Autocrine Growth of Epstein-Barr Virus-Positive Gastric Carcinoma Cells Mediated by an Epstein-Barr Virus-Encoded Small RNA

Dai Iwakiri, Yoshito Eizuru, Masayoshi Tokunaga, and Kenzo Takada

Department of Tumor Virology, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815 [D. I., K. T.], and Center for Chronic Viral Diseases, Kagoshima University, Kagoshima 890-8520 [Y. E., M. T.], Japan

Abstract

Although 5–10% of gastric carcinoma (GC) cases worldwide are associated with EBV, a human herpesvirus, it is still not clear what the precise contribution of the virus is to the pathogenesis of EBV-positive GC. Here we report that EBV infection induces expression of insulin-like growth factor 1 (IGF-I) in the GC-derived EBV-negative cell line NU-GC-3 and that the secreted IGF-I acts as an autocrine growth factor. Transfection of individual EBV latent genes into NU-GC-3 cells revealed that the EBV-encoded small RNA (EBER) was responsible for IGF-I expression. Addition of recombinant IGF-I accelerated growth of NU-GC-3 cells, whereas growth of the IGF-I-deficient NU-GC-3 cells was blocked by treatment with an anti-IGF-I antibody. These results suggest that IGF-I induced by EBER acts as an autocrine growth factor for EBV-positive GC. These findings seem to be operative in vivo, as EBV-positive GC biopsies consistently express IGF-I, whereas EBV-negative GC biopsies do not. EBER is invariably expressed in EBV-associated malignancies including GC. The present findings strongly suggest that EBV directly affects the pathogenesis of EBV-positive GC and underline the importance of RNA molecules on cell growth regulation.

Introduction

EBV is a ubiquitous virus, which infects the majority of the human population and is the causative agent of infectious mononucleosis (1). Increasing evidence has linked EBV infection to various epithelial malignancies as well as lymphoid ones recently. The very strong association between EBV and nasopharyngeal carcinoma is already well known (1). Moreover, the viral genome is detected in rare carcinomas with intense lymphoid stroma (termed lymphoepithelioma-like carcinoma) arising in the salivary glands, thymus, and stomach (2). In addition, an increasing number of studies have suggested a casual relationship between EBV and primary GC (3) of the more common adenocarcinoma type (2, 3). About 5–15% of patients with GC in all parts of the world have EBV DNA in 100% of carcinoma cells (2). Analysis of the terminal repeat sequence of EBV plasmid DNA in GC cells indicated that tumor cells arose from a single EBV-infected cell, thus suggesting that EBV infection had occurred in the very early stage of tumor development (4).

GC cells express a limited number of EBV genomes (4), similar to those in Burkitt’s lymphoma, which are EBNA1, small nonpolyadenylated RNA known as EBER, and the transcripts from the BamHI-A region (BARF0). In addition, LMP2A is weakly positive in some GC cases. This is different from the pattern in nasopharyngeal carcinoma in which LMP1 is also expressed in carcinoma cells in about half of the patients (1). Concerning the effects of EBV products on epithelial cells, LMP1 has some pleiotropic biological activities (5–7), but other gene products do not. We generated recently EBV recombinants with a selectable marker, which makes it possible to select EBV-infected cells even when the efficiency of infection is low or the EBV-uninfected population in culture is able to proliferate (8). Using the recombinant virus, we found that various carcinoma cell lines can be infected with EBV (9–11) and that their virus convertants consistently express a limited number of EBV latent genes, as EBV-positive GC cells do, thus indicating that the system could be a model for EBV oncogenesis. Using this system, we demonstrated that EBV promotes gastric epithelial cell growth in the absence of EBNA2 and LMP1 expression (11).

Here we report that EBV infection induces expression of IGF-I (12, 13) in the GC-derived EBV-negative cell line NU-GC-3 (14) and that the secreted IGF-I acts as an autocrine growth factor. Transfection of individual EBV latent genes into NU-GC-3 cells revealed that the EBER was responsible for IGF-I expression. EBER is ~170 nucleotides long and is the most abundant EBV RNA expressed in all of the EBV-associated malignancies (15). The present findings suggest that small RNA molecules directly affect the pathogenesis of EBV-positive GC.

Materials and Methods

Cell Lines and Tumor Biopsies. NU-GC-3 cells were cultured in DMEM (Sigma, St. Louis, MO). The medium was supplemented with 10% FBS (Sigma) and antibiotics. An Akata cell clone infected with rEBV, which carries the Neo’ gene, was cultured in RPMI 1640 supplemented with 10% FBS, antibiotics, and G418 (700 µg/ml; Sigma). GC tumor biopsies were obtained from patients diagnosed pathologically.

Establishment of EBV-infected NU-GC-3 Cells. NU-GC-3 cells were infected with rEBV (containing the Neo’ gene described above) using a “cell-to-cell” infection procedure as described previously (10). Cells were maintained in culture medium containing G418 (500 µg/ml).

Immunofluorescence. Expression of EBER was examined on acetone: methanol (1:1)-fixed cells by anticomplement immunofluorescence with reference human serum (titer, ×640).

Immunoblot Analysis. Cells lysates were resolved by 8% SDS-PAGE. For protein detection, membranes were probed with anti-LMP1 monoclonal antibody S-12 (kindly provided by Elliott Kieff, Harvard Medical School, Boston, MA) or human serum followed by antimouse or antihuman horseradish peroxidase-conjugated IgG. Membranes were visualized with an ECL Western blotting kit (Amersham Bioscience Corp., Piscataway, NJ).

RT-PCR Analysis. cDNA aliquots were then subjected to PCR analyses using primer pairs and probes specific for glyceraldehyde-3-phosphate dehydrogenase, and EBV transcripts such as BamHI W, C, and Q promoter (Wp, Cp, and Qp) -initiated mRNA, LMP2A, LMP2B, BARF0, and EBER as charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 To whom requests for reprints should be addressed, at Department of Tumor Virology, Institute for Genetic Medicine, Hokkaido University, N15 W7, Kita-ku, Sapporo 060-0815, Japan. Phone: 81-11-706-5071; Fax: 81-11-706-7540; E-mail: kentaka@med.hokudai.ac.jp.

3 The abbreviations used are: GC, gastric carcinoma; EBER, EBV-encoded small RNA; EBNA, EBV-determined nuclear antigen; LMP, latent membrane protein; IGF, insulin-like growth factor; IGF-IR, insulin-like growth factor 1 receptor; EGF, epidermal growth factor; HGF, hepatocyte growth factor; IL, interleukin; RT-PCR, reverse transcription-PCR; FBS, fetal bovine serum; Neo’, neomycin resistance; rEBV, recombinant EBV; PKR, RNA-activated protein kinase.
described previously (10). Sequences of primer pairs used for detection of growth factor mRNA expression were as follows (described as 5' end primer and 3' end primer, respectively), IGF-I: 5'-CACTGTCACTGCTAATTCA-3', 5'-CTGTGGGCTTGTTGAAATAA-3', IGF-IR: 5'-ACCCGGAGTACTTCAGCGCT, 5'-TTCTAAACAATGCAGCTTGAATCATG-3', EGF: 5'-CTATACATGCACACACACCATCATGGAGGCT, 5'-CCATGATACACACACACACCATGATACCACACG-3', HGF: 5'-GTCAAGAGTATAGCACCATGGCCT-3', and IL-1β: 5'-ATTGCA-
GAACTGTACCTAAGCTCGC-3', 5'-TTGATCTGAAATGTTACGTTAAACA-3'. For DNA amplification, cDNA was denatured at 94°C for 2 min (IGF-I, IGF-IR, HGF, and IL-1β) or 5 min (EGF), primer annealing 52°C for 1 min (IGF-I and HGF), 50°C for 1 min (IGF-IR), and then DNA extension at 72°C for 2.5 min (IGF-I and HGF) or 2 min (IGF-IR, EGF, and IL-1β) for 30–35 cycles (IGF-I, IGF-IR, and HGF) or 35–40 cycles (EGF and IL-1β).

ELISA. IGF-I production released from cells into the culture supernatant was assayed with an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Plasmids, Transfection, and Cell Cloning. We used the EBER plasmid that contained 10 tandem repeats of the EBER subfragment (6297–7325 bp) from the EcoRI K fragment of Akata EBV DNA and Neo′ gene driven by the SV40 promoter (16). The EBNA1 plasmid carries the SV40 promoter-driven Neo′ and the EBNA1 gene (17). The BARF0 plasmid carries the SV40 promoter-driven Neo′ and the FLAG-tagged BARF0 gene (17). Each of the plasmids was introduced into NU-GC-3 cells by the electroporation method. For isolation of stable transfectants, transfected cells were selected with complete culture medium containing 500 μg/ml of G418.

Reporter Plasmid Construction and Luciferase Assay. The IGF-I promoter from −1763 to +68 (18) was amplified from genomic DNA by PCR. PCR product was digested with MluI and BglII, and then subcloned upstream of the luciferase gene of PGV-basic vector 2 (Wako Pure Chemical Industries, Osaka, Japan). The IGF-I promoter-luciferase plasmid was cotransfected with the EBER plasmid into NU-GC-3 cells or transfected...
Results

IGF-I Expression in EBV-Infected GC Cells. To assess the role of growth factors in the growth of GC cells, the GC-derived EBV-negative cell line NU-GC-3 (14) was used. NU-GC-3 cells were infected with rEBV carrying the Neo\(^r\) gene, and G418-resistant cell clones were isolated by incubation in medium containing 500 \(\mu\)g/ml of G418. Ten representative cell clones were analyzed to ascertain whether cells were infected with EBV. All of the cell clones were 100% positive for EBNA by the immunofluorescence assay (Fig. 1A). Southern analysis of cell clones probed with the BamH I W fragment of EBV DNA also indicated that all of the clones were infected with EBV (data not shown). Immunoblot analysis demonstrated that the EBV-positive NU-GC-3 cell clones were positive for EBNA1 but negative for the other EBNAs and LMP1 (Fig. 1B). RT-PCR analysis revealed that EBV-positive cell clones used the Q promoter for EBNA transcription, and were positive for the EBER and BARF0, weakly positive for LMP2A, but negative for LMP2B (Fig. 1B). Their patterns of EBV expression were identical to those of EBV-positive GC, i.e., type I latency (1, 2).

EBV-positive and -negative NU-GC-3 cell clones were examined for the expression of various growth factors such as EGF, IGF, IL-1\(\beta\), and IGF-I, which are known to act as growth factors for epithelial cells. RT-PCR analysis revealed that expression of IGF-I was highly induced in EBV-positive NU-GC-3 cells (Fig. 1C). IGF-I induction was additionally confirmed by an ELISA assay of culture supernatants. After 3 days of culture of 2.5 \(\times\) 10\(^5\) cells in 1 ml of medium, EBV-positive NU-GC-3 cells secreted 6–9 ng of IGF-I. In contrast, the secreted IGF-I levels of EBV-negative NU-GC-3 cells were ~0.3 ng (Fig. 1D). As shown in Fig. 1E, the levels of IGF-1R expression did not differ between EBV-positive and EBV-negative NU-GC-3 cells.

Next we examined which of the three EBV genes expressed in these cells (EBNA1, EBER, and BARF0) was responsible for IGF-1 induction. NU-GC-3 cells were transfected with individual EBV latent genes, and cell clones that stably expressed similar levels of the gene as found in EBV-positive NU-GC-3 cells were selected and analyzed. The results indicated that all of the cell clones transfected with the EBER gene expressed higher levels of IGF-I than those transfected with the control Neo\(^r\) gene (Fig. 1F). EBNA1- and BARF0-transfected cell clones expressed IGF-I at levels similar to the Neo\(^r\)-transfected cell clones.

Role of IGF-I on Growth of EBV-Infected GC Cells. A comparison of growth between EBV-positive and -negative NU-GC-3 cells revealed that EBV clearly promotes growth yielding a higher saturation density in NU-GC-3 cells under low (0.1%) serum conditions (Fig. 2A). To assess the role of IGF-I, EBV-positive NU-GC-3 cells were cultured in the presence of anti-IGF-I antibody (R&D Systems). As a result, anti-IGF-I antibody inhibited the growth of EBV-negative NU-GC-3 cells in a dose-dependent manner (Fig. 2B). As well, addition of recombinant IGF-I to the culture medium of EBV-negative NU-GC-3 cells allowed these cells to grow at a rate similar to EBV-positive NU-GC-3 cells (Fig. 2C). These results indicated that IGF-I was an autocrine growth factor for NU-GC-3 cells.

IGF-I Expression in EBV-Positive GC Biopsies. To assess the role of IGF-I in GC, EBV-positive and -negative GC biopsies were examined for IGF-I expression by using the RT-PCR assay. All of the EBV-positive GC biopsies were positive for IGF-I, whereas IGF-I expression in EBV-negative GC biopsies was undetectable or marginal with the exception of case number 9 (Fig. 3).

Transcriptional Activation of the IGF-I Gene by EBER. To assess whether EBER modulates IGF-I transcription, transient transfection assays were performed in NU-GC-3 cells. We constructed a reporter plasmid containing the element ~1763 to +68 of human IGF-I 5' -franking region cloned in front of the luciferase gene (18). NU-GC-3 cells were cotransfected with the reporter plasmid and EBER or Neo\(^r\) plasmid. The results indicated that EBER slightly activated transcription from the IGF-I promoter (Fig. 4A). Next, to assess the effect of stable expression of EBER on IGF-I transcription, the IGF-I reporter plasmid was transfected into NU-GC-3 cell clones, which carried EBER plasmid and stably expressed EBER. As shown in Fig. 4B, the luciferase activity was significantly higher in EBER-expressing NU-GC-3 cells than in Neo\(^r\)-transfected NU-GC-3 cells. These results demonstrated that EBER activated transcription of the IGF-I gene.

Discussion

IGF-I consists of 70 amino acid residues (Mr, 7,650), and shares 40% homology in amino acid sequence with proinsulin. IGF-I is primarily produced by hepatocytes, serving an endocrine function, but is also produced by many other cells, where it may act in an autocrine or paracrine manner. The action of IGF-I is predominantly mediated through the IGF-IR. IGF-IR signaling can induce many diverse effects including differentiation, transformation, and prevention of apoptosis. Many studies have suggested an important role of IGF-I in neo-
plastic cell proliferation, showing a positive association between circulating IGF-I levels and risk of developing breast, prostate, and colorectal cancers (12, 13).

Regarding GC, several studies have shown that IGF-I receptor is expressed in GC-derived cells, and those cells have a mitogenic response to IGF-I (19, 20). Furthermore, normal and neoplastic cells are able to produce IGF-I (21). A recent study has shown that circulating IGF-I levels in GC are increased compared with control group, and radical surgery, with complete tumor ablation, induces a significant decrease in IGF-I levels (22). Although it is not known whether IGF-I is the sole determinant of GC development, what we have shown here is that EBER-induced IGF-I acts as an autocrine growth factor in GC cells, and EBV-positive GC biopsies consistently express IGF-I.

In previous articles, we reported that EBER contributes onco-genically to Burkitt’s lymphoma (16, 17, 23). EBER confers resistance to IFN-induced apoptosis via binding to double-stranded PKR and inhibiting its activation (23). Moreover, it induces IL-10 expression and secreted IL-10 acts as an autocrine growth factor for Burkitt’s lymphoma (16). The present results indicate that EBER makes key contributions to both lymphoid and epithelioid carcinogenesis.

Transient transfection assays indicated that transcription from the IGF-I promoter was activated in stably EBER-expressing cells, whereas it was less prominent in cotransfection studies of EBER with IGF-I promoter. These results suggest that EBER activates IGF-I transcription, but its effect is indirect, and other factors may be involved.

EBER has been reported to bind some cellular proteins such as La, EAP/L22, and PKR (15). Among them, the association of EBER with PKR has been most intensively studied (23, 24). However, treatment of NU-GC-3 cells with a PKR inhibitor did not induce IGF-I expression, making it unlikely that its inhibition by EBER is involved in IGF-I induction (data not shown). The mechanism by which EBER induces IGF-I expression will be fascinating to elucidate.

The present findings suggest a possible new therapeutic strategy against EBV-positive GC. Both existing drugs and various newly developed agents acting through the IGF-I pathways should have great potential as therapeutic or preventive agents for EBV-positive GC.

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References


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