INTRODUCTION

Recently there have been many reports suggesting the role of Epstein–Barr virus (EBV) in the development of certain cases of gastric carcinoma (Tokunaga et al., 1993; Fukayama et al., 1994; Imai et al., 1994; Shousha and Luqmani, 1994; Harn et al., 1995; Iezzoni et al., 1995). EBV has been found in most cases of rare gastric lymphoepithelioma-like carcinoma (LELC) (Min et al., 1991; Shibata et al., 1991; Ohfuji et al., 1996) and a small but significant proportion of common gastric adenocarcinoma (Shibata and Weiss, 1992; Tokunaga et al., 1993). The presence of EBV in gastric carcinoma has been shown by polymerase chain reaction (PCR) for EBV DNA (Satoh et al., 1998) and in situ hybridization for EBV-encoded small RNA (EBER) (Shibata et al., 1991; Ohfuji et al., 1996). In general it has been reported that the gastric carcinoma cells express EBV nuclear antigen (EBNA) 1 and EBER1, but not the other EBNAs or latent membrane proteins (LMPs) (Fukayama et al., 1994; Imai et al., 1994).

To understand the role of EBV in epithelial cells, we tried to establish EBV-positive epithelial cell lines from EBV-positive gastric carcinoma tissues. Recently we established two EBV-infected epithelial cell lines, GT38 and GT39, from gastric tissues bearing carcinoma from the patients, although they were derived from noncancerous portions of gastric carcinoma-tissues (Tajima et al., 1998). We characterized the cell property and EBV infection in the cell lines (Tajima et al., 1998; Takasaka et al., 1998; Gao et al., 1999; Kanamori et al., 2000). Both cell lines were positive for cytokeratin, an epithelial marker, but not lymphocyte markers (Tajima et al., 1998). The spontaneous EBV reactivation was observed in small proportions in the cells and the infectious virus was produced from the cells (Takasaka et al., 1998). The EBV infection was referred to as latency type III because the expression of EBNA1, EBNA2, and LMP1 was detected in the cells by Western blotting. A constitutive, low-level NO synthase mRNA was expressed in the cell lines and the produced NO was a regulatory factor in maintaining virus latency via inhibiting EBV reactivation in the cells (Gao et al., 1999). Both cell lines formed colonies in soft agar (Kanamori et al., 2000).

In this study, we investigated the tumorigenesis of the cell lines in the severe combined immunodeficient (SCID) mouse to determine whether the cell lines have the character of tumor cells. The present study describes the tumorigenesis, the character of developed tumors, and the expression of EBV genes in the tumor cells.

RESULTS

Development of tumors in SCID mice

To study the tumorigenesis of SCID mice, we inoculated them under the skin of SCID mice.
The development of tumors in SCID mice was observed at 45 and 56 days after inoculation of GT39 and GT38 cells, respectively (Fig. 1A). Both tumors were located under the skin which had been inoculated with the cells. No metastasis was observed in the mice.

**Hematoxylin/eosin (H & E) staining and EBER in situ hybridization**

Both of the tumors with GT38 and GT39 cells were diagnosed as undifferentiated carcinoma by H & E staining (Figs. 1B and 1D). No production of mucus was observed by periodic-acid-Schiff staining in the tumors (data not shown). There were vigorous proliferative cells in the outside portion of the cardia of the tumors. EBV infection in the tumor cells was tested by EBER1 in situ hybridization (Figs. 1C and 1E). EBER1 was detected in the nuclei of the tumor cells and the strong signals were detected in the same location of the proliferative cells. Tubular structures which were expected as mouse tissues were seen in the surrounding tumor in the GT39-inoculated mouse (Fig. 1D). EBER1 was not detected in the structures (Fig. 1E).

**Judgment on karyotype of human chromosome in the tumors**

To confirm the human origin of tumors induced with GT38 and GT39 cells, we carried out Q-band staining of chromosomes in the tumor cells cultured in vitro (Fig. 2). As a rule the centromere of the human chromosome does not stain, although the mouse chromosome's cen-
tromere does. Moreover, the mouse chromosome does not have short arms, whereas human chromosome does. We found that the centromeres were not stained in the chromosomes of tumor cells induced by GT38 (Fig. 2A) or GT39 (Fig. 2B), while they were stained in the mouse chromosomes (Fig. 2C). Additionally, the chromosomes of the tumor cells had short arms (Figs. 2A and 2B). These results indicated that the tumors originated from the inoculated GT38 and GT39 cells, respectively.

Detection of EBV DNA by Southern blot hybridization

The clonality of EBV genomes indicates cellular clonality (Raab-Traub and Flynn, 1986). The clonality of EBV genomes was analyzed in the tumors of SCID mice by Southern blot hybridization (Fig. 3). Hybridization with an XhoI fragment identified a major 12-kb EBV DNA restriction enzyme fragment in both the GT38 tumor (GT38T) and the GT39 tumor (GT39T; data not shown) cells. No ladder of fragments representing linear termini was detected in either GT38T or GT39T (data not shown), whereas the ladder of fragments was detected strongly in GT38 and weakly in the cultured GT38T cells (GT38TC) on two passages at an interval of 5 days in vitro.

Expression of EBV proteins

The expressions of EBNA2 and LMP1 were analyzed in GT38, GT39, GT38T, GT39T, and GT39TC by Western blotting (Fig. 4). EBNA2 was detected in GT38 and GT38T, but not in GT38T, and was detected in GT39, GT39T, and GT39TC, although the level in GT39T was much lower. Thus the expression of EBNA2 clearly reduced in both tumors.

The expression of LMP1 was detected in GT38 and GT38T; however, it was detected faintly in GT38T. On the other hand, LMP1 was detected in GT39, GT39T, and GT39TC. The reduction of LMP1 expression was clearly observed in GT38T. The tumor cells were continuously...
cultivated in vitro and the expressions of EBNA2 and LMP1 were always detected in the cultured cells (data not shown). Lytic antigens ZEBRA and EA-D were detected in GT38 and GT39, but not in GT38T, GT39T, GT38TC, or GT39TC by Western blotting (data not shown).

EBNA gene transcription

To understand the reduced expression of EBNA2 protein in the tumor cells (GT38T and GT39T), the expression of EBNA2 mRNA was analyzed in GT38, GT39, GT38T, GT39T, GT38TC, and GT39TC by reverse transcription–polymerase chain reaction (RT-PCR; Fig. 5A). The transcripts of EBNA2 were detected equally in all the preparations. Transcription for all the EBNAs can initiate from BamHI C and/or W at the BamHI C and/or W promoters (Cp and/or Wp) in latency III, while in latency I and II only EBNA1 is expressed and the mRNA initiates at a promoter within BamHI Q (Qp) (Rickinson and Kieff, 1996). RNAs prepared from these cells were analyzed by RT-PCR for EBNA1 transcription from Cp/Wp (Fig. 5B). The Cp and Wp transcripts were detected in all preparations from the different sources, whereas no Qp transcript was detected in any of the preparations (data not shown). These results indicate that the reduction of EBNA2 protein in GT38T and GT39T and LMP1 protein in GT38T was not due to the change of latency pattern of transcription from latency III to latency I.

DISCUSSION

The oncogenic potential of GT38 and GT39 cell lines has been suggested by the ability of colony formation in soft agar (Kanamori et al., 2000). The present study demonstrated that the tumors developed in SCID mice that had been inoculated with GT38 and GT39 cells. The tumors were needed to prevent the possibility of mouse cells becoming infected by EBV from the inoculated cell lines and growing as tumors, although EBV infection is restricted to human and other primates under normal circumstances. We detected the human karyotype in tumor cells. These facts demonstrate that GT38 and GT39 cells have oncogenic potential in the SCID mouse. This is the first evidence of the tumorigenesis in the SCID mouse on EBV-positive epithelial cell lines derived from gastric tissues, even though many previous studies reported on EBV-infected B cells in SCID mice (Rowe et al., 1991; Maria et al., 1992).

These tumors were solid carcinoma and were clearly different from EBV-positive B-cell lymphoma in the SCID mouse (Rowe et al., 1991; Maria et al., 1992). GT38T was morphologically similar to undifferentiated LELC, although the infiltration of lymphoid cells, which is seen in LELC, did not occur. The similarity to LELC is interesting, because EBV infection is detected with a high frequency in LELC (Min et al., 1991; Shibata et al., 1991; Ofuji et al., 1996). EBV DNA was detected in the tumors developed in SCID mice by Southern blot hybridization. The EBV DNA bands showed a monoclonal EBV genome by the terminal repeat (TR) analysis of EBV DNA (Fig. 3). The molecular size of TR was consistent with that of a previous report that both GT38 and GT39 had a single clonotype of EBV DNA (Takasaka et al., 1998). A ladder of fragments representing linear termini was not detected in the tumor cells; however, it was detected in the cultured cells in vitro. These results may suggest that EBV reactivation
dose not occur in vivo, but is induced in the condition in vitro. This phenomenon was observed in EBV-positive B cells. EBV was harbored in peripheral lymphocytes of infectious mononucleosis patients as a nonproductive latent infection, which is activated to produce virus in vitro (Rickinson et al., 1974). The common pathways for EBV reactivation may be transduced in the cultures in vitro.

The expression of EBNA2 and LMP1 proteins was also different between the cultured cells and the tumor cells (Fig. 4). In agreement with the classification of EBV latency type III (Takasaka et al., 1998). Interestingly, the EBNA2 and LMP1 expressions in GT38T were not detected by Western blotting, whereas the proteins were detected in both GT38 and the cultured tumor cells GT38TC. The decreased expression of EBNA2 was also seen in the serially passaged tumors of GT38T (data not shown). It was surprising because the EBV latency in GT38T was phenotypically latency type I, which is seen in gastric carcinoma (Fukayama et al., 1994; Imai et al., 1994; Iwasaki et al., 1998). However, the transcript of EBNA2 was detected equally in the cultured cells and tumor cells and the analysis of EBNA1 promoters demonstrated that Cp/Wp promoters were transcribed similarly in both cultured cells and tumor cells. The difference of EBV gene expression was also observed in EBER expression in the tumors. The signals of EBER were stronger in vigorous proliferative cells in the outside portion of cardia of tumors (Fig. 1). Necrosis was observed in the cardia portions of some tumors which grew over in SCID mice. The reduction of EBNA2, LMP1, and EBER expression may be the result of the reduced cell growth and cell death of tumors in SCID mice.

We have not tested in detail the efficiency of tumor development, but it seems to be high, because the tumor always developed in all mice inoculated with either GT38 or GT39 cells at 5 × 10^3 cells/mouse. Moreover, these tumors were serially passaged from mouse to mouse. EBV was always detected in the tumor cells which carried out several passages in SCID mice. This fact indicates that the oncogenic potential of GT38 and GT39 cells did not change during passage in vitro and in vivo.

Although GT38 and GT39 cell lines have the character of tumor cells, their origin from noncancerous portions and the latency type III suggest that GT38 and GT39 cell lines may not have originated from gastric carcinoma cells. What are the original cells? One of the possibilities is that they originate from EBV-infected noncarcinomatous gastric epithelium. Yanai and colleagues (1997) reported that gastric epithelium is frequently infected with EBV and suggested that prolonged EBV persistence may contribute to the development of gastric carcinoma. Another possibility is that they originate from normal gastric epithelial cells, which are infected in vitro with EBV from other EBV-infected cells, such as the carcinoma or non-carcinomatous gastric epithelium, and immortalized as the cell lines. Recently it was demonstrated that EBV infection causes a transformed phenotype on primary gastric epithelial cells in vitro (Nishikawa et al., 1999).

The tumor development in the SCID mouse will be a useful animal model for studying EBV-infected epithelial cell tumors, such as gastric carcinoma and nasopharyngeal carcinoma, and will give us valuable ideas for understanding EBV infection in epithelial cells on the molecular level.

MATERIALS AND METHODS

Cell cultures

GT38 and GT39 cell lines were maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum, 100 μg/ml of streptomycin, and 100 U/ml penicillin. Cultures were treated with 0.25% trypsin and 100 μM EDTA on passage, and incubated at 37°C in humidified air with 5% CO₂.

Inoculation of cell lines to SCID mice

SCID mice were purchased from Clea Japan (Tokyo, Japan). The mice were maintained in the infected animal laboratory (pathogen-free conditions) at the Animal Research Center at the Faculty of Medicine, Tottori University. Six-week-old female SCID mice were inoculated under the skin with 5 × 10^3 cells suspended in 3 ml of the cell culture supernatant.

Q-banding/Hoechst staining

Karyotype was checked by quinacrine/Hoechst double staining (Caspersson et al., 1970). Cells were treated with colcemid (0.05 μg/ml) for 2 h, washed with phosphate-buffered saline (PBS), and centrifuged at 1200 rpm for 5 min at room temperature. The pellet was mixed with 75 mM KCl reacted for 15 min, fixed with acetic acid and methanol at a ratio of 1:3, and then centrifuged at 1200 rpm for 5 min. The pellet was suspended in the acetic acid and methanol solution, then spread onto metaphase chromosome-prepared air-dried slides. The slides were stained as follows. First, the slides were soaked in Mcllvaine buffer I, soaked in quinacrine solution for 15 min, and washed with water. Second, the slides were soaked in Mcllvaine buffer II, soaked in Hoechst 33258 staining solution for 20 min, and washed with water. Third, the slides were soaked in Mcllvaine buffers II and III. They were then covered with a coverslip and observed under a fluorescence microscope.

In situ hybridization for EBER

In situ hybridization for EBER was carried out in paraffin-embedded tissue sections using an antisense probe for EBER as previously described (Tokunaga et al.,...
H&E staining was done in the sections for histological analysis of tumors. Southern blotting DNA was extracted from tumor tissues and cultured cells. To analyze the TR of EBV DNA in cells (Raab-Traub and Flynn, 1986), extracted DNA was digested with BamHI, and Southern blotting was done to detect the termini of EBV DNA with a 32P-labeled XhoI fragment probe as described previously (Takasaka et al., 1998).

Western blotting EBNA2 and LMP1 were detected by Western blotting. Tumor tissues were minced by razors in dishes with ice-cold PBS/10% trichloroacetic acid. The minced tumor cells and cultured cells were washed twice with ice-cold PBS/10% trichloroacetic acid, incubated on ice for 15 min, and centrifuged at 15,000 rpm at 4°C for 5 min. The pellets were lysed in 50 μl of lithium dodecylsulfate (LDS) sample buffer (125 mM Tris–HCl, pH 6.8, 2.3% [w/v] LDS, 10% [w/v] glycerol, 5% [v/v] 2-mercaptoethanol, 10 μg/ml bromphenol blue) and neutralized with 2 M Tris–HCl, pH 12 and boiled for 5 min. These samples were resolved by SDS–10% polyacrylamide gel electrophoresis (PAGE), and transferred to a PVDF membrane (Millipore, Bedford, MA). The membranes were reacted with mouse antibodies to LMP1 (Dako Japan, Kyoto, Japan), EBNA2 (Dako Japan), or ZEBRA (developed in our laboratory) for the first antibody and an alkaline-phosphatase-conjugated anti-mouse antibody (New England Biolabs, Beverly, MA) for the second antibody. The bands were visualized by using a combination of Nitro-block (Tropix, Bedford, MA) and CSPD (Boeringer Mannheim, Mannheim, Germany), and were exposed to X-ray film (Eastman Kodak, Rochester, NY).

RNA isolation Total cellular RNAs form cultured cells and tissues were extracted using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer’s protocol. Briefly, cells were lysed in the Isogen reagent. The homogenates of the cell lysates of the tissues were kept at room temperature for 5 min and then treated with 0.2 ml chloroform for 3 min. The mixture was centrifuged at 12,000 rpm for 10 min at 4°C. The upper aqueous phase was collected, treated with 0.5 ml isopropanol for 10 min, and centrifuged for 10 min at 4°C. The RNA pellets were washed in 75% ethanol, dried, and dissolved in diethyl pyrocarbonate-treated distilled water. All RNA samples had an OD260/OD280 ratio >1.50.

RT-PCR analysis For cDNA synthesis, 10 pmol of a 3′-primer specific for each transcript was added to the RNA sample (1 μg). Reverse transcription was performed using an M-MLV reverse transcriptase kit (Gibco BRL, Grand Island, NY) and Ready-To-Go first-strand cDNA kit (Pharmacia Bio-tech, Tokyo, Japan) as recommended by the manufacturer. Reverse transcription was performed at 37°C for 60 min in a total volume of 20 μl, then heated at 95°C for 5 min to stop the reaction and denatured the reverse transcriptase, and cooled on ice for 5 min. These products were used to analyze promoter and EBNA2 expression. The PCR products were electrophoresed in 5% acrylamid

<table>
<thead>
<tr>
<th>Transcripts</th>
<th>Primer sequences</th>
<th>Product size (bp)</th>
<th>EBV genome coordinates</th>
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<td>5′ primer</td>
<td>GCTGCTACGCATTAGAGACC</td>
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<td></td>
<td>probe (Uh)</td>
<td>GGTGAATCTGCTGCCAGGTC</td>
<td>67628–67609</td>
</tr>
</tbody>
</table>

* Oligonucleotide sequences used for PCR analysis for detection of EBV mRNA.
* Coordinates refer to genomic map of B95-8 virus.

1993). H&E staining was done in the sections for histological analysis of tumors.

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gels, stained with ethidium bromide, and photographed under a UV-transilluminator. Full details of the sequences and genome coordinates of primers and probes used to detect EBV transcripts are given in Table 1. Our primer pairs were designed in different exons for individual EBV transcripts so that occasional amplification of contaminated genomic DNA, if any, could be easily discriminated from the relevant RNA amplification by product size. They were blotted onto a nylon membrane (Amersham, Buckinghamshire, UK) by electric transfer and subjected to hybridization with \[^{32}P\]ATP 5'-end-labeled internal oligonucleotide probes.

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