Dysregulation of HER2/HER3 Signaling Axis in Epstein-Barr Virus-Infected Breast Carcinoma Cells

Jiun-Han Lin,1 Ching-Hwa Tsai,2 Jan-Show Chu,3,4 Jeou-Yuan Chen,5 Kenzo Takada,6 and Jin-Yuh Shew1*

Graduate Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan;
Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan; Department of Pathology, College of Medicine, Taipei Medical University, Taipei 11031, Taiwan; Department of Pathology, Taipei Medical University Hospital, Taipei 11031, Taiwan; Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan; and Department of Tumor Virology, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan

Received 1 January 2007/Accepted 9 March 2007

The role of Epstein-Barr virus (EBV) in the pathogenesis of breast cancer has been of long-standing interest to the field. Breast epithelial cells can be infected by EBV through direct contact with EBV-bearing lymphoblastoid cells, and EBV infection has recently been shown to confer breast cancer cells an increased resistance to chemotherapeutic drugs. In this study, we established EBV-infected breast cancer MCF7 and BT474 cells and demonstrated that EBV infection promotes tumorigenic activity of breast cancer cells. Firstly, we showed that the EBV-infected MCF7-A and BT474-A cells exhibited increased anchorage-independent growth in soft agar. The increased colony formation capacity in soft agar was associated with increased expression and activation of HER2/HER3 signaling cascades, as evidenced by the findings that the treatment of HER2 antibody trastuzumab (Herceptin), phosphatidylinositol 3-kinase inhibitor, or MEK inhibitor completely abolished the tumorigenic capacity. In the EBV-infected breast cancer cells, the expression of EBV latency genes including EBNA1, EBER1, and BARF0 was detected. We next showed that BARF0 alone was sufficient to efficiently up-regulate HER2/HER3 expression and promoted tumorigenic activity in MCF7 and BT474 cells by the use of both overexpression and small interfering RNA knock-down. Collectively, we demonstrated that EBV-encoded BARF0 promotes the tumorigenic activity of breast cancer cells through activation of HER2/HER3 signaling cascades.

Epstein-Barr virus (EBV), a ubiquitous human gammaherpesvirus, is characterized by its association with an array of malignancies, including Burkitt’s lymphoma, NK/T lymphoma, Hodgkin’s disease, nasopharyngeal carcinoma (NPC), gastric carcinoma, and salivary gland carcinoma (50). Although multiple extrachromosomal copies of the viral episome are present in cells in the biopsy tissues, only a limited set of viral gene products is constitutively expressed since viruses exist in a latent status. The latent proteins comprise six EBV nuclear antigens (EBNA-1, -2, -3A, -3B, -3C, and -LP), three latent membrane proteins (LMP-1, -2A, and -2B), two relatively abundant small, nonpolyadenylated RNAs (EBER1 and EBER2), and BamHI A rightward frame transcripts (BARTs) (29). EBV-encoded latent genes can induce B-cell transformation in vitro by altering cellular gene transcription and constitutively activating key cell-signaling pathways (71). One of the mechanisms for EBV to immortalize B lymphocytes is to up-regulate the expression of integrins (23). On the other hand, the increase of lyosomal phosphatidic acid resulting from up-regulation of autotaxin is the main reason that EBV infection can promote the growth of Hodgkin lymphoma cells (3). Among the epithelial-derived malignancies, reinfection with EBV enhances the tumorigenicity of NPC cells (62). In gastric carcinoma cells, EBV promotes cell proliferation through the induction of insulin-like growth factor-mediated signaling in an autocrine fashion (24).

In addition to the above-mentioned human malignancies, a growing body of evidence has indicated the possible involvement of EBV in other human cancers, such as carcinomas of breast and liver (50). The EBV genome has been detected in 0 to 50% of breast carcinomas (2, 5, 11, 12, 19, 22, 34, 48), and the variations may arise from the varying or uncertain specificity and sensitivity of the detection methods (16). In vitro, breast epithelial cells can be infected by direct contact with EBV-bearing lymphoblastoid cell lines (58). In addition, Arbach et al. have recently reported that the EBV infection of breast carcinoma cells confers increased resistance to chemotherapeutic drugs by facilitating the expression of a multidrug-resistance gene (2). These studies suggest a role of EBV in the pathogenesis of breast cancer and thus warrant further characterization.

Breast carcinoma is the most frequently diagnosed malignancy of women worldwide. The epidermal growth factor receptor (EGFR) family-mediated signaling pathway is known to play a crucial role in breast carcinoma formation and development. There are four members of the EGFR family: HER1 (EGFR), HER2 (also known as neu or ErbB2), HER3, and HER4 (68, 73). Amplification and overexpression of HER2 is observed in 20 to 30% of human breast cancers and is correlated with a poor prognosis (55, 56). Recently, increased ex-
pression of HER3 in breast cancer has been linked to decreased survival. Overexpression of HER3 is noted in about 20% of all breast cancers and is usually coexpressed with HER2 (1, 4, 44, 49, 66). Structurally, HER3 is an unliganded type of receptor while HER3 is deficient in kinase activity. However, coexpression and the formation of a HER2/HER3 heterodimer allow the efficient activation of potent oncogenic signaling cascades (10, 20), which may be particularly important in driving the malignant transformation and progression of mammmary tumors (1). To explore the involvement of EBV in breast cancer, we use the MCF7 and BT474 breast cell lines as study models for EBV infection and follow the biological outcomes of these two cell lines. Of note, EBV infection significantly increases the anchorage-independent growth of both cell lines, which is mediated through the overexpression of HER2 and HER3 followed by the activation of downstream extracellular signal-regulated kinase (ERK) and Akt. Most importantly, we further show that BARF0 alone is sufficient to confer on breast cancer cells increased transforming activity.

MATERIALS AND METHODS

Cell culture. Human breast carcinoma MCF7 and BT474 cell lines were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium-F12 (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biological Industries), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL) at 37°C in a 5% CO2 humidified atmosphere. Trastuzumab (Herceptin) was purchased from Genentech, Inc. (South San Francisco, CA). LY294002 and U0126 were obtained from Calbiochem (San Diego, CA).}

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Establishment of EBV-infected MCF7 and BT474 cells. MCF7 and BT474 cells were cotransfected with the rEBV-infected Akata cells which had been pretreated with 0.8% (vol/vol) rabbit anti-human immunoglobulin G (Cappel, Aurora, OH) to induce virus production. The plate was centrifuged at 2,000 × g for 5 min and then incubated at 37°C for 24 h. After incubation, the culture was washed five times with phosphate-buffered saline to remove residual Akata cells, and fresh medium was added. On day 3, the cells were reseded into 24-well plates in culture medium containing G418 (700 μg/ml) (Gibco BRL) for selection of pooled rEBV-infected clones.

Plasmids, transfection, and cell cloning. The EBNA1 plasmid pCEP4 (Invitrogen) carries the simian virus 40 (SV40) promoter-driven hygromycin resistance (Hyg) gene and the EBNA1 gene. The pcDNA3 and pcDNA3.1-Hyg (Gibco BRL) for selection of pooled rEBV-infected clones.

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EXPERIMENTS were repeated three times. The results were presented as relative activation by calculating the ratio of normalized luciferase activity of the MCF7-HA-BARF0 to that of the MCF7-Neo’ cells.

RESULTS

EBV-infected breast cancer cells display type I latency. To investigate the potential effects of EBV infection in human breast cancer, two human breast cancer cell lines, MCF7 and BT474, were infected with an rEBV (rAkata EBV) which contains the Neo’ gene (54). By a “cell-to-cell” infection procedure previously described (6), EBV-harboring cell clones were enriched by selection against G418. Genomic PCR assays were performed to verify the presence of EBV in the pooled Neo’ clones. PCR was performed using primers specifically amplifying three regions of the EBV genome: BamHI W, BZLF1, and BRLF1. All the PCR results indicated that EBV DNA BamHI W, BZLF1, and BRLF1 were detected in MCF7-A (EBV-infected MCF7 cells) and BT474-A (EBV-infected BT474 cells) but not in the vector control MCF7-Neo’ and BT474-Neo’ cells (data not shown). The status of EBV is known to lead to different gene expression patterns. We therefore determined the viral gene expression in the EBV-infected breast cancer cells by RT-PCR assays. As shown, the EBV latency I products such as EBNA1 and BARF0 transcripts and the EBER1 RNAs were readily detected but not RK-BARF0, EBNB2, LMP1, LMP2A, and Zta transcripts (Fig. 1A), indicating the status of EBV as type I latency in both cell lines. Consistent with these findings, Western blot analysis confirmed that EBNA1 protein but not EBNA2 or LMP1 was expressed in MCF7-A and BT474-A cells (Fig. 1B). We further determined the EBV copy numbers in each of the recipient cells by real-time PCR using Raji cells (50 copies of EBV DNA per cell) (59) as a standard. As a result, approximately one copy of EBV genome DNA was detected in each single MCF7-A and BT474-A cell (data not shown).

EBV enhances breast cancer cell growth in soft agar. To study the effect of EBV infection on breast cancer cells, we assessed the capacity for anchorage-independent growth of the EBV-infected breast cancer cells by testing their ability to form colonies while suspended in soft agar. Like most transformed cells, the parental MCF7 and BT474 cells formed a number of colonies after 2 weeks of growth in soft agar. Under the same conditions, MCF7-A and BT474-A cells formed a number of colonies after 2 weeks of growth in soft agar. Under the same conditions, MCF7-A and BT474-A cells exhibited an increased capacity for soft agar colony formation, with more than a twofold increase in colonies formed (P < 0.05), whereas there was no change in the control vector-harboring cells (Fig. 2A and B). These data suggest that EBV may enhance the tumorigenic capacity of breast cancer cells.

EBV infection leads to upregulation of HER2 and HER3 in breast cancer cells. We next investigated the mechanisms through which EBV enhanced breast cancer cell anchorage-independent growth. HER2 axis is one of the major signaling pathways that go awry in the pathogenic development of breast cancer. Overexpression of HER2 is found in 20 to 30% of cases of human breast cancer; moreover, it is correlated with a poor prognosis (55, 56). In vitro, ectopic expression of HER2 in breast carcinoma cells can promote anchorage-independent growth and enhance tumorigenicity (18, 45). We examined the status of HER2 in EBV-infected MCF7 and BT474 cells. Notably, HER2 was expressed at a strikingly elevated level in both MCF7-A and BT474-A cells, as demonstrated by RT-PCR analysis (Fig. 3A). The HER2 protein level was also significantly increased (Fig. 3B). Because HER2 is devoid of an activating ligand, it forms heterodimers with HER1, HER3, or HER4 to relay signals downstream upon ligand binding (68). We also examined the expression status of HER1, HER3, and HER4 and found that HER3 expression was significantly enhanced at both transcription and translation levels in the EBV-infected cells, whereas the expression of HER1 and HER4 remained unchanged (Fig. 3A and B). The formation of the HER2/HER3 heterodimer in these cells was detected by co-immunoprecipitation. When HER2 was immunoprecipitated from the MCF7-A cell lysates, a robust increase in the amount of HER3 was detected in the immunoprecipitates (Fig. 3C). Concomitantly, a dramatic increase of tyrosine phosphorylation of HER2 was observed, along with increased binding of p85 (PI3K subunit) to HER2/HER3 complex. All of these results suggest that EBV infection not only increases the expression but also enhances the functional activity of HER2/HER3.

FIG. 1. EBV-infected MCF7 and BT474 cells display type I latency viral gene expression profile. (A) RT-PCR analyses of EBV gene expression profiles in the parental (MCF7 and BT474), vector control (MCF7-Neo’ and BT474-Neo’), or EBV-infected (MCF7-A and BT474-A) cells using primers specific for the designated transcript. MCF7-A and BT474-A cells are positive for EBNA1, EBER1, and BARF0 but negative for RK-BARF0, EBNB2, LMP1, LMP2A, and Zta transcripts. RNA from B95.8 virus-transformed lymphoblastoid cells (LCL) was included as a positive control. S26 was detected as an internal control. (B) Western blot analysis of EBV-encoded proteins in MCF7-A and BT474-A cells. Fifty micrograms of protein extracts was detected by 8% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane for immunoblotting with specific antibodies. Both cells are positive for EBNA1 but negative for EBNB2 and LMP1. Cell lysate from LCL was included as a positive control. a-Tubulin was detected as an internal control.
ERK and PI3K are activated downstream of the HER2/HER3 signaling pathway to enhance tumorigenic activity in EBV-infected breast cancer cells. We next examined the downstream signals of the HER2/HER3 axis in EBV-infected MCF7 and BT474 breast cancer cells. As shown in Fig. 4A, the phosphorylation of ERK and Akt, representing the major proliferative and survival pathways downstream of the HER2/HER3 activation, was profoundly increased in MCF7-A and BT474-A cells but not in the control cells. To attest that ERK and Akt were activated downstream of HER2/HER3 but not other signaling pathways, these cells were treated with trastuzumab, a well-known monoclonal antibody directed against the extracellular domain of and an effective inhibitor of HER2. Upon treatment with trastuzumab (10 μg/ml) for a designated time, cell lysates were prepared and subjected to immunoblotting analysis. As shown in Fig. 4B, trastuzumab treatment suppressed phosphorylation of ERK and Akt in MCF7-A cells in a time-dependent manner (Fig. 4B), without affecting the protein levels of these two kinases. These results thus demonstrate
an essential role of activation of HER2/HER3 signaling for efficient activation of ERK and Akt in EBV-infected breast cancer cells.

We further evaluated the role of the HER2/HER3 signaling pathway in the EBV-enhanced transforming capability in the MCF7-A and BT474-A cells. An anchorage-independent assay of growth in soft agar was performed in the presence of trastuzumab. As shown in Fig. 5, trastuzumab treatment efficiently reduced the number of colonies formed by MCF7-A cells in soft agar to that observed in MCF7 and MCF7-Neor cells. At the indicated time points, the cells were washed and harvested in lysis buffer, and 50 μg of protein lysates was analyzed in immunoblotting with antibodies as indicated.

EBV-encoded BARF0 enhances HER2 and HER3 expression. Because expression of EBNA1, EBER1, and BARF0 was detected in EBV-infected MCF7 and BT474 cells (Fig. 1A), we further investigated which of the EBV-encoded products could be attributed to the enhanced expression and activation of HER2 and HER3. MCF7 cells were transfected with individual EBNA1-, EBER-, and BARF0-expressing plasmids, and transfectants harboring the designated EBV genes were established by antibiotic selection. Pooled clones stably expressing specific products at levels similar to the MCF7-A cells were selected for RT-PCR analysis of HER2 and HER3 expression (Fig. 6A). Of note, the transcripts of these two receptors were significantly upregulated in MCF7-HA-BARF0 (BARF0-transfected MCF7 cells), whereas MCF7-EBNA1 and MCF7-EBERs (EBNA1- and EBER-transfected MCF7 cells) expressed HER2 and HER3 at levels similar to those of parental cells. Consistently, the protein levels of HER2 and HER3 were also robustly increased in BARF0 transfectants compared to levels of parental cells (Fig. 6B). To further corroborate that BARF0 is responsible for EBV-mediated upregulation of HER2 and HER3 in breast cancer cells, we performed targeted knockdown of BARF0 expression in MCF7-A cells. After transfection of pSUPERpuroBARF0, the vector carrying the siRNA specifically targeting toward BARF0, the transfected MCF7-A-siBARF0 cells (Fig. 6C). The knock-down was specific toward BARF0 for the expression of EBER1, and EBNA1 remained highly expressed in the MCF7-A-siBARF0 cells. As shown in Fig. 6C, targeted knock-down of BARF0 in MCF7-A cells effectively reversed the expression of HER2 and HER3 to a basal level similar to that observed in the parental MCF7 cells. These data thus suggest that BARF0 may activate the transcription of HER2 and HER3 in MCF7 cells. This notion was further supported by promoter assays. The HER2 promoter-containing luciferase reporter plasmid (pNulit) or the HER3 pro-
A moter-containing luciferase reporter plasmid (pGL3-erb3) was transfected into MCF7-Neo or MCF7-HA-BARF0 cells, respectively. As shown, the luciferase activities driven by HER2 and HER3 promoters were greatly induced by 9.1-fold and 8.3-fold, respectively, in the MCF7-HA-BARF0 cells compared to levels in MCF7-Neo cells (Fig. 6D).

BARF0 enhanced breast cancer cell growth in soft agar through HER2/HER3 signaling. Next, we examined whether BARF0 could activate HER2/HER3 signaling. MCF7-HA-BARF0 and MCF7-Neo cells were treated with vehicle control (dimethyl sulfoxide [DMSO]) and trastuzumab for 48 h, and cell lysates were prepared for Western blot analyses. As shown in Fig. 7A, ERK and Akt were phosphorylated at higher levels in MCF7-HA-BARF0 than in the MCF7-Neo cells, and the treatment with trastuzumab blocked the phosphorylation of both ERK and Akt. As controls, the treatment with LY294002 and U0126 blocked the phosphorylation of ERK and Akt, respectively, in MCF7-HA-BARF0 cells. These data suggest that BARF0 alone is sufficient to induce the activation of HER2/HER3 which, in turn, drives the activation of ERK and Akt. We further determined the involvement of BARF0 in the process of malignant transformation of EBV-harboring breast cancer cells. An anchorage-independent growth assay was performed to measure the capacity of MCF7-HA-BARF0
cells for colony formation in soft agar. After culturing for 14 days, MCF7-HA-BARF0 cells formed significantly more colonies than the control MCF7-Neo cells, and the BARF0-elicited anchorage-independent growth in soft agar was abrogated when MCF7-HA-BARF0 cells were treated with trastuzumab (Fig. 7B). In agreement with these findings, treatment with LY294002 or U0126 also abolished BARF0-mediated colony-forming activity. We thus conclude that BARF0 facilitates tumorigenic activity in breast cancer cells by activating HER2/HER3 signaling axis.

**DISCUSSION**

EBV infection has been implicated in the pathogenic development of a variety of human cancers including NPC and stomach cancer (27, 39, 46, 69). It is of particular interest that EBV employs strategies different from those of other tumor viruses to enhance the transforming activities of the infected cells. EBV has been shown to directly promote cell cycle progression (37), to enhance the cancer cell growth through induction of interleukin-9 and insulin-like growth factor 1 (24, 25), to inhibit apoptosis by increasing the expression of anti-apoptotic protein bel-2 in B cells or up-regulating the expression of A20 in epithelial cells (17, 35), to activate the expression of a variety of matrix metalloproteinases, and to promote cell invasiveness (39, 69). Of interest, the EBV genome and its viral products have been commonly detected in many human carcinoma biopsies including breast cancer; however, the role of EBV in breast malignancy has only recently been studied (2, 5, 50). We have previously established a cell-to-cell cocultured system which has allowed us successfully to infect epithelial cells in vitro by EBV (6). By this cell-to-cell infection procedure, we obtained in this study two EBV-infected breast cancer cell lines and demonstrated the EBV-mediated overactivation of the HER2/HER3 signaling axis in these EBV-infected breast cancer cells.

Protein tyrosine kinases represent a major class of oncogene products. EBV-encoded gene products have been shown to activate a number of receptor and nonreceptor tyrosine kinases and control many crucial events in cell proliferation, differentiation, and development (9, 14, 21, 36, 38, 40, 42, 53). In particular, LMP1 can up-regulate the expression of c-Met through the activation of transcription factor Ets-1 and promote the expression of vascular endothelial growth factor via NF-κB activation (21, 43). LMP1 has also been shown to up-regulate the expression of EGFR both in vivo and in vitro (42, 53). The EB viral latent membrane protein LMP2A can act as a survival factor by inhibiting transforming growth factor β1-mediated apoptosis and promote cell survival through the PI3K pathway (14). LMP2A also activates Syk in epithelial cells and promotes cell migration (40). The viral lytic transactivators Zta and Rta can induce the expression of tyrosine kinase TKT and c-Mer, respectively (36, 38). On the other hand, ectopic EBNA1 expression has been shown to suppress the expression and transforming activity of the HER2/neu oncogene in HER2/neu-overexpressing human ovarian cancer cells and to sensitize cells to paclitaxel (Taxol)-induced apoptosis (8, 9). In this study, we report for the first time that EBV infection leads to the dysregulation of the HER2/HER3 signaling axis in breast cancer cells. Furthermore, we demonstrate that EBV-elicited activation of the HER2/HER3 cascade ushers the phosphorylation of ERK and Akt, which may in turn stimulate cell anchorage-independent growth in soft agar. It has been reported that EBV infection may contribute to paclitaxel resistance in breast carcinoma cell lines due to the increased expression of MDR1 (2). In this study, we have also found that EBV-infected MCF7 and BT474 cells displayed increased resistance to paclitaxel-induced apoptosis, and the resistance phenotype is associated with a concomitant increase in HER2-mediated phosphorylation of p34(Cdc2) at tyrosine-15 (data not shown). These data are in consistent with previous findings that overexpression of HER2 or MDR1 renders breast cancer cells highly resistant to paclitaxel (72). These findings therefore suggest that EBV infection may impede clinical chemotherapy in breast cancers by inducing drug resistance.

To further dissect the molecular mechanisms through which EBV up-regulates HER2/HER3 signaling in breast cancer cells, we examined the involvement of the major EBV gene products expressed in the infected cells, including EBNA1, EBERR1, and BARF0 (Fig. 1A). Notably, we found that BARF0 alone confers increased expression and activation of HER2 and HER3 in breast cancer cells (Fig. 6). In addition, using siRNA specifically targeted to knock down the expression of BARF0 in the EBV-infected MCF7-A cells reversed EBV-elicited HER2 and HER3 expression, demonstrating that BARF0 alone is sufficient to induce the constitutive activation of the HER family in EBV-infected breast cancer cells. BARF0 belongs to the BART family (51). Other than BARF0, RK-BARF0, RPMS1, and A73 also represent the main products of these transcripts (13, 15, 26, 33, 51, 52, 57). The transcripts of this differentially spliced RNA family share unique characteristics, and their expression is constantly detected in several types of EBV-infected cells and EBV-associated malignant biopsies (64). Among them, RK-BARF0 is proposed to encode a 279-amino-acid protein with a possible endoplasmic reticulum-targeting sequence. It interacts with Notch, and reduced expression of Notch is found in EBV-positive cell lines that correlate with RK-BARF0 expression (33). RPMS1 is proposed to encode a nuclear protein named RPMS. It interacts both with CBF1 and corepressor CIR and interferes with NotchIC and EBNA2 activation of CBF1-repressed promoters (57, 74). It has been demonstrated that A73, a cytoplasmic protein, can interact with the cell protein RACK1 (57). In contrast to BARF0, RK-BARF0 (Fig. 1A) and RPMS1 transcripts (data not shown) were not detected in MCF7-A or BT474-A cells by RT-PCR. Actually, it is still not clear how the BART family would contribute to EBV infection or pathology. In this study, we provide another line of evidence that BARF0 can enhance the cell clonability through dysregulation of the HER2/HER3 signaling cascade. This data provide insights into the roles of...
EBV and BARF0 in EBV-associated malignancies, in particular, breast cancer.

ACKNOWLEDGMENTS
We thank Mien-Chie Hung of the University of Texas M.D. Anderson Cancer Center for kindly providing pNult plasmid and Frederick E. Domann of the University of Iowa for kindly providing pGL3-erbb3 plasmid.

This work was supported by the National Science Council, Taiwan (grants NSC 92-2321-B-002-007 and NSC 95-2320-B-002-006).

REFERENCES


49. Quinn, C. M., J. L. Ostrowski, S. A. Lane, D. P. Loney, J. Teasdale, and F. A. Rol.
59. Sternas, L., T. Middleton, and B. Sugden. 1990. The average number of Epstein-Barr virus nucleic antigen 1 per cell does not correlate with the average number of Epstein-Barr virus (EBV) DNA molecules per cell among different clones of EBV-immortalized cells. J. Virol. 64:2407–2410.