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## Original Paper

# A Novel Method to Culture Laryngeal Human Papillomavirus-positive Epithelial Cells Produces Papilloma-type Cytology on Collagen Rafts

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**A novel method to culture human papillomavirus (HPV) positive laryngeal epithelial cells is described. Biopsies of laryngeal papillomas and of HPV-positive laryngeal mucosa were first cultured as a monolayer in which irradiated laryngeal fibroblasts originally derived from a papilloma (PPLF-XR) patient served as feeder cells. When these fourth or fifth passage epithelial cells were transferred to allow growth on an organotypic growth base (collagen raft containing unirradiated PPLF), they grew as a multilayer. This layer showed features typical of HPV infection with koilocytosis, parakeratosis, and isolated dyskeratotic cells. Based on *in situ* hybridisation, the original tumour sections and epithelial cells from each monolayer passage, as well as the collagen raft sections, contained HPV DNA. Our results show that HPV-infected epithelial cells can be maintained during passages in monolayer culture and that PPLF can support the growth of these cells well. The monolayer cell culture and the collagen raft, the latter providing differentiation-promoting effects, appears to facilitate maintenance of the infected cells and of the viral genome.** © **1998 Elsevier Science Ltd. All rights reserved.**

**Key words: collagen raft, epithelial cells, HPV, laryngeal neoplasms, monolayer cell culture**
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## INTRODUCTION

LARYNGEAL PAPILLOMAS are benign neoplasms caused by human papillomavirus (HPV). Of the approximately 100 known genotypes of HPV, HPV 6 and HPV 11 are, in laryngeal papillomas, the most common. Clinically, the disease is divided into two groups: juvenile-onset and adult-onset forms [1]. HPV DNA is also found in normal laryngeal mucosa by polymerase chain reaction (PCR) in 25% of cases [2]. Cytologically typical findings in papilloma lesions are parakeratosis, koilocytosis, and isolated dyskeratotic cells. Dysplasia of the epithelial architecture is sometimes seen and malignant transformation is possible [3]. The course of the disease varies greatly. Some patients recover after only one laser treatment, but others may have frequent multiple relapses. The reason for this is unknown, and thus far no special curative treatment to prevent recurrences is available.

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Papillomavirus infection, known to be involved in the generation of various forms of benign and malignant human tumours, has been extensively studied [4, 5]. The fact that the virus does not replicate in cell culture has, however, made this work difficult; the differentiation of the epithelium, a critical factor for papillomavirus replication, cannot be fully duplicated in cell culture [6]. Efforts to develop culture models to study HPV replication have involved immortalising primary squamous epithelial cells or culturing them directly from papillomatous lesions. Immortalisation of epithelial cells with HPV 16 and 18 DNA [7–10] results in the integration of the viral genome into host cell DNA. In the case of transfection with HPV 11, which does not integrate, episomal viral replication is detected, but only a portion of the virus reaching the nucleus undergoes replication [11]. A method that allows laryngeal papilloma-derived squamous epithelial cells containing HPV 6 or HPV 11 DNA to be cultured has been introduced by Steinberg and colleagues [12], but the viral DNA is rapidly lost from the cell cultures with passaging,

presumably because the infected cells show a selective disadvantage in plating efficiency compared with that of uninfected cells [13].

Epithelial–mesenchymal interactions and mesenchymal influences are important for the homeostatic equilibrium of epithelial cells [14–16]. Keratinocyte growth factor (KGF), a cytokine produced by dermal fibroblasts and other mesenchymal cells, stimulates the growth of HPV 16 DNA transfected cells, but suppresses HPV 16 early gene expression [17]. Human embryo skin (HES) fibroblasts co-cultured with these cells induce their anchorage-independent growth [18]. To utilise the stimulating effect of mesenchymal tissue, HPV-infected epithelial cells have been cultured in nude mice [19] and on an organotypic growth base [20, 21]. When small pieces of a condyloma, containing HPV 11, were explanted on a collagen raft, HPV production was observed [22].

We now describe a novel method to culture HPV-positive laryngeal epithelial cells without losing them with passaging in monolayer cultures and show how these cells can be made to duplicate the *in vivo* cytology of a papilloma tumour.

## PATIENTS AND METHODS

*Patients and biopsies*

Biopsies from 30 laryngomicroscopies (corresponding to 15 adult-onset and 2 juvenile-onset laryngeal papilloma patients) and of one total laryngectomy (from a patient with an oesophageal carcinoma infiltrating the trachea) were obtained for the present study. All papilloma patients had relapsing disease treated with $CO_2$-laser. The oesophageal carcinoma patient was a heavy smoker and alcohol drinker but had no history of manifest laryngeal disease. The oesophageal carcinoma had infiltrated his trachea adjacent to the larynx. To remove carcinoma tissue with a sufficient margin, total laryngectomy was performed, although his larynx was macroscopically intact. The biopsy for this study was derived from this macroscopically normal-appearing laryngeal mucosa (false vocal fold).

All patients were treated at Helsinki University Hospital. The study was approved by the institutional ethics committee of the Department of Otorhinolaryngology, Helsinki University Hospital, and informed consent was obtained from the study subjects. The biopsies were cultured first in monolayers in which irradiated (XR) fibroblasts (dose of irradiation 6000 rad, radiation source $Ce^{137}$) served as feeder cells. For a mini plate (8.8 $cm^2$; Nunc A/S, Roskilde, Denmark) $2.5\times10^5$ irradiated fibroblasts were seeded.

*Fibroblasts*

In the first attempts to culture HPV-positive laryngeal epithelial cells, irradiated mouse fibroblasts (3T3-J2; kindly provided by Dr Michele De Luca, Genoa, Italy) and HES fibroblasts were used as feeder cells. Because these attempts were unsuccessful, we then used fibroblasts, which were originally derived from a papilloma tumour, as feeder cells and labelled them PPLF (papilloma-patient laryngeal fibroblasts). The patient who supplied these laryngeal fibroblasts was a 24-year-old male with adult-onset disease of a low-risk HPV type. The biopsy from his laryngeal papilloma tumour was passaged initially on 3T3-XR cells, but, as usual, the epithelial cells disappeared after two passages and the fibroblasts took over; in the third passage the culture had a typical fibroblastic appearance.

The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (henceforth referred to as supplemented DMEM). The medium was changed three times a week. Prior to being used as feeders, the fibroblasts were irradiated as described above.

*Monolayer cell cultures*

The biopsies were brought to the laboratory in supplemented DMEM, sectioned into small pieces, and set to grow on irradiated fibroblasts. Irrespective of the type of irradiated fibroblasts used, the supplemented DMEM, with or without epithelial cells, was supplemented with 5 μg/ml insulin (Sigma, St Louis, Missouri, U.S.A.), 5 μg/ml transferrin (Sigma) 400 ng/ml hydrocortisone (Sigma), $10^{-10}$ M cholera enterotoxin (ICN, Aurora, Ohio, U.S.A.), and 10 ng/ml epidermal growth factor (EGF) (Austral Biologicals, San Ramon, California, U.S.A.). This medium and method used to propagate the epithelial cells is modified from that of Rheinwald and Green [23]. The medium was changed three times a week. Before the fibroblasts were seeded, coverslips coated with 1% 3-aminopropyltriethoxysilane (Sigma) were placed on the culture dishes to obtain cells from the monolayers for *in situ* hybridisation. At each passage, coverslips were removed, rinsed in phosphate-buffered saline, pH 7.4, and fixed in cold acetone at −20°C for 10 min. The cultures were maintained in a 5% $CO_2$ incubator at 37°C, and if not used for experiments at once, the cells were frozen in medium containing 10% dimethyl sulphoxide (Merck, Darmstadt, Germany) at −70°C. On average, the cells reached the fourth passage on the 28th day of culture.

*Collagen rafts*

The procedure described previously by Zheng and colleagues [10] was used. Twelve-well plates (4 $cm^2$; Costar, Cambridge, U.K.) were precoated with 1.6% sterile agarose in 2×MEM containing glutamine and antibiotics to prevent the fibroblasts from adhering to the plates. Fibroblasts were suspended in 2×MEM containing 20% FCS, glutamine and antibiotics and mixed with type I collagen (rat tail; UBI, Lake Placid, New York, U.S.A.). For each raft, $4\times10^5$ PPLF fibroblasts were used; the final collagen concentration was 0.7 mg/ml. The total volume of the mixture of cells and collagen was 2.5 ml per well. The plates were then cultured in a 5% $CO_2$ incubator at 37°C for 1 day. The shrunken collagen gels were then transferred to a 24-well plate (1.75 $cm^2$; Greiner, Frickenhausen, Germany) into which the fourth or fifth passage HPV-positive epithelial cells ($2\times10^5$ cells from 90% confluent culture dishes) were seeded (Table 1). The next day, the collagen rafts were transferred on to metal grids and cultured at the air–medium interface for 2 weeks. The medium (DMEM with 10% FCS, antibiotics, glutamine, insulin, hydrocortisone, transferrin, cholera toxin and EGF) was changed three times a week. The rafts were then fixed in formalin, embedded in paraffin and sectioned for detection of HPV DNA. Sections were stained with haematoxylin and eosin (H&E) for histological evaluation.

In situ *hybridisation*

Both the tissue biopsies from the larynx and the collagen rafts were embedded in paraffin and sectioned. From these sections, as well as acetone-fixed cells from monolayers,

*Table* 1. *Origin of epithelial cells grown on collagen rafts. Carcinoma patient's biopsy from normal appearing laryngeal mucosa*

| Patient No./sex/age (years) | Passage of cells | Type of tumour | HPV | |
|---|---|---|---|---|
| | | | In tumour | In collagen raft |
| 1/F/5 | 5 | Juvenile-onset papilloma | Low risk* | + |
| 2/M/33 | 5 | Adult-onset papilloma | Low risk | + |
| 3/M/25 | 4 | Adult-onset papilloma | Low risk | + |
| 4/M/48 | 4 | Oesophageal carcinoma | N.D. | + |

*Low risk corresponds to HPV types 6, 11, 42, 43 or 44 in chemiluminescent molecular hybridisation assay. HPV, human papillomavirus; N.D., not determined; +, positive result in HPV *in situ* hybridisation screening test.

HPV DNA was tested by *in situ* hybridisation (HPV *in situ* Screening Test®; Biohit, Helsinki, Finland). The details of this method have been described previously by Syrjänen [24]. Five micrometre sections were deparaffinised, digested with proteinase and hybridised with a cocktail of probes (corresponding to 32 HPV types) at 37°C overnight. The biotinylated hybrids were detected by alkaline phosphatase conjugated to streptavidin, with 5-bromo-4-chloro-3-indoyl-phosphate as the substrate and nitroblue-tetrazolium as the chromogen. The incubation in substrate solution was 3 h for paraffin-embedded sections and 30 min for cell samples. Fuchsin was used as a counterstain. Fixed human uterine cervical carcinoma CaSki cells (ATCC CRL-1550; American Type Culture Collection, Rockville, Maryland, U.S.A.), which contain HPV 16 DNA, were used as positive controls when biotinylated HPV DNA probes were added and as negative controls when biotinylated pBR 322 control probes were used for hybridisation. Moreover, our own additional controls included paraffin-embedded biopsies of ovarian tube (negative controls), uterine cervix with condyloma and dysplasia, and a laryngeal papilloma known to be of low-risk HPV type (positive controls). Adjacent sections were hybridised to a negative control probe to rule out non-specific staining.

*Chemiluminescent molecular hybridisation assay*

The detection of HPV DNA in PPLF cell cultures and in original tissue biopsies was performed by means of the Hybrid Capture™ System (Digene Diagnostics, Beltsville, Maryland, U.S.A.). It is a sandwich capture molecular hybridisation assay that utilises chemiluminescent detection [25]. Biopsies of 2–5 mm in diameter or cell suspensions containing 1–2 million cells from a culture dish served as samples. The reported sensitivity of the test is 1 copy/cell when 1–2 million cells are assayed.

## RESULTS

*Growth of HPV-positive laryngeal epithelial cells in monolayer co-cultures with mouse 3T3-J2 fibroblasts, HES fibroblasts and PPLF*

Our initial attempts to grow epithelial cells from laryngeal papillomas on 3T3-J2-XR and HES-XR fibroblasts were unsuccessful. After irradiation, 3T3-J2-XR cells detached easily from the culture dish and died, and poorly supported the growth of HPV-positive epithelial cells. HES-XR fibroblasts were used in a few experiments and although they were more hardy, laryngeal epithelial cells did not grow well on them. The epithelial cells could be cultured successfully on 3T3-J2-XR or HES-XR feeder cells, but usually for one to two passages only. After that, the fibroblasts, which always accompany epithelial cells in the biopsies, overwhelmed the cultures.

We then decided to use fibroblasts (PPLF), which were originally derived from a papilloma tumour, as feeder cells. Staining of the cells for cytokeratin was negative, which indicated that no cells of epithelial origin remained. A chemiluminescent molecular hybridisation assay showed no HPV DNA in a cell sample derived from the culture. These PPLF

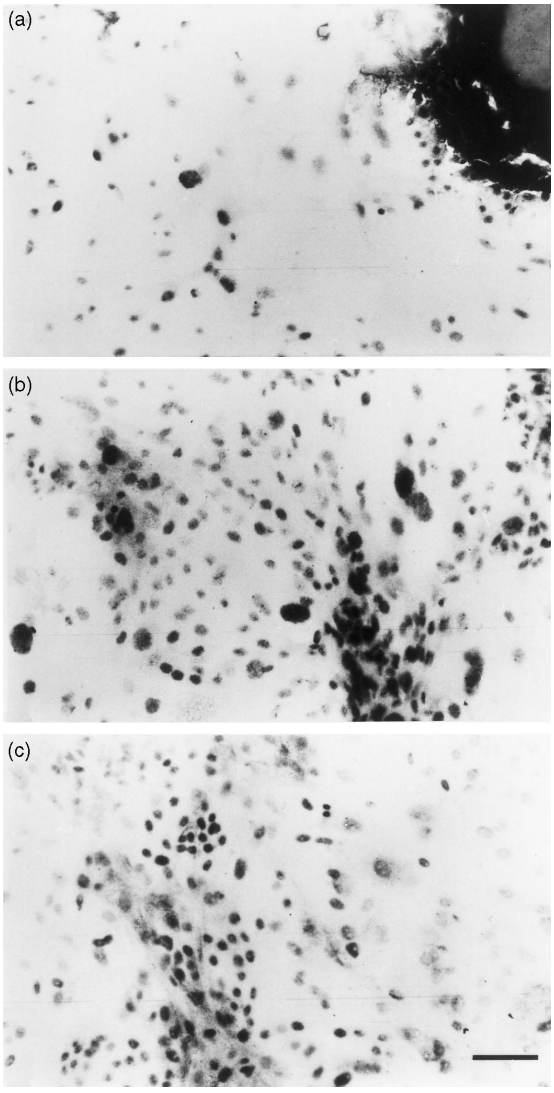


**Figure 1. *In situ* hybridisation of laryngeal epithelial cells (from patient no. 1, see Table 1) grown on coverslips during different passages in monolayer. A positive reaction is seen as black stain in the nuclei of squamous epithelial cells. (a) First passage cells, part of original tumour explant in right corner of picture, (b) third passage, (c) fifth passage. Fuchsin-counterstained negative nuclei are seen as grey. Scale bar, 100 μm.**

cells have been cultured for 27 passages and the HPV DNA assay was performed on the 3rd, 13th and 18th passages with negative results. Of the 30 laryngeal papilloma tumour biopsies, 20 biopsies were not co-cultured with PPLF-XR cells, with 19 lost during early passages. They did not grow on either 3T3-XR or HES-XR cells and a few cultures were lost because of fungal infection. We tried to culture single biopsies without feeders or in soft agarose. Moreover, some specimens were lost when inhibition of the growth of accompanying fibroblasts on calcium-free growth medium was attempted without success. Only once were HPV-positive laryngeal papilloma cells able to grow up to the fourth passage on 3T3-XR cells. We then decided to culture all our biopsies on PPLF-XR cells.

Of the 30 biopsies of laryngeal papilloma tumours, 10 were cultured on PPLF-XR cells and five of them at least until the fourth passage. The biopsy derived from a carcinoma patient was also cultured on PPLF-XR cells, and grew successfully until the fourth passage. Of these monolayer cultures, *in situ* hybridisation was performed on those four which were later transferred to grow on a collagen raft. These cultures showed persistence of HPV-positive epithelial cells during passages (Figure 1).

*Collagen raft cultures*

Of the HPV-positive laryngeal epithelial cells which were first cultured in a monolayer up to the fourth or fifth passage, cells from 4 patients (Table 1) were transferred to grow on an organotypic growth base. These cells maintained their viral genome: *in situ* hybridisation showed a black stain in the nuclei of squamous epithelial cells (Figures 2 and 3). They produced a cytology typical of a laryngeal papilloma tumour.

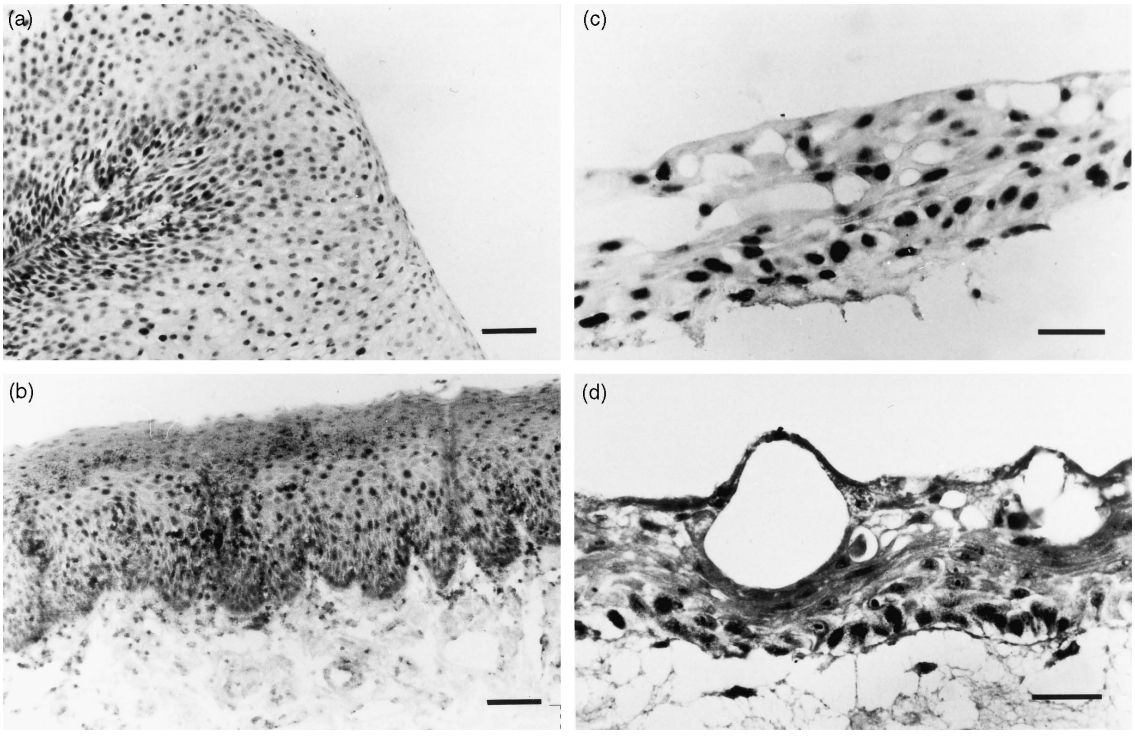


**Figure 2. *In situ* hybridisation of original laryngeal specimens: (a) papilloma tumour, (b) macroscopically normal-appearing mucosa of oesophageal carcinoma patient and of corresponding collagen rafts (c, d). (a) and (c) are from patient no. 3, (b) and (d) are from patient no. 4, see Table 1. A positive reaction is seen as black stain in the nuclei of squamous epithelial cells. Scale bar in each picture, 100 μm.**

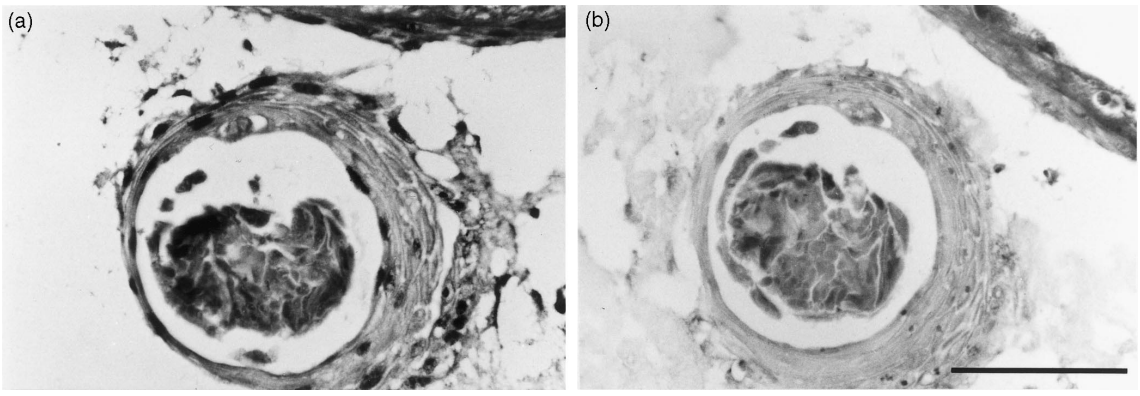


**Figure 3. Adjacent sections hybridisation with HPV DNA probes (a) and pBR 322 probes (b). No black stain is seen in the nuclei of the squamous epithelial cells (b). Sections are derived from rafts shown in Figures 2(d) and 4(d). Scale bar, 100 μm.**

In H&E sections, these collagen rafts showed koilocytes, parakeratosis and dyskeratotic cells (Figure 4).

## DISCUSSION

A major problem in studies on laryngeal papillomavirus infection has been the lack of a good experimental model of the disease. All attempts to culture HPV-positive laryngeal epithelial cells continuously in monolayer have failed, having resulted in the disappearance of HPV genome within three to four passages [13]. The effect of retinoid acid on laryngeal papilloma cells has been tested on a collagen raft consisting of NIH 3T3 cells [26]. However, the epithelial cells for that experiment derived from papilloma tumours were cultured in monolayer only for one passage (2 weeks). Because laryngeal papilloma tumours are often small and the disease is rare, with an incidence of only 3.8/million/year [27], it would be of benefit to expand the cells in monolayer and if needed, store them frozen until use in experiments. Our method to culture laryngeal papilloma cells makes this possible. However, to culture these cells is not easy. Cellular density should be optimal, i.e. small biopsies should be seeded on small dishes. From our experience, the cells should be subcultured even when the culture is not more than 20–30% confluent in the first passages. One critical factor for successful culturing of cells is their adherence to the culture substratum. A difference in the organisation of cytoskeletal proteins is a possible explanation for the difficulties in attachment [28] and even with this method not all epithelial cells adhered to the culture dish.

Feeder cells are important for epithelial growth in a monolayer. When HPV-positive laryngeal epithelial cells are considered, our data show that human fibroblasts perform the feeder function better than mouse fibroblasts. Our experience in the use of HES cells as feeders is limited to a few unsuccessful attempts. It seems possible that the laryngeal origin of the feeder cells is an advantage, possibly providing some as-yet-undefined tissue-specific effects, but whether it is significant that the feeder cells are derived from a papilloma patient remains unknown.

The role of HPV in carcinogenesis of the larynx is not as clear as is its role in anogenital cancers [4]. The most frequently detected HPV types in the larynx (types 6 and 11) are in gynaecological lesions associated with benign tumours. However, in squamous cell carcinomas and pre-existing laryngeal papillomas, HPV 11 DNA has been present in high copy numbers [29]. Malignant progression of HPV 6-associated laryngeal papilloma has also been reported [30]. Moreover, both HPV 16 and 18 DNA are found in laryngeal carcinomas [31, 32]. Human papillomavirus infection alone is not oncogenic [33]. Of all laryngeal carcinoma patients, smokers comprise 95% [34]. Because conversion of papilloma to carcinoma is often associated with smoking or laryngeal irradiation, it is possible that in these malignancies viral infection is less a primary carcinogenic agent than a cofactor. HPV has also been detected in oesophageal carcinoma [35]. Our patient's oesophageal carcinoma infiltrated the upper part of the trachea and both the carcinoma tissue

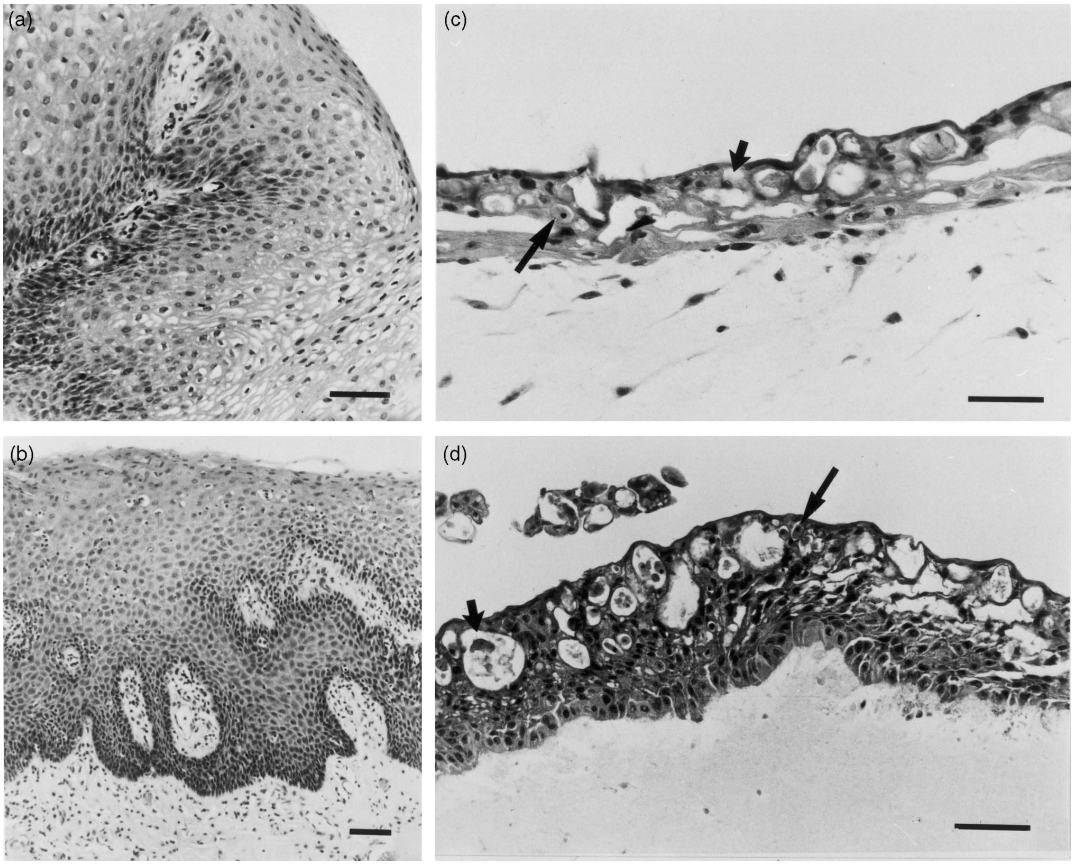


**Figure 4. Haematoxylin and eosin staining of sections of original laryngeal specimens: (a) papilloma tumour, (b) macroscopically normal-appearing mucosa of oesophageal carcinoma patient and of corresponding collagen rafts (c, d). (a) and (c) are from patient no. 3, (b) and (d) are from patient no. 4, see Table 1. Parakeratosis on the surface layer, koilocytes (small arrow) and occasional dyskeratotic cells (large arrow) were reproduced on collagen rafts. Scale bar in each picture, 100 μm.**

as well as the macroscopically normal-appearing laryngeal mucosa were HPV positive. In addition, although the mucosa was macroscopically normal, histology showed typical features of HPV infection. It is possible that the patient's immunological defence prevented manifestation of a papilloma tumour. This patient was a heavy alcohol drinker and smoker, so HPV was perhaps a cofactor in this case as well. To investigate the significance of HPV infection in these malignancies, HPV-positive epithelial cell culture on a collagen raft could prove a useful tool.

Mesenchymal influences are crucial for the differentiation of epithelial cells, and the differentiation of these cells is essential for HPV replication. When HPV-positive epithelial cells derived from laryngeal papilloma or from the normal-appearing mucosa of a carcinoma patient were first cultured in monolayer and then later transferred to grow on a collagen raft, they exhibited cytology typical of papilloma lesions. These morphological changes were even stronger than those seen in our laboratory when epithelial cells from an HPV 16-positive cell line were cultured on collagen rafts [18]. Perhaps the cytology of these rafts appeared more normal because of the fact that the HPV 16-immortalised cervical epithelial cells do not produce viral particles. The sections of the original tumours and of the collagen rafts, as well as the majority of the epithelial cells grown on coverslips, were HPV positive according to *in situ* hybridisation. Thus, it appears that it is possible to maintain HPV DNA in epithelial cells in a monolayer cell culture. We propose that this new method to use PPLF fibroblasts as feeder cells in a monolayer for HPV-positive laryngeal epithelial cells and to transfer the cells to a collagen raft after the fourth or fifth passage is a useful experimental model in investigating laryngeal HPV infection.


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