Epstein-Barr Virus Infection of Human Gastric Carcinoma Cells: Implication of the Existence of a New Virus Receptor Different from CD21

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Received 30 December 1996/Accepted 19 March 1997

Epstein-Barr virus (EBV) is a human herpesvirus that causes infectious mononucleosis and has been associated with various lymphoid and epithelial malignancies (11). There is increasing evidence that links EBV infection to epithelial cells. EBV is detected in desquamated cells shed from the oropharyngeal epithelia of patients with infectious mononucleosis (20). Hairy leukoplakia is a productive EBV infection of epithelial cells of the tongue (5). The very strong association between EBV and nasopharyngeal carcinoma is already well known. Moreover, the viral genome is detected in rare carcinomas with intense lymphoid stromata (lymphoepitheliomatous carcinomas) arising in the salivary glands (7, 23), thymus (8), and stomach (15) and also in gastric carcinomas of the more common adenocarcinoma type (16).

The virus preferentially infects B cells in vitro, owing in large part to restricted expression of the EBV receptor CD21 (3), which also serves as the receptor for the complement C3d fragment. The major envelope protein of EBV, designated gp350/220, is the ligand that mediates attachment of the virus to CD21 on B cells (10, 22).

Unlike B lymphocytes, epithelial cells have displayed a remarkable resistance to EBV infection in vitro. The earliest reported success is of EBV-replicative-antigen expression in a primary epithelial cell culture of a nasopharyngeal carcinoma after superinfection with EBV strain P3HR-1 (4). Successful infection of a small number of cultured cells from the uterine cervix occurred when EBV was taken directly from throat washings (21). These results suggest that EBV receptors do exist on epithelial cells. Two monoclonal antibodies, HB5 and B2, against different determinants on CD21 were shown to react with a 200-kDa glycoprotein on normal and transformed epithelial cells (19, 26). However, this protein does not interact with gp350 (2). Although CD21 RNA is detected in epithelial cells, including nasopharyngeal carcinoma cells, CD21 has not yet been shown to be the EBV receptor of epithelial cells (2).

Recently, we isolated EBV-negative cell clones from the Akata Burkitt’s lymphoma line (17) and demonstrated that EBV-negative Akata cells (Akata−) are suitable for clonal propagation of EBV recombinants (18). With this system, we have generated 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. The validity of using this virus was shown by isolating EBV-positive cell clones from a human T-cell line, MT2 (24). In this study, we used recombinant EBV carrying a selectable marker for infecting human carcinoma cell lines. The results indicate that some carcinoma cell lines are susceptible to EBV infection and that the infection is mediated via a new receptor different from CD21.

EBV infection of human gastric carcinoma cells. Three gastric carcinoma cell lines, AGS (1), MKN28 (9), and MKN74 (14), were used for EBV infection. AGS is derived from signet ring cell carcinoma, and MKN28 and MKN74 are derived from well-differentiated tubular adenocarcinomas.

For EBV infection, we used a recombinant EBV containing the neomycin resistance (Neo+) gene at the BXLF1 site of EBV DNA, which is nonessential for infection and replication (18). The EBV-converted Akata− cell clone which was used to produce drug-resistant EBV was infected only with the recombinant virus. To induce virus production, cells (5 × 106) were suspended in 5 ml of complete medium containing 1% (vol/vol) goat antibodies specific for human immunoglobulin G (IgG; Cappel). After 4 h of incubation, cells were washed, suspended in fresh medium, and incubated for 44 h. Then, the culture fluid was harvested, filtered through a 0.45-μm-pore-size membrane, and stored at −152°C until use.

Carcinoma cells were removed from culture flasks with EDTA (1 mM for 5 min). Cells (106) were then suspended in 1 ml of diluted (1:10) culture supernatant from recombinant EBV-infected Akata cells. After a 90-min incubation with gentle shaking, cells were washed and resuspended in fresh medium and incubated for 2 days. Cells were then plated in...
96-well, flat-bottom plates at 10,000 cells per well (AGS cells, 420 μg/ml; MKN28 or MKN74 cells, 560 μg/ml) with complete medium containing G418. Cells were fed every 5 days until colonies emerged (3 weeks).

All three lines were susceptible to EBV infection. After 3 days of infection, EBNA was expressed in up to 1% of the cells when they were studied by anticomplement immunofluorescence with a polyvalent human antiserum. After 3 weeks of incubation in the selective medium, G418-resistant clones appeared from each of the cell cultures.

The six representative cell clones from each gastric carcinoma cell line were analyzed to ascertain whether cells were infected with EBV. All clones were virtually 100% positive for EBNA (Fig. 1A). Southern analysis of cultures probed with the BamHI W fragment of EBV DNA indicated that all clones were infected with EBV (Fig. 1B). Further analysis of EBV-infected epithelial cell clones by immunoblot analysis demonstrated that all clones were positive for EBNA 1 but negative for EBNA 2. LMP 1 expression was negative in most clones, but there were a few exceptions. Among each of the six clones, one MKN28 and two MKN74 cell clones were weakly positive for LMP 1 (Fig. 1C).

Among the three gastric carcinoma lines, AGS was most susceptible to EBV infection. The susceptibility of the gastric carcinoma lines was 14 to 70% of the susceptibility of Akata cells (Table 1). CNE1 (an EBV-negative epithelial cell line derived from a nasopharyngeal carcinoma [27]), HeLa (a cell line derived from a uterine cervical carcinoma), BHK, and NIH 3T3 cells were also used for EBV infection and were not at all susceptible to EBV infection.

Absence of CD21 expression on gastric carcinoma cells. EBV infection of B lymphocytes starts from virus binding to cell surface CD21 molecules through the envelope protein gp350/220. Thus, we examined whether the gastric carcinoma cells expressed CD21 molecules on their cell surfaces by fluorescence-activated cell sorting analysis. As shown in Fig. 2A, CD21 was not expressed on gastric carcinoma cells.

We then examined the expression of CD21 in the gastric carcinoma cell lines by reverse transcription-PCR analysis (24). Serially diluted RNA samples were reverse transcribed and subjected to 30 cycles of PCR amplification with the sense primer 5'-GTTGTTCAGGTACCTTCGC-3' and the antisense primer 5'-TAGGAAGTGCTGGACACTCG-3'. PCR products were detected with an oligonucleotide of CD21 with the sequence 5'-CGACACGACTACCAACCTGTGT-3'. The results are shown in Fig. 2B. In Akata cells, CD21 mRNA was amplified from a 15.6-ng RNA sample. On the other hand, in AGS and MKN28 cells, CD21 mRNA was not detected even when 250-ng RNA samples were used for amplification. Under these conditions, a weak band was detected in MKN74 cells.

To confirm that EBV did not utilize CD21 for infection of gastric carcinoma cells, AGS cells were preincubated with a

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**TABLE 1. EBV infection of gastric carcinoma cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of G418-resistant cell clones per 10⁶ infected cells in a</th>
<th>Expt 1</th>
<th>Expt 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akata</td>
<td>50</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>AGS</td>
<td>35</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>MKN28</td>
<td>7</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>MKN74</td>
<td>9</td>
<td>10</td>
<td></td>
</tr>
</tbody>
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a Representative results of five separate experiments are shown.
monoclonal antibody, OKB7, which recognizes the EBV binding site on CD21 and then infected with recombinant EBV. After 3 weeks of incubation in selective medium, the appearance of EBV-positive cell clones was determined. As shown in Fig. 2C, OKB7 did not block EBV infection of AGS cells though it did block EBV infection of Akata cells. OKB7 also did not block EBV infection of MKN28 and MKN74 cells (data not shown).

EBV replication in gastric carcinoma cells. The immunofluorescence assay showed that 6 to 16% and 7 to 25% of gastric carcinoma cells expressed the immediate-early BZLF1 protein and the viral capsid antigen, respectively. The Western blot analysis showed that all of the recombinant EBV-infected gastric carcinoma cell clones spontaneously produced EBV lytic antigens (Fig. 3).

Release of infectious EBV from gastric carcinoma cells was assayed by transformation of cord blood lymphocytes. The 50% transforming doses were 10^{3.4} to 10^{3.7} per ml (Table 2).

In this study, we tested five human carcinoma cell lines and two rodent fibroblast lines for their susceptibilities to EBV infection. Among them, all three gastric carcinoma lines were susceptible to EBV infection. This was clearly proved by isolating 100%-EBV-positive carcinoma cell clones with recombinant EBV carrying a selectable marker. Although we have shown here that only gastric carcinoma cells are susceptible to EBV, our preliminary study indicated that some carcinoma lines are also susceptible.

**FIG. 2.** CD21 expression in gastric carcinoma cells. (A) Fluorescence-activated cell sorting analysis. Cells (10^6) were incubated with an anti-CD21 monoclonal antibody, OKB7, and subsequently incubated with FITC-conjugated anti-mouse Ig. Histograms are plotted as log fluorescence intensity (x axis) versus cell number (y axis). Fluorescence-activated cell sorting analysis indicated that 11% of Akata^- cells were positive for CD21. (B) Quantitative reverse transcription-PCR assay for CD21 mRNA. Amounts of RNA samples used for the assay were 250 ng (lane 1), 62.5 ng (lane 2), 15.6 ng (lane 3), 3.9 ng (lane 4), and 1.0 ng (lane 5). (C) Effect of OKB7 on EBV infection. Cells (10^6) were preincubated with various amounts of OKB7 monoclonal antibody for 60 min at 4°C. The cells were washed and suspended in 1 ml of diluted (1:10) culture supernatant from recombinant EBV-infected Akata cells. After a 90-min incubation with gentle shaking, cells were washed, resuspended in fresh medium, and plated in 96-well, flat-bottom plates at 10^3 cells per well. After 2 days of infection, G418 was added to cell culture. Cells were fed every 5 days until colonies emerged (3 weeks). The experiments whose results are presented in panel C were done at the same time. All 10 wells produced drug-resistant clones in both Akata and AGS cultures that had not been pretreated with OKB7.

**TABLE 2.** EBV production in recombinant EBV-infected gastric carcinoma cells

<table>
<thead>
<tr>
<th>Virus source</th>
<th>Transforming efficiency on cord blood lymphocytes (TD⁵₀/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akata</td>
<td>10^{3.5}</td>
</tr>
<tr>
<td>AGS</td>
<td>10^{3.7}</td>
</tr>
<tr>
<td>MKN28</td>
<td>10^{3.9}</td>
</tr>
<tr>
<td>MKN74</td>
<td>10^{3.5}</td>
</tr>
</tbody>
</table>

*To determine transforming activity, the virus preparation was titrated on cord blood lymphocytes. One milliliter of cell suspension (2 × 10^⁶) was incubated with a serially diluted 20-ml suspension of EBV and incubated at 37°C. The cultures were fed weekly by changing half of the medium and observed on an inverted microscope every 5 days for 6 weeks to detect the appearance of growing cell aggregates. The presence of EBNA in the transformed cells was taken as a proof of the role of EBV in the transformation. The 50% transforming dose (TD⁵₀) was calculated by the Reed-Muench formula.*

**FIG. 3.** Production of EBV lytic antigens in recombinant EBV-infected gastric carcinoma cells. The blot was treated with an early antigen (EA)-positive human serum (early antigen titer, 1:1,280) and with FITC-conjugated goat anti-human Ig. Lane Akata + anti-IgG contains the EBV-positive Akata cells treated with anti-IgG for 48 h. cl., clone.
cells that originated from other tissues are also susceptible to EBV infection.

Flow-cytometric analysis failed to detect CD21 on the surfaces of carcinoma cells, and pretreatment of carcinoma cells with an anti-CD21 monoclonal antibody, OKB7, did not inhibit EBV infection of carcinoma cells, though it did inhibit infection of B cells completely. These results indicate that EBV utilizes a receptor other than CD21 for infection of epithelial cells.

EBV-infected gastric carcinoma cells were positive for EBNA 1 but negative for EBNA 2. Most cell clones were also negative for the expression of LMP 1. Although we have not yet examined them precisely, these patterns seem to correspond to type I latency (13), which is seen in gastric carcinoma (6). Therefore, the present system should become a model for investigating the pathogenic role of EBV in EBV-associated gastric carcinoma. We are now studying whether primary epithelial cells are immortalized by EBV.

All of the cell clones spontaneously produced EBV. It remains to be clarified whether virus production reflects their approach toward terminal differentiation, as was indicated in the infection of cervical epithelial cells (20).

Gastric carcinoma cell lines were obtained from the Japanese Cancer Research Resources Bank.

This work was supported in part by grants-in-aid from the Ministry of Education, Science, Sports, and Culture, Japan, and from the Vehicille Racing Commemorative Foundation.

REFERENCES