Expression of Epstein-Barr nuclear antigen 1 in gastric carcinoma cells is associated with enhanced tumorigenicity and reduced cisplatin sensitivity

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Abstract. Epstein-Barr nuclear antigen 1 (EBNA-1) is consistently expressed in all EBV-associated gastric carcinomas. We explored its biological effects in gastric carcinoma cells by expressing the protein in two Epstein-Barr virus (EBV)-negative gastric carcinoma cell lines (SCM1 and TMC1). EBNA1-expressing SCM1 and TMC1 cells displayed no significant differences in growth rates, respectively, compared to those of vector-transfected SCM1 and TMC1 cells *in vitro*. However, EBNA1 was able to enhance tumorigenicity, the growth rate and the malignant histopathological grade in a xenograft nude mice test. We also evaluated whether EBNA1 caused EBNA1-expressing cells to have enhanced tumorigenicity in an immunocompetent host. We showed that EBNA1-expressing LL/2 cells (derived from lung carcinoma of a Swiss mouse) had enhanced tumorigenicity and growth ability in the immunocompetent allograft Balb/c mice test. These results support the expression of EBNA1 in EBV-associated gastric carcinoma being able to provide advantages of EBV-mediated cell growth and transformation, and to enhance the malignant potential *in vivo*. In a clonogenic assay, we showed that EBNA1 could reduce the sensitivity of gastric carcinoma cells (SCM1 cells) harboring wild-type p53 to cisplatin, but this was not found in mutant p53-bearing TMC1 cells. In addition, we demonstrated that EBNA1-expressing SCM1 cells, but not EBNA1-expressing TMC1 cells, were associated with reduced expression levels of p53. These findings are compatible with EBNA1 efficiently competing with p53 for binding to ubiquitin-specific protease 7, which causes p53 to degrade by the ubiquitin/proteasome system. These findings suggest that EBNA1 expression is able to reduce the p53 protein level, resulting in the inhibition of its functional activities. Finally, our results suggest that EBV infection with EBNA1 expression in gastric carcinomas provides advantages for host cell survival, growth ability and transformation potential involving escape from immunosurveillance and a reduction in the sensitivity to DNA damage or other apoptotic stress stimuli mediated by suppression of the wild-type p53 protein level; these are distinct from the pathogenesis of EBV-negative gastric carcinomas.

Introduction

Gastric carcinoma is a common cancer and results in about 876,000 new cases per year making it one of the leading causes of cancer deaths worldwide (1). Among gastric carcinomas, 2-16% (mean 10%) of conventional gastric adenocarcinomas (2), more than 80% of lymphoepithelioma-like carcinomas (LELCs) of the stomach (3,4), and 35% of adenocarcinomas of the gastric stump are associated with Epstein-Barr virus (EBV) infection (5). The worldwide absolute number of EBV-associated gastric carcinomas makes it the largest group of EBV-associated malignancies (2). This association is characterized by the presence of EBV infection in nearly all tumor cells (6), clonality of the EBV genome in tumor cells and an elevation of EBV-specific antibodies in patients (7,8). The molecular characteristics of EBV-associated gastric carcinomas have been explored. Aberrant hypermethylation of CpG islands is one mechanism of tumor suppressor gene inactivation in EBV-associated gastric carcinomas (9). Allelic loss at some chromosomal markers such as 5q (APC), 17p (p53) and 18q (smad 4) was inversely correlated with an EBV association. Allelic loss at the p53

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Abbreviations: EBNA1, Epstein-Barr nuclear antigen 1; USP7, ubiquitin-specific protease 7; LELC, lymphoepithelioma-like carcinoma; LMPs, latent membrane proteins; GAr, glycine-glycine-alanine repeat sequence

locus is not encountered in EBV-associated gastric carcinomas, but occurs in 51% of EBV-negative gastric carcinomas (10). Microsatellite instability, a hallmark of a defective DNA mismatch repair system, is not related to the EBV status in gastric carcinomas (11). By comparative genomic hybridization, loss of 4p, 11p and 18q was found to be more frequent in EBV-associated gastric carcinomas than in EBV-negative gastric carcinomas (12). By a tissue array analysis, EBV-associated gastric carcinomas were shown to exhibit frequent loss of expression of some tumor suppressor genes such as p16, smad 4, FHIT and KAI-1, but usually retained the expressions of APC, DCC and some DNA-repair proteins (13). The clinicopathological features of EBV-associated gastric carcinomas are found more frequently in male and younger patients, and in the proximal part of the stomach with a tubular histopathological subtype together with prominent activated CD8+ lymphocyte infiltration and a lower frequency of lymph node metastasis (14,15). EBV-associated adenocarcinomas do not obviously differ in prognosis compared to EBV-negative adenocarcinomas (16,17), but the prognosis of EBV-associated LELCs of the stomach is significantly better than that of conventional EBV-negative gastric adenocarcinomas (18,19). All of these findings suggest that EBV-associated gastric carcinomas exhibit specific clinicopathological features, novel genomic and epigenetic aberrations, and a distinct protein expression profile. Therefore, EBV-associated gastric carcinomas have a distinct pathogenic pathway in which EBV infection and its Epstein-Barr nuclear antigen 1 (EBNA-1) might play crucial roles.

In EBV-associated gastric carcinomas, EBV shows a unique latent pattern characterized by transcription of the transforming BARF1 gene, EBER1/2, Q-promoter-driven EBNA1, BARF0 and LMP2A, but not EBNA-2, -3A, -3B, or -3C, leader protein, or other latent membrane proteins (LMPs) due to gene methylation (7,20,21). EBNA1 is a DNA-binding nuclear phosphoprotein essentially required for replication and maintenance of the episomal EBV genome through the binding of EBNA1 to the origin of viral replication (22). EBNA1 is consistently expressed in EBV-associated gastric carcinomas and can act as a transcriptional transactivator upregulating the Cp and LMP1 promoters (23). EBNA1 also interacts with the Qp promoter and immediately downstream regulatory region III to positively and negatively regulate its own expression (24). The EBNA1 protein can be separated into amino- and carboxy-terminal domains by a glycine-glycine-alanine repeat (GAr) sequence, which acts as a cis-acting inhibitor of MHC class I-restricted presentation and appears to function by inhibiting antigen processing via the ubiquitin/proteasome pathway (25,26). In addition, EBNA1 has a well-defined modular structure with no apparent enzymatic activity, but may fulfill its functions through interactions with cellular proteins. Many cellular-interacting proteins such as importin-α, karyopherin-α1, P32/TAP, EBP2, TAF-Iα, TAF-Iß, CK2, PRMT5 and USP7 were shown to interact with EBNA1 (27-32), but their exact biological effects are not well clarified.

Several reports supported the hypothesis that EBNA1 has oncogenic potential, and these include the fact that EBNA1 is required as a survival factor in Burkitt's lymphoma (33), that only EBNA1 is consistently expressed in EBV-associated Burkitt's lymphoma and gastric carcinomas (34), and that EBNA1 is able to induce B-cell neoplasia in transgenic mice (35). Moreover, we showed that the expression of EBNA-1 in an EBV-negative NPC cell line resulted in increased tumorigenic and metastatic capabilities *in vivo* (36), thus suggesting that EBNA-1 may enhance the malignant progression of EBV-associated epithelial tumors. These findings suggest that EBNA-1 is the EBV-encoded protein consistently expressed in all EBV-associated malignancies, which has biological effects and as such would seem to play a critical role in viral persistence and EBV-mediated cellular transformation (37). In contrast, it was also shown that EBNA1 is unable to induce lymphomas in transgenic FVB mice (38) and may act as a transforming suppressor of the HER2/neu oncogene by its N-terminal domain (39). Furthermore, it is able to sensitize HER2/neu-overexpressing ovarian cancer cells to topoisomerase II-targeted and paclitaxel drugs (40). In addition, the expression of EBNA1 in epithelial cells requires an undifferentiated cellular environment and may induce cytotoxic effects in some cell lines (41). All of these divergent findings suggest that the exact biological activities of EBNA1 may be complicated and result from a diverse range of biological effects on different cellular systems together with interactions with various extracellular environmental factors.

Based on the restricted EBV gene expression in gastric epithelial cells, EBNA1 is the latent protein consistently expressed in all EBV-associated gastric carcinomas. Although EBNA1 would seem to contribute at least some pathogenic factors to tumorigenesis and/or the modulation of clinico-pathological features of EBV-associated gastric carcinomas, its biological effects in gastric carcinoma are still not well understood. In this report, using *in vivo* and *in vitro* studies, we evaluated the oncogenic potential and alterations in cisplatin sensitivity between two gastric carcinoma cell lines (SCM1 and TMC1) that were transfected with a gene expressing EBNA1.

Materials and methods

Cells and animals. The EBV-negative gastric carcinoma cell lines SCM1 and TMC1 used in this study are derived from gastric adenocarcinomas and were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) as previously described (42). The LL/2 (LLC1) cell line derived from a primary Lewis lung adenocarcinoma (43) was cultured in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine and 1 mM sodium pyruvate containing 10% fetal bovine serum (FBS). All cell lines were incubated at 37˚C in an incubator with 5% CO_2 and water-saturated air. Pathogen-free nude mice (Balb/c nu/nu) and immunocompetent Balb/c mice were used for the tumorigenic test, which involved subcutaneous transplantation of either EBNA1-expressing gastric carcinoma cells, EBNA1-expressing LL/2 cells, or vector-transfected cells. All procedures involving animal experiments and the care of animals in this study carried out in accordance with an established animal protocol and were approved by the Experimental Animal Care and Use Committee at National Defense Medical Center.

Plasmid construction and DNA transfection. The EBNA1 gene was introduced into the pCP4/EBNA1 plasmid and the control pCP4 vector, and the DNA transfection methods are as previously described (44). The pCP4/EBNA1 plasmid was used to transfect SCM1, TMC1 and LL/2 cells to establish the EBNA1-expressing cells to evaluate the biological effects of EBNA1 in transfected cells. pCP4 was used to establish vector-transfected control cells. After transfection for 24 h, transfected SCM1 and TMC1 cells were subcultured at a 1:10 dilution into selection medium consisting of growth medium containing 100 μg/ml hygromycin, while transfected LL/2 cells were subcultured in selection medium consisting of growth medium containing 20 μg/ml hygromycin. After long-term selection, stable transfected cells were established.

Immunocytochemical staining. EBNA1 expression in transfected SCM1, TMC1 and LL/2 cells was detected by *in situ* immunocytochemical staining, as previously described with minor modifications (42). For immunocytochemical staining, normal goat serum (at a 1:20 dilution in TBS) was used as the blocking reagent. Cells were then incubated with antiserum (at a 1:30 dilution) from an EBV-positive donor with nasopharyngeal carcinoma (NPC) for 2 h; this was followed by incubation with a biotin-labeled goat anti-human IgG antibody (Santa Cruz, CA, USA), then streptavidin was linked to horseradish peroxidase (Vector, Burlingame, CA, USA). Slides were washed three times in TBS for 5 min each. The color was developed in AEC solution (Vector), and slides were slightly counterstained with hematoxylin and mounted for examination. Substitution of the primary antibody with neonatal umbilical cord serum or omission of the primary antibody served as a negative control for all immunostaining.

Western immunoblotting. Western immunoblotting was used to evaluate EBNA1 expression in the established EBNA1-expressing SCM1, TMC1 and LL/2 cells, as previously described with minor modifications (44). In brief, blots were incubated with blocking buffer containing 2% BSA, 1% normal goat serum and 0.1% Tween-20 in TBS for 45 min and then incubated with antiserum (at a 1:200 dilution) from an EBV-positive donor with NPC for 2 h. The antigen-antibody complex was detected by an HRP-coupled goat anti-human immunoglobulin G (IgG) antibody (Santa Cruz) followed by Western Blotting Luminol Reagent (Santa Cruz). The same procedures were used to evaluate the expressions of GSTπ, MDR1, topoisomerase I and II, Her-2/neu, and p53 in EBNA1-expressing gastric carcinoma cells by respectively substituting the primary antibodies with anti-human GSTπ (Novocastra, UK), MDR1 (Santa Cruz), topoisomerase I and II (NeoMarkers, CA USA), Her-2/neu (NeoMarkers) and p53 (DO7, NeoMarkers).

Immunoprecipitation of EBNA1. Immunoprecipitation plus Western immunoblotting was used to evaluate EBNA1 expression in tumor masses derived from the tumorigenic assay in nude mice or Balb/c mice as described in a previous report with minor modifications (36,44). In brief, the extracted protein concentration was determined by a bicinchonic acid assay (Pierce, Rockford, IL, USA), and an appropriate amount of protein was used for immunoprecipitation. Proteins were pre-cleared by adding neonatal umbilical cord serum (at a 1:30 dilution) and 1.0 μg of normal goat IgGs together with 30 μl of protein A/G-agarose (25% v/v); this was followed by incubation at 4°C for 30 min, and then centrifugation at 13,000 rpm for 5 min at 4°C. The supernatant was transferred to a fresh tube on ice, and immunoprecipitation was initiated by the addition of antiserum (at a 1:30 dilution) from an EBV-positive donor with NPC, followed by incubation overnight at 4°C. Incubation was then carried out for an additional 4 h in the presence of protein A/G agarose. The immunoprecipitate was washed four times in lysis buffer. For sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), pellets were resuspended in 30 μl of 3x SDS sample buffer and then heated at 98°C for 5 min, followed by SDS-PAGE. For the Western immunoblot analysis, proteins were transferred to nitrocellulose; membranes were blocked with 3% BSA and 0.1% Tween-20 in TBS for 90 min and incubated with antiserum (at 1:300 dilution) from an EBV-positive donor with NPC. The antigen-antibody complex was detected by HRP-coupled goat anti-human IgG antibody (Santa Cruz) followed by luminol reagent (Santa Cruz).

MTT assay. The MTT assay procedures used to evaluate the difference in growth rates between EBNA1-expressing cells and vector-transfected control cells were the same as those previously described (45).

Tumorigenic assay in nude and Balb/c mice. The tumorigenic assay in nude mice was used to evaluate variations in tumorigenesis between EBNA1-expressing SCM1 and TMC1 cells and vector-transfected SCM1 and TMC1 cells, respectively, as previously described (42). In brief, $4x10^6$ cells were subcutaneously transplanted into the right and left back regions of each nude mouse. Animals were sacrificed on the 60th day of the experiment, and the tumor mass was excised, weighed and divided into two parts. One of them was fixed in 10% neutralized formalin for the histopathological examination, and the other was quickly frozen in liquid nitrogen then stored at -80°C for studies such as the detection of EBNA1 expression in tumor cells by immunoprecipitation and Western immunoblotting. The tumorigenicity of EBNA1-expressing LL/2 cells and vector-transfected LL/2 cells was evaluated by an allograft in Balb/c mice according to the same experimental procedures.

Morphological examination. The morphological characteristics of cultured EBNA1-expressing cells and vector-transfected control cells were examined under subconfluent culture conditions. The morphological features of each tumor mass derived from the tumorigenicity assay were evaluated by serial tissue sections using hematoxylin and eosin (H&E) staining.

Clonogenic assay. A clonogenic assay was used to evaluate differences in cisplatin sensitivity between EBNA1-expressing and vector-transfected gastric carcinoma cells as previously described with modifications (46). Cells growing in the log phase were trypsinized, and viable cells were counted. On a 60-mm culture dish, appropriate cell numbers (1000 SCM1 cells and 2000 TMC1 cells) were seeded in selection medium.
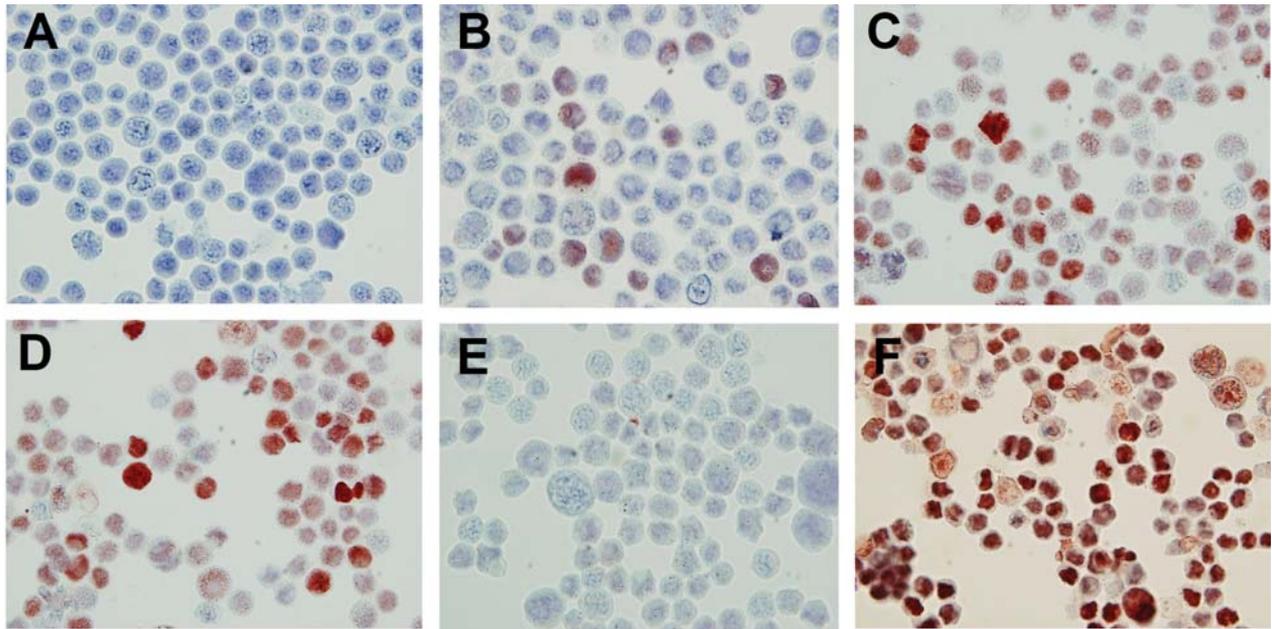
Figure 1. Immunocytochemical staining for EBNA1 expression in EBNA1-expressing gastric carcinoma cells. (A) Vector-transfected SCM1 cells. (B) EBNA1-expressing SCM1 cells. (C) Subclonal EBNA1-expressing SCM1 cells (B8). (D) Subclonal EBNA1-expressing SCM1 cells (D23). (E) Vector-transfected TMC1 cells. (F) EBNA1-expressing TMC1 cells.

After a 24-h culture, cells was treated with various concentrations of cisplatin (0, 1, 2, 4, 8 and 16 μg/ml) for 20 min, and then washed with serum-free medium five times to remove the drug. Cisplatin-treated cells were cultured in selection medium, and the medium was refreshed every 2-3 days. After 2 weeks of incubation, cultured cells were washed with PBS, fixed in 10% neutral buffered formalin and stained with a 1% solution of crystal violet to determine the colony number. Each assay was carried out in triplicate and consisted of three independent experiments. Colonies that consisted of more than 20 cells were scored under an inverted microscope, and the number was compared to the untreated control. Cell survival curves were drawn by plotting the means of three independent experiments with the SD as an error bar.

Single-stranded conformation polymorphism (SSCP) analysis. The SSCP analysis was used to detect mutations of the p53 gene in SCM1 and TMC1 cells. It was performed using the same procedures as previously reported (47). In brief, each exon 5-8 of the p53 gene was amplified by 35 cycles of PCR using 5'-end-labeled primers and Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT, USA) and analyzed on 6% polyacrylamide gels after denaturation of the PCR products by heating at 95˚C for 7 min in the presence of a 15% urea solution. Then, abnormal band shifts were demonstrated if the p53 gene had mutated exons.

Statistical analysis. Variations in tumorigenesis between EBNA1-expressing cells and vector-transfected cells were tested by Pearson's Chi-square test. Growth rates of tumors (assessed by tumor weight) induced by EBNA1-expressing cells and vector-transfected cells were evaluated by the Wilcoxon signed-rank test. Probability values of <0.05 were considered statistically significant.

Results

Establishment of EBNA1-expressing cells. After transfection and selection for 2 weeks, more than 300 drug-resistant colonies were obtained from the pCP4/EBNA1-transfected SCM1 and TMC1 cells, and only about 80 colonies were obtained from pCP4-transfected SCM1 and TMC1 cells. These colonies were harvested and expanded in selection medium to give EBNA1-expressing SCM1 and TMC1 cells and vector-transfected SCM1 and TMC1 cell lines. The expression of EBNA1 in EBNA1-expressing SCM1 and TMC1 cells was evaluated by *in situ* immunocytochemical staining and Western blotting. About 30 and 90% of EBNA1-expressing SCM1 and TMC-1 cells, respectively, showed detectable EBNA1 expression in the nucleus by *in situ* immunocytochemical staining (Fig. 1). By Western blotting, EBNA1-expressing SCM1 and TMC1 cells expressed EBNA1 at 83 kDa (Fig. 2A). Because the expression level of EBNA1 in tumor cells was too low for detection by *in situ* immunocytochemical staining or the Western blotting method, EBNA1 expression in the tumors derived from the tumorigenic assay of EBNA1-expressing SCM1 and TMC1 cells was evaluated by immunoprecipitation plus Western blotting (Fig. 2B). Vector-transfected SCM1 and TMC1 cells showed no EBNA1 expression in either system. In addition, EBNA1-expressing LL/2 cells and vector-transfected LL/2 cells were established by the same procedure. EBNA1 expression in EBNA1-expressing LL/2 cells was undetectable by *in situ* immunocytochemical staining due to its lower-level expression. However, EBNA1 expression could also be detected as an 83-kDa protein in EBNA1-expressing LL/2 cells by Western blotting and in the tumor mass derived from the tumorigenic assay by immunoprecipitation plus Western blotting (Fig. 2A and B). These results confirmed that SCM1,

Table I. Tumorigenesis of EBNA1-expressing gastric carcinoma cells and vector-transfected gastric carcinoma cells in nude mice, and tumorigenesis of EBNA1-expressing LL/2 cells and vector-transfected LL/2 cells in Balb/c mice.

Mouse number	EBNA1-expressing SCM1 cell-derived tumors (g)	Vector-transfected SCM1 cell-derived tumors (g)	EBNA1-expressing TMC1 cell-derived tumors (g)	Vector-transfected TMC1 cell-derived tumors (g)	EBNA1-expressing LL/2 cell-derived tumors (g)	Vector-transfected LL/2 cell-derived tumors (g)
1	1.03	0.3	2.39	0.38	5.17	0
2	0.14	0	0.89	0.03	0	0
3	0.16	0.40	0.76	0	0	0
4	2.31	0.45	1.05	0	0	0
5	0.27	0	0.3	0	0	0
6	0.59	0.54	0.55	0.46	1.85	0
7	1.09	0.82	1.09	0	3.22	0
8	2.68	1.10	0	0	0	0
9	3.42	2.01	0	0	0	0
10	1.52	0.74	0	0	0	0
11	0.59	0	0.59	0	2.65	0
12	1.25	0.83	1.25	0.83	0	0
Mean of tumor weight (g)[a]	1.25	0.74	0.98	0.43	3.78	0
Tumorigenicity (%)[b]	100	75	75	33	33	0

[a]Wilcoxon signed-rank test for the growth rate (assessed by tumor weight); the difference was significant at $p<0.05$. [b]Pearson Chi-square test for tumorigenicity; the difference was significant at $p<0.05$.
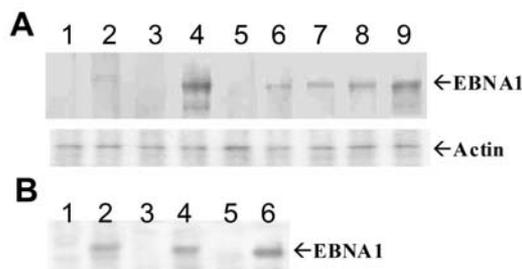

Figure 2. Western blot analysis of EBNA1 expression in EBNA1-expressing LL/2, TMC1 and SCM1 cells. (A) EBNA1 expression in EBNA1-expressing cells demonstrated by Western blotting; aliquots of 20 μg of cell extract from each representative cell line were used. Lane 1, vector-transfected LL/2 cells; lane 2, EBNA1-expressing LL/2 cells; lane 3, vector-transfected TMC1 cells; lane 4, EBNA1-expressing TMC1 cells; lane 5, vector-transfected SCM1 cells; lane 6, EBNA1-expressing SCM1 cells; lane 7, subclonal EBNA1-expressing SCM1 cells (B15); lane 8, subclonal EBNA1-expressing SCM1 cells (D8); lane 9, subclonal EBNA1-expressing SCM1 cells (D23). (B) EBNA1 expression in tumors derived from EBNA1-expressing cells in a tumorigenic assay demonstrated by immunoprecipitation plus Western blotting; aliquots of 20 mg of tumor tissue extract from each representative tumor were used except in lane 1. Lane 1, whole-cell lysate of vector-transfected LL/2 cells; lane 2, tumor derived from EBNA1-expressing LL/2 cells; lane 3, tumor derived from vector-transfected SCM1 cells; lane 4, tumor derived from EBNA1-expressing SCM1 cells; lane 5, tumor derived from vector-transfected TMC1 cells; lane 6, tumor derived from EBNA1-expressiong TMC1 cells.

TMC1 and LL/2 cells transfected with EBNA1 gene are able to express EBNA1 *in vitro* and *in vivo*, but no such expression occurred in vector-transfected SCM1, TMC1 or LL/2 cells.

Growth rate of EBNA1-expressing cells. In the MTT assay, EBNA1-expressing SCM1 and TMC1 cells displayed no significant difference in growth rates when, respectively, compared to vector-transfected SCM1 and TMC1 cells (data not shown). These results suggest that EBNA1 does not obviously influence the growth ability of gastric carcinoma cells *in vitro*. A similar result was also obtained for EBNA-expressing LL/2 cells and vector-transfected LL/2 cells by the same assay.

Increased tumorigenicity and growth rate of EBNA1-expressing cells. Results of the tumorigenic assay are summarized in Table I. Although the difference did not reach statistical significance (p=0.06), tumorigenesis of EBNA1-expressing SCM1 cells (100%) showed a higher frequency than that of vector-transfected SCM1 cells (75%). The tumorigenesis frequency of EBNA1-expressing TMC1 cells (75%) was significantly higher than that of vector-transfected TMC1 cells (33%) (p=0.04). Growth rates of tumors derived from EBNA1-expressing SCM1 and TMC1 cells, as assessed by the tumor weight, were respectively significantly higher than those of tumors derived from vector-transfected SCM1 (p=0.005) and TMC1 (p=0.008) cells. These results suggest that EBNA1-expressing gastric carcinoma cells are able to enhance tumorigenicity and growth rates compared to vector-transfected gastric carcinoma cells in an immunocompromised nude mice model. In the clinic, patients with EBV-associated gastric carcinoma always have a normal immune function (7). To clarify whether EBNA1 causes EBNA1-expressing cells to have increased tumorigenicity in an immu-

nocompetent host, we evaluated the tumorigenicity and growth rates of EBNA1-expressing LL/2 cells using allografts in immunocompetent Balb/c mice. We found that EBNA1-expressing LL/2 cells were able to induce tumors (33%), and these had the ability to grow aggressively. In contrast, no tumorigenesis was observed in vector-transfected LL/2 cells (p=0.01). These results suggest that EBNA1-expressing LL/2 cells have increased tumorigenicity and growth ability compared to vector-transfected LL/2 cells. Therefore, we concluded that the expression of EBNA1 is able to help host cells escape from allograft rejection and enhance tumorigenicity in an immunocompetent host.

Morphological alterations in EBNA1-expressing TMC1 cells in vivo. Morphological alterations of EBNA1-expressing gastric carcinomas and LL/2 cells were examined *in vitro* and *in vivo*. Under subconfluent culture conditions, there was no obvious difference in morphological alterations between EBNA1-expressing SCM1, TMC1 and LL/2 cells and the respective vector-transfected SCM1, TMC1 and LL/2 cells (data not shown). Morphological alterations of induced tumors in the tumorigenic tests were also evaluated using serial sections with H&E staining. The morphological features of tumors derived from EBNA1-expressing SCM1 cells were similar to those of tumors derived from vector-transfected SCM1 cells (data not shown), whereas tumors derived from EBNA1-expressing TMC1 cells displayed a higher histological grade than tumors derived from the vector-transfected TMC1 cells, and this was characterized by greater cellular polymorphism with occasional anaplastic large cell formation, conspicuous nucleoli and frequent mitosis (Fig. 3). These findings suggest that the expression of EBNA1 in gastric carcinoma cells has the potential to induce tumors of a higher histological grade compared to tumors derived from vector-transfected gastric carcinoma cells (as observed in EBNA1-expressing TMC1 cells) in the nude mouse model used.

Reduced sensitivity to cisplatin of EBNA1-expressing SCM1 cells. We also evaluated the potential of EBNA1 to modulate the chemodrug sensitivity of gastric carcinoma cells by a clonogenic assay, because patients with gastric carcinoma usually receive cisplatin as adjuvant therapy. For this assessment, three additional subclonal EBNA1-expressing SCM1 cell lines (B15, D8 and D23) were isolated from EBNA1-expressing SCM1 cells. In each subclonal cell line, about 80% of tumor cells expressed EBNA1 as an 83-kD protein in the nuclei as demonstrated by *in situ* immunocytochemical stain and Western immunoblotting (Figs. 1 and 2A). In the clonogenic assay, each subclonal EBNA1-expressing SCM cell line (B15, D8 and D23) and EBNA1-expressing SCM1 cells displayed reduced sensitivity to cisplatin compared to vector-transfected SCM1 cells as measured by their IC_{50} values (a concentration that inhibited 50% of the colony-forming ability) (Fig. 4A and C). EBNA1-expressing TMC1 cells did not show an obvious change in sensitivity to cisplatin compared to vector-transfected TMC1 cells (Fig. 4B and C). These findings suggest that the expression of EBNA1 in gastric carcinoma cells has the potential to down-modulate sensitivity to cisplatin (as observed in EBNA1-expressing SCM1 cell lines).
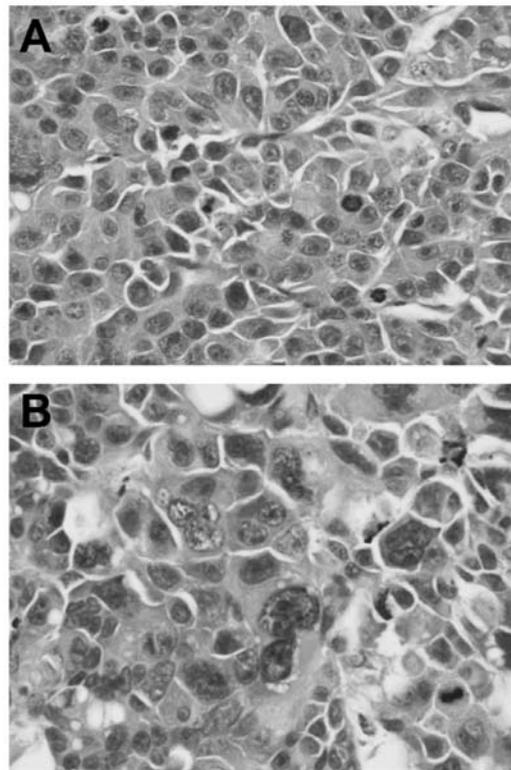

Figure 3. Histopathology of induced tumors derived from vector-transfected TMC1 cells showing a homogeneous appearance of tumor cells (A) and from the EBNA1-expressing TMC1 cells showing a higher grade of pleomorphic and anaplastic features of tumor cells (B).

Expression of drug resistance-related proteins in EBNA1-expressing gastric carcinoma cells. To clarify the mechanism responsible for the reduced cisplatin sensitivity in EBNA1-expressing gastric carcinoma cells, expressions of proteins related to chemodrug resistance such as GSTπ, MDR1 (P-glycoprotein), topoisomerase I and II, Her-2/neu and p53 were evaluated by Western blotting (48). No obvious changes in the expressions of these drug resistance-related proteins were respectively demonstrated between EBNA1-expressing SCM1 and TMC1 cells and vector-transfected SCM1 and TMC1 cells except for the p53 protein. We found that the expression level of p53 was much lower in SCM1 cells than that in TMC1 cells (Fig. 5). Immunohistochemical staining demonstrated p53 as a diffuse strong nuclear positive signal in induced tumors derived from TMC1 cells in nude mice, but in induced tumors derived from SCM1 cells, the results were negative for p53 staining (data not shown). An SSCP analysis was used to evaluate p53 mutations in SCM1 and TMC1 cells, and results showed an abnormal band shift in exon 5 of the p53 gene in TMC1 cells, but none was found in SCM1 cells (Fig. 6). These findings indicate that the p53 gene in TMC1 cells is a mutant, but it is a wild-type in SCM1 cells.

Reduced p53 expression in EBNA1-expressing SCM1 cells. EBNA1 was demonstrated to compete with p53 for binding with ubiquitin-specific protease 7 (USP7). This binding allows p53 ubiquitination and degradation by the proteosome system. Such competition is able to protect cells from apoptotic challenge and provide a mechanism to explain how
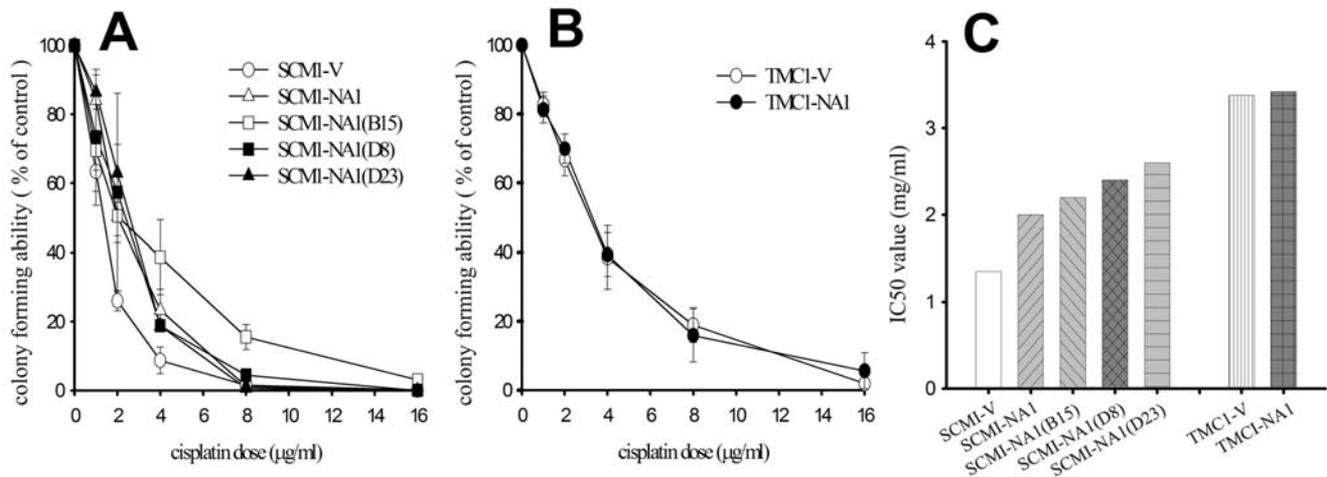
Figure 4. Clonogenic assay demonstrating the difference to cisplatin sensitivity (IC$_{50}$ value) between vector-transfected gastric carcinoma cells and EBNA1-expressing gastric carcinoma cells. (A) SCM1-V, vector transfected SCM1 cells; SCM1-NA1, EBNA1-expressing SCM1 cells; SCM1-NA1(B15), SCM1-NA1(D8) and SCM1-NA1(D23), different subclones of EBNA1-expressing SCM1 cells. (B) TMC1-V, vector-transfected TMC1 cells; TMC1-NA1, EBNA1-expressing TMC1 cells. (C) The IC50 value of cisplatin for the EBNA1-expressing SCM1 cells and each subclonal EBNA1-expressing SCM1 cells and vector transfected SCM1 cells, and the EBNA1-expressing TMC1 cells and vector-transfected TMC1 cells are presented in the bar chart.
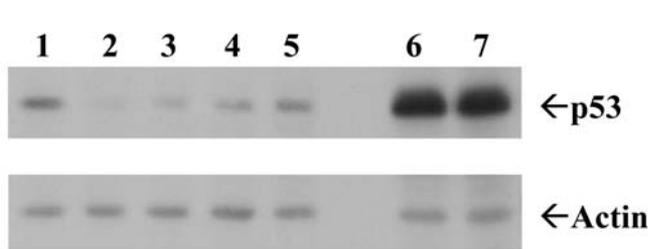

Figure 5. p53 expression in the EBNA1-expressing SCM1 cells and each subclonal EBNA1-expressing SCM1 cells and vector-transfected SCM1 cells, and the EBNA1-expressing TMC1 cells and vector-transfected TMC1 cells were demonstrated by Western blotting. Lane 1, vector-transfected SCM1 cells; lane 2, subclonal EBNA1-expressing SCM1cells (D23); lane 3, subclonal EBNA1-expressing SCM1 cells (D8); lane 4, subclonal EBNA1-expressing SCM1 cells (D15); lane 5, EBNA1-expressing SCM1 cells; lane 6, vector-transfected TMC1cells; lane 7, EBNA1-expressing TMC1 cells.
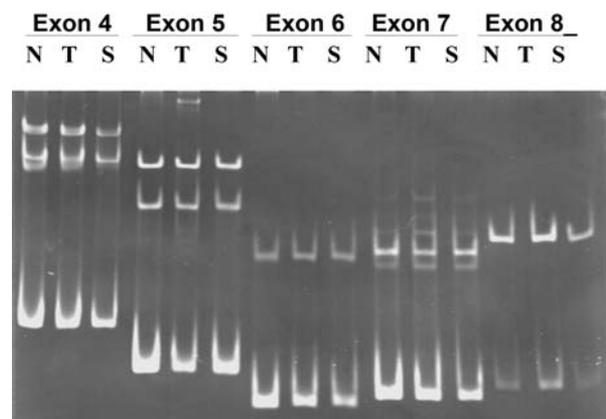

Figure 6. Single-stranded conformation polymorphism (SSCP) analysis of the p53 mutation in exons 4, 5, 6, 7 and 8 of SCM1 and TMC1 cells. p53 gene mutation in exon 5 was observed in TMC1 cells. N, normal control; T, TMC1 cells; S, SCM1 cells.

EBNA1 might contribute to the survival and transformation potential of EBV-infected cells (49-52). We demonstrated that EBNA1-expressing SCM cells have reduced sensitivity to cisplatin compared to vector-transfected SCM1 cells, which possibly resulted from interactions between EBNA1 and p53. To elucidate this possibility, we evaluated the difference in p53 expression levels between EBNA1-expressing gastric carcinoma cells and vector-transfected gastric carcinoma cells. In Fig. 5, we found that the expression level of p53 was reduced in EBNA1-expressing SCM1 cells and in each subclonal EBNA1-expressing SCM1 cell line (D23, D8 and D15). In addition, the reduced p53 level was correlated with the expression level of EBNA1 in EBNA1-expressing SCM1 cells and each subclonal EBNA1-expressing SCM1 cell line (D23, D8 and D15) as shown in Fig. 2A. These findings are compatible with EBNA1 being able to compete with p53 for binding with USP7 and enhance p53 ubiquitination and degradation by the proteosome system.

Discussion

In the present study, we assessed the biological effects of EBNA1 in gastric carcinoma cells *in vitro* and *in vivo*. We found that the expression of EBNA1 in SCM1 and TMC1 cells did not obviously influence their growth ability *in vitro*. Similar studies demonstrated that the expression of EBNA1 is able to induce cytotoxicity in squamous epithelial cells but not in glandular epithelial cells (41). The cytotoxicity of EBNA1 was also shown in SKOV3 ovarian cancer cells which exhibit growth inhibition and G2/M arrest (39,40). Based on previous reports and our results, we suggest that the expression of EBNA1 in epithelial cells seems to be able to induce diverse cytotoxicity possibly resulting from various states of the cellular environment.

With xenografts in nude mice, the expression of EBNA1 in SCM1 and TMC1 gastric carcinoma cells was able to

enhance the tumorigenicity and growth rate, but, however, induced a higher histopathological grade of the tumor only in TMC1 cells. These results suggest that EBNA1 expression has the potential to provide advantages that promote tumorigenesis and the growth ability of EBV-associated gastric carcinomas *in vivo* (22,33). Using allografts into immunocompetent Balb/c mice, we also showed that the expression of EBNA1 in LL/2 cells was able to induce tumors (33%) with aggressive growth ability, but that there was no observable tumorigenesis in vector-transfected LL/2 cells. These results further suggest that EBNA1 also helps EBNA1-expressing cells escape from allograft rejection in an immunocompetent host, and this is possibly mediated by down-modulation of allograft rejection responses (53), avoidance of tumor immune surveillance mediated by the suppression of HLA molecules/ TAA on the tumor cell surface, the production of immuno-suppressive cytokines, or the expression of lymphotoxic molecules (i.e., FAS ligand) by cancer cells (54-56), but these remain to be clarified in future studies. The experimental tumorigenicity results provide evidence that supports the hypothesis that expression of EBNA1 in EBV-associated gastric carcinoma may play an important role in tumorigenesis and a tumor's ability to escape from host immune eradication during EBV-mediated cell transformation. In previous reports, the oncogenic potential of EBNA1 was also identified in other cellular systems including the induction of lymphomagenesis in transgenic mice (35,57) and the enhancement of malignant progression of NPC cells *in vivo* (36) at low-level expression only detected by immunoprecipitated Western blotting. However, EBNA1 failed to induce lymphomas in transgenic FVB mice at an expression level similar to that of latent EBV infection in human B lymphocytes (38). These discrepant findings might have resulted from different expression levels of EBNA1. The GAr of EBNA1 can protect EBNA1 from proteasomal degradation and results in a failure of presentation of EBNA1-derived epitopes on MHC class I molecules and therefore in CD8+ T cell recognition. This helps latently infected cells escape from cell-mediated immune responses during the transformation or long-term survival *in vivo* (25,26,58). Importantly, GAr also inhibits mRNA translation of EBNA1 in *cis* and minimizes the expression of EBNA1, both *in vitro* and *in vivo*, thus avoiding efficient presentation of respective MHC class I and II molecules for CD8+ and CD4+ T cell recognition (59-61). The protective effects of GAr regulation of EBNA1 expression to a minimized stable level may allow the persistence of the virus in latently infected cells and make it more suitable to mediate cell transformation (62).

The oncogenic mechanisms of EBNA1 are not well clarified, but there are some reports of biological activities of EBNA1 that provide some reasonable explanations. EBNA1 was shown to have oncogenic potential through different action mechanisms: a) it interacts with Nm23-H1 and inhibits its ability to suppress cell migration in lymphoblastoid cells, which highlights a novel function of EBNA1 that contributes to malignant transformation and tumor progression (63); b) it modulates the AP-1 transcription factor pathway in nasopharyngeal carcinoma cells and enhances angiogenesis which implicates EBNA1 in the angiogenic process and might contribute to the development and aggressively metastatic

nature of NPC (64); c) it promotes genomic instability *via* induction of reactive oxygen species, highlighting a novel function of EBNA-1 which can contribute to malignant transformation and tumor progression (65); and d) it modulates the STAT1 and TGF-ß signaling pathways to induce a lower steady-state level of SMAD2 protein, downregulates the tumor growth factor (TGF)-ß target gene, PTPRK, and contributes to the growth and survival of Hodgkin lymphoma cells, suggesting that EBNA1 can influence cellular gene transcription resulting in effects that may contribute to the development of EBV-associated tumors (49,50). These observations support the view that the expression of EBNA1 in EBV-associated tumors might not only reflect a requirement for viral persistence but also results in a selective growth advantage and increased oncogenic potential that enhances the malignant transformation and progression of tumor cells.

By a clonogenic assay, EBNA1-expressing SCM cells were shown to have reduced sensitivity to cisplatin compared to vector-transfected SCM1 cells, but the same results were not observed in EBNA1-expressing TMC1 cells. The underlying molecular mechanisms of these findings are not easily explained based on the present study, because no upregulated expression of the drug resistance-related proteins GSTπ, MDR1 (P-glycoprotein), topoisomerase I and II, or Her-2/neu was demonstrated in EBNA1-expressing SCM1 or TMC1 cells. However, we found that the p53 expression level was much lower in SCM1 cells than in TMC1 cells as demonstrated by the Western blot assay. By the SSCP analysis, we showed that a point mutation in the p53 gene had occurred in TMC1 cells, but the p53 gene remained a wild-type in SCM1 cells.

EBNA1-expressing SCM cells had reduced sensitivity to cisplatin compared to vector-transfected SCM1 cells, but that was not found for EBNA1-expressing TMC1 cells which is compatible with EBNA1 being demonstrated to play a role as a survival factor in Burkitt's lymphoma by being able to inhibit p53-induced apoptosis *in vitro* (34). These findings can be explained by EBNA1 possibly competing with p53 for binding to USP7. This binding allows p53 ubiquitination and degradation. Such competition may therefore protect cells from apoptotic challenge and provide a mechanism to explain how EBNA1 might contribute to the survival and transformation potential of EBV-infected cells (66,67). Such protection from apoptotic challenge is predicated to protect cells from DNA damage induced by apoptosis mediated through p53 activity. Thus, we suggest that the expression of EBNA1 in SCM cells in parallel with wild-type p53 ubiquitination and degradation by the ubiquitin/proteasome system reduces DNA damage by apoptosis induced by cisplatin treatment. But this was not observed in TMC1 cells because the mutant p53 in TMC1 cells may be insensitive to EBNA1's ubiquitination modulation and/or the mutant p53 has greater potential than EBNA1 to reduce DNA damage induced by apoptosis. This rationally explains the expression of EBNA1 in SCM1 cells (with wild-type p53) conferring reduced sensitivity to cisplatin and why no similar finding was observed in EBNA1-expressing TMC1 cells (with mutant p53). In addition, this explanation is also compatible with clinical findings that in EBV-positive gastric carcinomas,

infrequent overexpression of p53 protein (68) and much lower frequency of p53 gene mutation (10,69) are displayed, which are significantly distinct from EBV-negative gastric carcinomas.

Finally, the present study shows that the expression of EBNA in gastric carcinoma cells has the potential to enhance tumorigenicity and induce tumors with higher-grade histo-pathological features (observed in TMC1 cells) in a xenograft nude mouse model. EBNA1-expressing LL/2 cells also showed enhanced tumorigenicity and growth ability in the allograft immunocompetent Balb/c mouse model. These results support the hypothesis that the expression of EBNA1 in EBV-associated gastric carcinoma may play an important role in EBV-mediated cell transformation and allow such cells to escape from a patient's immune eradication, possibly by down-modulation of tumor immune surveillance and/or rejection responses. In addition, EBNA1 is able to reduce the sensitivity to the DNA damage cytotoxicity induced by cisplatin in gastric carcinoma cells harboring wild-type p53 and suggests that EBV infection with EBNA1 expression reduces the sensitivity to apoptotic stimuli of host cells. Together, our results provide an additional example highlighting that EBV infection with EBNA1 expression provides host cells with advantages in terms of survival and transformation potential, escape from host immunosurveil-lance, and reduced sensitivity to the DNA-damage stress and/or apoptotic stimuli from the extracellular environment. These findings may characterize the pathogenesis of EBV-positive gastric carcinomas, which is distinct from that of EBV-negative gastric carcinomas.

Acknowledgments

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