Epstein-Barr Virus (EBV) Infection in Epithelial Cells In Vivo: Rare Detection of EBV Replication in Tongue Mucosa but Not in Salivary Glands

Phroso Frangou, Maike Buettner, and Gerald Niedobitek
Institute for Pathology, Friedrich-Alexander-University, Erlangen, Germany

Epstein-Barr virus (EBV) is transmitted through saliva, but the cellular source is controversial. Putative reservoirs include oral epithelium and salivary glands. Tongue mucosal samples, salivary glands, and tongue carcinomas were studied, by immunohistochemistry and in situ hybridization, for evidence of EBV infection. EBV replication was seen in 1.3% of tongue mucosal samples. No latent infection was found at this site. EBV infection was detected neither in normal salivary glands nor in tongue carcinomas. Thus, EBV replication occurs infrequently in tongue epithelial cells, and salivary glands are unlikely to harbor EBV. EBV is unlikely to be involved in the pathogenesis of tongue cancer.

Primary Epstein-Barr virus (EBV) infection is followed by lifelong persistence of the EBV in B lymphocytes. EBV is spread through saliva, and it is believed that replication in oropharyngeal epithelial cells is the source of this infectious virus [1]. However, evidence supporting this view is limited. EBV replicates in epithelial cells in oral hairy leukoplakia, an AIDS-associated epithelial lesion at the lateral margin of the tongue, and in normal tongue mucosa of HIV-infected patients [2]. EBV replication has been reported to occur in oral epithelial cells in primary infection and in healthy carriers of the virus, but these results could not be reproduced by subsequent studies [3, 4]. Rare EBV replication in tongue epithelial cells has been demonstrated in an autopsy series [5]. However, all patients with evidence of EBV replication were either receiving immunosuppressive therapy or terminally ill patients with cancer, which suggests that an impaired immune system may have allowed EBV replication to occur [5]. Another possible site of EBV replication is the salivary glands. Infectious virus has been recovered from saliva obtained from parotid-gland ducts, and EBV genomes have been detected in parotid glands [1, 6]. We have examined a large series of biopsy specimens from the lateral margin of the tongue and from salivary glands, for evidence of EBV replication. Because EBV has been suggested to play a role in the pathogenesis of oral cancer [7], we have also studied squamous-cell carcinomas from the tongue, for evidence of EBV infection.

Patients, materials, and methods. Paraffin blocks containing 232 mucosal samples obtained, for diagnostic purposes, from the lateral margin of the tongues of 217 patients (155 men and 62 women, who were 22–86 years old) were studied, including 116 histologically normal samples from resection margins obtained during carcinoma surgery, 34 samples of leukoplakias, 17 samples from ulcers, 30 samples from cases with nonspecific inflammation, 2 samples of hemangiomas, 15 samples of normal tongue mucosas, and 3 samples of minor epithelial dysplasia. Tumor samples from 59 cases (4 well differentiated, 37 moderately well differentiated, and 18 poorly differentiated) of invasive squamous-cell carcinomas and from 4 cases of in situ carcinoma primary to the lateral margin of the tongue were studied (these 63 samples were obtained from 55 men and 8 women, who were 43–87 years old). Also included were tissue samples from 241 salivary glands (190 parotid and 51 submandibular, from 115 women and 126 men, who were 9–92 years old); these were either histologically normal (54 samples), comprised normal salivary-gland tissues adjacent to either benign (108 samples) or malignant (47 samples) tumors, or showed chronic inflammation (32 samples).

Monoclonal antibodies (mAb) BZ1, which is specific for the BZLF1 transactivator protein of EBV, PE-2, which is directed against the EBV-encoded nuclear antigen–2 (EBNA2), and CS1-4, which is specific for the EBV-encoded latent membrane protein 1 (LMP1) were obtained from Dakocytomation. EBNA1-specific mAb 1H4 was a gift from E. Kremmer (Munich, Germany). mAb OT41A, which recognizes the EBV viral capsid antigen (VCA) p40 (BdRF1), OT6, which is specific for the membrane antigen (MA) gp 350/220 (BLLF1), and OT14E, which recognizes the p47 early antigen (EA) (BMRF1) were from Dr. J. Middeldorp (Amsterdam, The Netherlands) [5]. Before immunohistochemistry (IH) analysis was performed, tissue sections were subjected to antigen retrieval in a pressure cooker, and IH analysis was performed as described elsewhere [8].
Figure 1. Immunohistochemical detection of Epstein-Barr virus lytic-cycle antigens. Expression of the BZLF1 immediate early antigen (A), the early antigen (B), the viral capsid antigen (C), and the membrane antigen (D) is detected in upper epithelial cells of tongue mucosa (red nuclear labeling in A–C and red membrane staining in D); in contrast, immunohistochemistry analysis reveals that BZLF1 expression is absent from a parotid gland (E) and a submandibular gland (F).

Expression of the EBV-encoded nuclear RNA–1 and –2 (EBER1 and EBER2, respectively) was detected by in situ hybridization (ISH) using 35S- or digoxigenin-labeled RNA probes as described elsewhere [8]. Bound probes were detected by either autoradiography using Ilford G5 emulsion (Ilford) or IH analysis using an anti-digoxigenin mAb (Sigma), as appropriate [8]. EBV DNA ISH was performed by use of a 35S-labeled plasmid harboring the BamHI W internal repetitive fragment of the EBV genome [8]. This method can detect a single copy of the EBV genome [9].

For polymerase chain reaction (PCR), DNA was extracted from dewaxed paraffin-embedded sections by digestion with proteinase K, and 5-μL aliquots of DNA extracts were subjected to PCR (5 min at 94°C, 10 cycles of 20 s at 94°C, 20 s at 58.5°C and 30 s at 72°C, followed by 25 cycles of 20 s 94°C, 20 s at 53.3°C and 30 s at 72°C, completed by 150 s at 72°C). The reaction contained 1X polymerase buffer, 1.5 mmol/L MgCl₂, 200 μmol/L dNTP, 1 U Taq polymerase and 400 nmol/L primer mix (5′-CAC TTT AGA GCT CTG GAG GA-3′ and 5′-TAA AGA TAG CAG CAG CAC AG-3′). To serve as control, the β-globin gene was amplified.

Results. Samples from the lateral margins of the tongue were screened for evidence of EBV replication, by BZLF1 IH analysis, a procedure that yielded positive results in 3 (1.3%) of the samples, from 3 (1.4%) of the patients. Staining was confined to nuclei of superficial squamous epithelial cells (fig-
Figure 2. Results of in situ hybridization (ISH). ISH using radiolabeled probes reveals the presence of replicating Epstein-Barr virus (EBV) DNA (black nuclear labeling, arrowheads) in upper epithelial cell layers of tongue mucosa near an ulceration (A). ISH used for the detection of the EBV-encoded nuclear RNAs (EBERs) results in black nuclear labeling in the same compartment (B, arrowheads). This represents hybridization of RNA probes to replicating viral DNA [9]. No EBER expression was detected in basal epithelial cells (figure 2B). Because BZLF1 IH analysis was used as an initial screen for EBV replication, we wanted to exclude the possibility that a latent or lytic EBV infection undetectable by this approach might be present, and we therefore randomly selected 40 mucosal samples from the BZLF1-negative cases and subjected them to EBV DNA PCR, EBNA1 IH analysis, and EBER-specific ISH; of these 40 samples, 5 were EBV DNA positive by PCR, but EBNA1 IH analysis, EBER-specific ISH, and subsequent EBV DNA ISH yielded negative results (not shown).

BZLF1 IH analysis also yielded negative results in all 241
salivary-gland samples (figure 1E and 1F). A total of 47 randomly selected salivary-gland samples (35 parotid-gland samples and 12 submandibular-gland samples) were also subjected to ISH for the detection of EBV DNA and of the EBERs; again the results were negative in all samples (figure 2C and 2D).

EBER-specific ISH of squamous-cell tongue carcinomas yielded negative results in all samples (figure 2E), including the carcinoma from patient 1 (see below). To exclude the possibility of an EBER-negative form of latent EBV, all samples were subjected to EBV-DNA PCR, which yielded specific bands in 11 (17.5%) of the 63 (data not shown). Further analysis of these samples by use of both EBV DNA ISH and EBNA1 IH analysis yielded negative results in all samples, confirming that EBV was absent from the epithelial tumor cells (figure 2F).

The clinicopathological features of patients with EBV-positive tongue mucosa samples were evaluated. Patient 1, a 56-year-old man, presented with a small squamous-cell carcinoma at the left margin of the tongue. Simultaneously, a 1-cm² ulcer was excised from the right edge of the tongue. This patient had not received radiotherapy or chemotherapy before surgery, and there was no evidence of immunodeficiency. Histological evaluation revealed an ulcer with active inflammation and regenerative changes. No fungal infection was detectable. IH analysis and ISH showed focal EBV replication in squamous epithelial cells near the ulcer (figures 1A, 1B, 2A and 2B).

Patient 2, an 84-year-old woman, presented with a leukoplakia at the right margin of the tongue. This patient had not received chemotherapy or radiotherapy, and there was no evidence of immunodeficiency. Histological evaluation revealed epithelial hyperplasia and nonspecific chronic inflammation. No fungal infection was detectable. IH analysis and ISH revealed a small focus of EBV replication in differentiated squamous epithelial cells.

Patient 3, a severely ill 60-year-old man with a history of rheumatoid arthritis, hepatitis B virus infection, and liver failure, had drug-induced bone-marrow aplasia, acute renal failure, and herpes simplex virus-induced encephalitis. Because this patient had intraoral bleeding, a biopsy specimen was taken from the lateral margin of the tongue; this specimen showed nonspecific chronic inflammation. No fungal infection was detectable. IH analysis and ISH revealed extensive EBV replication in differentiated squamous epithelial cells.

Discussion. EBV persists in memory B cells and is transmitted through saliva, but the cellular source of infectious virus has remained controversial. Oral epithelial cells have been implicated as a likely site of virus production, EBV replication occurs in epithelial cells in oral hairy leukoplakia, and the detection of replicating EBV in oropharyngeal epithelial cells has been reported [2, 3], although this observation could not be reproduced in other studies [4]. Finally, salivary glands are a possible site of EBV replication [6].

Replication of EBV can occur in B lymphocytes during infectious mononucleosis [4]; however, it is uncertain whether this is sufficient to explain virus transmission. In a previous report, we have demonstrated EBV replication in squamous epithelial cells in 2.4% of tongue mucosa samples from autopsy cases [5]. The significance of this observation has remained uncertain, because all samples with EBV replication have displayed some evidence of immunosuppression [5]. In the present study, we have analyzed biopsy specimens from patients who, with 1 exception (sample 3), showed no evidence of immunosuppression. Normal tongue mucosal samples from patients with oral cancer were obtained during primary surgery before administration of potentially immunosuppressive treatment. There was also no evidence of HIV infection. Serological data on EBV infection were not available, but testing of a total of 1667 patients in our hospital in 2001 showed that the rate of seropositivity was 90.4% [5]. Thus, we are confident that our series is as representative of EBV infection in the general population as can reasonably be expected in a hospital-based study.

BZLF1 expression usually precedes the expression of other lytic viral genes [10], and BZLF1 IH analysis was used as an initial screening method for the detection of EBV replication. Lytic replication in epithelial cells can also be triggered by the BRLF1 protein [11]. Because this induces BZLF1 expression, this route of EBV induction should also be detectable by our approach [11]. We identified BZLF1 expression in epithelial cells at the tongue margins in 3 (1.4%) of the 217 patients studied. One of these 3 patients (i.e., patient 3) was severely ill, with bone-marrow failure and infectious complications; when this patient was excluded from the analysis, EBV replication was found to be detected in 2 (0.9%) of 216 patients without immune defects. This proportion is lower than that (3.6%) found in our previous autopsy study [5], but we believe that it provides a more representative picture of EBV replication at this site. When EBER-specific ISH and EBNA1 IH analysis were used, no evidence of latent EBV infection was detected in epithelial cells at this site.

The present study was restricted to samples from the tongue margin because this is the predominant site for oral hairy leukoplakia, and we reasoned that EBV replication also was most likely to occur at this site in nonimmunocompromised individuals; nevertheless, we cannot exclude the possibility that EBV replication may occur at other mucosal sites. Analysis of larger numbers of samples from different sites will be necessary to address this issue.

One patient in the present study showed EBV replication near an ulcer. The detection of infectious EBV in genital ulcers has been reported previously, but the cellular source has remained uncertain [12]. However, because, in the case of the patient in the present study, EBV replication was not detected immediately at the edge of the ulcer (figure 2B), we believe
that this finding was coincidental and that it does not indicate that EBV caused the ulceration.

Cell-free EBV has been detected in saliva obtained from the parotid duct, and detection of EBV has been reported in a small series of parotid glands, which suggests that EBV may replicate in salivary glands [1, 6]. We found no evidence of lytic or latent EBV infection in salivary glands. Thus, our results do not support the idea that salivary glands are a reservoir of EBV infection.

Because EBV can replicate in epithelial cells at the lateral margin of the tongue, we reasoned that, if EBV were to be involved in the pathogenesis of oral carcinomas [7], it should be detectable in carcinomas at this site. Using EBER-specific ISH, we did not find evidence of EBV infection in the neoplastic epithelial cells. The possibility that an EBER-negative form of EBV latency exists has been suggested [13], and we therefore studied our samples by using EBV DNA ISH and EBNA1 IH analysis, which yielded negative results. Thus, our results suggest that EBV is not involved in the pathogenesis of tongue carcinomas.

In summary, the present study suggests that EBV only rarely replicates in normal squamous epithelial cells at the tongue margin. The biological significance of this observation remains uncertain. We found no evidence of EBV infection in salivary glands, which indicates that salivary glands are not a reservoir of EBV replication. Finally, our findings suggest that EBV is not involved in the pathogenesis of squamous-cell carcinoma of the tongue.

Acknowledgments

We are grateful to E. Kremmer and J. Middeldorp for the donation of antibodies.

References