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Human Herpes Simplex-1 and Papillomavirus Type 16 Homologous DNA Sequences in Normal, Potentially Malignant and Malignant Oral Mucosa

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We have tested a range of normal, potentially malignant and malignant oral mucosal biopsies tissues by Southern blot hybridisation analysis for the simultaneous presence of HSV-1 and HPV type 16 DNA sequences, both of which have been implicated as risk factors in oral carcinogenesis. The results show that: (1) 2/4 patients with lichen planus, 2/4 patients with non-specific keratosis, 1/8 patients with oral carcinoma and 3/5 biopsy specimens of normal oral mucosa contained DNA sequences homologous to the HSV-1, *Bam* HI-G fragment. (2) HPV-16 homologous DNA sequences were detected in 3/4 patients with lichen planus, 4/4 non-specific keratosis, 4/8 oral carcinomas and in 3/5 biopsy specimens of normal oral mucosa. (3) Overall, only 5 patient biopsy specimens were positive for both HSV-1 and HPV-16 homologous DNA sequences; 2 lichen planus, 2 non-specific keratosis and 1 normal. The data cannot exclude a "hit and run" oncogenic mechanism for HSV but suggest that if HSV-1 and HPV-16 play a synergistic role in the development of oral cancer this may be an early event. Indeed, the data suggest HSV might be more frequently found in potentially malignant lesions than in carcinoma.
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INTRODUCTION

ORAL SQUAMOUS cell carcinoma is a common malignancy with a multifactorial aetiology including particularly tobacco and alcohol [1]. Over the past decade evidence has accumulated implicating certain viruses in the aetiology of human oral carcinogenesis, most notably human herpes simplex virus (HSV) and human papillomavirus (HPV) [2]. The strongest evidence associating HSV with oral carcinoma has been the detection by *in situ* hybridisation of RNA complementary to HSVDNA in tumour tissues [3]. However, it is possible that this technique may have detected areas of homology between the HSV genome and human cellular DNA, transcribed to excess in proliferating cells [4]. Nevertheless, HSV-1 antigens have been demonstrated in biopsy samples from oral carcinoma [5].

HPV type 16 DNA has been detected in tissue biopsy samples from oral cancer patients [6–16] but such sequences can also be detected in other proliferating oral tissues [4]. Therefore, the roles that either HSV or HPV may play in oral carcinogenesis remain unclear.

In cervical carcinogenesis it has been postulated that HSV and HPV may act as co-carcinogens, HSV as an initiating agent and HPV as a promoting agent [17]. In support of this hypothesis, *in vitro* studies have shown that both HSV and HPV-16 can transform mammalian cells [18–21] and that HSV can be a potent mutagen as well as mediating gene amplification in simian virus 40 (SV40) transformed cells [23]. Furthermore, recent studies have reported that HSV can, at least *in vitro*, influence the expression of both cellular [24] and HPV genes [25]. *In vivo* studies have also shown an association between the presence of both HSV and HPV-16 genomes in cervical tumour cells [26, 27] though not in all cases [28, 29].

In the oral mucosa HSV and HPV-16 infections are common [4, 6, 7, 30, 31] and risk factors similar to those for cervical cancer have been implicated in oral carcinogenesis [2]. However, there have been no studies examining the simultaneous presence of HSV-1 and HPV-16 in the oral mucosa.

We have therefore addressed this question. Specifically, a series of normal, potentially premalignant (keratosis, lichen planus) and malignant (squamous cell carcinoma) oral tissues have been analysed for the presence of HPV-16 DNA and a fragment of the HSV-1 DNA genome, representing the morphological transforming region [18].

MATERIALS AND METHODS

Biopsy material

Cold knife biopsy specimens of normal oral squamous epithelium were taken from the buccal mucosa of 5 control patients, with no history or evidence of oral mucosal disease. Case tissues were obtained from the buccal mucosa of 4 individuals with oral keratosis, 4 with lichen planus and 8 individuals with carcinoma of the lateral tongue border/floor of the mouth. Upon excision, one half of the biopsy specimen was used for histological diagnosis, while the other half was immediately snap-frozen and stored in liquid nitrogen at −70°C until use.

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DNA extraction, restriction enzyme cleavage, Southern blotting, and high (Tm-15°C), stringency hybridisations were performed as previously described [8, 32].

Probe preparation

HPV-16 cloned into the *Bam* HI site of pBR322 was a kind gift from Professor H. zur Hausen (Heidleberg, Germany). The *Eco*RI F-fragment of HSV-1 (map units 0.315–0.421) was cloned into the Lambda bacteriophage. From this clone the *Bam* HI-G fragment (map units 0.346–0.397) was purified, representing part of the morphological transforming region [18], see Fig. 1.

Following *Bam* HI digestion, both HPV and HSV DNAs were purified and radioactively labelled with ^{32}P-labelled dCTP to a specific activity of 1×10^9 cpm/μg as previously described [8, 32].

Hybridisation reconstruction tests

Reconstruction experiments were carried out in order to estimate the sensitivity of our hybridisation techniques. Each reconstruction test contained 10 μg of virus negative human DNA mixed with appropriate amounts of purified HPV-16 DNA or the HSV-1 *Bam* HI-G fragment. Under the experimental conditions used in this study, all the labelled probes detected DNA sequences with homology to viral DNA, present at 0.5 copies per cell (data not shown).

Probe removal and re-use of DNA blots

Probes were removed by incubating nylon membranes (Hybond N, Amersham) at 45°C for 30 min in 0.4 mol/l sodium hydroxide, followed by incubation in 0.1 × sodium saline citrate (SSC), 0.1% sodium dodecyl sulphate (SDS), and 0.2 mol/l Tris-HCl, pH 7.5 for 30 min at 45°C.

RESULTS

Evidence of herpes simplex DNA

The presence of HSV type 1 DNA was assayed by probing *Pst*-I-digested DNA extracted from 21 oral tissue samples for sequences homologous to the HSV-1, *Bam* HI-G fragment under conditions of high stringency. The results, summarised in Table 1 and Fig. 1, indicate that 2/4 (50%) patients with lichen planus (LP), 2/4 (50%) patients with non-specific keratosis (NSK), 1/8 (17%) patients with oral squamous cell carcinoma (SCC) and 3/5 (60%) of the biopsy specimens of

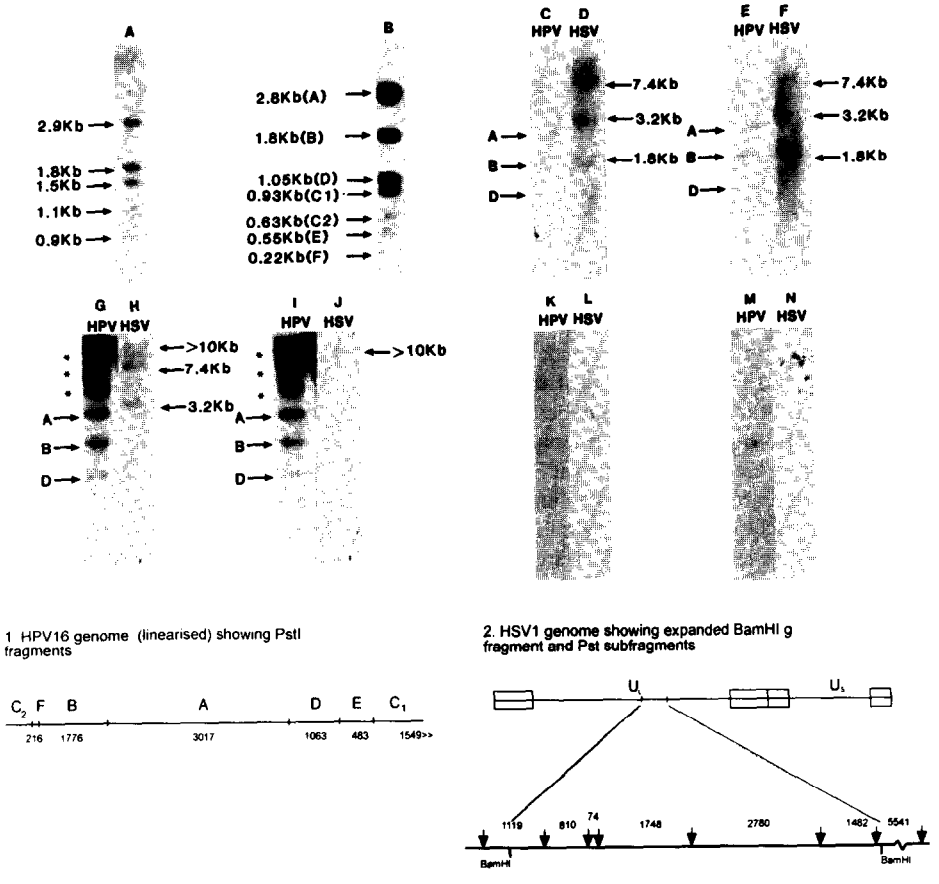

Fig. 1. Detection of HPV-16 and HSV-1 homologous DNA sequences in oral tissue biopsies using Southern blot hybridisation. The sizes of the fragments detected, are in kilobase pairs and have been calculated from the EMBL data base DNA sequences for HPV-16 and the HSV *Bam* HI-G fragment. Lanes A–H show the results of hybridisation of viral DNA probes to DNA extracted from lichen planus #71 (lanes A and B), non-specific keratosis #32 (lanes C and D), normal # (lanes E and F) and lichen planus #11 (lanes G and H). The viral probe used is indicated at the top of each individual lane. The A, B and D HPV fragments detected correspond in size with the A, B and D *Pst*-I fragments of prototype HPV-16 DNA. Asterisks represent "off sized" HPV-16 homologous DNA fragments—see text for further details.

Table 1. Characteristics of oral patients positive for HSV-1 or HPV-16 DNA homologous sequences

Patient number	Age	Sex	Diagnosis	Homology to HSV *Bam* HI-G DNA	Homology to HPV-16 DNA
7	68	F	Normal	+	−
2	17	F	Normal	+	+
11	19	M	Normal	−	+
9	23	F	Normal	+	−
4	21	F	Normal	−	+
71	10	F	Lichen planus	+	+
1	91	F	Lichen planus	−	+
11	47	M	Lichen planus	+	+
13	61	M	Lichen planus	−	−
24	63	M	NS keratosis	−	+
32	65	M	NS keratosis + dysplasia	+	+
16	30	F	NS keratosis	−	+
17	41	F	NS keratosis	+	+
34	71	F	SCC	−	+
39	63	F	SCC	−	+
33	71	F	SCC	−	+
38	87	F	SCC	−	−
51	60	M	SCC	−	−
57	58	M	SCC	+	−
60	59	M	SCC	−	−
36	83	F	SCC	−	+

NS = non-specific.
SCC = squamous cell carcinoma.
+ = positive for virus DNA; − = negative for virus DNA.

normal oral mucosa contained DNA sequences homologous to the HSV-1 probe employed in this study. Interestingly, duplicate biopsy specimens obtained from SCC #34 and LP #1 were both negative for HSV DNA homologous sequences, whereas NSK #17 gave a heterogeneous result; with biopsy sample #17a being negative for HSV sequences and biopsy sample #17b giving a positive result.

Evidence of human papillomavirus type 16 DNA

The distribution of HPV-16 DNA homologous sequences obtained using a 7.9 Kb HPV-16 insert probe, using high stringency hybridisation, is summarised in Table 1. HPV-16 homologous DNA sequences were detected in 3/5 (60%) of biopsy specimens from normal oral mucosa, 3/4 (75%) lichen planus, 4/4 (100%) non-specific keratosis and 4/8 (50%) oral carcinomas. Only in 1 patient did the HPV-16 DNA sequences resemble the prototype *Pst*-I pattern as described by Durst *et al.* [33]. A different *Pst*-I pattern, which appears to have lost the *Pst*-I-C fragment, encompassing the L2/L1 open reading frames was detected in the remaining oral tissues. This has been described in detail elsewhere [4, 10, 30] and represents a deletion mutant of HPV-16 in which the late genes have been replaced with repetitive cellular DNA sequences. In a number of the *Pst*-I digestions, extra 'off-sized' HPV DNA fragments were detected of both high and low molecular weights (represented by asterisks in Fig. 1). Such bands have been taken by many to represent virus-cell junction fragments resulting from the integration of the HPV genome into the host cell chromosome [34, 35]. We have evidence, using subgenomic probe hybridisation, that in a significant number of the present oral tissues, the HPV DNA homologous sequences have integrated into the host cell DNA (Cox, Maitland, Scully, in preparation).

Concurrence of herpes simplex and human papilloma viruses

Overall, only five oral biopsy specimens positive for the presence of HSV DNA homologous sequences were also positive for HPV-16 DNA sequences (one biopsy of normal mucosa, two of lichen planus and two of non-specific keratosis). No carcinomas contained both HSV DNA and HPV DNA.

DISCUSSION

We have previously reported the presence of a human papilloma virus (HPV), closely related to HPV type 16, in normal, premalignant and malignant oral tissues and were unable to support the hypothesis that HPV-16 infection represented a clear risk factor in oral carcinogenesis [4]. RNA complementary to HSV DNA has previously been demonstrated in tumour tissues from human oral carcinomas [3, 36]. The present study, prompted by the hypothesis that HSV and HPV may act as co-carcinogens in the development of mucosal carcinomas [17] has found no evidence of simultaneous HSV and HPV infection in oral carcinomas.

The present study has demonstrated DNA sequences homologous to the HSV-1 *Bam* HI-G fragment in three of five biopsy specimens of normal oral tissues, two of four lichen planus lesions, two of four non-specific keratosis, and in one of eight oral carcinomas. The HSV-1 *Bam* HI-G probe (0.346–0.397 map units) represented a proportion of the viral genome between 0.31 and 0.42 map units, an area which can transform BALB 3T3 and hamster embryo cells *in vitro* [18, 19]. In addition, the glycoproteins VP 143 and A/B encoded by this region have been detected in virally transformed cells and DNA sequences from this region have been shown to persist in some cells transformed by UV-inactivated HSV [37]. Other

groups have, however, been unable to detect this viral fragment in HSV-1 transformed cell lines, a result which has been attributed to a lack of hybridisation sensitivity because these studies used relatively large viral DNA probes to detect small viral DNA sequences [37, 38]. The lack of hybridisation sensitivity was overcome in this study by the use of the smaller HSV-1, *Bam* HI-G DNA probe.

Figure 1 shows typical hybridisation patterns obtained with both the HPV-16 and HSV-1 DNA probes. The *Pst*-I hybridisation pattern of the HSV-1 homologous sequences was not uniform in all of the positive patients, with a single fragment detected in some (Fig. 1, lane J) and multiple bands detected in others (Fig. 1, lanes D, F and H). The 3.2-, and 1.8 kb species were similar to the 2.8, and 1.8 kb DNA fragments generated by *Pst*-I cleavage of the HSV-I, *Bam* HI-G region (Fig. 1; line diagram 2), and may have represented unrearranged viral DNA sequences. However, the higher molecular weight fragments could have represented junctions of viral and cellular DNA sequences (i.e. integration events) or rearranged HSV-1 sequences.

The detection of single high molecular weight bands of approximately the same size as the HSV-1 *Bam* HI-G probe (8.2 kb) suggested that either a proportion of the HSV-1 genome had integrated into the host cell DNA, or that these fragments had lost all *Pst*-I sites. Such bands were not thought to have arisen from incomplete *Pst*-I digestion, since a 4-fold excess of restriction enzyme was used and the completeness of digestion was also monitored by hybridisation of the filters with a cellular gene probe (see below).

Negative results in the remaining 13 patients' DNAs were not due to the lack of DNA sequences since some were strongly positive for HPV-16 DNA homologous sequences (see below) and rehybridisation with a *c-H-ras* DNA gene probe gave a positive signal in all lanes (data not shown) which also confirmed the complete restriction endonuclease digestion of the cellular DNA. The negative results for HSV-1 detection, like those for HPV-16, should be seen in the context of the stated hybridisation sensitivity of the experiments, which were estimates, on the basis of reconstruction experiments to be 0.5 genomes/cell of vial DNA. A biopsy specimen which contained large numbers of uninfected cells relative to the virus-positive cells could appear negative at this level of sensitivity.

For several reasons it is unlikely that the HSV-1 homologous DNA sequences detected here resulted from homology to unrelated cellular DNA sequences [8], or to guanine and cytosine rich DNA sequences of normal human DNA [39] which might bind the probe. First, the *Bam* HI-G probe has not been shown to have any sequence homology to normal cellular DNA. Second, only 8 of 21 of the oral tissues examined gave positive results with the HSV-1 *Bam* HI-G probe. Third, only one of the two biopsy specimens from NSK #17 was positive for sequence homology to the HSV-1 probe. Finally, in a number of tissues, some of the HSV-1 homologous DNA sequences closely resembled their *Pst*-1 counterparts in the prototype HSV-1 *Bam* HI-G fragment.

The unusual pattern of hybridisation of the *Bam* HI-G probe suggested that, in a number of tissues, the HSV-1 DNA sequences may have undergone integration events or have been extensively rearranged, although we were not able to distinguish between these possibilities in this present study. It is equally likely that the HSV-1 genome could undergo a recombination, perhaps with G+C rich cellular repetitive sequences, and remain in the cell in this form (as suggested for the deletion mutant of HPV-16 above). However, the present data is not able to confirm that HSV-1 DNA has been permanently integrated into the cell chromosome, and the lack of evidence for this type of integration event *in vivo*, including the well established neuronal latency of HSV-1 argues against this as the explanation for the heterogeneous hybridisation patterns observed in Fig. 1. However, there does exist the *in vitro* precedent of integrated HSV DNA in a number of transformed cell lines. Another possible source of the discrepancy between the theoretical sizes of the *Pst*I subfragments of HSV-1 *Bam* HI-G and the fragments observed in the tissue biopsy specimens could have been shrinkage in the nylon filter after washing to remove the HPV-16 probe. The theoretical *Pst*-I fragment size from the HSV-1 reference strain are shown in Fig. 1 as 5541, 2780 and 1748 bp could translate into the observed 7.4, 3.2, 1.8 kb fragments with even a 2 mm shrinkage in the filter. Finally, there have been many restriction endonuclease polymorphisms in cleavage sites of HSV-1 strains [40] relative to the reference strain whose sequence has been determined [41], which could also account for these differences. Similar observations have also been made with regard to HSV-2 DNA sequences in cervical carcinoma tissues [27, 29]. Furthermore, we were not able to determine whether or not the tissues analysed contained the entire HSV-1 genome, which would be indicative of an infectious or latent viral infection. Clearly further studies with subfragments of the *Bam* HI-G fragment and other regions of the HSV-1 genome as a hybridisation probe are called for at this stage to map the precise amount of HSV-1 DNA present in each tissue fragment. The detection of only single viral DNA fragments in some of the tissues analysed argued against this latter possibility and in this respect it has been suggested that, following HSV infection, cell survival may only result from the selection of cells which have lost specific virus DNA sequences which might be incompatible with host cell survival [37, 42].

Our present results for HSV-1 and oral cancer therefore agree with those reported for HSV-2 and cervical cancer, i.e. that HSV DNA can only be detected in a small percentage of carcinomas [3, 30, 43, 44]. The detection of HSV-1 homologous DNA sequences in normal oral tissues, lichen planus and non-specific keratoses has, however, not been previously documented. Any functional importance of these sequences therefore remains unknown at this time.

Our failure to detect the simultaneous presence of HPV-16 and HSV-1 homologous sequences in any of the eight oral carcinomas, despite their demonstration in one of five normal biopsy specimens, two or four lichen planus biopsies and two of four non-specific keratosis biopsy samples, might argue against a co-carcinogenic role for these two viruses in oral cancer as suggested by Prakash *et al.*, who were unable to detect HPV-16 DNA sequences in 2 of 13 cervical cancers which harboured HSV-2 DNA sequences [28]. However, it must be stressed that, even though the oral tissues in this study have been analysed with a mixed HPV DNA probe (HPV types 1, 2, 4, 6, 11, 13, 16, 18) under reduced stringency conditions [4], it is possible that other 'oncogenic' HPV types might be present, or that oral tissues harboured HSV-1 sequences which were not detected by the *Bam* HI-G probe. Furthermore, the absence of HSV-DNA by no means excludes the involvement of HSV in a hit and run mechanism of carcinogenesis [44]. Indeed, HSV sequences were if anything detected more frequently in potentially malignant lesions than in carcinomas.

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