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Analysis of HPV16 E6 and mutant p53-transfected keratinocytes in reconstituted epidermis suggests that wild-type p53 inhibits cytokeratin 19 expression

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SUMMARY

Using a reconstituted skin culture model we have analysed the effects of oncogenic human papillomavirus (HPV) and mutant *TP53* genes on the proliferation and differentiation of human keratinocytes. Immortal cell lines generated by transfection of early passage normal human keratinocytes with HPV16 *E7* plus mutant human *TP53* (KN #1), HPV16 *E7/E6* (KN #2), or HPV16 *E7* plus murine *p53* (KN #3) were examined. KN #1 and KN #2 behaved identically, reconstructing a tumor-like epidermis characterized by the lack of differentiation and the presence of an aberrant epidermal architecture. In contrast, KN #3 reconstructed an epidermis that was more similar to that obtained with normal keratinocytes. KN #1 and KN #2 were further characterized by the inversion of the proliferative compartment and the abnormal expression of cytokeratin 19 (CK19). Because p53 function is reduced in these cells, either by heterocomplex formation between endogenous wild-type p53 and transfected mutant p53 or by E6-induced degradation of wild-type p53, we hypothesized that CK19 expression may be normally repressed by wild-type p53. This hypothesis was supported by the strict correlation observed between *TP53* mutation and CK19 expression in a set of human skin tumors. CK19 was detected in all eight carcinomas containing a mutated *TP53* gene but in none of the 16 carcinomas containing only wild-type *TP53.* These results illustrate the utility of the in vitro reconstituted skin model for investigating the consequences of genetic alterations in human keratinocytes.

Key words: p53, HPV, keratinocyte

INTRODUCTION

Skin can serve as a useful model for investigating genes involved in cell proliferation and differentiation because these events occur in different compartments of the tissue. In vitro reconstituted epidermal cultures (RE) of normal keratinocytes mimic many of the features of normal skin, and, because individual genes can be transfected into the cells prior to reconstitution, RE cultures can be used to study the phenotypic consequences of specific genetic alterations in keratinocytes.

Characterization of the p53 protein has shown that it has DNA binding properties and acts as a transcriptional modulator (Unger et al., 1992) via specific DNA sequences (El-Deiry et al., 1992). Genes under p53 control are numerous, from cytokine (Santhanam et al., 1991) to oncogenes (Ginsberg et al., 1991) showing a broad spectrum of p53 action. At the cellular level, p53 has been implicated in the regulation of the cell cycle, particularly in stressed cells (Kastan et al., 1991; Kuerbitz et al., 1992). p53 has also been implicated in cell differentiation, e.g. T lymphocytes (Khochbin and Lawrence, 1989), and in apoptosis (Shaw et al., 1992; Yonish-Rouach et al., 1993). Alterations in the human tumor-suppressor gene *TP53* are the most frequent genetic changes found in human cancers (Hollstein et al., 1991). The inactivation of *TP53* is often the result of point mutation in certain conserved domains and/or due to allelic losses of the gene (Hollstein et al., 1991). In carcinomas, most frequently cervical, inactivation of p53 can also occur through the activity of E6 protein of HPV16 or other oncogenic human papillomaviruses (HPVs) (Sheffner et al., 1991; Wrede et al., 1991). The interaction of E6 with p53 induces the degradation of p53 by an ATP-dependent ubiquitin pathway (Scheffner et al., 1990).

Human papillomaviruses are small DNA viruses that infect squamous epithelia. Examination of cervical tumors and cell lines derived from them has suggested that the E6/E7 region of the HPV genome is important in cervical carcinogenesis, since tumors and cell lines preferentially transcribe E6 and E7 genes (Schwarz et al., 1985; Smotkin and Wettstein, 1986). The mechanism by which the HPVs function in malignant progression appears to be related to the activity of these two viral oncoproteins, E6 and E7, which, respectively, form complexes with p53 and Rb, two cell proteins normally involved in cell

growth control (Werness et al., 1990; Dyson et al., 1989). The *E6* gene of HPV16 cooperates with HPV16 *E7* to immortalize normal human keratinocytes (Hawley-Nelson et al., 1989; Munger et al., 1989). Human and mouse mutant p53 can functionally substitute for E6 in the induction of immortalization (Sedman et al., 1992). However, E6 appears to have activities that do not rely upon its ability to inactivate p53 (Sedman et al., 1992), raising the possibility that lines immortalized by *E6* plus *E7* may differ phenotypically from those immortalized by *TP53* plus *E7* genes. Lines immortalized by *E6* and *E7* genes have been shown to undergo abnormal differentiation when grown on collagen rafts at the air-liquid interface (McCance et al., 1988; Schlegel et al., 1988; Rader et al., 1990; Hudson et al., 1990; Hurlin et al., 1991) but their growth and differentiation properties in RE cultures have not been analysed, and the effect of substituting mutant *TP53* for *E6* has not been evaluated. In this study we have examined *E6* plus *E7* and mutant *TP53* plus *E7* immortalized keratinocytes in RE cultures, and have used a panel of antibodies to examine the in situ distribution of specific markers of keratinocyte proliferation and differentiation. The results were compared with those obtained from biopsies of skin carcinomas that had been previously analysed for *TP53* gene mutation (Molès et al., 1993).

MATERIALS AND METHODS

Cells

Normal human keratinocytes (NHK) were obtained after digestion of neonatal foreskins overnight at 4°C in trypsin 0.25%. Keratinocyte cell lines were generated in previous studies (Sedman et al., 1991, 1992) by co-transfection of primary human keratinocytes with a long terminal repeat (LTR) HPV16 *E7* gene plus human mutant (exon 5; codon 135) *TP53* cDNA (KN #1), with HPV16 *E6/E7* (KN #2) and with a long terminal repeat (LTR) HPV16 *E7* gene plus murine mutant (exon 5, codon 153) *p53* cDNA (KN #3). All keratinocytes and cell lines were cultured with the defined medium SFM (Gibco, BRL) in the presence of bovine pituitary extract (70 µg/ml) and EGF (5 ng/ml).

Skin tumor biopsies

Twenty four tumor samples were collected at the Dermatological Department of Hôpital Saint Charles, after informed consent. Fourteen were basal cell carcinomas and 10 were squamous cell carcinomas. Tissues were rapidly embedded in OCT compound (Miles, USA), frozen in liquid nitrogen and stored at −80°C until used. This series has been previously analysed for *TP53* gene mutation by PCR-SSCP (single-stranded conformation polymorphism) techniques. Among them, seven were single point mutations in codon 151, 174, 177, 196, 277, 282, 300 and one was a 28 bp deletion in exon 5 leading to a stop codon.

Reconstituted epidermis culture

Reconstituted epidermis was generated according to the Prunièras model (Prunièras, 1979). Briefly, cell suspensions (200,000 cells in 10 µl of culture medium) were deposited on a mortified de-epidermized dermis (1 cm^2) and maintained at the air-liquid interface using a metallic support. Culture medium contained DMEM/Ham's F12 (3/1, v/v; Gibco BRL), foetal calf serum (10%, Gibco BRL), penicillin/streptomycin (1%, Gibco BRL), fungizone (1%, Gibco BRL), hydrocortisone (0.4 µg/ml, Sigma), insulin (5 µg/ml, Sigma), cholera toxin (0.1 nM, Sigma) and EGF (10 ng/ml, Sigma). After 7 to 28 days of culture, all reconstituted epidermis were collected, embedded in OCT compound (Miles, USA), frozen in liquid nitrogen and stored at −80°C until used. REs at 14 days of cultures showed complete maturation and were routinely used in this study. Two series of REs were obtained using lines at 30th and 50th passage. Two independently derived KN #1 cell lines were examined.

Table 1. Antibody specificity and origin

Antibody	Specificity	Source	Working dilution
LL017	CK1	ICRF	Pure
LH6	CK5 and -14	ICRF	1/20
LL020	CK6	ICRF	1/20
LE41	CK8	ICRF	1/50
LH2	CK10	ICRF	1/20
LH8	CK14 (basal)	ICRF	1/20
LL001	CK14 (basal and suprabasal)	ICRF	1/20
LL025	CK16	ICRF	1/50
LE61	CK18	ICRF	1/50
LP2K	CK19	ICRF	1/10
Anti-human involucrin	Involucrin	Euromedex	Pure
Anti-human filaggrin	Filaggrin and profilaggrin	Euromedex	1/50

Antibodies

Monoclonal anti-cytokeratin antibodies were obtained from Dr I. Leigh. Their production and characterization has been described elsewhere (Stasiak et al., 1989; Purkis et al., 1990; Leigh et al., 1993). The specificity and working dilution of the different antibodies are presented in Table 1. Proliferating cells were detected using Ki67 antibody (Dakopatts, Denmark). Anti-filaggrin antibody came from Euromedex (France).

Immunofluorescence and immunohistochemical analysis

Antigen detection was performed on 4 µm cryostat sections of reconstituted epidermis. Non-specific sites were blocked by incubation in PBS/0.5% BSA. The first antibody was applied for 1 hour at 37°C in a moist chamber and then visualized using an anti-mouse biotinyled antibody (dilution 1/50, Sigma, France) and with Streptavidin-Texas Red (dilution 1/100, Amersham, UK). After each incubation, slides were washed three times in PBS/0.5% BSA for 5 minutes. Slides were then mounted and examined with an Axioscop microscope (Zeiss, Germany) equipped with camera. CK19 expression was also examined on tumor sections using an Immustain Universal kit (DPC, France) according to the manufacturer's recommendations. No fixation was used for the differentiation markers whereas for Ki67, slides were fixed 10 minutes in formaldehyde 3.7%/PBS and permeabilized for 30 seconds in cold acetone.

Bromodeoxyuridine incorporation in reconstituted epidermis

After two weeks of reconstruction, neo-epidermis were starved for 48 hours in culture medium containing 0.5% of foetal calf serum and without growth factors. Epidermis were then stimulated with complete medium containing BrdU (Amersham, UK) for 3 hours, after which RE samples were collected, embedded in OCT compound (Miles, USA) and frozen into liquid nitrogen. BrdU incorporation was revealed by immunostaining according to the manufacturer's instructions (Amersham, UK).

RESULTS

Morphology of the reconstituted epidermis generated by the immortalized keratinocyte lines

NHKs were able to rapidly reconstruct an epidermis that was very similar to that observed in vivo. The REs exhibited

properly stratified layers and the presence of a stratum corneum, indicating that a relatively normal differentiation program had been obtained (Fig. 1A). KN #1 and KN #2 produced similar aberrant REs. The architecture was disorganized and lacked a defined basal layer and a stratum corneum. Numerous cellular atypies were observed as well as the presence of dyskeratosic cells (see arrows in Fig. 1B,C). REs obtained from these cells resembled severe dysplasias or in situ carcinoma. KN #3 had a reconstructed epidermis with a more normal appearance in that a basal layer and a stratum corneum were present. However, cell atypies and the absence of flattening keratinocytes in the intermediate layers were observed (Fig. 1D).

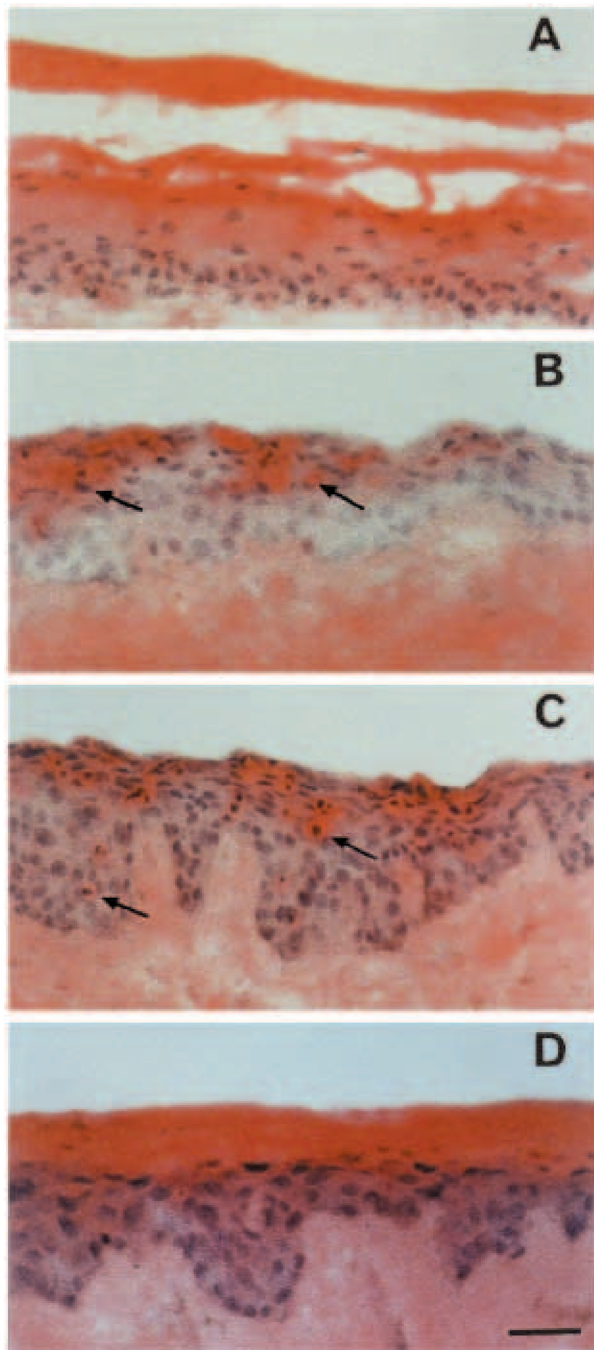

Fig. 1. Morphologies of the different REs obtained. Hematoxylin- and eosin-stained slides showed that normal human keratinocytes (NHK) reconstruct an epidermis similar to that observed in vivo (A) whereas KN #1- and KN #2-derived REs were characterized by an abnormal and tumor-like architecture (B,C): lack of differentiation (absence of intermediate compartment and stratum corneum), no defined basal layer and presence of numerous cellular atypies and dyskeratosic cells (see arrows). The KN #3-derived RE differs from the two others essentially by the presence of a basal layer and a stratum corneum (D). Similar results were obtained with transfected keratinocytes taken at the 30th and 50th passage, and with two independent KN #1 cell lines. Bar, 50 μm.

Analysis of REs proliferative compartment

In order to determine the location of the proliferating cells in the RE, we stained sections with antibodies against Ki67, which is a protein expressed from late G_1 phase to S phase of the cell cycle (Gerdes et al., 1984). Proliferating cells in the NHK-derived REs localized in the basal layer of the epidermis and were restricted to a only few cells (Fig. 2A). In KN #1- and KN #2-derived REs, the number of proliferating cells was increased and, surprisingly, they were mostly detected in superficial layers (Fig. 2B,C). Conversely, KN #3-derived REs showed a similar pattern to that observed in NHK-derived REs, although the number of proliferating cells was greater (Fig. 2D). Similar results were obtained in BrdU incorporation experiments (data not shown).

Analysis of RE differentiation

Antibodies to basal cell-associated cytokeratins (CK5 and CK14) gave a diffuse signal in all epidermal layers of the REs derived from all three lines (Fig. 3C to H) whereas in NHK-derived REs, the staining was restricted to the basal layer (Fig. 3A,B) as seen in normal human skin (data not shown).

Antibodies to terminal differentiation-associated cytokeratins (CK1 and CK10) were detected only in NHK and KN #3-derived REs (Fig. 4A,B and 4G,H). However, their appearance was delayed when compared to that observed in normal skin (data not shown, unpublished results). No expression was detected in KN #1- and KN #2-derived REs (Fig. 4C,D,E,F).

Two other terminal differentiation markers (filaggrin and involucrin) gave similar results (data not shown). They were detected in the superficial layers in NHK- and KN #3-derived REs but not in KN #1- and KN #2-derived REs (except for a few involucrin positive cells).

Antibodies to hyperproliferation-associated cytokeratins (CK6 and CK16) strongly stained all REs, in both basal and more superficial layers (Fig. 5A,B, D,E, G,H and J,K).

For CK8, CK18 and CK19 expression, cytokeratins associated with simple epithelia, human embryogenesis and epithelial transformation; no reactivity was observed in NHK- and KN #3-derived REs (see Fig. 5C,L for CK19 stainings). However, in KN #1- and KN #2-derived REs, a strong positive staining with CK19-specific antibody was observed mainly in superficial layers (Fig. 5F,I), corresponding to the proliferative compartment, whereas no CK8 and CK18 could be detected (data not shown).

Analysis of the CK19 expression in a human skin tumor series

In order to determine if CK19 expression could be associated

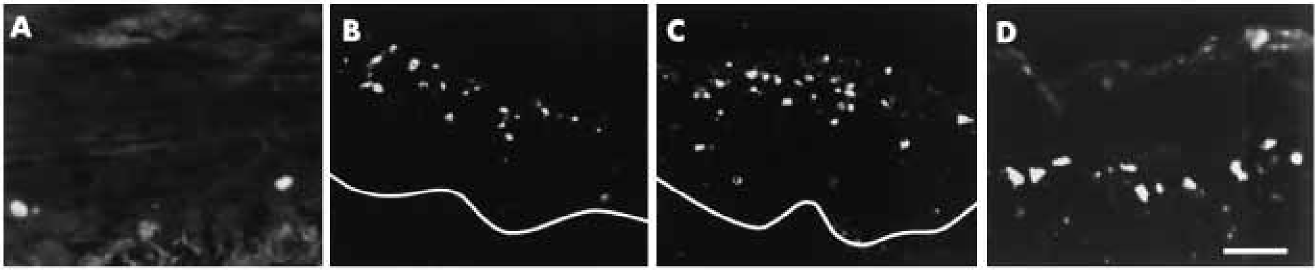

Fig. 2. Localization of the proliferative compartment in REs. Results were obtained by detection of the antigen Ki67. In all transfected cell-derived REs, the number of proliferating cells is greater than in NHK-derived RE (A). In KN #1- and KN #2-derived REs (B,C), these cells localized in the superficial layers of the neo-epidermis (basement membranes are underlined). Bar, 50 µm.

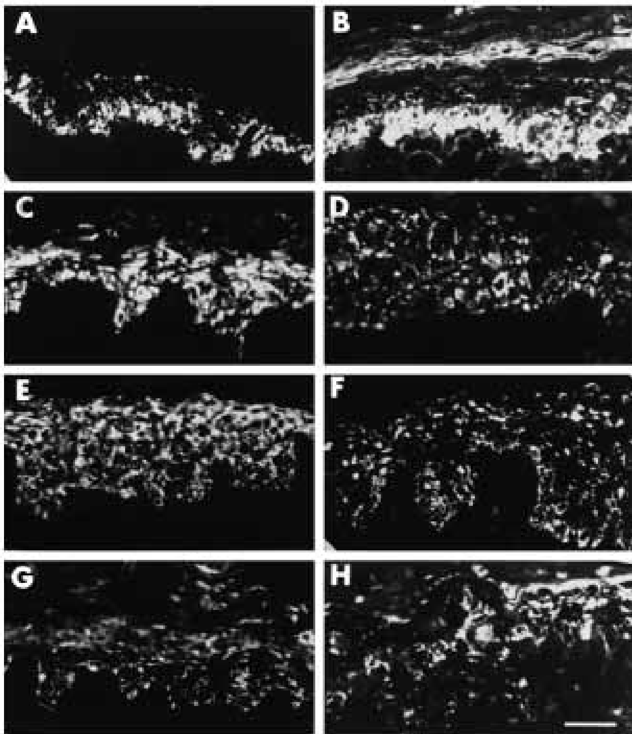

Fig. 3. Expression of basal-associated cytokeratins in REs. Detection of CK5 (left column) and CK14 (right column) was observed in the basal layer of NHK-derived RE (A and B). In KN #1- (C and D), and in KN #2- (E and F) and in KN #3-derived REs (G and H) all epidermal layers were reactive. Bar, 50 µm.

with the presence of mutant *TP53* gene in vivo, we have analysed CK19 expression by immunohistochemistry in a series of skin tumors previously characterized for *TP53* gene mutation (Molès et al., 1993). Among eight tumors with the mutant *TP53* gene (basal cell carcinomas n=7, squamous cell carcinomas n=1), all were positive for CK19 expression (results are shown in Fig. 6A,B). Conversely, none of the 16 skin tumors containing only the wild-type *TP53* gene (basal cell carcinomas n=6, squamous cell carcinomas n=10), showed CK19 expression (for example, see Fig. 6C). In control experiments, positive stainings were observed in several normal adnexal skin structures such as sweat glands and the outer root sheath (Fig. 6D,E). Normal human epidermis as well as supratumoral epidermis were negative in all cases except for one sample that showed two positive cells (see arrows in Fig. 6A).

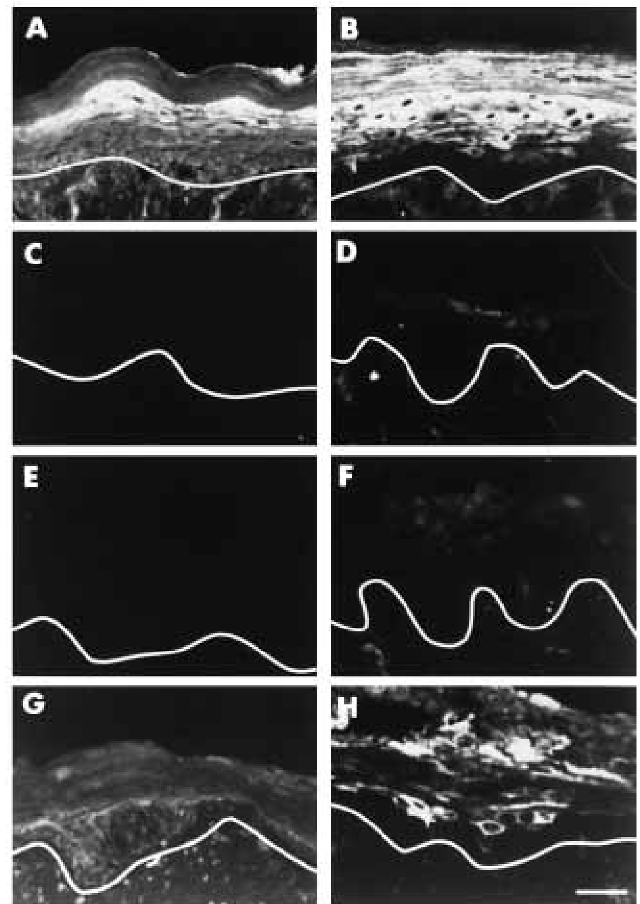

Fig. 4. Expression of intermediate-associated cytokeratins in REs. Detection of CK1 (left column) and CK10 (right column) was observed in the suprabasal layers of NHK- (A and B) and in KN #3-derived REs (G and H) whereas no staining was observed in KN #1- (C and D) and KN #2-derived REs (E and F). Basement membranes are underlined. Bar, 50 µm.

DISCUSSION

This study reports the phenotypic consequences of HPV16 *E7* plus mutant human *TP53* (KN #1), HPV16 *E6/E7* (KN #2), or HPV16 *E7* plus mutant murine *p53* (KN #3) transfections in human keratinocytes using a reconstituted epidermis model. Our objectives were to analyse the proliferation and the differentiation properties of REs generated by the Prunièras system

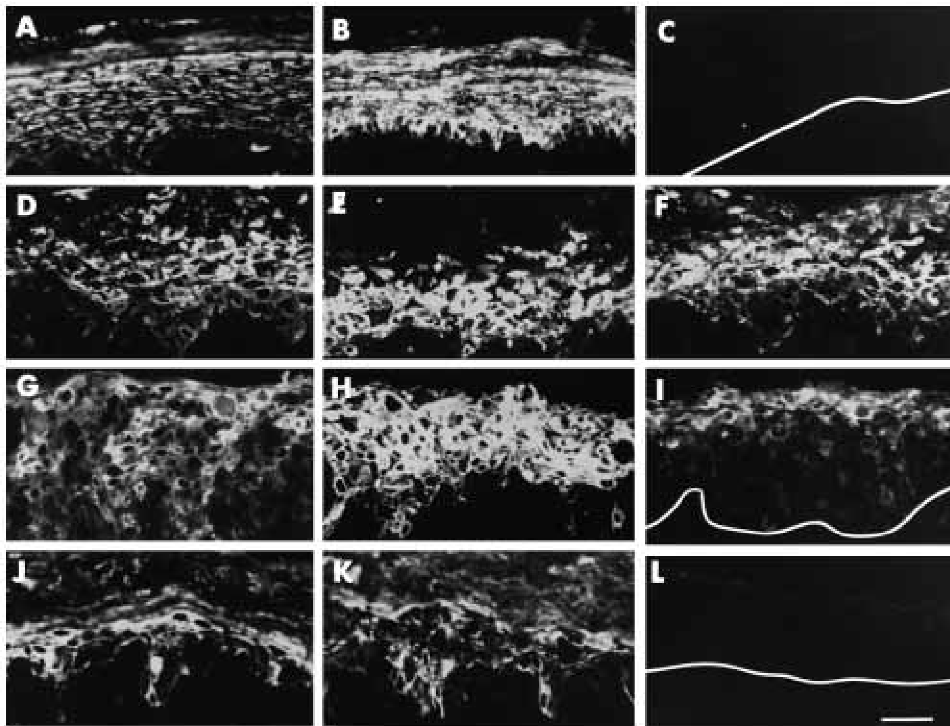

Fig. 5. Expression of hyperproliferation-associated and tumor-associated cytokeratins in REs. Detection of CK6 (left column) and CK16 (middle column) was observed in all layers of NHK- (A and B), and KN #1- (D and E), and KN #2- (G and H) and KN #3-derived REs (J and K). Expression of the CK19 (right column) was observed only in KN #1- (F) and KN #2-derived REs (I) whereas no staining could be detected in NHK- (C) and KN #3-derived REs (L); in these cases, basement membranes are underlined. Bar, 50 µm.

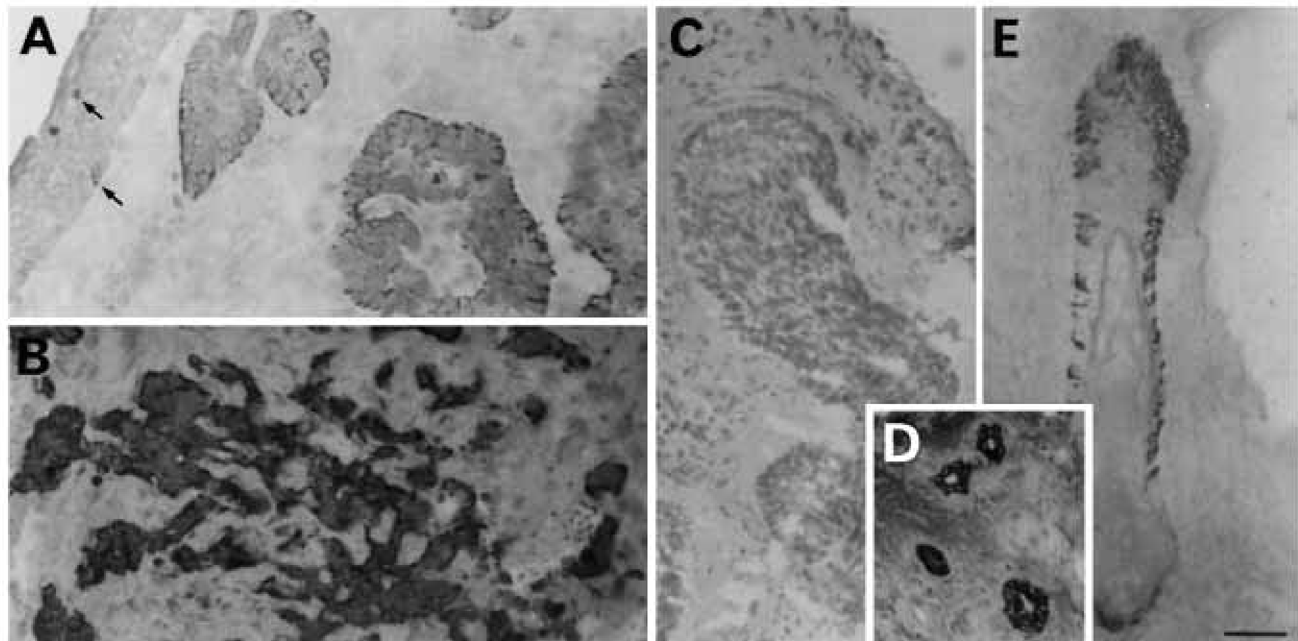

Fig. 6. CK19 expression in a skin tumor series. Tumor cells were positive in some basal cell carcinomas (A) and squamous cell carcinomas (B) whereas no staining could be detected in others tumors (C). Normal CK19 expression was also observed in cutaneous adnexal skin structures such as sweat gland (D) and outer root sheath (E). Note that two cells were positive in the supratumoral epidermis (A, arrows). Bar, 50 µm.

(Prunièras, 1979). REs have proven to be a powerful and efficient model system for studying skin physiopathology (Regnier et al., 1988; De Dobbeleer et al., 1989; Basset-Séguin et al., 1993). Our results indicate that this system is also useful for analysing the consequences of specific gene transfer into keratinocytes and are correlated with those observed on a series of 24 skin tumors previously characterized for *TP53* gene mutation.

Under the culture condition employed, the three cell lines did not behave identically. The morphology of the REs was clearly abnormal and tumor-like for KN #1- and KN #2-derived REs. They lacked the normal epidermal architecture in that a distinct basal layer, intermediate keratinocyte compartment and stratum corneum were absent (Fig. 1). Conversely, the KN #3-derived RE produced a neo-epidermis similar to that observed with NHK-derived RE, in that a distinct basal layer

and stratum corneum were present. However, the KN #3-derived RE did contain numerous cell atypies (Fig. 1).

Proliferation marker analysis showed additional differences between these cell lines as inversion of the proliferative compartment was observed in KN #1 and KN #2-derived REs whereas in KN #3-derived REs this compartment was localized, like that of NHK-derived REs, in the basal layer (Fig. 2). The abnormal distribution of proliferative cells seen in KN #1- and KN #2-derived REs has been previously reported for keratinocytes transfected with the HPV16 genome in a raft culture system (Hudson et al.,1990; Rader et al., 1990). This inversion suggests that in these REs have become independent of the presence of and/or the attachment to the basal lamina, in contrast to what is observed in normal epidermis. Perhaps the cells in the uppermost layer tend to proliferate preferentially because it is the most extensible compartment of the RE.

Differentiation marker analysis substantiated the morphological characterizations. All epidermal REs expressed basal-associated cytokeratins (CK5 and CK14) (Fig. 3) as well as hyperproliferation-associated cytokeratins (CK6 and CK16) (Fig. 5). The absence of significant morphological differentiation in KN #1- and KN #2-derived REs correlated with the absence of terminal differentiation markers (Fig. 4). Additionally, KN #1- and KN #2-derived REs, but not KN #3-derived REs, were characterized by expression of an additional cytokeratin, CK19 (Fig. 6), which is not expressed in the normal epidermis (Van Muijen et al., 1987), but can be found in simple epithelia (Purkis et al., 1990) and foetal epidermal keratinocytes (Van Muijen et al., 1987). CK19 expression was detected only in the superficial layers of KN #1 and KN #2 REs, suggesting that its expression requires nuclear factors present in proliferative cells. Therefore, the expression of CK19 could reflect a de-differentiation of the cells to an embryonic stage during the immortalization process.

The complete concordance of results obtained with KN #1 and KN #2 in the RE cultures indicates that mutant human *TP53* can not only substitute for HPV16 *E6* in the immortalization of normal human keratinocytes as observed in in vitro experiments (Sedman et al., 1992) but fully mimic the effects of *E6* in the disruption of normal epidermal differentiation. Somewhat surprisingly, mutant mouse *p53* appeared to have less drastic effects on human keratinocyte differentiation even though it binds to human wild-type p53 (Milner and Medcalf, 1991) and can immortalize keratinocytes in conjunction with E7 (Sedman et al., 1992). The result raises the possibility that the mutant p53 activities required for immortalization and differentiation inhibition may be partially separable and that the mutant mouse protein is more active in the former. Two pools of intracellular p53 have been proposed to explain the E6-induced degradation rate of this protein (Hubbert et al., 1993). Inactivation of these pools could differ between human and mouse mutant p53-transfected cell lines and be implicated in these differential biological activities.

In these cell lines, the p53 function might be abolished or reduced by either heterocomplex formation between endogenous wild-type p53 and transfected mutant p53 (Milner and Medcalf, 1991) or E6-induced degradation of wild-type p53 (Scheffner et al., 1990). The detection of CK19 in KN #1RE suggests that either human mutant p53 induces or wild-type represses CK19 expression. The finding that CK19 is also expressed in KN #2RE, which contains E6 but not mutant p53, supports the conclusion that wild-type p53 is a repressor. However, if inactivation of *TP53* is able to inhibit wild-type p53-dependant repression of CK19 expression, it does not appear to be sufficient to induce CK19 expression, since all cell layers do not express this cytokeratin. Wild-type p53 has previously been shown to bind to specific DNA sequences (El-Deiry et al., 1992) and act as a transcriptional modulator (Unger et al., 1992) but it has not been previously implicated in the regulation of CK19 expression. Unfortunately, genomic CK19 has not been sequenced, thus we have not been able to determine if the gene is associated with a consensus p53 binding motif (El-Deiry et al., 1992). We excluded the participation of the E7 protein in the induction of CK19 as it is present in the KN #3 line, which do not express this cytokeratin.

CK19 expression has been reported in human cutaneous tumors and shows a heterogeneous distribution (Habets et al. 1988, 1989). These carcinomas generally have a relative high incidence of *TP53* gene mutation (Brash et al., 1991; Pierceall et al., 1991; Rady et al., 1992; Molès et al., 1993; Ziegler et al., 1993) but are very rarely associated with HPV infection (Kawashima et al., 1990). Our finding that 8/8 tumors, which had been previously been shown to contain *TP53* gene mutations, expressed CK19, while none of the 16 tumors that contained only wild-type *TP53* gene expressed CK19, strongly supports the hypothesis that wild-type p53 is a repressor of CK19 expression. Additionally, these results together with those observed on KN #3 cell line, also make it very unlikely that HPV16 *E7* plays a critical role in the induction of CK19 expression.

In conclusion, using this in vitro system, the consequences of gene transfections for cell proliferation and differentiation can be examined. Our data suggest that a close relation might exist between p53 inactivation and CK19 expression in immortalized keratinocytes. This relation could help to investigate *TP53* gene mutation in skin cancers and must be extended to other cancers.

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