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Properties of Two Epithelial Cell Lines Derived From HPV-associated Cervical and Vulvar Lesions

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Two epithelial cell lines were established from human papilloma virus (HPV) 18 or 16 associated tumours, characterised as poorly and well differentiated squamous cell carcinomas of the cervix uteri (EC) and the vulva (GC), respectively. The cell lines are described by their morphology, biological parameters, and immunological markers. Both cell lines have undergone approximately 35 passages *in vitro*. HPV16 and 18 DNA are maintained integrated into the host cell DNA. Expression of epithelial cell markers—cytokeratins K1, K10, K13, K14 and involucrin, proliferation-specific proteins, proliferating cell nuclear antigen (PCNA) and Ki67 as well as the epidermal growth factor (EGF) receptor were monitored by indirect immunofluorescence studies. The cytoplasmic and membrane-associated locations of EGF receptor molecules in EC and GC cells, respectively, suggest a differently regulated expression. Studies of the HPV18 oncogene transcription revealed marked differences of amplimers between HeLa and EC cells, such as an additional fragment, probably corresponding to a E6 ^ E7 splice product, and a radical shift in transcription pattern observed in various sections of the tumour tissue. Injected subcutaneously into nu/nu mice both cell lines were non-tumorigenic.**
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INTRODUCTION

HUMAN PAPILLOMA VIRUSES (HPV) are known as strictly epitheliotropic viruses. More than 65 different types have been described infecting mucosal and cutaneous epithelia. Among them HPV 16 and 18 are the most prominent because of their strong association with human anogenital neoplasia [1]. Both types are found in a high percentage of malignant cervical, vulvar and anal lesions. Whereas in intraepithelial lesions the viral DNA predominantly persists in an extrachromosomal form, in invasive neoplasias either episomal and integrated forms of viral DNA coexist or, more often, this solely integrates into the host genome [2]. A number of cell lines containing HPV 16 or 18 have been established from tumour biopsy specimens and proven to be valuable tools to analyse HPV gene expression. Recently, several groups have reported the isolation of cell lines from biopsy samples which contain HPV 16 or 31b DNA in an episomal form [3, 4]. Most of the cell lines decribed so far contain only integrated copies of viral DNA [5, 6] and perhaps are representative of the malignant state of the lesions.

As shown in a number of studies, epithelial cell growth in HPV 16 and 18 associated cultures is tightly linked to the expression of viral oncogenes E6 and E7[1, 7, 8]. Viral and virus cell fusion transcripts have been found in intraepithelial neoplasia and carcinoma-derived cell lines. Transcripts contained coding capacity for the E6 and E7 open reading frames (ORF) and an E6* ORF, generated by internal splicing within E6 [9]. Expression of E6/E7 genes of the "high risk" HPV types was shown to be necessary and sufficient for *in vitro* immortalisation of primary human epithelial cells of genital and non-genital origin and some rodent fibroblasts [11, 12]. Available data emphasise an important role of E6/E7 proteins in maintenance of cell proliferation and tumour progression [14, 15]. However, viral oncogenes E6/E7 are not sufficient for progression from an immortal to the malignant type of growth [1]. Initially, immortal cell lines, in contrast to most cancer-derived lines, are non-tumorigenic in nu/nu mice and need additional events, either spontaneous (long term cultivation; [16]) or experimental (*ras*-oncogene transfection; [15]) to convert to malignancy.

Data obtained with permanent cell lines are believed to characterise the malignant stage of progression, unless the cell lines have already been kept *in vitro* over a long period of time and, therefore, certainly have undergone a number of changes during establishment.

To further elucidate basic principles of HPV-associated tumorigenesis, comparative studies seem to be indicated, including analysis of viral DNA and RNA transcription in tumour biopsy specimens, cell lines isolated from them and different experimental models, such as raft cultures and grafting in nude mice.

In this context, we describe the establishment of two cell lines from a cervical and a vulvar carcinoma and their initial characterisation.

MATERIALS AND METHODS

Cell culture

Small biopsy specimens were obtained from surgically resected tumours and transported from the operating theatre in MCDB 153 (Sigma) containing penicillin and streptomycin. The

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samples were washed in phosphate buffered saline (PBS) and finely minced with scissors. A single-cell suspension was produced by warm trypsinisation (0.1% trypsin/0.1% versene, 30 min at 37°C). The resulting cells initially were plated together with lethally irradiated Swiss 3T3 cells (clone J2) in 25 cm^2 flasks (Falcon). Feeder cells before irradiation were grown in Dulbecco's modified Eagles medium (DMEM) with 10% fetal calf serum (FCS) (Gibco/BRL). Epithelial cell cultures were maintained alternatively in keratinocyte-serum-free medium (SFM) (Gibco/BRL) or KGM (Clonetics). Cultures were maintained in a humidified incubator at 37°C containing 5% CO_2. After approximately 10 passages cells grew in serum-free media without feeder cells.

DNA extraction and Southern blot analysis

Cells were washed in monolayer with ice-cold PBS and lysed in 10 mmol/l Tris–HCl, pH 7.5, 10 mmol/l EDTA, 0.5% sodium dodecylsulphate (SDS). 100 μg/ml proteinase K were added and the suspension was incubated at 37°C. After phenol, phenol: chloroform and chloroform extractions (each twice) DNA was precipitated with 2 volumes cold ethanol. RNA was removed by RNase treatment (20 g/ml, 30 min at 37°C). DNA was resuspended in TE buffer (10 mmol/l Tris–HCl/1 mmol/l EDTA) and stored at 4°C. DNA was digested overnight with restriction enzymes according to the manufacturer's instructions (Boehringer Mannheim). DNA, electrophoresed in a 0.8% agarose gel, was transferred to nylon filters (Hybond, Amersham) using Stratagene's PosiBlotter. Hybridisation was performed in 50% formamide, 5 × SSPE, 5 × Denhardt's solution [10], 1% SDS and 100 g/ml denatured herring sperm DNA at 42°C for 24–48 h in a hybridisation oven (Biometra). A radioactively labelled HPV probe was prepared by the random primer technique [10] with a [^{32}P]dATP (222 mBq/mmol, Amersham). After hybridisation membranes were washed, air-dried and exposed to Kodak XOMAT films with DuPont intensifying screens.

Reverse transcription polymerase chain reaction (RT-PCR)

10 μg of GuHCl/CsCl purified total RNA were reverse transcribed using random primer and 200 units of cloned MuLV-RT (Gibco/BRL) at 42°C as described previously [13].

One tenth of the RT-PCR product was amplified in a thermocycler (Biomed 60) with Amplitaq™ at standard conditions (Perkin Elmer Cetus). The following sets of nested primers were utilised to amplify HPV16 E6/E7-specific cDNAs from SiHa cells and tumour biopsy specimens: 5′-CTGCAATGTTTCAGGACCCAC-3′ (nt. 99–119), 5′-ACAGGAGAGCGACCCAGAAAGTTACCACA (nt. 118–144) and 5′-CCCTCTTCCCCATTGGTACC (nt. 899–880). Primers specific for HPV18 E6/E7-cDNAs were 5′-ACTATGGCGCGCTTTGAGGATCCAACACGG (nt. 102–131), 5′-ACCCTACAAGCTACCTGATCTGTGCAC (nt. 134–160) and 5′-TGCTTACTGCTGGGATGCAC (nt. 910–891). After primer annealing at 56°C the PCR reaction was carried out for two times 30 cycles. Amplimers were analysed by agarose gel electrophoresis (1.6%, wt/vol) and Southern blot hybridisation using HPV16 and HPV18 E6/E7-specific radioactive probes labelled by random priming with a [^{32}P]dATP (222 mBq/mmol, Amersham).

Indirect immunofluorescence

Cells, grown in 60 mm Falcon Petri dishes, were washed with ice-cold PBS and fixed with 100% methanol for 10 min at −20°C [13]. After rehydration cells were incubated with primary antibodies in PBS containing 0.5% NP40 at room temperature for 60 min. The primary antihuman antibodies were rabbit pan anti-cytokeratin, mouse anti-CK1, anti-Ki67 (all from Dakopatts) and anti-CK13 (Sigma). Antibodies directed against proliferating cell nuclear antigen (PCNA) and epidermal growth factor (EGF) receptor were obtained from Dianova and Oncogene Science, respectively. Anti-involucrin antibodies, anti-CK10 (LH2) and antibodies against CK14 (LH8) were kindly provided by Dr Fionna Watt (ICRF, London) and Dr Irene Leigh (The London Hospital), respectively. Secondary fluorescein isothiocyanate-conjugated antimouse and antirabbit antibodies were purchased from Sigma and diluted according to the manufacturer's instructions. Cells were embedded in Citifluor AF1, and fluorescence photographs were obtained with a Zeiss Aksioskop microscope using Kodak TMAX 3200 black and white films.

Collagen raft cultures

Collagen gels containing Swiss 3T3 feeder cells at 3 × 10^5/ml were prepared in 24- or 6-well microtitre plates. Gels were kept overnight in DMEM containing 10% fetal bovine serum (FBS), 0.5 μg/ml hydrocortisone, 10 μg/ml EGF and 10^{-10} mol/l cholera toxin. 2–5 × 10^5 epithelial cells were seeded on to the gels and grown to confluency. Approximately 24 h later collagen plugs were lifted on to stainless steel grids, and epithelial cells were allowed to stratify at the air–liquid interface for 9–12 days [17]. Rafts were then fixed in 4% paraformaldehyde, embedded in paraffin, and 5 μm sections were cut and stained with haematoxylin/eosin to view growth characteristics.

Tumorigenicity assays

Cells (5 × 10^6–10^7) were injected subcutaneously in the flank of athymic mice (nu/nu) and allowed to grow for up to 10 weeks. At various time intervals animals were killed for inspection. The resulting cell formations were fixed in 4% paraformaldehyde, paraffin-embedded, cut into 5 μm sections and stained with haematoxylin–eosin for microscopical examination.

RESULTS

Cell lines were isolated from two tumours characterised pathologically as poorly and well differentiated squamous cell carcinoma of the cervix uteri and the vulva, respectively (Fig. 1a–d). Cell lines were designated EC and GC, and were found to contain HPV DNA of type 18 or 16 (Fig. 2). Although peripheral parts of the biopsies (Fig. 1a/c) morphologically resembled apparently normal squamous epithelium, viral DNA sequences were detectable and, as shown in Fig. 3, actively expressed. Both cell lines showed a typical epithelial cell morphology with numerous mitotic figures, giant bi- and multinucleated cells (Fig. 4). A number of cells had a high nuclear-to-cytoplasmic ratio and multiple nucleoli. The main phenotypic characteristics of both cell lines were seen to be independent of the passage number and whether they were cultivated in the presence of fibroblast feeders or not (Fig. 4). Whereas in the early passages, cell growth was strictly feeder-dependent after 8–10 passages *in vitro*, both cell lines became able to grow in serum-free keratinocyte media without feeder support. Cell lines EC and GC, however, appeared morphologically distinct from each other. EC cells generally appeared smaller. Phenotypically the GC culture appeared heterogeneous in itself. Attempts were made to subclone the culture to obtain homogeneous subpopulations. The analysis of those is currently underway. We mentioned that the phenotype of both cell lines was highly dependent on the culture media used. The growth properties

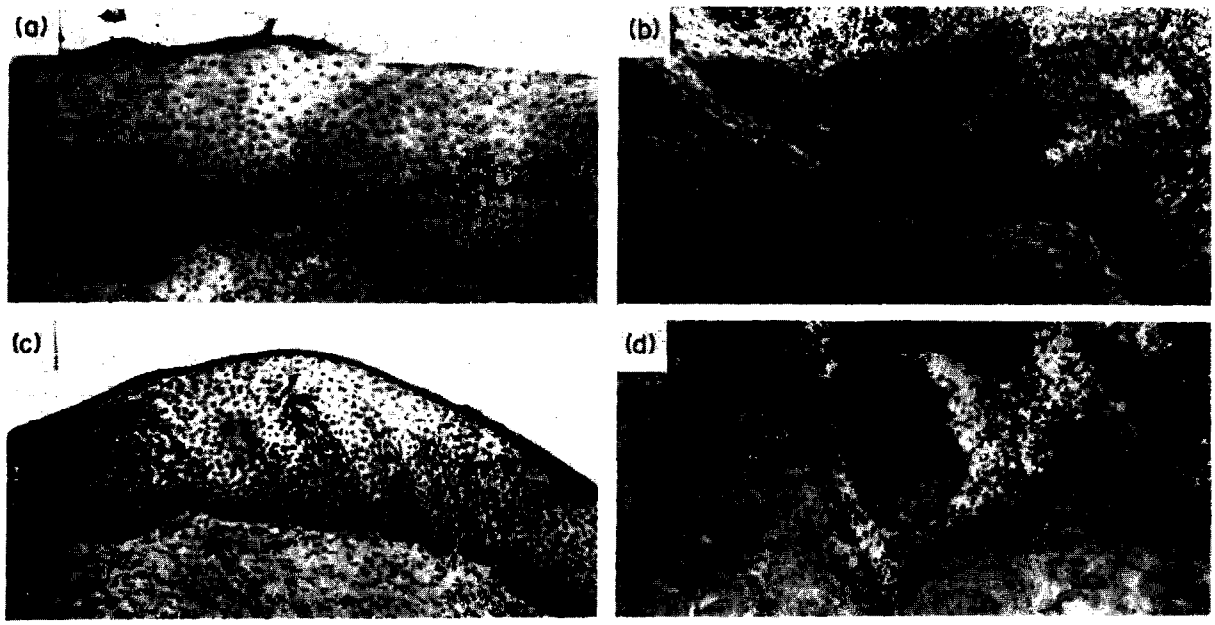

Fig. 1. Haematoxylin–eosin (HE) stains of paraffin sections showing the histopathology of different biopsies from squamous cell carcinomas of the cervix uteri (EC a/b) and the vulva (GC c/d) (×20). Panels a/c and b/d represent sections from peripheral and central parts of the tumour tissues obtained.

and cell morphology changed when we cultivated cell lines in KGM (Clonetics) or Keratinocyte SFM (Gibco/BRL) (Fig. 4a, b). Whether this will be reflected in the expression of certain viral or cellular proteins is as yet unknown. A major difference in growth properties is illustrated in Fig. 5. The population doubling time was determined from triplicate dishes of exponentially growing cells. 2.5×10^4 cells were seeded per dish and counted after the time intervals indicated. EC cells were rapidly dividing cells with a doubling time of approximately 20–22 h (Fig. 5a). In contrast, the growth rate of GC is slow, especially at low seeding densities. The generation time was estimated from the growth curve to be 68–72 h (Fig. 5b). These cells showed a higher tendency to differentiate in culture and formed at a higher percentage giant cells with bi- or multinucleated cells. The differences in growth rates might reflect various degrees of transformation.

To characterise the cell lines further, EC and GC were analysed by indirect immunofluorescence. Antibodies were chosen which identify epithelial cells on the one hand and distinguish proliferating and non-dividing cells on the other.

Unless there was some quantitative variation, there did not seem to be any obvious visual differences between EC and GC in

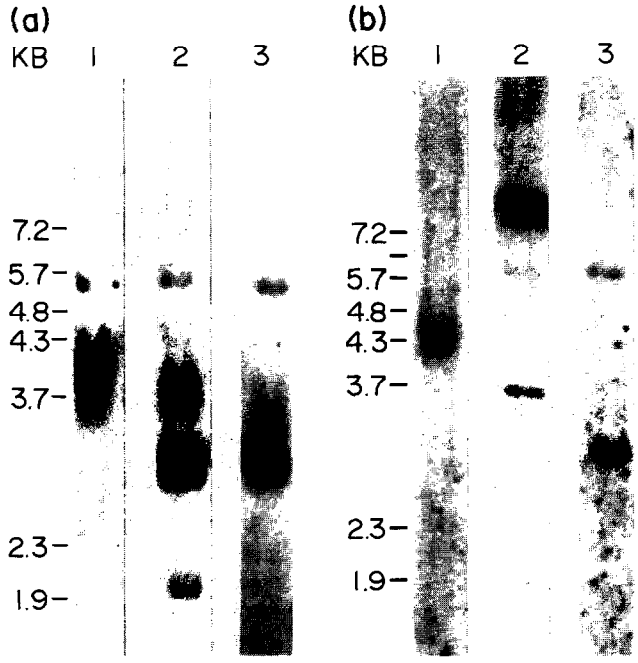

Fig. 2. Southern blot of DNA, isolated from EC (a) or GC (b) cells. DNAs were digested with *Hin*dIII (a1, b1), *Bam*HI (b2), *Eco*RI (a2), and *Pst*I (a3, b3). Blots were hybridised with a ^{32}P-labelled HPV18- (a) or HPV16-DNA (b). Endlabelled k-*Bst*EII-fragments were run as length standard.

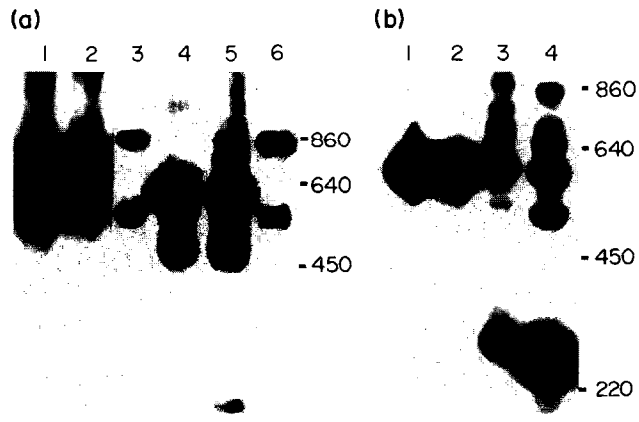

Fig. 3. Southern blot hybridisation of RT-PCR products from HPV18- (a 1–6) and HPV16-positive (b 1–4) tissue samples and cell lines with the appropriate HPV-type, labelled by random priming. Lanes represent: a1–HeLa, A2-361 cells, a3-biopsy EC1, a4-EC2, a5-EC cells, passage 15, a6-EC cells passage 22; b1-biopsy GC1, b2-biopsy GC2, b3-GC cells, passage 5, b4-GC cells, passage 16. Biopsies EC1/2 and GC1/2 correspond with HE stains a/b and c/d in Fig. 1, respectively.

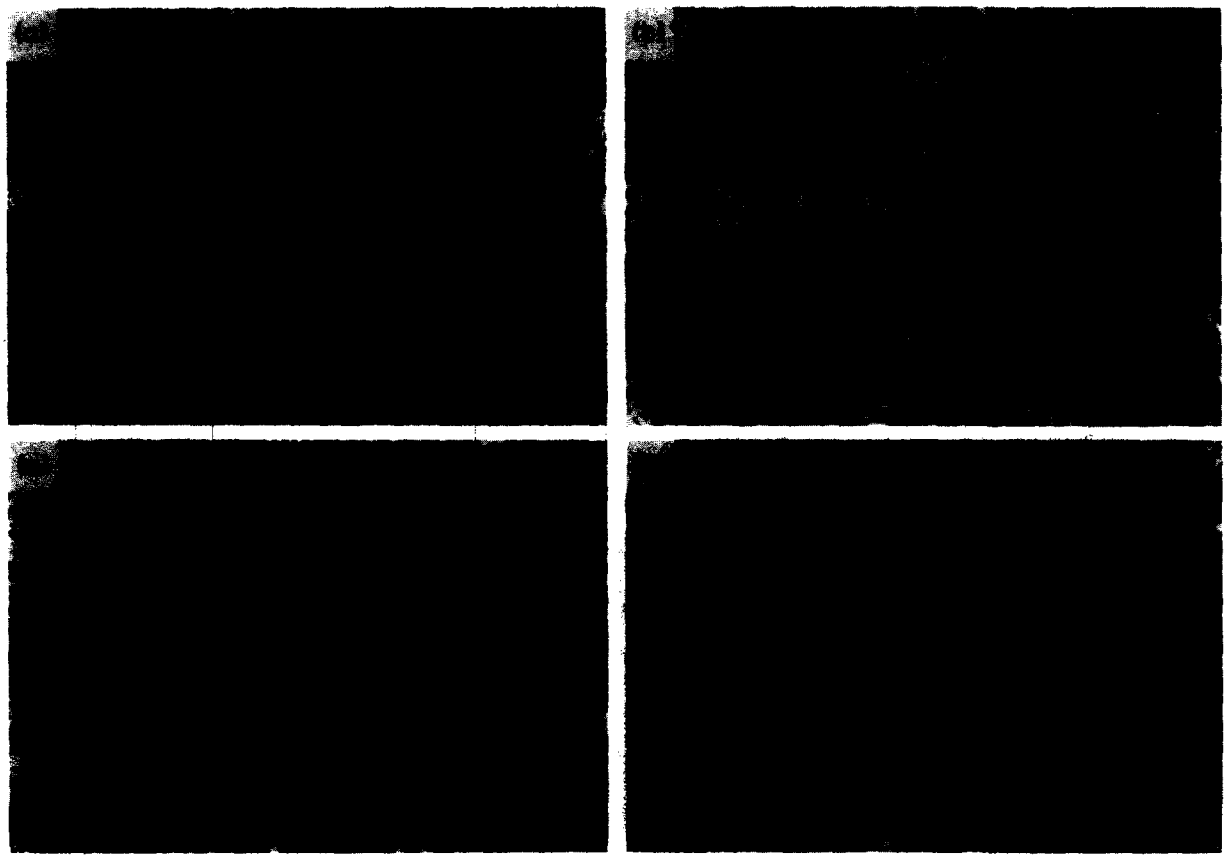

Fig. 4. Cultured human vulvar (GC a,b) and cervical (EC c,d) epithelial cells. GC cells were grown with feeder support (a) upon passage 10 and then maintained in serum-free medium (b). EC cells grown in Gibco-SFM (c) or Clonetics-KGM (d) at passage 22. [×20, ×40 (b)].

the expression of epithelial cell markers such as cytokeratins 1, 10, 13 and 14 (Figs 6 and 7 CK1-f, CK10-b, CK13-g and CK14-c, respectively). Involucrin, usually found in cells that have initiated terminal differentiation, was detected at low levels and only occasionally was raised in single differentiating cells (Fig. 6, 7h). To obtain information concerning the proliferative properties, monoclonal antibodies (Mab) to nuclear antigens were applied. The PCNA/cyclin, an auxiliary protein of DNA polymerase δ, was present in all cells as a strong nuclear fluorescence (Figs 6, 7d). The proliferating cell antigen Ki67 was mostly found as dot-like fluorescence in dividing but not in resting cells (Figs 6, 7i). Interestingly, there was an obvious difference in EGF receptor expression. Although the protein was present and easily detectable in both cell lines, there was considerable diversity in its location (Figs 6, 7e). HPV18-associated EC cells revealed a granular cytoplasmic fluorescence. In contrast, the EGF receptor was found exclusively in association with cell membranes of GC cells (Fig. 7e).

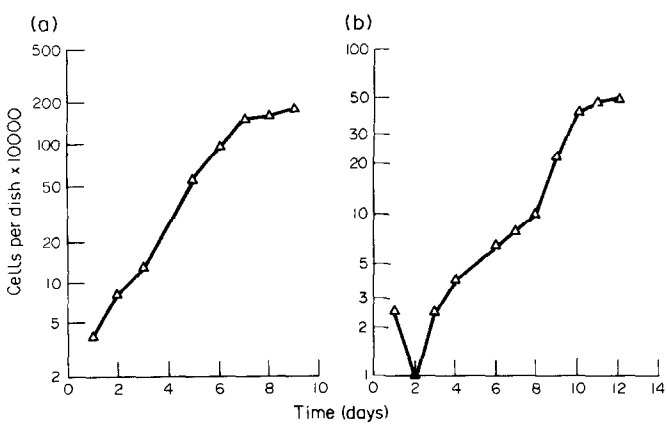

Fig. 5. Growth curves of cell lines EC (a) and GC (b). 2.5 × 10^4 cells were seeded per 35 mm dish, cells were counted after trypsinisation at times indicated.

Previous reports demonstrated that epithelial cells are able to stratify and differentiate *in vitro* when cultured on top of a collagen plug at a liquid–air interface [17]. Using this strategy the cell lines EC and GC were first grown to confluency on the feeder-containing collagen gel submerged in DMEM supplemented with 10% FCS, hydrocortisone, EGF and cholera toxin. The confluent cultures were allowed to stratify on stainless steel grids for 9–12 days. Haematoxylin–eosin (HE) stains of 5 μm cross sections show the histopathological features of the GC raft—mainly dedifferentiated epithelial cells, polymorphic spinal cells throughout cell layers with hornpearles and a tendency to form keratinised structures (Fig. 8a). In EC raft cultures keratinization was absent (Fig. 8c). Comparing the HE stains with those of the tumour tissues where the cell lines were derived from (Fig. 1), it is noteworthy that, at least concerning keratinization pattern, EC and GC rafts were similar to the original tissue. However, GC initiated from a peripheral part of the tumour biopsy sample, in culture showed a clear tendency to a more profound dedifferentiation (Fig. 8c). A detailed comparative analysis of viral gene expression in the tumour and raft culture will be published later.

The presence of HPV-DNA was first detected by PCR. The electrophoretic separation of amplimers revealed fragments characteristic of HPV18 in the case of EC and HPV16 in the case of GC, respectively (data not shown). The viral integration pattern became evident from Southern blot and hybridisation studies. EC cells contain one to two copies of incomplete HPV18 DNA integrated into the host genome (Fig. 4), and GC was shown to be HPV16-positive. The viral DNA in GC cells was integrated at low copy number. These integration patterns were obtained at passages 17 and 22 for EC and GC, respectively. It is a subject of future work to study the viral DNA starting from earlier passages.

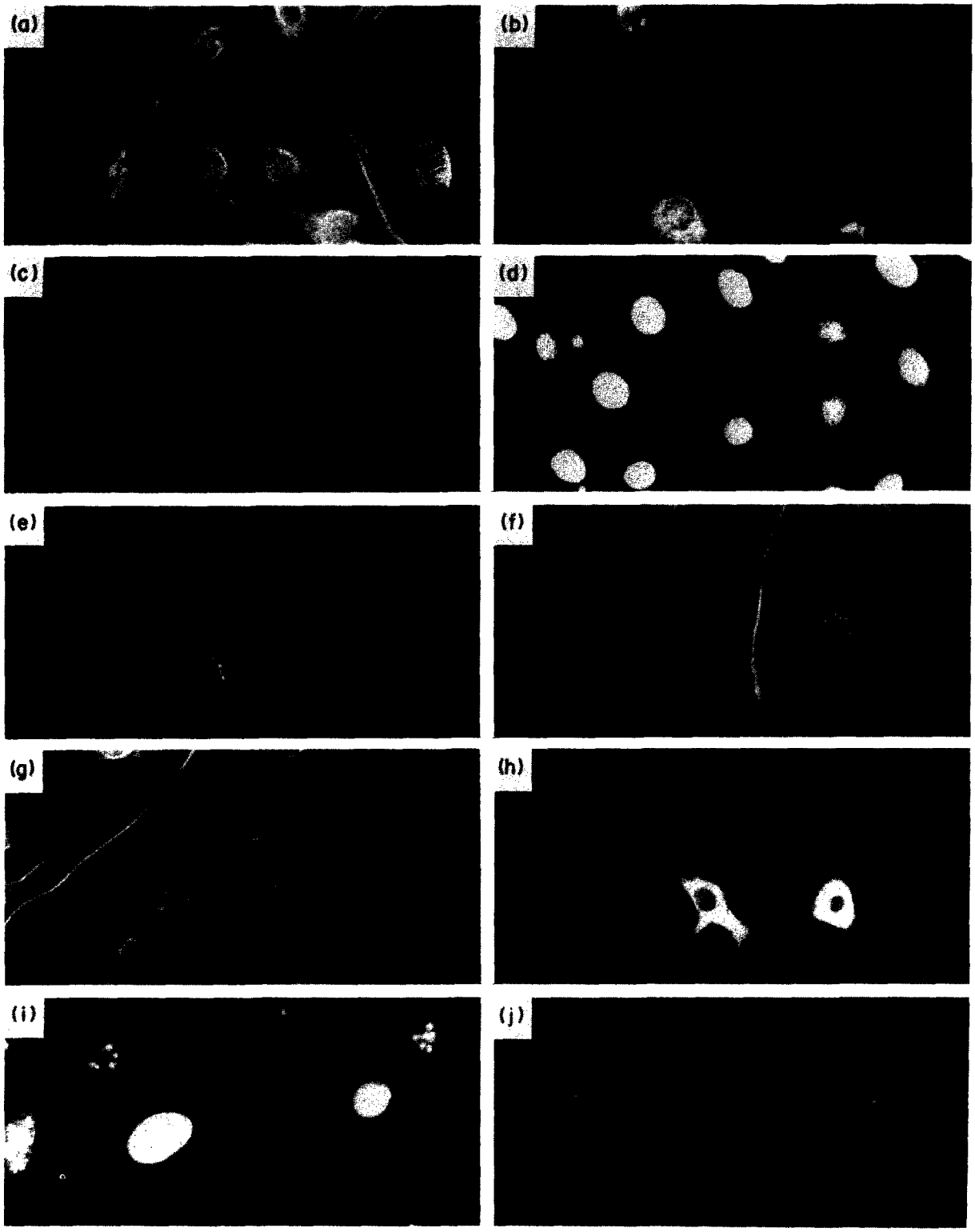

Fig. 6. Indirect immunofluorescence of marker proteins in EC (5) cells. Primary antibodies were directed against pan cytokeratin (a), keratins 10 (b) and 14 (c), PCNA (d), EGF receptor (e), keratins 1 (f) and 13 (g), involucrin (h) and Ki67 (i). Background staining of FITC-conjugated antibodies is shown in (j) [×40 except 6c (×100)].

There are several lines of evidence that the expression of viral oncogenes E6 and E7 of HPV16 and 18 is the main prerequisite for initiation and malignant conversion of lesions associated with papillomavirus infections [1]. The transcription of ORF E6 and E7 is therefore a representative feature of a given cell line or tumour. Transcription was analysed by reverse transcription of random primed mRNA followed by two times 30 cycles of PCR with HPV16 or 18 E6/E7-specific nested primer pairs (Fig. 3). Amplimers obtained from RNA of the original tumours were compared with the cell lines EC and GC at different passages and the two standard cell lines HeLa (HPV18) and SiHa (HPV16) (Fig. 3). As one can see, there were almost no differences in transcription pattern, as revealed by amplimer comparison, between GC and SiHa cells. We have found the two major

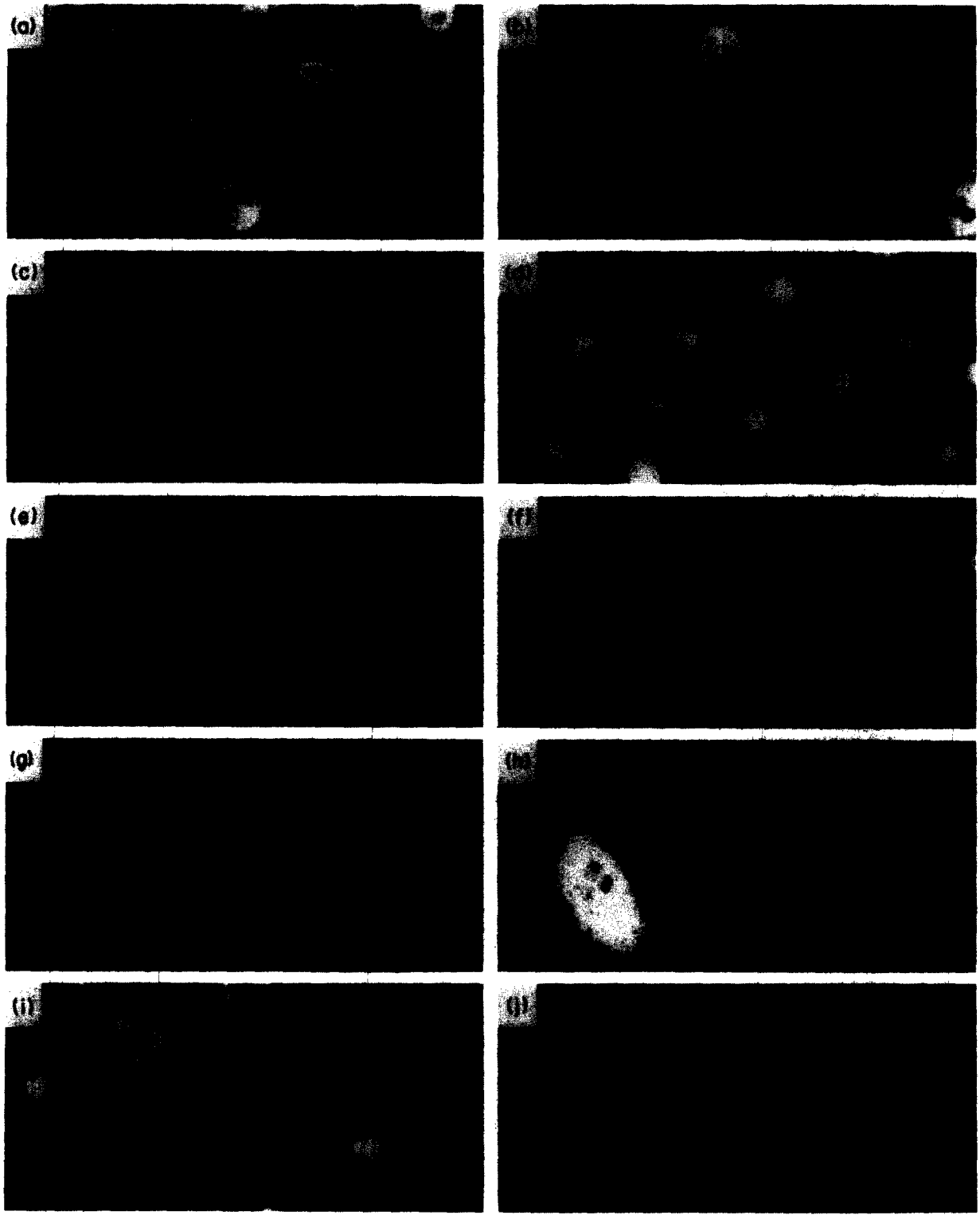

Fig. 7. Indirect immunofluorescence of marker proteins in GC (6) cells. Methods and panels as in Fig. 6.

transcripts E6* ^E7 and E6** ^E7 in GC cells as described for Caski cells from the Wettstein lab [9]. In hybridisation studies from amplimers of HPV18-EC cell line, additional fragments, not yet identified in HeLa cells, were visible in case of EC (HPV18). Surprisingly, transcription patterns were not uniform between several passages (Fig. 3, lanes 5 and 6) but corresponded with transcripts found in different parts of the tumour (Fig. 3, lanes 3 and 4). Although further work is required to locate conclusively the 5'- and 3'-termini, it seems likely that the pattern observed represents additional E6/E7-specific messengers generated from distinct splice donor and/or acceptor sites. Whether this is somehow connected with the evolution of the cell line remains to be elucidated.

The tumorigenicity of cell lines EC and GC was tested by injecting 5×10^6–10^7 cells subcutaneously into the flank of each of three nude mice. After 1 week cysts began to grow out. After approximately 2–3 weeks of progressive growth they completely regressed. No tumours were found after 8 weeks of continuous inspection. In parallel mice were injected with primary cervical epithelial cells, which formed cysts of similar size. This is in agreement with recent reports [18]. It should be noted that cysts, formed after injection of tumour-derived cells lines, generally appeared earlier and grew faster than those from comparable normal cultures. HE stains of the cysts (Fig. 8b) were different from those of tumour-derived cell lines grown in raft cultures, which consist of narrow layers of polymorphic spinal epithelia (Fig. 8a/c) with nuclear atypia. Surprisingly, at passages 14 and 20 cell lines EC and GC, respectively, despite some morphological changes were not really tumorigenic. Taking into account that both cell lines grew out of various locations of the respective tumours one would expect differences in tumorigenicity. It will be an interesting subject of future work to analyse the tumorigenicity in nu/nu mice during evolution of the cell lines.

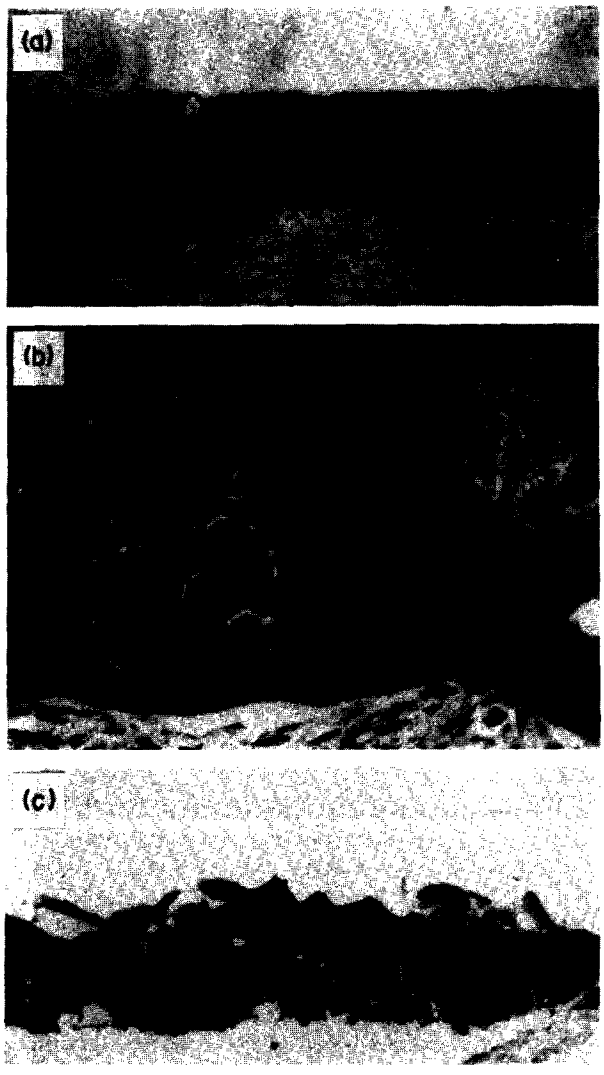

Fig. 8. HE stains of paraffin sections demonstrating the morphology of GC- (a) and EC-raft cultures (c), and of a cyst (b) obtained from injection of GC cells into nude mice (×50).

DISCUSSION

We have established two HPV-associated epithelial cell lines, designated EC (HPV18) and GC (HPV16). Here we presented the initial characterisation of the cell lines, describing their morphological, immunological as well as molecular parameters.

Cells were maintained in serum-free culture media. They are immortal, since they have undergone more than 35 passages *in vitro*. Depending on the type of media used, a more or less typical epithelial cell morphology was observed. Phenotypically, cells differed if they were kept in two low-Ca-media KGM (Clonetics) and Keratinocyte-SFM (Gibco, BRL) or MCDB 153 with supplements (Sigma). After at least 10 passages *in vitro* both cell lines grew well without feeder support. There is little doubt that cells even at low passages have undergone to a certain degree cytogenetic changes, which might influence growth characteristics. In the case of GC cells, a stronger feeder dependence was expected because the culture was initiated from a rather dysplastic part of the tumour biopsy sample. But, from normal cervical or vulvar epithelial cells EC and GC cells are clearly distinguished by the presence of HPV18 or 16 DNA, respectively. There is increasing evidence that the expression of HPV16 and 18 oncogenes encoded by the E6/E7 ORF in the early region is necessary to initiate and maintain the immortal or proliferative phenotype [12, 14]. However, it is widely excepted that HPV-associated transformation results from a complex interaction of viral and cellular gene activities and additional events other than viral infection appear to be necessary for malignant conversion and cancer progression both *in vitro* and *in vivo* [1]. Viral DNA, present at approximately one genome equivalent per cell, was found to be exclusively integrated into the cellular genome. Southern blot analysis revealed the presence of partially deleted copies of the viral DNA, probably leaving the E6/E7 genes intact. This is indeed supported by results of transcription studies conducted using reverse transcription PCR and hybridisation (Fig. 3). Interestingly, amplimers were observed in EC cells, different from the known HeLa transcripts. In addition to the E6* ^E7 splice product of HeLa [8] and cervical epithelial cells immortalised *in vitro* by transfection of an E6/E7-expression vector (Platzer *et al.*, manuscript in preparation) (Fig. 3a, lanes 1 and 2) a second fragment was detected, perhaps corresponding to the E6** ^E7 splice described in the HPV16 system [9] and confirmed in the GC cell line (Fig. 3b). Moreover, we have found in the tumour and adjacent tissues fully independent transcription patterns. Amplimers (Fig. 3a, lanes 3 and 4) reflecting these tissue parts revealed a shift in splicing not described so far. Surprisingly, the same was true for the cell line EC analysed at the 15th and 22nd passages (lanes 5 and 6). The detailed analysis of this transcription variant in context with growth behaviour of the cell line is the subject of current studies.

Generally, the E6* protein does not seem to exert a biologically important function, but rather E6* ^E7 splicing seems to improve the 'translatability' of the mRNA thereby increasing the E7 protein levels [19]. A shift in splicing as deduced from our RT-PCR analysis might therefore be of functional importance.

We further performed indirect immunofluorescence studies with several antisera directed against proteins of the cytoskeleton, characteristic of epithelial cells. We have analysed the expression of cytokeratins K1, K10, K13, K14 and involucrin. In non-neoplastic cervical epithelia keratin K14 is found in cells of the basal layer, K1 in maturing, suprabasal cells and K10/K13 in upper parts of the epithelium, from intermediate to superficial layers [20]. In our cell cultures the expression of keratins, despite some minor quantitative variations, was almost common to all epithelial cells; there were no obvious differences between proliferating cells EC and GC in low-calcium, serum-free media. Involucrin in normal squamous epithelium is usually first observed in the intermediate layers with progressively increasing intensity towards superficial layers. Its expression is indicative of initiation of terminal differentiation [21]. The absence of involucrin or, alternatively, the presence in only a few cells, indicates a lack of squamous cell maturation, which was described in association with early neoplastic transformation [22]. In low-calcium media involucrin has been shown in individual cells with a keratinocyte-like cell morphology and may represent a disturbance in cellular function, because phenotypically cells were not committed to squamous cell differentiation. A simultaneous expression of cytokeratins K13 and K14 is in support to this thesis; usually both were not found in the same cell layers of non-neoplastic epithelia.

The majority of cells in culture showed an intensive immunofluorescence staining for PCNA/cyclin, identified as an auxiliary protein of DNA polymerase δ [23]. The staining pattern was of two different types: a granular, dot-like and a more diffuse fraction. The granular staining as concluded by Bravo *et al.* [23] is S-phase specific and associated with replicon clusters. A diffuse nucleoplasmic staining was previously shown to be present in $G_{1,0}$ and G_2/M phases [24]. Ki67, an antigen reported to be present in cycling cells only [25], was demonstrated in a subpopulation of cells and characterised by a dot-like immunofluorescent staining. The immunostaining of cervical and vulvar epithelial cells revealed that there is only a little variation in PCNA expression during the cell cycle. This observation is in agreement with previous reports [26].

The importance of EGF for growth of normal and malignant human keratinocytes has been evident for a long time. The expression of EGF-receptors on the surface of keratinocytes was demonstrated and found to be related to terminal differentiation [27]. Moreover, many squamous cell carcinomas show elevated levels of EGF receptor expression [28]. The homology of the human EGF receptor gene with the avian erythroblastosis virus transforming oncogene v-*erb*-B and its cellular counterpart c-*erb*-B further emphasises the potential importance of the EGF receptor in control of normal and abnormal cell proliferation [29]. We have shown a considerable difference in compartmentalisation of EGF receptor molecules in GC and EC cells (Figs 6, 7e). Whereas in EC cells there was predominantly a granular cytoplasmic immunostaining, in GC cells the EGF receptor was concentrated at the surface membrane. Consequently, our findings suggest that due to the changes in membrane location both cell lines are differently regulated by EGF. This is considered to be reflected in their differentiation capacity. The differences in the EGF receptor expression in cell cultures might reflect the differentiation capabilities of the original tumour. In EC cells an alteration of regulation of EGF receptor expression may have occurred at the post-translational level. Various strategies of regulation of EGF receptor expression have been reported recently [30]. There were no obvious differences in growth characteristics of EC cells kept in EGF-free or in complete serum-free medium (not shown).

With the initial characterisation of EC and GC cells we have demonstrated that both cell lines can serve for the future elucidation of the variability of viral gene expression in tumour biopsy samples, cell lines derived from them and experimental models, such as raft cultures and development of neoplasias in nude mice.

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