using TRIzol reagent (Invitrogen), oligonucleotide microarray analysis (GeneChip; Affymetrix) was carried out as described previously (22), according to the instructions from the Affymetrix GeneChip Expression Analysis Technical Manual. In Affymetrix U133plus2.0 array used here, signals from one transcript (= probe set) were measured by 11 probes, which allowed statistical evaluation for signal difference between comparative two objects. The intensities (Table 1) indicate the summed signals from 11 probes and are calculated as described in the Web site. Briefly, to assess the significance of the intensity, the one-sided Wilcoxon’s signed rank test was used where the signals from almost 11 probes needed to be different. When there was little difference in the intensities from several probes among all 11 probes for a particular gene, the gene expression difference between these two samples were regarded as “not statistically significant,” even if the summed signals seemed to be different.

http://www.affymetrix.com/support/technical/whitepapers.affx
http://design.rnai.jp/sidirect
with an anti-FLAG antibody (Sigma). To further confirm LMP2A-mediated survivin expression in EBV-infected gastric carcinoma cell lines, TMK1 and MKN7 were transfected with the LMP2A expression vector.

**Effect of Bay 11-7082 on the LMP2A-mediated survivin expression.**
A total of 7.5 × 10\(^5\) per well gastric carcinoma cells (MKN-1, TMK1, and MKN7) expressing LMP2A or FLAG (control) were incubated in RPMI1640 with 10% FCS overnight. Cells were then maintained by 1% FCS medium for 60 h. Fresh medium containing 5 \(\mu\)mol/L Bay 11-7082 or vehicle control, DMSO (Sigma), was added to the cells, which were further incubated for 12 h. The inhibitory effects of 5 \(\mu\)mol/L Bay 11-7082 on gastric carcinoma cells expressing LMP2A or FLAG were evaluated by Western blotting analysis. Nuclear proteins were extracted by the standard protocol and blotted with P65 antibody. Histone H1 served as a loading control of nuclear extracts. Total RNA (2 \(\mu\)g) was reverse transcribed using SuperScript III First-strand Synthesis System (Invitrogen) and assessed by quantitative real-time RT-PCR for survivin.

**Immunohistochemistry and ISH.** The survivin protein expression was evaluated by immunohistochemistry in gastric carcinoma tissues in vivo, which were resected for the treatment of gastric cancer at Tokyo University Hospital between 1993 and 1997. TMA was constructed from 10% formalin-fixed and paraffin-embedded blocks of gastric carcinoma. All cases of gastric carcinoma were histologically diagnosed according to the Japanese Classification of Gastric Carcinoma (23) and Lauren’s classification (24).

The presence of EBV in carcinoma tissues was evaluated by ISH targeting EBV-encoded small RNA (EBER-ISH) with an EBER-PNA probe (Dako-Cytomation). Immunohistochemical analysis of survivin was applied to TMA sections using an LSAB2 Kit (Dako) with a mouse monoclonal antisurvivin antibody at a dilution of 1:500. To obtain negative controls, the primary antibody was omitted. As criteria to evaluate survivin expression, >10% positivity in carcinoma cells was considered as overexpression.

**Statistical analysis.** Statistical analyses were appropriately performed using the \(\chi^2\) test or \(t\) test. Differences were considered significant at \(P < 0.05\).

### Results

**Establishment of EBV-infected gastric carcinoma cell lines showing type I latency.** EBV-infected gastric carcinoma cell lines were established after G418 selection, and stable infection was confirmed by EBER-ISH. These cells showed the same cytologic appearance as the original cells. RT-PCR analysis confirmed that all gastric carcinoma cell lines infected with EBV belonged to latency I.

### Table 1. GeneChip analysis of apoptosis-associated gene expression in MKN-1 with and without EBV infection at 96 h under serum deprivation

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<th>Increase/decrease of signals (P)</th>
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**NOTE:** Signals from one transcript (= probe set) were measured by 11 probes, which allowed statistical evaluation for signal difference between comparative two objects.

**Abbreviations:** I, statistically increased intensity in EBV–MKN-1 relative to MKN-1; D, statistically decreased intensity in EBV–MKN-1 relative to MKN-1; NS, not significant.
showing the RNA expression of EBNA1, BARF0, and LMP2A, but not EBNA2 or LMP1 (Fig. 1A).

Resistance to serum deprivation–induced apoptosis is one of the characteristics of EBV-infected gastric carcinoma cells. To explore the changes closely associated with EBV infection of gastric carcinoma epithelial cells, comparative studies were performed on the growth rate (Fig. 1B), apoptosis (data not shown), and cell migration (Fig. 1C) of gastric carcinoma cell lines with and without EBV infection under the condition of 10% FCS. There were no significant differences observed in any gastric carcinoma cell lines with and without EBV infection. Because apoptosis is induced by serum deprivation, we evaluated the apoptotic profile under serum-free conditions. Apoptosis increased over time, and became marked 72 h after the cells were placed under 0% serum. The apoptotic index at 96 h after serum deprivation (Fig. 1D) was significantly lower in three of four EBV-infected gastric carcinoma cell lines compared with the respective original, such as in MKN-1 (71.2 ± 6 versus 17.4 ± 14, \( P = 0.003 \)), TMK1 (54.3 ± 4 versus 5.9 ± 9, \( P = 0.015 \)), and MKN-74 (47.8 ± 10 versus 24.8 ± 7, \( P = 0.042 \)).

Survivin as a candidate molecule for resistance to serum deprivation–induced apoptosis in EBV-associated gastric carcinoma cell lines. We compared the expression of apoptosis-related genes in MKN-1 and EBV–MKN-1, under serum-free conditions.

Figure 1. Establishment and characterization of EBV-infected gastric carcinoma cells. A, RT-PCR analysis of RNA expression of EBV latent genes (BARF0, EBER1, EBNA1, EBNA2, LMP1, and LMP2A and LMP2B) in gastric carcinoma cell lines with and without EBV infection. Note that all EBV-infected gastric carcinoma cell lines show latency I type of latent gene expression; positive for EBNA1, BARF0, and LMP2A, but negative for EBNA2 and LMP1. Lane 1, a positive control; lane 2, MKN-1; lane 3, EBV-MKN-1; lane 4, TMK1; lane 5, EBV-TMK1; lane 6, MKN-74; lane 7, EBV-MKN-74; lane 8, MKN-7; lane 9, EBV-MKN-7. B, comparative studies of cell growth under 10% of FCS. No difference in gastric carcinoma cell lines with and without EBV infection. C, comparative studies of cell migration under 10% of FCS. No difference in gastric carcinoma cell lines with and without EBV infection. D, serum deprivation–induced apoptosis. The pair of figures showing fluorescent microscopy of TUNEL assay of MKN-1 and EBV-MKN-1 at 96 h after serum deprivation. Nearly all of the cells show apoptosis (green) in MKN-1, but most of the cells do not show apoptosis (red) in EBV-MKN-1. Apoptotic index at 96 h after serum deprivation is significantly lower in three of four EBV-infected gastric carcinoma cell lines compared with the respective original.
conditions, using oligonucleotide microarray analysis (Table 1). In Affymetrix U133plus2.0 array used here, the signal from one transcript was measured by 11 probes, which allowed statistical evaluation for signal difference between comparative two objects. Among antiapoptotic IAP family proteins, only signals from survivin gene were significantly different and the survivin probe signals in EBV–MKN-1 were statistically higher than that in MKN-1. With respect to other apoptosis-related genes, the levels of caspase-3/9 mRNA did not show any changes. The expression of Bcl-2 family members showed no difference between MKN-1 gastric carcinoma cell lines with and without EBV infection.

The time course of survivin mRNA expression, determined by quantitative RT-PCR, confirmed the increased level in EBV-MKN-1 at 72 and 96 h after serum deprivation, in contrast to its decrease in MKN-1 (* and **, P < 0.01). Agarose gel electrophoresis shows no change in the configuration of 28S and 18S RNA of MKN-1 and EBV-MKN-1 at 0, 24, 48, 72, and 96 h after serum deprivation. B, comparative studies of survivin mRNA expression at 72 h after serum deprivation in gastric carcinoma cell lines with and without EBV infection. C, immunoblot analysis of survivin and cIAP-1 and cIAP-2 at 72 h after serum deprivation in MKN1 and EBV-MKN1 cells. D, inhibition of survivin expression by siRNAs. Evaluation was performed in MKN-1 and EBV-MKN-1 transfected with siRNAs (SVV-A and SVV-B), each against different sequences of the survivin gene, respectively, 72 h after serum starvation.

Survivin as a responsible molecule for resistance to serum deprivation–induced apoptosis in EBV-associated gastric carcinoma cell lines. siRNAs were used to inhibit survivin expression in EBV–MKN-1 cells. After transfection of siRNAs, gastric carcinoma cell lines were placed under serum starvation for 72 h. Transfection of SVV-B caused a slight decrease of the protein level of survivin in MKN1 cells, but transfection of SVV-A greatly decreased the protein level (Fig. 2D). When apoptotic cells

Figure 2. Survivin up-regulation confers resistance to serum deprivation–induced apoptosis in EBV-infected gastric carcinoma cells. A, time course of mRNA expression of survivin under serum deprivation in MKN1 cells with and without EBV infection, evaluated by quantitative RT-PCR. EBV-MKN-1, which shows increase of survivin mRNA at 72 and 96 h after serum deprivation, in contrast to its decrease in MKN-1 (* and **, P < 0.01). Agarose gel electrophoresis shows no change in the configuration of 28S and 18S RNA of MKN-1 and EBV-MKN-1 at 0, 24, 48, 72, and 94 h after serum deprivation. B, comparative studies of survivin mRNA expression at 72 h after serum deprivation in gastric carcinoma cell lines with and without EBV infection. C, immunoblot analysis of survivin and cIAP-1 and cIAP-2 at 72 h after serum deprivation in MKN1 and EBV-MKN1 cells. D, inhibition of survivin expression by siRNAs. Evaluation was performed in MKN-1 and EBV-MKN-1 transfected with siRNAs (SVV-A and SVV-B), each against different sequences of the survivin gene, respectively, 72 h after serum starvation.
were evaluated by TUNEL, apoptotic cells dramatically increased with SVV-A, but only slightly with SVV-B at 72 h after serum deprivation. Importantly, the percentage of apoptosis in EBV–MKN-1 with SVV-A was close to that in MKN-1 under comparable conditions.

**LMP2A as a responsible viral molecule for up-regulation of survivin.** As the first step to evaluate the mechanisms underlying survivin up-regulation in EBV–MKN-1, the expression pattern of EBV-latent genes was evaluated along the time course of serum deprivation. The intensities of the amplified band of RT-PCR of EBER, BARF0, and LMP2A RNAs were nearly equal during 72 h under serum deprivation (Fig. 3A).

To identify the EBV gene responsible for survivin up-regulation, vectors containing the **LMP2A, EBER, EBNA1, or BARF0** gene were transfected into MKN-1, and each stable gastric carcinoma cell line was obtained with G418 selection. The expression level of each gene in transfected MKN-1 cells was comparable with that in EBV–MKN-1 by semiquantitative RT-PCR, respectively (Fig. 3A).

Survivin mRNA expression in each latent gene–transfected MKN1 cells was then evaluated by quantitative RT-PCR after 0, 24, 48, and 72 h incubation under serum-deprived conditions (Fig. 3B). Survivin mRNA expression increased in LMP2A-transfected MKN-1, whereas it was stable in EBER–, EBNA1–, and BARF0–MKN-1 during serum deprivation (Fig. 3B).

To further confirm LMP2A as a responsible viral molecule for the up-regulation of survivin in gastric carcinoma cells, we transfected LMP2A expression vector or control FLAG vector to TMK1 and MKN-7. EBV-infected TMK1 showed resistance to serum deprivation–induced apoptosis, whereas EBV-infected MKN-7 did not show any differences in apoptotic cell number under serum deprivation. The increase of survivin mRNA expression after serum deprivation (72 h) was observed in LMP2A-transfected TMK1.

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**Figure 3.** LMP2A causes survivin up-regulation in EBV-infected gastric carcinoma cells under serum deprivation through the NF-κB pathway. **A,** EBV latent gene expressions in EBV-MKN1 under serum deprivation were evaluated by RT-PCR analysis along the course of serum deprivation. Lane 1, positive control; lanes 2 to 4, MKN-1 at 0, 24, and 72 h after serum deprivation; lanes 5 to 7, EBV-MKN-1, at 0, 24, and 72 h after serum deprivation. **B,** effect of viral latent genes on cellular survivin gene expression. The time course of survivin expression was evaluated under serum deprivation in MKN-1 cells, which were transfected with each of **EBNA1, EBER, LMP2A, BARF0,** and FLAG tag. The longitudinal axis indicates the relative fold of survivin mRNA expression. Survivin copy number per 1,000 GAPDH copies is normalized by the corresponding number of FLAG-transfected MKN-1 cells. **C,** Western blotting evaluation of Akt and phosphorylated Akt (Ser473) in MKN-1 and EBV-MKN-1 under serum deprivation (72 h). pAkt, phosphorylated Akt; tAkt, total Akt. **D,** effect of Bay 11-7082 on LMP2A-mediated survivin expression in MKN-1, TMK1, and MKN-7. The immunoblot analysis reveals that the nuclear localization of NF-κB is decreased with Bay11-7082. By quantitative real-time PCR analysis, the increased level of survivin mRNA expression is markedly reduced with Bay 11-7082 in LMP2A-transfected MKN-1 and TMK1, but not in LMP2A-transfected MKN-7.
showed the latency I typeof EBV-latent gene expression similar to the experimental condition, we showed that three of four gastric carcinoma cells expressed EBNA1, or without nuclear staining.

To evaluate the possible involvement of the NF-κB pathway in LMP2A-mediated survivin up-regulation, the NF-κB inhibitor Bay 11-7082 was applied to MKN-1 gastric carcinoma cell lines transfected with LMP2A vector (LMP2A-transfected MKN-1) and control FLAG vector (FLAG-transfected MKN1). Because Bay 11-7082 was apparently toxic to EBV–MKN-1 for 24 h, we adopted the condition of 1% of serum starvation and incubation of Bay 11-7082 in the last 12 h of the 72-h incubation under serum starvation. Under this condition, 5 μmol/L of Bay 11-7082 decreased the p65 protein level and reduced the up-regulation of survivin mRNA expression in LMP2A-transfected MKN1 (Fig. 3D). Incubation with Bay 11-7082 similarly reduced the up-regulation of survivin mRNA expression in LMP2A-transfected TMK1 but not in LMP2A-transfected MKN7.

Survivin expression in carcinoma tissues of surgically resected gastric carcinoma. To clarify the clinical significance of survivin expression in EBV-associated gastric carcinoma, immunohistochemical staining of survivin was applied to the tissue microarray of gastric carcinoma; the results are summarized in Table 2. Positive staining for survivin was predominantly observed in the cytoplasm (Fig. 4A) in 80 of 117 tumor tissue specimens of gastric carcinoma (68.4%), 21 of 27 EBV-associated gastric carcinomas (77.8%), and 59 of 90 EBV-negative gastric carcinomas (65.6%). The nuclear staining of survivin (Fig. 4B) was observed in 8 of 80 cases showing survivin expression, 1 EBV-associated gastric carcinoma, and 7 EBV-negative gastric carcinomas. As for the clinicopathologic factors affecting the survivin expression in gastric carcinoma, the frequency of survivin expression was significantly higher in EBV-associated gastric carcinoma (12 of 13) than in EBV-negative gastric carcinoma (31 of 50) at the advanced stage of gastric carcinoma (P < 0.0307). Positive ratio of survivin expression was similarly high in intestinal and diffuse-type histology in EBV-associated gastric carcinoma, but the ratio was relatively lower in the diffuse type of EBV-negative gastric carcinoma (P = 0.1339). All of the gastric carcinoma cases showing nuclear staining of survivin were diffuse type in histology, although there was no morphologic difference between carcinoma cells with or without nuclear staining.

Discussion

EBV infection in stomach epithelial cells in vitro invariably showed the latency I type of EBV-latent gene expression similar to the neoplastic cells of EBV-associated gastric carcinoma in vivo (22). EBV-infected gastric carcinoma cells expressed EBNA1, EBERs, LMP2A, and BARF0, but not EBN2A or LMP1. In this experimental condition, we showed that three of four gastric carcinoma cells of latency I EBV infection showed resistance to serum deprivation–induced apoptosis, although the growth rate and cell mobility analyses did not show any differences. Serum deprivation, mimicking the microenvironment of cancer tissue in vivo, is a strong inducer of apoptosis through the intrinsic pathway. Interestingly, a lower rate of apoptosis has also been well documented in EBV-associated gastric carcinoma in vivo (25); therefore, the mechanisms of resistance to apoptosis may be important in this type of gastric carcinoma and should be further investigated in relation to EBV infection.

Subsequent oligonucleotide array analysis of apoptosis-associated genes of the representative gastric carcinoma cell line, MKN-1, with and without EBV infection has identified survivin as a candidate for this resistance, among seven members of IAPs—NAIP, cIAP-1, cIAP-2, XIAP, survivin, livin, and ILP2 (26–28). RT-PCR in other gastric carcinoma cell lines and Western blotting of EBV–MKN-1 cells validated the array data. Knockdown of the endogenous survivin level by siRNA-mediated RNA interference (RNAi) in EBV–MKN-1 cells increased apoptosis to the level of the original MKN-1 cells. These results indicate that increased survivin expression causes resistance to serum deprivation–induced apoptosis in EBV-infected gastric carcinoma cell lines. In parallel with the results in vitro, survivin was highly expressed in nearly all EBV-associated gastric carcinomas in the advanced stage (12 of 13), and the frequency was significantly higher than that in EBV-negative gastric carcinomas (31 of 51). Recently, Shi et al. (29) showed the role of survivin in nasopharyngeal carcinoma by demonstrating its overexpression in nasopharyngeal carcinoma biopsies and the increase of irradiation-induced apoptosis by siRNA treatment of survivin in the nasopharyngeal carcinoma model C666-1 cell line. This nasopharyngeal carcinoma result is in accordance with the results in the present study of gastric carcinoma.
Survivin is abundantly expressed during development, but it is undetectable in nonproliferating adult tissue (15). Its up-regulation has been observed in various carcinomas with or without the association of viral infection, but the mechanisms of survivin overexpression are only partially understood. In normal ovaries, survivin exon I is silenced by methylation, but it becomes demethylated and transcriptionally active in ovarian carcinomas. We also evaluated exon 1 methylation of survivin according to Hattori et al. (30), but the CpG sites remained unmethylated in gastric carcinoma cell lines with EBV infection (data not shown).

T-cell leukemia virus type I tax has the potential of transcriptional activation of survivin through the NF-κB pathway (31). The oncoprotein E6 of human papilloma virus type 16 up-regulates survivin expression through interaction with p53. Survivin, up-regulated by hepatitis B virus X protein (HBx), forms complexes with HBx-interacting protein and binds pro-caspase-9, preventing its recruitment to Apaf1 in hepatoma cells. Thus, survivin up-regulation by viral protein may be a common denominator in the mechanisms of human viral oncogenesis.

LMP2A is capable of transforming human keratinocyte cell line HaCaT (32, 33) through the activation of Akt. Fukuda and Longnecker (34) have shown that LMP2A inhibits transforming growth factor-β–mediated apoptosis by activating the phosphatidylinositol 3-kinase/Akt pathway in the gastric cancer cell line Hsc-39; however, the induction of Akt phosphorylation under serum deprivation was not remarkable in EBV-MKN1 compared with that in MKN-1 cells. Alternatively, the expression level of p65, a subunit of NF-κB, in EBV–MKN-1 was significantly higher than that in MKN-1. We showed that LMP2A-mediated survivin up-regulation was inhibited by a NF-κB inhibitor in both MKN-1 and TMK1. On the other hand, the up-regulation was not observed or inhibited with a NF-κB inhibitor in MKN-7, in which EBV-infection did not inhibit apoptosis after serum deprivation. These facts indicate that LMP2A-NFkB pathway plays a primary role for survivin up-regulation in EBV-associated gastric carcinoma and also suggest that there are some prerequisites inherent in the parental cells for the activation of this pathway. Because LMP2A is constitutively expressed in EBV–MKN-1 cells, serum deprivation may activate or elicit the LMP2A-NFkB pathway after the priming period of 48 h, resulting in the up-regulation of survivin. Recently, Stewart et al. (35) reported that LMP2A modulated NF-κB activity in a nasopharyngeal carcinoma cell line, HONE-1, although the effect was reportedly inhibitory. Further studies are necessary to investigate the interaction of LMP2A with the NF-κB pathway as a possible mechanism for resistance to apoptosis.

In conclusion, up-regulation of survivin, induced by LMP2A, confers resistance to serum deprivation–induced apoptosis in EBV-infected gastric carcinoma cells and may play a role in the progression of EBV-associated gastric carcinoma in vivo. EBV uses its latent protein, LMP2A, to activate the NF-κB–survivin pathway to rescue EBV-infected epithelial cells from apoptotic threatening of serum deprivation. Further studies are necessary to clarify the function of LMP2A and its downstream pathway for therapeutic intervention in this specific type of gastric carcinoma infected with EBV.

Figure 4. Immunohistochemistry of survivin in carcinoma tissues of gastric carcinoma. Carcinoma tissues from patients’ stomachs were fixed in formalin and embedded in paraffin, and their sections were subjected to survivin immunostaining. A, cytoplasmic survivin expression in neoplastic epithelial cells of EBV-associated gastric carcinoma. B, nuclear and cytoplasmic survivin expression in neoplastic epithelial cells of EBV-associated gastric carcinoma. C, negative expression of survivin in EBV-negative gastric carcinoma.

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