Is Herpes Simplex Virus Associated with Peptic Ulcer Disease?[†]

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Received 8 November 1989/Accepted 12 January 1990

To test the hypothesis that herpes simplex virus type 1 (HSV-1) may be associated with peptic ulcer disease, we examined ulcerative lesions of the distal stomach and proximal duodenum for the presence of nucleic acids and antibodies specific for HSV-1. Utilizing in situ hybridization, immunocytochemistry, and polymerase chain reaction with sequencing, gastric or duodenal tissues from 4 of 22 patients (18%) with documented peptic ulcer disease demonstrated the presence of both specific HSV-1 nucleic acid sequences and proteins. HSV-1 was found restricted in clusters of cells near the margin of the ulcer but was absent at sites distal to the lesion. Several of such HSV-1-infected cells also contained cholecystokinin. These cholecystokinin-containing cells are of neuroendocrine origin and receive contact from the vagal nerve. *Campylobacter pylori* bacteria were not found in three of the four peptic ulcer tissues that harbored HSV-1. Further, none of the stomach or duodenal tissue samples from 33 patients undergoing clinical evaluation, but having no evidence of peptic ulcer disease, had HSV-1 materials. Thus, our data suggest that a subset of peptic ulcer disease may be associated with HSV-1 and raise the possibility that some peptic ulcers may be caused by this virus.

Observations by several investigators have suggested an association between herpes simplex virus type 1 (HSV-1) and peptic ulcer disease (5, 17, 27; Editorial, Lancet i:705–706, 1981). First, the clinical picture and course of peptic ulcers can resemble those of other herpetic infections. For example, viral lesions occur most often at the same site, may recur a few times per year, and the incidence of both peptic ulcers and herpetic lesions peaks in the spring and autumn (5, 27). Both lesions are junctional in origin, appearing at the intersection between two different types of epithelium (15). Second, the fever blister of the lip follows activation of latent HSV-1 in the trigeminal ganglia, and surgical cutting of that nerve prevents reactivation (3, 4, 23, 24, 28). Correspondingly, HSV-1 can cause a latent infection of the vagus nerve ganglia (28), and cutting of the vagus nerve (vagotomy) is a frequently used treatment to prevent recurrence of peptic ulcer disease (2). These observations and the relationship of vagus nerve fibers with the gastric mucosa (6; L. Rinaman, M. E. Whealy, J. S. Schwaber, A. Robbins, J. P. Card, and L. Enquist, Book of Abstr. Soc. Neurosci., part 1, vol. 239, abstr. 17A, p. 140, 1988) suggest that reactivated virus may travel by way of the vagus nerve to the peptic ulcer site. Third, carbonoxolene and cimetidine, two common pharmacological remedies beneficial in the treatment of peptic ulcer, also inhibit HSV replication (7, 16, 26). In this study, we test for a direct association of HSV-1 and peptic ulcer. We conclude that a subset of patients with peptic ulcer disease have evidence of HSV-1 infection at the ulcer site. HSV-1 nucleic acids and proteins were found in neuroendocrine cells bordering the ulcer, inferring that HSV-1-vagal nerve activation may play a role in some cases of peptic disease.

MATERIALS AND METHODS

Patients and clinical materials. Biopsied or surgically removed tissue samples from 22 patients with clinically and histopathologically confirmed peptic ulcer disease as well as

samples from a control group of 33 patients who underwent clinical evaluation of the stomach or duodenum but had no evidence of peptic ulcer disease were obtained from the departments of pathology at the University of California Medical School, San Diego (K. Benirschke), Rush University and Medical Center, Chicago (V. E. Gould), Free University of Brussels, Belgium (G. Klöppel), and University of Hamburg, Federal Republic of Germany (T. Löning). The classification of peptic ulcer disease was based on patient history, clinical data, and both location and histological appearance of the lesions. The peptic ulcer patients did not have ulcerative lesions in other parts of the upper gastrointestinal tract or oral cavity, they had no other debilitating diseases, and they were not on immunosuppressive medication. The control group (nonpeptic ulcer) included patients with gastritis (*n*, 3) and gastric carcinoma (*n*, 2). The mean age of the ulcer group (*n*, 22) was 59.6 ± 16.3 years, with a range of 22 to 75 years, and the control group (*n*, 33) was 60.1 ± 15.8 years, with a range of 19 to 82 years, respectively. The male-female ratio was 12 to 10 and 15 to 18, respectively. Serum samples were not available for the majority of patients studied. Screening for *Campylobacter pylori* was performed by using Giemsa stains (13) and evaluated by microscopy.

Molecular and immunocytochemical analysis. Polymerase chain reaction (PCR) analysis was done on DNA extracted from paraffin-embedded peptic ulcer tissues, utilizing oligonucleotide primers from a conserved region in the small subunit of the ribonucleotide reductase gene of HSV-1 (14). Twenty to thirty 5- μ m sections per sample were collected from the paraffin-embedded tissue and were rehydrated and digested with proteinase K (8). After phenol-chloroform extraction and ethanol precipitation, the DNA was suspended in TE (10 mM Tris hydrochloride [pH 8.0] and 0.1 mM EDTA) and measured and adjusted to 100 ng/ μ l. The primers for the PCR (19) were derived from the small subunit of HSV-1 ribonucleotide reductase, encoded by UL40. The sequence of the primers (Genetic Designs Inc., Houston, Tex.) was as follows: 5' CTCATGATCCTCATCGAGGG CATC 3' (rightward) and 5' CGCACGTAGTTTTTCGATG

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[†] Publication no. 5620-IMM from the Department of Neuropharmacology, Research Institute of Scripps Clinic, La Jolla, Calif.

GCCGCC 3' (leftward), yielding a 322-base-pair (bp) fragment between nucleotides 90369 and 90791 of the HSV-1 sequence (14) (see Fig. 1a). Amplification of DNA was performed with the GeneAmp kit (Perkin-Elmer Corp., Norwalk, Conn.). The reaction was carried out with 100 ng of DNA and 300 pmol of primers. The reaction mixture (1×) contained 50 mM Tris hydrochloride (pH 8.3), 10 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 200 μM (each) dATP, dGTP, dTTP, and dCTP in a final volume of 100 μl; 5 U of *Taq* polymerase was used for each assay. Each cycle of amplification consisted of 2 min of denaturation at 94°C, a 1-min annealing cycle at 50°C, and 3 min of extension at 72°C. Typically, 40 cycles were performed. Each assay was performed at least twice. A 20-μl sample of each PCR mixture, containing 20 ng of DNA, was run on a 1.8% agarose gel and transferred in 0.4 M NaOH to Zetabind (Bio-Rad Laboratories, Richmond, Calif.) after treatment with 0.25 M HCl for 10 min, two rinses in water, and a 10-min soak in 0.4 M NaOH. Prehybridization and hybridization were in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% deionized formamide–0.5% nonfat dry milk–0.5% sodium dodecyl sulfate (SDS)–0.1 mg of sonicated salmon sperm DNA per ml, with 10⁶ cpm of the *Fnu*4HI fragment of the HSV-1 UL40 PCR product. To achieve a probe for the PCR product, the gel-purified 322-bp PCR product from HSV-1-infected Vero cells was treated with Klenow polymerase (New England BioLabs, Beverly, Mass.), precipitated, and blunt-end ligated in the *Hinc*II site of pUC19. After being cloned, the fragment was released with *Eco*RI–*Hind*III, gel purified, digested with *Fnu*4HI to yield a 253-bp fragment from the middle portion (nucleotides 90513 to 90766) lacking the priming oligonucleotides (see Fig. 1a). The sequence of the cloned fragment was confirmed by direct double-stranded sequencing of this plasmid with the Sequenase kit (Promega). Labeling of this gel-purified fragment was with random hexoprimers and [³²P]dATP and [³²P]dCTP. After hybridization, filters were washed at 37°C in 2× SSC–0.1% SDS and 0.1× SSC–0.1% SDS twice, respectively. Filters were exposed to film for 6 to 12 h at –70°C with an intensifying screen. Sequencing of the PCR products was performed on single-stranded DNA obtained by PCR (11), using the chain-terminating method of Sanger (20) for sequencing; the leftward primer was used (see Fig. 1a).

Nucleic acid in situ hybridization studies. Nucleic acid in situ hybridization studies on paraffin-fixed tissues utilized probes to an HSV-1 immediate-early α gene (ICP0) and a late γ gene (VP-5) (24). Briefly, 3-μm serial sections were attached to acid-cleaned and poly-L-lysine-coated glass slides with 0.1% Elmer's white glue (Borden Inc., Columbus, Ohio). The specimens were permeabilized with 0.2 N HCl (10 min)–1% Triton X-100 (1.5 min)–proteinase K (1 μg/ml for 20 min at 37°C). Sections were rinsed in phosphate-buffered saline (PBS) and dehydrated in 100% ethanol. In situ hybridization was performed as described previously (21). In brief, the hybridization mix consisted of 50% deionized formamide, 5× Denhardt solution, 5× hybridization salts (0.9 mol of NaCl per liter, 50 mmol of NaH₂PO₄ per liter, 5 mmol of EDTA per liter), 10% dextran sulfate, 30 U of heparin per ml, 500 μg of salmon sperm DNA per ml, 250 μg of HeLa cell RNA per ml, and 0.1% SDS. ³⁵S-labeled DNA probes (double gel-purified fragments of ICP0, VP-5, or pCM3, nick translated to a specific activity of greater than 10⁸ cpm/μg) were added to a concentration of 10⁵ cpm/μl of mixture. The entire solution was boiled for 2 min and chilled on ice; dithiothreitol was added to a concentration of 10

mmol/liter. The hybridization mixture was placed on the sections, covered with sheets of Gel-Bond film, and sealed with rubber cement. Hybridization was carried out at 37°C for 18 h. Cover slips were then removed, and slides were washed with 2× SSC for 30 min at 21°C, followed by washes in 0.1× SSC at room temperature for 30 min, at 60°C for 10 min, and at 37°C for 15 min. Slides were rinsed in 2× SSC for 10 min, dehydrated through ethanol, and dried. They were then dipped in Kodak NTB-2 emulsion, exposed for 4 days at 4°C, and developed with Kodak D19 and standard fixer followed by counterstaining with hematoxylin. The specificity of the HSV-1 nucleic acid probes was detailed previously (24). These reagents failed to hybridize with either uninfected Vero cells or human foreskin fibroblasts infected with human cytomegalovirus, but both probes hybridized to HSV-1-infected Vero cells. The probe to human cytomegalovirus (pCM3) (18) failed to hybridize to the gastric tissues or HSV-1-infected Vero cells but did hybridize to human cytomegalovirus-infected human foreskin fibroblasts.

Immunocytochemistry studies. Immunocytochemistry studies were performed on paraffin-fixed sections as described previously (29). Briefly, 3-μm serial sections were cut and mounted on acid-cleaned poly-L-lysine coated glass slides with 0.1% Elmer's white glue. After the paraffin was removed, the endogenous peroxidase was blocked with 3% H₂O₂ in methyl alcohol. After further rehydration and washes in PBS, the slides were treated with 1 μg of proteinase K (Boehringer Mannheim Biochemicals) per ml for 15 min at 37°C followed by washes in PBS with 0.1% bovine serum albumin. The slides were then incubated with 10% normal swine serum (DAKO) in a humid chamber at 21°C for 30 min. After the blocking serum was shaken off, the monoclonal antibody was applied diluted in PBS containing 0.1% bovine serum albumin. Incubation took place at room temperature for 18 h. After several washes in PBS, the secondary biotinylated antibody (goat anti-mouse immunoglobulin G; Boehringer Mannheim) was applied at a 1:50 dilution for 30 min at 21°C. After several washes and a 30-min incubation with the ABC complex (Vectastain) at 21°C, the slides were rewashed in PBS, reacted with 2.2 diaminobenzidine, rewashed, dehydrated, and mounted for microscopic evaluation. Identical results were obtained with mouse monoclonal antibodies against HSV-1 glycoproteins gC and gG. The specificities of these reagents were detailed previously (1, 10). Reagents used failed to stain either uninfected Vero cells or human foreskin fibroblasts infected with human cytomegalovirus but did stain HSV-1-infected Vero cells. Normal preimmune serum used as primary antibody failed to react with the tissue. The cholecystokinin antibody is a rabbit anti-cholecystokinin-eight antiserum (INC, Stillwater, Minn.). For the double staining, immunocytochemistry was performed first and in situ hybridization was done directly after the final PBS washes.

RESULTS

HSV-1-specific nucleic acid sequences are found in peptic ulcer tissue. DNA extracted from paraffin-embedded peptic ulcer tissues was examined for the presence of HSV-1 by the PCR (19), utilizing oligonucleotide primers from a conserved region in the small subunit of the ribonucleotide reductase gene of HSV-1 (14). PCR amplification of DNA from the peptic ulcer tissue with these HSV-1 primers and analysis of the products on an agarose gel (Fig. 1b) resulted in the identification of a predicted 322-bp fragment. Results were

FIG. 1. (a) Strategy and results of the PCR-directed amplification of HSV-1 UL40 that encodes the small subunit of the ribonucleotide reductase (14). A1 and A2 priming oligonucleotides were used for PCR. B, The cloned fragment of 253 bp (shown at the top of panel a) used for probing Southern blots (panel b); C, DNA structure obtained by direct sequencing of the PCR product, using the leftward PCR primer (A2). The DNA sequences from patients with peptic ulcer disease showed homology with the sequence of HSV-1 UL40 wild type (strain 17+). The consensus sequence (C) is:

5'CCGGGTCAGG.ATATGGCTGT.CCGTCGGCGC.CTGGGATCGG.
ATAAATCCGA.TCTCGATCTC.GACCGCCTGG.AACAGC3'

(b) Demonstration of HSV-1 nucleic acid sequences by Southern blot hybridization using the 253-bp probe shown in panel a. Lanes: 1 and 2, results of PCR using DNA extracted from uninfected (negative control) and HSV-infected (positive control) Vero cells, respectively; 3 through 5, DNA from paraffin-embedded HSV-1-positive peptic ulcers. Results are from patients 6576, 740, and 4561 (Table 1). Similar results were obtained with patient 2329. Lanes 6 through 8, Results for DNA extracted from nonpeptic ulcer stomach; 9, DNA from HSV-1-infected mouse brain. DNA was extracted from Formalin-fixed paraffin-embedded tissue. Lane 10, Plasmid pUL40 containing the probe used (panel a).

seen with DNA obtained from tissues of four peptic ulcer patients, and results for patients 6576, 740, and 4561 (see Table 1) are recorded in Fig. 1b. The DNA band (Fig. 1b, lanes 3, 4, and 5) comigrated with a fragment produced from PCR amplification of DNA extracted from HSV-1-infected cells (lane 2). HSV-1-specific PCR-amplified products were not detected in tissues from the nonpeptic ulcer tissues (lanes 6, 7, and 8) nor the other peptic ulcers studied. To ascertain that the PCR product was the ribonucleotide reductase of HSV-1, direct DNA sequencing was employed as shown in Fig. 1a. The sequence obtained through PCR in patients 6576 and 740 was identical (Fig. 1a) to that of the wild-type HSV-1 sequence (14, 24).

HSV-1 nucleic acid sequences occur in cells bordering the peptic ulcer lesion. In order to identify the cells harboring the virus, we next examined peptic ulcer tissue for expression of HSV-1 nucleic acid sequences, using in situ hybridization with probes specific to an HSV-1 immediate-early α gene (ICP0) and a late γ gene (VP-5) (24). Both HSV-1 probes hybridized to tissue samples in 4 of 22 patients, with the same 4 patients being positive by PCR (Table 1). Signal (Fig. 2) was detected at the margins of the peptic ulcer lesion. Neither probe hybridized to tissues from nonpeptic ulcer patients (Fig. 2E), and no signal was detected in these tissues with a human cytomegalovirus probe (data not shown).

HSV-1 proteins occur in cells bordering the peptic ulcer lesion. HSV-1-specific monoclonal antibodies to gG and gC demonstrated the presence of HSV-1 proteins (Fig. 3C and D), with the same four peptic ulcer specimens positive by PCR and by in situ hybridization (Table 1). The distribution of cells positive for HSV protein was similar to the pattern observed by in situ hybridization. The majority of the staining occurred at the border of the ulcerative lesion (Fig. 3A). The HSV-1 monoclonal antibodies did not react with control tissues from 33 normal patients or patients with gastric or duodenal diseases other than peptic ulcer, despite similar degrees of injury and necrosis (i.e., from patients with gastritis or tumor).

Several neuroendocrine cells containing cholecystokinin contain HSV-1 nucleic acid sequences. Cells positive for HSV-1 nucleic acid and proteins were commonly found at the base of the crypt. The morphology and location of the HSV-1-positive cells strongly suggested that they were of neuroendocrine origin. To determine whether the HSV-1-infected cells were of neural origin, double-label immunocytochemistry-in situ hybridization techniques were employed,

TABLE 1. Clinical and microbiological data from four male patients exhibiting evidence of HSV-1 gene expression in peptic ulcer tissue^a

Patient data		Ulcer localized to tissue type	In situ hybridization to probe ^b :		Immunocytochemistry ^c		<i>Campylobacter</i> detection ^d
Case no.	Age		ICP0	VP-5	H1379	3-G-11	
6576	56	Prepyloric	+	++	+	+	Nil
740	45	Pylorus	+	++	+	+	Nil
4561	38	Antrum	++	+	+	+	Nil
2329	68	Prepyloric	+	+	+	+	+

^a DNA amplification was obtained for all patients with the PRC (19) using the UL40 primer.

^b In situ hybridization as described in Materials and Methods and shown in Fig. 2. Probe ICP0 is complementary to the immediate-early α gene and probe VP-5 to the late γ gene (24).

^c Immunocytochemistry as described in Materials and Methods and shown in Fig. 3. Monoclonal antibody H1379 is against HSV-1 glycoprotein gGI, while monoclonal antibody 3-G-11 is against HSV-1 glycoprotein gC (1, 10).

^d Analysis for *C. pylori* was performed by using Giemsa stains.

FIG. 2. Demonstration of HSV-1 nucleic acid sequences by in situ hybridization in the gastric mucosa of two patients having peptic ulcer disease. Signal occurred in the immediate vicinity of the ulcer. Panels A and B are from patient 4561; panel C and D are from patient 740 (Table 1). Panels A and C were probed with ICP0; panels B and D were probed with VP-5. Panel E records the mucosa of a control (nonpeptic ulcer) patient probed with ICP0. When this or other samples from controls were hybridized with ^{35}S -DNA probe to VP-5, no signal was observed (data not shown).

utilizing an antibody specific for cholecystokinin in conjunction with HSV-1 nucleic acid probes. Cholecystokinin is a neuropeptide localized in such neural cells. By the double-label technique, several single cells were found that contained both HSV-1 and cholecystokinin (Fig. 3E, F, G, H, and I).

DISCUSSION

Our results indicate that HSV-1 is present in cells bordering some peptic ulcer lesions and can be associated with a subset of patients having peptic ulcer disease (Table 1; Fig. 1 to 3). HSV can reside latently in the vagal ganglia (28). Utilizing a model of another virus, pseudorabies in rats, Enquist and associates (Rinaman et al., Abstr. Soc. Neurosci. 1988) have shown transneuronal transport of virus from the stomach musculature to neurons in the dorsal motor nucleus of the vagus and the nucleus of the solitary tract. Similarly, Ugolini et al. (25) recently provided evidence of the transneuronal transfer of HSV from peripheral nerves to the central nervous system. We hypothesize that HSV-1, latent in vagal ganglia, may be activated through a local event in the stomach-duodenum or through nerve impulses

FIG. 3. Demonstration of HSV-1 antigens in the gastric mucosa of three patients having peptic ulcer disease. (A) Cartoon of the area in which cells positive for HSV-1 antigens are located. HSV-1 was not detected at sites distal to the ulcer lesion nor in 33 healthy controls. (B) Histological appearance of a peptic ulcer lesion in the distal stomach from patient 4561. (C and D) Photomicrographs of tissue adjacent to the peptic ulcer lesion from patients 4561 and 740, respectively, demonstrating HSV-1 in the gastric mucosa. (E, F, G, H, and I) Localization of cholecystokinin (immunocytochemistry) and HSV-1 (^{35}S -DNA to VP-5, panels E, G, H, and I) in a single cell. Panels E and F are 3- μm consecutive sections from the same paraffin block. After being stained for cholecystokinin, tissue in panel E was probed with ^{35}S -DNA to HSV VP-5, while panel F tissue was probed with ^{35}S -DNA to late genes of cytomegalovirus (18). Arrows point to a doubly stained cell (HSV-1 and cholecystokinin); photomicrographs also demonstrate the specificity of ^{35}S -labeled HSV probes.

FIG. 3—Continued.

from cerebral connections to the vagal nerve ganglia. The herpesvirus may have initially entered the vagus ganglia through the oral pharynx or other peripheral connecting sites. Upon activation, the virus would travel down the vagal nerve to the potential site of the peptic ulcer lesion, presumably able to reach the target tissue within a few days (22; Rinaman et al., Abstr. Soc. Neurosci. 1988). The vagal nerve endings terminate on cells, like those containing cholecystokinin. Presumably, virus is released from these neuroendocrine cells, replicates in epithelial cells, and causes the ulcer. Further, stimulation of the vagus nerve itself increases acid secretion in the stomach, thus contributing to the local pathology (12). Neuroendocrine cells near the peptic ulcer site contained HSV-1. However, they showed no evidence of lysis (Fig. 3), despite expressing viral proteins. We have no explanation for this observation, although others have noted that HSV can persist in neuronal cells in vivo without killing them (22).

In conclusion, HSV-1 itself might cause the ulcerative lesion in selected cases of a subset of peptic disease by directly infecting the mucosal cells in the stomach following

virus release from neuroendocrine cells or vagal nerve terminals or both. These events would help explain the clinical and histopathological characteristics of the lesion, its usual recurrent location of a restricted area in the stomach-duodenum, and the successful role that vagotomy and cimetidine medication play in therapy. Additionally or alternatively, the peptic ulcer might activate latent HSV-1 in vagal ganglia, therefore making replication of HSV-1 a contributing but not an initiating factor of the ulcer. Whatever the mechanism involved, HSV-1 is an additional infectious agent, like *C. pylori* (13), that should now be suspected as playing a role in peptic ulcer disease. While both microbes may act synergistically, our inability to find *C. pylori* in three of four peptic ulcer tissues that express HSV-1 (Table 1) suggests that each agent might independently be associated with peptic ulcer disease. The availability of a selective anti-HSV drug like acyclovir (9) and, alternatively, a variety of antibacterial drugs with no antiviral activity as well as the establishment of an animal model of HSV latency and reactivation from the vagal ganglia should help to resolve these questions. Such studies are currently under way.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grants AI-07007 (M.B.A.O.) and AI-21640 (J.A.N.) from the National Institutes of Health. J.M.L. is the recipient of a postdoctoral fellowship from the Juvenile Diabetes Foundation, New York, N.Y. J.A.N. is the recipient of an American Cancer Society Faculty Award.

We are grateful to Katsu Miyai, Department of Pathology, University of California, San Diego, for assistance in identifying neuroendocrine cells in the gut. We thank John Subak-Sharpe for identifying suitable HSV sequences for PCR and Etienne Joly and Maria Salvato for thoughtful comments and advice.

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