Oral Oncol, Eur J Cancer, Vol. 28B, No. 2, pp. 145–152, 1992
Printed In Great Britain

0964–1955/92 $5.00+0.00

Feature Articles

In vitro and Animal Studies of the Role of Viruses in Oral Carcinogenesis

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The linkage of herpes simplex virus (HSV) and human papillomavirus (HPV) to the development of oral cancer has been studied. In spite of the presence of viral nucleic acids in some human oral cancer specimens, HSV alone is not carcinogenic in animals: repeated viral inoculation to mouse or hamster oral mucosa fails to produce tumours or histopathological evidence of malignancy. However, HSV demonstrates co-carcinogenicity *in vivo*: viral inoculation significantly enhances the oncogenic capacity of chemical carcinogens in the oral cavity of mice and hamsters. Though the detailed mechanisms of HSV cocarcinogenicity are unknown, HSV promotes the chemical carcinogen-induced activation of certain cellular proto-oncogenes and inactivation of p53 tumour suppressor gene. Human papillomaviruses type 16 (HPV-16) and 18 (HPV-18) demonstrate oncogenicity by transforming normal human oral keratinocytes *in vitro*. While normal cells exhibit a limited life-span, cells transformed by these viruses show immortality and altered morphology in comparison with their normal counterparts. The HPV-immortalised cells contain multiple copies of intact viral genome integrated into cellular chromosomes. These cells also express several viral-specific mRNAs including viral E6/E7 mRNAs. Notably, these cells contain low levlels of p53 protein and overexpressed cellular *myc* proto-oncogene compared to their normal counterpart; however, the immortilised cell lines are non-tumorigenic in nude mice.

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INTRODUCTION

ORAL CANCER (including oropharynx) accounts for 2.1% of all cancers and 1% of cancer deaths [1]. Nationally, oral cancer incidence rates from the 1940s to early 1980s have increased more than 50% among women of all ages and in men aged 54–64 years. In 1988, approximately 30 000 people developed oral cancer and approximately 9000 died of this disease in the United States. Like many cancers, oral cancer is a disease of the elderly; rare under the age of 30, but it represents 0.05–0.06% of the 65–69-year-old group. Based on statistics collected in the early 1980s, the 5-year survival rate for oral cancer patients is 54% for Caucasians and 31% for African–American. Oral cancer is an important problem not only because of the significant mortality associated with the disease, but also because of the functional defects and disfigurement often associated with its treatment. Efforts aimed at early diagnosis, identification of risk factors, and mechanisms of oral carcinogenesis are warranted.

Clearly cigarette, cigar and pipe smoking are causally associated with oral cancer as is the use of smokeless tobacco. Though there has been a general decline in cigarette smoking in Western countries during the last decade, there has been an increased use of smokeless tobacco during the same period. Epidemiological studies support the contention that smokeless tobacco causes oral cancer in humans [2]: A recent consensus conference also concluded a strong association between smokeless tobacco use and the development of oral cancer. The constituents of tar from smoked tobacco and smokeless tobacco responsible for oral cancer are tobacco specific *N'*-nitrosamines (TSNAs) and benzo(*a*)pyrene (B(*a*)P). TSNAs are formed from nicotine and minor tobacco alkaloids during aging, curing and fermentation of tobacco. Among TSNAs, high levels of *N'*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are found in tobacco. B(*a*)P is an ubiquitous environmental pollutant with metabolites that are mutagenic and carcinogenic in a variety of biological systems. Though the carcinogenicity of NNN, NNK, and B(*a*)P has been demonstrated in mice, rats and golden Syrian hamsters, oral carcinogenicity of these compounds has not been documented sufficiently in laboratory animals. Some studies indicate a tenuous linkage between malignant changes and the use of smokeless tobacco in humans [3]. Moreover, several laboratory studies have reported the failure to produce oral malignancies with repeated intraoral placement of smokeless tobacco in animals [4, 5]. Possible involvements of other factors such as alcohol, caffeine, and viruses have, therefore, been postulated in the development of tobacco-related oral malignancies in humans. Among these factors, the role of viruses, especially herpes simplex virus (HSV) and human papillomavirus (HPV), in

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Received 14 July 1992; accepted 17 July 1992.

oral carcinogenesis have been studied in our laboratory. In this review article, we summarise the investigations associated with the *in vitro* and animal studies of the role of HSV and HPV in oral carcinogenesis.

HERPES SIMPLEX VIRUS (HSV)

General characteristics and tumorigenicity of HSV

Herpes simplex virus (HSV) is an enveloped virus containing a double-stranded DNA as genetic material. The virus exists in two closely related forms, type 1 (HSV-1) and 2 (HSV-2). HSV-1 predominantly causes infectious diseases in the orofacial area, while HSV-2 is mostly responsible for genital infections. HSV infection is very prevalent; up to 90% of individuals have antibodies to HSV-1 by 10 years of age. Primary HSV infection is followed by latent viral infection in the sensory or autonomic ganglia. More than 80% of the population in the USA is thought to have latent HSV infection in the trigeminal ganglia. More than one third of the world's population suffers from recurrent intraoral or orofacial herpetic infections resulting from the activation of latent virus from the trigeminal ganglia. Individuals with latent HSV-1 infection in the ganglia actively shed infectious virions onto the oral mucosa without clinical symptoms [6], resulting in repeated exposure of mucosal cells to the virus. This could induce certain molecular biological changes in those cells and provide an opportunity for HSV to interact with many environmental chemicals in the oral cavity.

There is ample evidence linking HSV to human malignancies. Many epidemiological studies indicate a close association of HSV-1 with the genesis of oral cancer [7, 8] and HSV-2 in the development of uterine cervix and vulval malignancies [9]. Expression of HSV-1 genes has been detected in some oral cancer tissue specimens. HSV-2 antigens, RNA, and DNA have also been detected in certain cancer tissues from the uterine cervix or vulva. Malignant transformation of mammalian cells can be experimentally induced by ultraviolet (UV)-irradiated HSV, fragments of HSV DNA, and photodynamically inactivated HSV. In fact, the transforming region of HSV-1 [minimum transforming region 1, (mtr-1)] and HSV-2 (mtr-2 and mtr-3) were found to be located in the left third of the HSV-1 genome and close to the centre of the HSV-2 genome, respectively [10]. However, unlike other DNA tumour viruses, a given set of HSV genes are not consistently retained or expressed in the transformed cells, and viral nucleic acids and antigens are not always detected from human cancer specimens. The failure to detect viral nucleic acids and/or proteins in transformed cells and cancer tissue led to the "hit-and-run" hypothesis of HSV transformation, indicating the mutagenic capacity of HSV.

Carcinogenicity of HSV in animals

Despite HSV's *in vitro* mutagenic and cell-transforming activities, viral nucleic acids and antigens are not consistently detected from most human oral and genital cancer specimens. Furthermore, repeated inoculation of animal oral mucosa with either infectious or UV-inactivated HSV, does not induce tumours. The failure to detect viral nucleic acids and/or antigens in a high percentage of human oral and genital tumours and unsuccessful development of tumours with HSV inoculation has made it difficult to implicate HSV in the aetiology of these neoplasias. The cocarcinogenic role of HSV in conjunction with other factors, especially tobacco or tobacco-related chemical carcinogens, in oral carcinogenesis has been investigated, since oral mucosa could be a unique target tissue being directly exposed to both tobacco and HSV. Our studies indicate that tobacco and HSV are synergistic in the development of precancerous lesions in mice. Repeated HSV-1 inoculations or chronic exposure of oral mucosa to water-extractable components of snuff (snuff-extract) or smoked-tobacco tar does not induce preneoplastic changes in mice, but topical application of snuff-extract or tobacco tar in conjunction with HSV-1 inoculation produces epithelial dysplasia and other premalignant histomorphologic changes [11]. Repeated HSV infections in combination with simulated snuff-dipping leads to the development of oral cancer in rats [12] and of micro-invasive squamous-cell carcinoma in hamster buccal pouch tissue [5]. Neither HSV infection nor simulated snuff-dipping alone produces similar results. Similarly, HSV inoculation significantly enhances the carcinogenicity of chemical carcinogens such as 7,12-dimethylbenz(*a*)anthracene (DMBA) and B(*a*)P. The appearance of preneoplastic and neoplastic changes caused by the chemical carcinogens is significantly hastened and the carcinogenicity of these chemical carcinogens is notably enhanced by HSV. These experimental data suggest that HSV alone may not be carcinogenic, but it enhances the oncogenicity of tobacco or tobacco-related chemical carcinogens in the oral cavity of animals.

Mechanisms of HSV carcinogenicity

The mode of *in vivo* carcinogenicity (or *in vitro* cell transforming activity) is largely speculative, but many studies have suggested that HSV carcinogenicity is associated with its mutagenicity and its ability to induce chromosomal aberrations in cells. Furthermore, HSV induces expression and amplification of certain genes in virus-infected cells probably through the transactivation of those particular genes. For example, HSV-1 and cloned fragments of HSV-1 DNA can activate latent type C retroviruses and induce gene amplification, particularly of sequences harboring an origin of replication such as SV40. Inasmuch as DNA amplification is a common feature of tumours and the amplification of known oncogenes has been detected in a number of human cancer tissue and cell lines, the ability of HSV to induce an amplification of foreign DNA in cells allows us to assume that HSV may induce an amplification and overexpression of certain oncogenes. Yet no known proto-oncogenes are reported to be activated by HSV. Nevertheless, HSV notably increases the carcinogenicity of chemical carcinogens in hamster buccal pouches, and significantly hastens and enhances chemical carcinogen-induced amplification and overexpression of c-*erb*B-1/EGFR in the pouch epithelium. These data indicate that HSV may elicit its carcinogenicity by, in part, accelerating the chemical carcinogen-induced activation process of certain proto-oncogenes.

Carcinogenesis is a multistep process involving at least two classes of genes, proto-oncogenes and tumour suppressor genes. Considerable circumstantial evidence exists that the activated and overexpressed oncogenes play an important role in oral carcinogenesis: overexpression of c-*erb*B-1/epidermal growth factor receptor (EGFR), *bcl*, and c-*myc* genes is found in oral cancer tissue. In the hamster buccal pouch tumour model, the overexpression of transforming growth factor-α (TGF-α), c-*erb*B-1/EGFR, c-Ha-*ras*, and c-Ki-*ras* is also associated with the development of tumours with topical DMBA application. Cancer also results from a recessive mutation,

rearrangement, or deletion of tumour suppressor genes. Among these genes, overexpression of mutant p53 is often found in squamous-cell carcinomas, such as non-small cell lung cancers, oesophageal cancer, colorectal cancers, and uterine cervix cancers. Recent studies also indicate a close association between p53 gene mutation and the development of oral cancer.

We have established several oral squamous-cell carcinoma cell lines derived from hamster buccal pouch tumours induced by topical DMBA, alone or in conjunction with HSV inoculation. Subsequent immunocytochemical studies showed that these cell lines overexpressed TGF-α, c-Ha-*ras*, c-*erb*B-1/EGFR, c-Ki-*ras*, and mutant p53. Inasmuch as tumorigenesis is a multistep process and sequential activation of proto-oncogenes and inactivation of tumour suppressor genes may be involved in the process, we hypothesise that the onset of the aberrant overexpression of these genes could be associated with specific stages of multistep tumorigenesis in hamster buccal pouch epithelium. HSV-1 might elicit cocarcinogenicity by hastening and enhancing the activation or inactivation of these genes.

To test this hypothesis, hamsters were divided into five groups and the bucal pouches were treated as follow: group 1, mock inoculation and topical application of mineral oil (control); group 2, HSV-1 inoculation and topical application of mineral oil; group 3, mock inoculation and topical application of 0.1% DMBA in mineral oil; group 4, HSV-1 inoculation and topical application of 01.% DMBA in mineral oil; group 5, vaccinia virus inoculation and topical application of 0.1% DMBA in mineral oil (viral control). The development of precancerous and cancerous lesions was monitored for 16 weeks after the initiation of mock or viral inoculation. Furthermore, expression of c-*erb*B-1/EGFR, TGF-α, c-Ha-*ras*, c-Ki-*ras*, and p53 genes was examined from the pouch tissue on 0, 4, 8, 12, and 16 weeks after the inoculation using immunohistochemical analysis to localise the cellular sources of the expression of these genes. We further investigated the linkage, if any, between HSV-1 inoculation, duration of DMBA application, the histopathologic changes, and the expression of proto-oncogenes and tumour suppressor p53 gene.

Our result showed that HSV-1 significantly increased the carcinogenicity of DMBA and hastened the appearance of precancerous lesions and tumours induced by DMBA (Tables 1 and 2). Unlike HSV-1, vaccinia virus, a DNA virus, did not alter the oncogenicity of DMBA in hamsters, indicating that enhanced oncogenicity of DMBA by HSV-1 is not due to a simple exposure of tissue receiving DMBA to foreign DNA. Overexpression of TGF-α and c-Ha-*ras* resulting from the activation of the corresponding genes and enhanced expression of p53 protein presumably by mutation of p53 gene were noticed from the hamster pouch epithelial cells at early (hypertrophy and dysplasia) and later stages (carcinoma *in situ* and invasive cancer formation) of carcinogenesis. These data indicate that these genes might play a role in early stage transformation of cells and maintenance of cell malignancy (Tables 3–5). Increased translation of c-*erb*B-1/EGFR and c-Ki-*ras* resulting from possible activation of the genes was noticed only at later stages of cancer development (carcinoma *in situ* and invasive cancer formation), suggesting that the latter two genes might be involved in the malignant transformation of premalignant cells and invasion of cancer cells into connective tissue (Tables 6 and 7). HSV-1 notably hastened the onset of the overexpression of all tested gene products, suggesting that HSV-1 may increase the tumorigenicity of DMBA by, in part, hastening and enhancing the expression of DMBA-induced overexpression of these genes.

Present data show that HSV-1 enhances the carcinogenicity of DMBA. Though the exact mechanism of HSV-1 cocarcinogenicity remains speculative, HSV-1 clearly enhances the activation of some proto-oncogenes and inactivation of p53 gene in hamster buccal pouch epithelium. HSV-1 may stimulate the oncogenicity of chemical carcinogens by impairing the immunological response of the host, by interfering with cellular chemical detoxification, by altering target cell permeability, or by causing proliferation of latent tumour cells [13]. Since HSV infection is associated with chromosomal aberrations, mutations, and selective DNA amplification, HSV may alter cellular DNA, making interaction with DMBA more favourable. However, rigorous laboratory investigations must be carried out to further understand the mechanism of HSV cocarcinogenicity.

HUMAN PAPILLOMAVIRUS (HPV)

General characteristics and tumorigenicity of HPV

Over the past two decades, research with papillomaviruses, a group of small DNA viruses that induce papillomas in animals, has been exploded. Papillomaviruses containing circular DNA (approximately 8.0 kilobase pairs in size) as the genome,

Table 1. Effect of HSV-1 inoculation and topical DMBA application on the development of leukoplakia in hamster buccal pouch mucosa

Groups	Weeks after mock or viral inoculation			
	4	8	12	16
1. Control (mock inoculation + topical application of mineral oil)	0/8	0/8	0/8	0/8
2. HSV-1 inoculation + topical application of mineral oil	0/8	0/8	0/8	0/8
3. Mock inoculation + topical application of 0.1% DMBA	0/14	2/14	5/14	7/14
4. HSV-1 inoculation + topical application of 0.1% DMBA	3/14*	7/14*	12/14*	14/14*
5. Vaccinia virus inoculation + topical application of 0.1% DMBA	0/14	3/14	5/14	6/14

Hamster pouch tissue was scarified and inoculated with culture medium (0.1 ml; mock inoculation), HSV-1 (0.1 ml of 10^8 plaque-forming units per milliliter [PFU/ml]), or vaccinia virus (0.1 ml of 10^8 PFU/ml). After inoculation, 0.1 ml of mineral oil or 0.1% DMBA solution was applied to the pouches three times per week for 15 consecutive weeks. The pouches were examined once a week to detect the appearance of leukoplakia and tumours.

Numerators: Number of pouches with leukoplakia by 4, 8, 12, or 16 weeks after the initiation of topical mineral oil or 0.1% DMBA solution.

Denominators: Number of pouches tested.

*Significantly different from group 3 receiving both mock inoculation and 0.1% DMBA.

Table 2. Effect of HSV-1 inoculation and topical DMBA application on the development of tumours in hamster buccal pouch mucosa

Groups	Weeks after mock or viral inoculation			
	4	8	12	16
1. Control (mock inoculation + topical application of mineral oil)	0/8	0/8	0/8	0/8
2. HSV-1 inoculation + topical application of mineral oil	0/8	0/8	0/8	0/8
3. Mock inoculation + topical application of 0.1% DMBA	0/14	0/14	0/14	3/14
4. HSV-1 inoculation + topical application of 0.1% DMBA	0/14	0/14	7/14*	14/14*
5. Vaccinia virus inoculation + topical application of 0.1% DMBA	0/14	0/14	0/14	2/14

Hamster pouch tissue was scarified and inoculated with culture medium (0.1 ml; mock inoculation), HSV-1 (0.1 ml of 10^8 PFU/ml), or vaccinia virus (0.1 ml of 10^8 PFU/ml). After inoculation, 0.1 ml of mineral oil or 0.1% DMBA solution was applied into the pouches three times per week for 15 consecutive weeks. The pouches were examined once a week to detect the appearance of leukoplakia and tumours.
Numerators: Number of pouches with tumours by 4, 8, 12, or 16 weeks after the initiation of topical mineral oil or 0.1% DMBA solution.
Denominators: Number of pouches tested.
*Significantly different from group 3 receiving both mock inoculation and 0.1% DMBA.

Table 3. Expression of TGF-α from hamster buccal pouch epithelium receiving HSV-1 inoculation and topical DMBA, alone or in combination

Groups	Weeks after mock or viral inoculation				
	0	4	8	12	16
1. Control (mock inoculation + topical application of mineral oil)	+	+	+	+	+
2. HSV-1 inoculation + topical application of mineral oil	+	+	+	+	+
3. Mock inoculation + topical application of 0.1% DMBA	+	+	+	++	+++
4. HSV-1 inoculation + topical application of 0.1% DMBA	+	++	+++	++++	++++
5. Vaccinia virus inoculation + topical application of 0.1% DMBA	+	+	+	++	++

Hamster pouch tissue was scarified and inoculated with culture medium (0.1 ml; mock inoculation), HSV-1 (0.1 ml of 10^8 PFU/ml), or vaccinia virus (0.1 ml of 10^8 PFU/ml). After inoculation, 0.1 ml of mineral oil or 0.1% DMBA solution was applied into the pouches three times per week for 15 consecutive weeks. The expression of TGF-α was detected on 0, 4, 8, 12, and 16 weeks after the initiation of topical DMBA application with immunohistochemical staining. The immunohistochemical staining of paraffin sections was performed with a monoclonal antibody to TGF-α (Oncogene Sciences, Manhasset, New York) using DAKO LSAB® Kit, peroxidase system 40 (DAKO Corp., Carpinteria, California). The amount of TGF-α expression was arbitrarily indexed by the intensity of immunostaining as follows: no expression (−), minimum (+), moderate (++), high (+++), and highest (++++).

Table 4. Expression of c-Ha-ras from hamster buccal pouch epithelium receiving HSV-1 inoculation and topical DMBA, alone or in combination

Groups	Weeks after mock or viral inoculation				
	0	4	8	12	16
1. Control (mock inoculation + topical application of mineral oil)	+	+	+	+	+
2. HSV-1 inoculation + topical application of mineral oil	+	+	+	+	+
3. Mock inoculation + topical application of 0.1% DMBA	+	+	+	++	++
4. HSV-1 inoculation + topical application of 0.1% DMBA	+	+	++	++	+++
5. Vaccinia virus inoculation + topical application of 0.1% DMBA	+	+	+	++	++

Hamster pouch tissue was scarified and inoculated with culture medium (0.1 ml; mock inoculation), HSV-1 (0.1 ml of 10^8 PFU/ml), or vaccinia virus (0.1 ml of 10^8 PFU/ml). After inoculation, 0.1 ml of mineral oil or 0.1% DMBA solution was applied into the pouches three times per week for 15 consecutive weeks. The expression of c-Ha-*ras* was detected on 0, 4, 8, 12, and 16 weeks after the initiation of topical DMBA application with immunohistochemical staining. The immunohistochemical staining of paraffin sections was performed with a monoclonal antibody to c-Ha-*ras* (Oncogene Sciences) using DAKO LSAB® Kit, peroxidase system 40 (DAKO Corp.). The amount of c-Has-*ras* expression was arbitrarily indexed by the intensity of immunostaining as follows: no expression (−), minimum (+), moderate (++), high (+++), and highest (++++).

are non-enveloped viruses that replicate in the nucleus of epithelial cells. About 66 types of human papillomaviruses (HPVs) have been identified.

Several types of HPVs are associated with different types of human papillomas, premalignant lesions, and malignant cancers. HPV DNA is frequently found in condylomata acuminata of the anogenital areas such as the cervix, vulva, and anus. Of the 66 genotypes of HPV, high-risk HPVs such as types 16 (HPV-16) and 18 (HPV-18) are most frequently associated with the most malignant genital lesions, while HPV-6 and HPV-11 are mostly found in benign tumours [14]. Evidence that high-risk HPVs are involved in the genesis of anogenital cancer is as follows: (1) More than 90% of anogenital cancer tissue contains HPV-16 or 18 DNA; (2) The cancer cells contain viral DNA as integrated form with the disruption of early gene 2 open reading frame (E2 ORF), whereas benign

Table 5. Expression of p53 from hamster buccal pouch epithelium receiving HSV-1 inoculation and topical DMBA, alone or in combination

Groups	Weeks after mock or viral inoculatin				
	0	4	8	12	16
1. Control (mock inoculation + topical application of mineral oil)	—	—	—	—	—
2. HSV-1 inoculation + topical application of mineral oil	—	—	—	—	—
3. Mock inoculation + topical application of 0.1% DMBA	—	—	+	++	+++
4. HSV-1 inoculation + topical application of 0.1% DMBA	—	++	+++	++++	++++
5. Vaccinia virus inoculation + topical application of 0.1% DMBA	—	—	+	++	++

Hamster pouch tissue was scarified and inoculated with culture medium (0.1 ml; mock inoculation), HSV-1 (0.1 ml of 10^8 PFU/ml), or vaccinia virus (01. ml of 10^8 PFU/ml). After inoculation, 0.1 ml of mineral oil or 0.1% DMBA solution was applied into the pouches three times per week for 15 consecutive weeks. The expression of p53 was detected on 0, 4, 8, 12, and 16 weeks after the initiation of topical DMBA application with immunohistochemical staining. The immunohistochemical staining of paraffin sections was performed with a monoclonal antibody to p53 (Oncogene Sciences) using DAKO LSAB® Kit, peroxidase system 40 (DAKO Corp.). The amount of p53 expression was arbitrarily indexed by the intensity of immunostaining as follows: no expression (−), minimum (+), moderate (++), high (+++), and highest (++++).

Table 6. Expression of c-erbB-1/EGFR from hamster buccal pouch epithelium receiving HSV-1 inoculation and topical DMBA, alone or in combination

Groups	Weeks after mock or viral inoculation				
	0	4	8	12	16
1. Control (mock inoculation + topical application of mineral oil)	—	—	—	—	—
2. HSV-1 inoculation + topical application of mineral oil	—	—	—	—	—
3. Mock inoculation + topical application of 0.1% DMBA	—	—	—	—	+
4. HSV-1 inoculation + topical application of 0.1% DMBA	—	—	—	++	+++
5. Vaccinia virus inoculation + topical application of 0.1% DMBA	—	—	—	—	+

Hamster pouch tissue was scarified and inoculated with culture medium (0.1 ml; mock inoculation), HSV-1 (0.1 ml of 10^8 PFU/ml), or vaccinia virus (0.1 ml of 10^8 PFU/ml). After inoculation, 0.1 ml of mineral oil or 0.1% DMBA solution was applied into the pouches three times per week for 15 consecutive weeks. The expression of c-*erb*B-1/EGFR was detected on 0, 4, 8, 12, and 16 weeks after the initiation of topical DMBA application with immunohistochemical staining. The immunohistochemical staining of paraffin sections was performed with a monoclonal antibody to c-*erb*B-1 (Oncogene Sciences) using DAKO LSAB® Kit, peroxidase system 40 (DAKO Corp.). The amount of c-*erb*B-1/EGFR expression was arbitrarily indexed by the intensity of immunostaining as follows: no expression (−), minimum (+), moderate (++), high (+++), and highest (++++).

Table 7. Expression of c-Ki-ras from hamster buccal pouch epithelium receiving HSV-1 inoculation and topical DMBA, alone or in combination

Groups	Weeks after mock or viral inoculation				
	0	4	8	12	16
1. Control (mock inoculation + topical application of mineral oil)	—	—	—	—	—
2. HSV-1 inoculation + topical application of mineral oil	—	—	—	—	—
3. Mock inoculation + topical application of 0.1% DMBA	—	—	—	—	+
4. HSV-1 inoculation + topical application of 0.1% DMBA	—	—	—	+	++
5. Vaccinia virus inoculation + topical application of 0.1% DMBA	—	—	—	—	+

Hamster pouch tissue was scarified and inoculated with culture medium (0.1 ml; mock inoculation), HSV-1 (0.1 ml of 10^8 PFU/ml), or vaccinia virus (0.1 ml of 10^8 PFU/ml). After inoculation, 0.1 ml of mineral oil or 0.1% DMBA solution was applied into the pouches three times per week for 15 consecutive weeks. The expression of c-Ki-*ras* was detected on 0, 4, 8, 12, and 16 weeks after the initiation of topical DMBA application with immunohistochemical staining. The immunohistochemical staining of paraffin sections was performed with a monoclonal antibody to c-Ki-*ras* (Oncogene Sciences) using DAKO LSAB® Kit, peroxidase system 40 (DAKO Corp.). The amount of c-Ki-*ras* expression was arbitrarily indexed by the intensity of immunostaining as follows: no expression (−), minimum (+), moderate (++), high (+++), and highest (++++).

cells contain episomal form of viral DNA; (3) Most cancer cell lines derived from cervix cancer containing high-risk HPVs actively transcribe viral E6 and E7 mRNAs that are able to transform normal cells *in vitro*. Though the exact mechanisms of action of high-risk HPVs on carcinogenesis are largely speculative, HPV may induce cell transformation by altering the expression pattern of c-*myc* gene. Overexpression of c-*myc* gene is frequently found in cancer cells derived from the cancer tissue containing high-risk HPV DNA.

Further evidence of HPV carcinogenicity comes from the *in vitro* cell transforming capacity of HPV DNA; Transformation of human skin fibroblasts, skin keratinocytes, cervical epithelial cells transfected with cloned HPV-16 and 18 DNA has been reported [15–18]. Though the molecular mechanisms of HPV-induced cell transformation is unknown, early viral gene products such as E6 and E7 proteins may play a crucial role in HPV-induced carcinogenesis. Transfection of normal cells with either cloned HPV-16 or 18 E6/E7 gene induces immor-

talisation of the cells. Recently the molecular biological functions of E6 and E7 proteins of high-risk HPVs were partly unveiled. The E6 protein binds and promotes the degradation of wild-type cellular p53, while the E7 protein binds and inactivates the function of retinoblastoma (Rb) tumour suppressor protein.

Benign and malignant oral lesions appear also to be associated with HPV infection. Many studies have provided evidence that HPV is involved in the development of oral mucosal lesions, including squamous-cell papillomas, chondylomas and focal epithelial hyperplasia [19]. DNA hybridisation studies have shown the presence of viral DNA in premalignant and malignant oral lesions [20]. This association is based on the findings of HPV DNA in oral cancer biopsy specimens. Up to 40–50% of cancer tissues contain the viral DNA (Personal communication with Dr Edward Shillitoe, University of Texas, Houston, U.S.A.). Inasmuch as epithelium of the oral mucosa is histologically similar to that of the female genital tract, and since it is also continuously challenged by many environmental factors, close association of HPVs with the development of oral malignancies has been anticipated. In addition, a number of cell lines derived from cervical and oral cancers contain both high-risk HPV DNA and RNA [21, 22].

In vitro *model for HPV oral carcinogenicity*

Our laboratory recently transformed normal human oral keratinocytes—one of the major *in vivo* target cells for HPV infection—with HPV-16 and 18 DNA [22, 23]. The cells transformed by HPV-16 DNA were named human oral keratinocytes-16A (HOK-16A) and 16B (HOK-16B). Cells transformed by HPV-18 DNA were called human gingival keratinocytes-18 (HGK-18). These cells were immortal, contain HPV DNA integrated into the cellular genome, and transcribed viral E6 and E7 genes, but they are non-tumorigenic in nude mice.

The cells displayed keratinocyte morphology and were characterized by a lack of stratification. These cells continued to proliferate and to retain an undifferentiated morphology. The

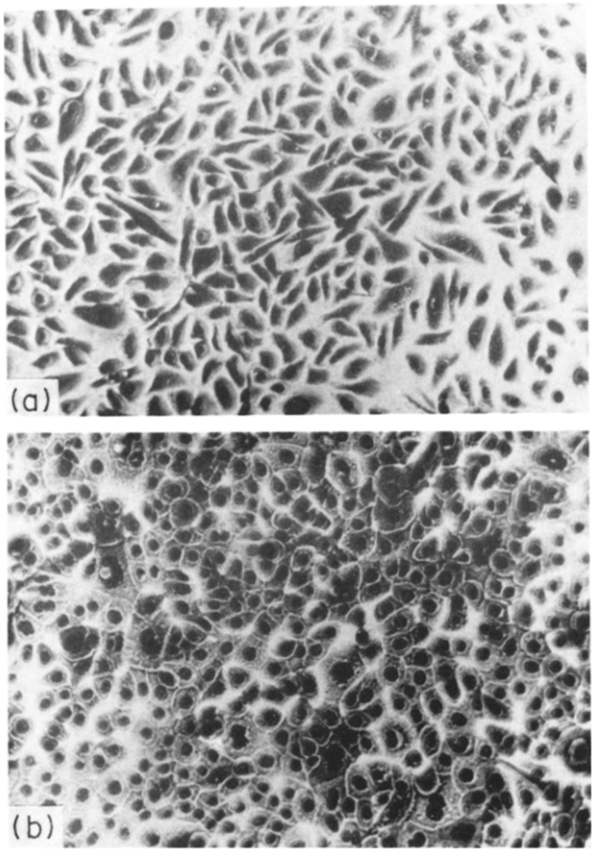

Fig. 1. Microscopic features of Giemsa-stained minolayers of NHGK (a) and immortal HGK-18 cell line (b). The HGK-18 cell line show denser growth pattern and morphological alterations. × 130.

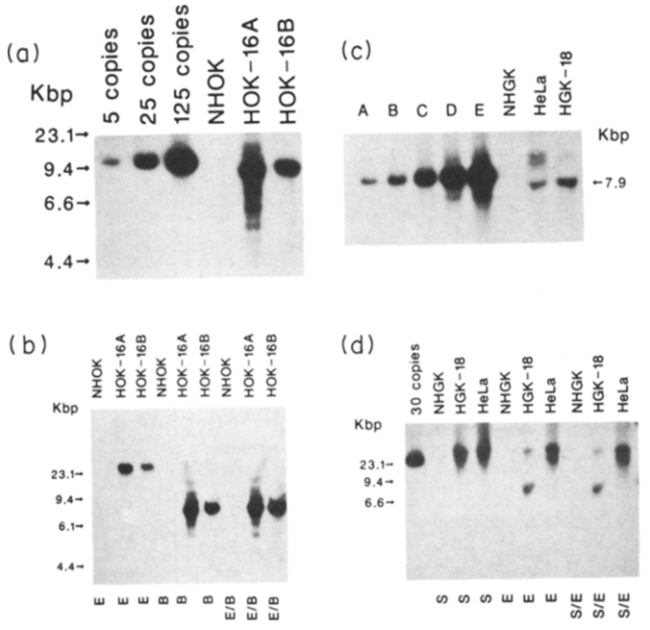

Fig. 2. Southern blot hybridisation analysis of cellular DNA of NHOK, NHGK, HOK-16A, HOK-16B, and HGK-18 cell lines. (a) Determination of the presence and copy numbers of HPV-16 DNA per cell in HOK-16A and HOK-16B cell lines. HPV-16 DNA (corresponding with 5, 25, and 125 copies of viral DNA per cell which were mixed with carrier DNA [*Bam*HI-digested 10 μg of NHOK DNA]) and *Bam*HI-digested cellular DNA (10 μg) extracted from NHOK, HOK-16A, and HOK-16B were electrophoresed in 1% agarose gel. The fragmented DNA was then transferred to a nitrocellulose filter and hybridised to ^{32}P-labelled 7.9-kbp total HPV-16 DNA. The filter was washed and exposed to Kodak SB-5 X-ray film. (b) Determination of physical state of HPV-16 DNA in HOK-16A and HOK-16B cell lines. 10 μg of high molecular weight cellular DNA were digested with *Bam*HI (B) and/or *Eco*RV (E) restriction enzymes. *Bam*HI enzyme separates vector from HPV-16 sequences, while *Eco*RV does not digest pMHPV-16d. The fragmented DNA was then transferred to nitrocellulose filter and hybridised to ^{32}P-labelled 7.9 kbp HPV-16 DNA. The filter was washed and exposed to X-ray film (from Park *et al. Carcinogenesis* 1991, 12, 1627). (c) Determination of the presence and copy numbers of HPV-16 DNA per cell in HGK-18 cell lines. HPV-18 DNA (corresponding with 5, 20, 50, 100 and 200 copies of viral DNA per cell which were mixed with carrier DNA [*Bam*HI-digested 10 μg of NHGK DNA]) and *Eco*RI-digested cellular DNA (10 μg) extracted from NHGK, HeLa, and HGK-18 cell lines were electrophoresed in 1% agarose gel. The fragmented DNA was then transferred to a nitrocellulose filter and hybridised to ^{32}P-labelled 7.9 kbp total HPV-18 DNA. (d) Determination of the physical state of HPV-18 DNA in HGK-18 and HeLa cell lines. SalI (S) and/or *Eco*RI (E)-digested NHGK, HGK-18, HeLa cell DNAs were electrophoresed in 1% agarose gel. *Eco*RI enzyme separates vector from HPV-18 sequences, while SalI does not digest pSHPV-18m. The fragmented DNA was transferred to a nitrocellulose filter and hybridised to ^{32}P-labelled 7.9 kbp total HPV-18 DNA. The filter was washed and exposed to X-ray film. (From Park *et al. Carcinogenesis* 1991, 12, 1627.)

transformed cell lines proliferated faster than normal human oral keratinocytes (NHOK) or normal human gingival keratinocytes (NHGK) and had a cobblestone-like morphology and established a higher density at confluence in comparison with the normal counterpart (Fig. 1). HOK-16A and HOK-16B cell lines contain approximately 40 and 25 copies of HPV-16 DNA per cell, respectively, while the HGK-18 cell line harbors about 10 copies of HPV-18 DNA per cell. HPV-16 DNA exists as an integrated form, not an episomal form, in the HOK-16A and HOK-16B cell lines. Furthermore, the cell lines contained rearranged HPV-16 DNA sequences. Like the HOK-16A and HOK-16B lines, the HGK-18 cell line also contained intact and integrated HPV-18 DNA (Fig. 2). These cell lines expressed numerous viral genes including E6/E7 region, overexpressed cellular c-*myc* gene compared with the normal counterparts (Figs 3 and 4)

The p53 mRNA level in the HPV-immortalised cells was higher than their normal counterpart, but the p53 protein level was notably lower in the immortalised cells [24]. The half-life of p53 protein in the normal and immortalised cells was shorter than 1 h, and the p53 cDNA sequence of these cells showed no mutation. The immortalised cells transcribed high amounts of E6/E7 mRNAs encoded by HPV, but normal cells did not. These observations suggest that the HPV-immortalised keratinocytes may translate normal level of wild-type p53 protein, and the low p53 level in these cells may be due to the enhanced degradation of the protein by HPV-16 E6 protein.

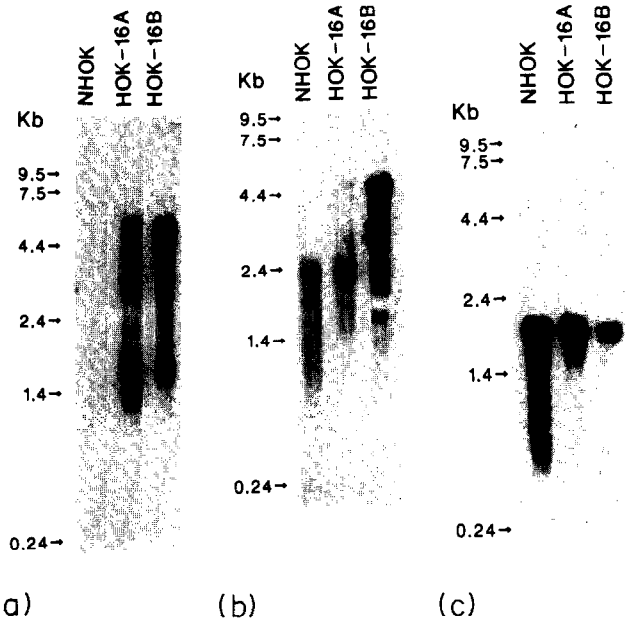

Fig. 3. Northern blot hybridisation analysis of poly(A$^+$)RNAs of NHOK, HOK-16A, and HOK-16B cell lines. (a) Determination of the expression of HPV-16 from HOK-16A and HOK-16B cell lines. Poly(A$^+$)RNAs extracted from NHOK, HOK-16A, and HOK-16B cells were electrophoresed in 1.2% agarose gel containing 2.2 mol/l formaldehyde, transferred to a nylon filter, and hybridised to ^{32}P-labelled 7.9 kbp total HPV-16 DNA. The filter was washed and exposed to X-ray film. (b) The hybridised [^{32}P]HPV-16 DNA was stripped from the nylon filter and rehybridised with ^{32}P-labelled v-*myc* oncogene probe. The filter was washed and exposed to X-ray film. (c) Hybridised [^{32}P]v-*myc* DNA was stripped from the filter and rehybridised to ^{32}P-labelled human β-actin gene. The filter was washed and exposed to X-ray film. (From Park *et al.* *Carcinogenesis* 1991, 12, 1627.)

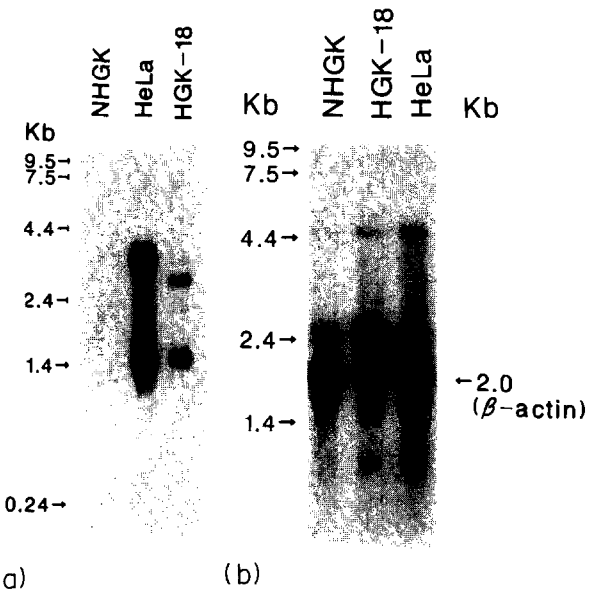

Fig. 4. Northern blot hybridisation analysis of poly(A$^+$)RNAs of NHGK, HGK-18, and HeLa cell lines. (a) Determination of the expression of HPV-18 from HGK-18 and HeLa cell lines. Poly(A$^+$)RNAs extracted from NHGK, HGK-18, and HeLa cells were electrophoresed in 1.2% agarose gel containing 2.2 M formaldehyde, transferred to a nylon filter, and hybridised to ^{32}P-labelled *Eco*RI-*Bam*HI 2.4 kbp fragment of HPV-18 DNA. This 2.4 kpb HPV-18 DNA fragment contains intact HPV-18 E6 and E7 genes which are responsible for cell transformation. The filter was washed and exposed to X-ray film. (b) The hybridised [^{32}P]HPV-18 DNA was stripped from the nylon filter and rehybridised with [^{32}P]-labelled v-*myc* oncogene and ^{32}P-labelled human β-actin gene probes. The filter was washed and exposed to X-ray film.

Though the HPV-transformed cells are immortal, express viral E6/E7 proteins, and contain extremely low amounts of wild-type p53 level, the cells are non-tumorigenic in nude mice. The non-tumorigenicity of HPV-transformed cells suggests that, in addition to HPV infection, other environmental or genetic factors may be involved in the complete transformation of cells. In fact, studies have shown that sequential transfection of HPV-immortalised cells with active c-Ha-*ras* or c-*fos* oncogenes results in the generation of tumorigenic cells. This is perhaps not surprising, recognising that it is now generally accepted that carcinogenesis is a multistep process. Thus immortal human oral keratinocytes harboring HPV DNA can provide a suitable model system for studying the role of other factors such as alcohol or tobacco in carcinogenesis. Because of the ubiquity of HPV infections and the long incubation period between initial infection and development of cancer, it is quite clear that HPV infection by itself is not sufficient for neoplastic conversion of the affected cell. Various environmental factors, including chemical cofactors such as cigarette smoking, may be important for malignant progression of HPV infected cells. In fact, our ongoing studies have shown that chemical carcinogens found in tobacco can further transform the HPV-immortalised cells into tumorigenic cells. Though the detail mechanisms of malignant conversion of these cells are still unknown, overexpression of TGF-α and viral E6/E7 genes were observed from the tumorigenic cells in comparison with the immortalised cells [25]. These data

indicate that further genetic changes are induced by chemical carcinogens, and those changes are necessary for complete transformation of HPV-immortalised cells.

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Acknowledgement—This study is, in part, supported by a grant from the Smokeless Tobacco Research Council., No. 0052 and 0231.