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Original article

Requirement of E7 oncoprotein for viability of HeLa cells

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Abstract

Most human papillomavirus (HPV)-positive cervical cancers contain integrated copies of the viral genome in their chromosomes and express the viral oncoproteins E6 and E7. A virus-encoded transcription factor, E2, is known to repress E6/E7 expression in HPV-positive cancer cells, leading to growth inhibition, which indicates that E6/E7 is required for the survival of the cells. We found that the E2-mediated growth inhibition of HeLa cells, an HPV18-positive cancer cell line, was coupled with a reduction in telomerase activity, an effect which was rescued by the complementation of E7 expression, but not E6 expression, indicating that the cell viability and the telomerase activity in HeLa cells are maintained by an E7-associated function. Analysis of E7 mutants suggested that the binding to the pRB family of pocket proteins was involved in the ability of E7 to rescue the growth potential and telomerase activity inhibited by E2 expression. We also showed that the telomerase activity upregulated by E7 expression was determined by the hTERT promoter activity, and that c-Myc upregulation caused by pRB inactivation could account for the promoter activity. The activation of p53 and consequent accumulation of p21Cip1, which were triggered by the downregulation of E6, appeared not to be essential for the E2-mediated growth arrest.

Keywords: Human papillomavirus; E6 gene; E7 gene; Telomerase; Senescence

1. Introduction

Human papillomaviruses (HPVs) cause a variety of benign squamous epithelial proliferative lesions, such as warts and condylomas. Lesions induced by specific types of HPVs occasionally become cancers after a long incubation period; such HPVs are categorized as high-risk types [1]. Cervical cancer is clinically the most important of the HPV-induced cancers; 90% of those with this cancer are positive for the viral genome and express viral major transforming genes, *E6* and *E7*. E6 and E7 are known to interfere with cell-cycle checkpoint regulators. E6 associates with p53 in combination with a cellular ubiquitin ligase, E6AP, which directs ubiquitin-mediated degradation of p53 [2,3]. E7 binds to pRB and functionally inactivates it [4]. Both p53 and pRB are important for the tumor suppressor function and involved in cell-cycle checkpoints, and their inactivation plays a pivotal role in cancer progression [5]. E6 is also known to activate cellular telomerase activity, leading to an expansion of the cellular life span [6,7]. E7 interacts with other pocket proteins, p107 and p130 [8]. It has been also reported that E7 binds to many cellular proteins, including a cdk inhibitor, p21Cip1 [9,10].

The HPV genome is maintained as episomal copies in productively infected cells. In most HPV-positive cancer cells, however, the viral DNA is integrated in cellular chromosomes [11,12], and often found disrupted within the E2ORF [13–15]. E2 protein is a DNA-binding transcription factor and functions as a negative regulator for *E6/E7* expression, thus the inactivation of E2 results in the deregulated expression of *E6/E7*,

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which might be favorable for cancer progression [16,17]. The continued expression of E6/E7 observed in most HPV-positive cancer cells raised a possibility that their functions are required not only for cancer progression but also for the maintenance of cancer cells. The introduction of E2 into HPV-positive cancer cells was reported to cause growth inhibition through suppression of *E6/E7* expression [18–23], which is also supportive evidence for this hypothesis. These observations are important to the development of therapeutic methods for HPV-associated cancer in addition to the understanding of molecular mechanisms being the acquisition of malignancy.

Recently, several studies showed that the E2-mediated growth inhibition of HPV-positive cancer cells was coupled with a senescence-like state, where telomerase activity was suppressed [24–26]. Although several possible mechanisms to induce the senescence were proposed in these reports, the individual contribution of E6 and E7 to the maintenance of cancer cell proliferation, as well as the involvement of telomerase activity in cell viability, is still controversial and further investigation is required. In this report, the biological significance of E6/E7 functions in HPV-induced cancer was analyzed by using a combination of E2, E6 and E7 expression plasmids in HeLa cells, which allowed selective expression of E6 and E7. Although previous experiments indicated the requirement of E6/E7 expression for HeLa cell proliferation ([27]: Wells, 2000 #86), our experiments indicated that only E7 expression could rescue HeLa cells from E2-mediated growth inhibition, and that the inactivation of pRB could account for this effect. The E7-mediated modification of the pRB pathway was also involved in telomerase activation in HeLa cells through the modification of hTERT promoter activity.

2. Materials and methods

2.1. Cell culture and transfection

HeLa, CV1, and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). DNA transfection was performed using a standard calcium phosphate precipitation method [28]. Cells (2×10^5) were seeded in a 6-cm dish 1 day prior to transfection. Plasmid and carrier DNA (total 10 μg) were incubated with 500 μl of Hepes-buffered saline (HBS) transfection buffer (140 mM NaCl, 0.75 mM Na_2HPO_4, 25 mM Hepes, and 110 mM $CaCl_2$; pH 6.90) for 30 min at room temperature, and then added to a culture dish. Transfection efficiency was monitored using green fluorescence protein (GFP), which was expressed from the co-transfected control plasmid. All results were obtained from samples with more than 90% transfection efficiency.

2.2. Plasmids

The construction of FLAG-tagged HPV16 E2 and mutant E2 expression plasmids was described in a previous report [22]. A DNA fragment containing the *E6*, *E7* or *E6/E7* sequence was obtained by polymerase chain reaction (PCR)-amplification with a DNA template containing the entire genome of HPV18 (GenBank accession no. X05015), and cloned into pCMV4. The sequences were confirmed by sequencing analysis. Two E7 mutants, d24g and c27g, were constructed by PCR-directed mutagenesis [29]. A codon for aspartic acid (GAC) at position 24 is changed to that for glycine (GGG) in the d24g mutant. A codon for cysteine (TGT) at position 27 is changed to glycine (GGG) in the c27g mutant. A GFP-expression plasmid, pGreenLantern-1 (Invitrogen Corp., Carlsbad, CA), was used to monitor the transfection efficiency. pSVβ is available commercially (Clontech Laboratories Inc., Palo Alto, CA). Herring sperm DNA (Roche Diagnostics GmbH, Mannheim, Germany) was used as carrier DNA for transfection experiments. The puromycin selection plasmid, pPUR (Clontech), was obtained commercially. The hTERT expression plasmid, pcDNA3-hTERT, was obtained from Dr. Fuyuki Ishikawa [30]. The hTERT-reporter plasmids, p3328-GL3 and p181-GL3, are described in a paper by Kanaya et al. [31]. p181-GL3 contains the core promoter for hTERT, and p3328-GL3 contains the entire promoter region.

2.3. Cell growth assay

At 24 h post-transfection, the cells were trypsinized and spread into multiple 6 cm dishes at a density of 5×10^4 cells/dish. The cells were maintained with selection in the presence of puromycin (Sigma–Aldrich Co., St. Louis, MO) at a concentration of 0.3 μg/ml, and then the number of cells per dish was counted at the indicated time after transfection. Trypan blue exclusion was used for counting only living cells. For each assay, the experiments were performed at least three times and a representative result is indicated in the figures. For each experiment, the data were obtained with triplicate samples and the standard deviation was less than 15% (not shown in figures).

As inhibitors for cell growth, double thymidine block and mitomycin C treatment were employed. Double thymidine block was performed according to a standard protocol [32]. Thymidine (Sigma–Aldrich) was added to exponentially growing cells (5×10^4 cells/dish) to a final concentration of 2 mM and the cells were incubated for 12 h. The medium was then replaced with fresh growth medium and the cells were incubated for 12 h, before thymidine was added again (2 mM). For mitomycin C treatment, mitomycin C (0.5 μg/ml) (Sigma–Aldrich) was added to exponentially growing cells (1×10^5 cells/dish).

2.4. Senescence associated (SA)-βGal assay

Staining for SA-βGal activity was performed as previously described [33]. Briefly, cells were washed in PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde for 5 min, washed, and incubated with SA-βGal staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal)/40 mM citric acid/sodium phosphate (pH 6.0)/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM $MgCl_2$) at 37 °C overnight.

2.5. Telomeric repeat amplification protocol (TRAP) assay

Telomerase activity was assayed with protein extracts prepared from transfected cells by using a TeloChaser Kit (Toyobo Inc., Osaka, Japan), which was based on modified "stretched PCR methods" originally developed by Dr. Ishikawa [34]. Using 5 μg of protein of cell lysate, telomere extension and PCR amplification were performed by following the manufacturer's instructions. The reaction product was resolved on 10% polyacrylamide gel and detected by staining with SYBR Green I (Molecular Probes Inc., Eugene, OR).

2.6. Luciferase assay

Cells were transfected with 2 μg of E2 expression plasmid, 2 μg of E6, E7 or E6/E7 expression plasmid, 1 μg of hTERT reporter plasmid, 0.5 μg of pGreenLantern-1, 0.5 μg of pSVβ and 4 μg of herring sperm DNA as a carrier. At 48 h after transfection, cells were harvested using Reporter Lysis Buffer (Promega Corp., Madison, WI), and then the lysates were subjected to a luciferase assay according to the manufacturer's directions. The activity of β-gal was used to normalize the transfection efficiency of each sample. The same lysates were used to measure β-gal activity with a Luminescent β-galactosidase Reporter system (Toyobo).

2.7. Immunoblotting and antibodies

Nuclear extracts were prepared from transfected cells [35]. The cells were washed once with PBS, collected into microtubes, and resuspended in 400 μl of buffer A (10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 16 μg/ml benzamidine HCl, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM PMSF) for 20 min on ice and mixed with 25 μl of 10% NP40, and centrifuged at 5000 rpm for 5 min at 4 °C. The nuclear pellet was resuspended in 50 μl of buffer C (20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 16 μg/ml benzamidine HCl, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM PMSF) for 20 min on ice and centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was taken as the nuclear extract, and the protein concentration were quantitated using Protein Assay CBB Solution (Nakarai Tesuque Inc., Kyoto, Japan).

The lysates with an equal amount of protein were separated by 15% SDS–polyacrylamide gel electrophoresis, and transferred to a Hybond-P PVDF membrane (Amersham Biosciences UK Ltd, Little Chalfont, UK). Anti-FLAG M5 monoclonal antibody (mAb) (Sigma–Aldrich) was used for the detection of FLAG-tagged proteins. Ab-6 (Oncogene Research Products, San Diego, CA), anti-human Rb 14001A (BD Biosciences Pharmingen, San Diego, CA), anti-p21(C-19), and anti-cMyc (Santa Cruz Biotechnology Inc., Santa Cruz, CA), were used for the detection of p53, pRB, p21Cip1, and c-Myc, respectively. For visualization, a chemiluminescence detection reagent (Roche Diagnostics) was used.

3. Results

3.1. E2 expression caused inhibition of both cell growth and telomerase activity in HeLa cells

HeLa is an HPV18-positive cervical cancer cell line, the growth potential of which is inhibited by the expression of E2 protein encoded by HPV16 [22], HPV18 [26], or BPV1 [20,21,23]. We demonstrated previously that the transcriptional transactivation activity of E2 was essential for the HPV16 E2-mediated growth inhibition by using a long-term colony reduction assay [22], which took several weeks to provide results; during such a long assay period, the level of E2, which was ectopically expressed in the cells, could be altered. The cells might also suffer unexpected modifications such as cytogenetic and epigenetic alterations during this period. Therefore, here we used a short-term growth suppression assay, where the effect of E2 expression on cell proliferation is measured directly by monitoring actual cell numbers and the growth inhibitory effect can be evaluated in less than a week. With the short-term assay, the requirement of the transactivation activity of E2 to inhibit the proliferation of HeLa cells could be confirmed: R37A and I73A mutants, both of which are defective in the transactivation function [22], were also defective in the growth inhibitory effect, whereas the transactivation-competent mutant E39A could inhibit cell proliferation similar to the wild-type E2 (Fig. 1A).

The HeLa cells expressing wild-type E2 showed remarkable growth retardation and an enlarged flatten cell shape, which is typical of senescence-like growth arrest. The induction of senescence was confirmed with a senescence-associated β-galactosidase (SA-βGal) assay (Fig.1B). Senescence is considered to be a sign of cellular aging, and it is proposed that the telomere structure plays an important role in limiting the cellular replicative life span [36,37]. Most somatic cells do not express telomerase activity, thus the telomere region erodes away with every cell division, eventually resulting in the induction of "replicative senescence". In contrast, telomerase activity is upregulated in many cancer cells including HeLa cells, which is considered critical to allow the infinite proliferation of cancer cells. To test whether the regulation of telomerase activity was involved in the induction of senescence in the E2-expressing HeLa cells, the activity was examined using a quantitative telomeric repeat amplification protocol (TRAP) assay (Fig. 2A). A high level of telomerase activity was observed in control cells, and the activity was remarkably reduced by the expression of wild-type E2 and the E39A mutant. The expression of R37A or I73A did not affect the telomerase activity significantly. These results suggested that the reduction in telomerase activity induced by E2 expression was involved in the E2-mediated growth inhibitory effect.

It remained possible, however, that the reduction in telomerase activity was a consequence of the retardation of cell proliferation, as it has been shown that exit from the cell cycle

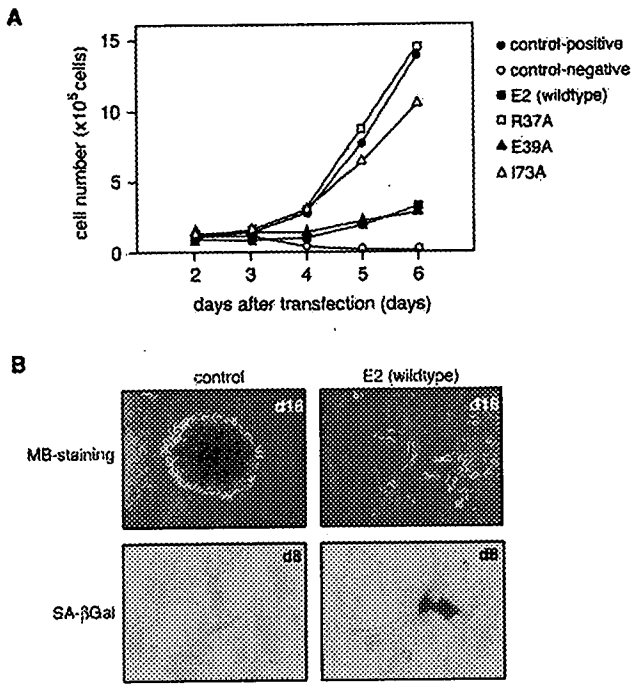

Fig. 1. Growth inhibition of HeLa cells by E2 is associated with senescence and a reduction of telomerase activity. (A) HeLa cells were transfected with the expression plasmid for HPV16 E2 or its mutants (R37A, E39A, and I73A) together with the pPUR selection plasmid, and the cells were maintained with puromycin-based selection. The numbers of cells in the dishes were counted on the indicated days after transfection. Positive and negative controls were the cells transfected with pPUR and pCMV4 and with pCMV4 alone, respectively. (B) HeLa cells were transfected as described in (A). For the SA β-galactosidase assay, the cells were treated with the SA-βGal staining procedure at 8 days after transfection (SA-βGal). At 16 days after transfection, the cells were fixed with ice-cold methanol and stained with methylene blue staining solution (MB-staining). The microscopic images were obtained with a ×40 and ×200 magnification for MB- and SA-βGal staining, respectively.

causes the downregulation of telomerase activity [38–40]. In order to examine this possibility, the telomerase activity in HeLa cells was monitored under other growth inhibitory conditions: double-thymidine block and mitomycin-C treatment. Even though both treatments induced an acute and significant growth inhibition, their effects on the telomerase activity were not significant (Fig. 2B,C). Thus the reduction in telomerase activity was considered not a consequence of growth inhibition, per se, but due to E2 expression in HeLa cells.

3.2. E7 function is essential to rescue both E2-mediated growth suppression and reduction of telomerase activity

E2 expression in HeLa cells suppresses the expression of viral oncoproteins E6 and E7, which has been considered critical for the E2-mediated growth inhibition [23,26]. In order to evaluate the individual role of E6 and/or E7 in the growth potential of HeLa cells, the E6 or E7 expression plasmid was introduced into the E2-expressing HeLa cells and growth potential was monitored with the short-term growth suppression assay as in Fig. 1A. E2 expression in HeLa cells suppresses E6/E7 expression from the integrated HPV18 copies,

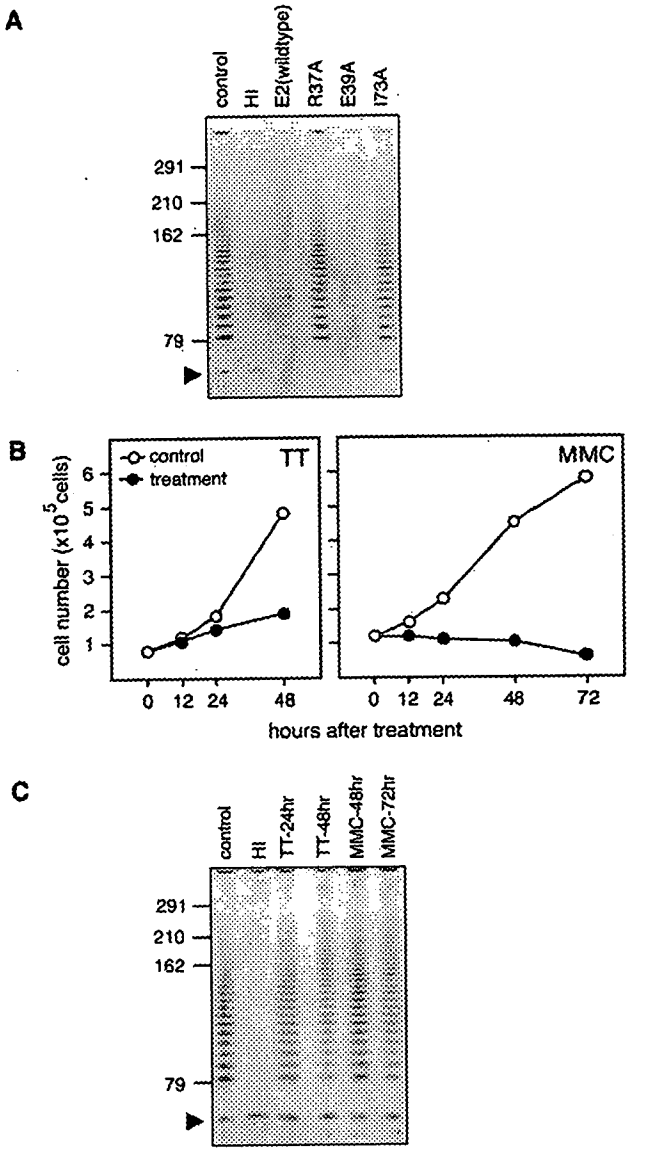

Fig. 2. Telomerase activity in HeLa cells was repressed during E2-mediated growth inhibition. (A) HeLa cells were transfected with expression plasmids for wild-type E2 or the E2 mutants (R37A, E39A, and I73A). Three days after transfection, the cells were harvested and telomerase activity in the cell extracts was analyzed with the TRAP assay. The positive control sample (control) was obtained from the HeLa cells transfected with the pCMV4 empty plasmid and the negative control was the cell extract following heat inactivation treatment (HI). The signal from the internal control for the TRAP assay is indicated by the arrowhead. The positions of DNA size markers are indicated at the left. (B) Growth inhibition of HeLa cells caused by double thymidine block (TT) or mitomycin C treatment (MMC). HeLa cells (1×10^5/dish) were treated with either TT or MMC, and enumerated at the indicated time points after treatment. (C) The telomerase activity of the cells indicated in (B) was analyzed as described in (A). The cells were harvested at the indicated time points after transfection. The positive control and heat-inactivated extracts (control and HI) were prepared as described in (A). The positions of DNA size markers are indicated at the left.

while the expression from the plasmids, which is driven by the cytomegalovirus (CMV) immediate early (IE) promoter, is not affected by E2 expression (data not shown). The growth potential of the E2-expressing HeLa cells could be restored to the control level by complementation with E7, but not E6

(Fig. 3A). In addition, the SA-βGal assay also showed that the co-expression of E7 with E2 could block the senescence while the co-expression of E6 failed to prevent the senescence (data not shown). Taken together, E7 expression seemed to be critical to the proliferation of HeLa cells, while E6 was not required.

To further analyze the role of E7 in rescuing HeLa cells from E2-mediated growth arrest, we used mutant HPV18 E7s (18E7s) which differed in the potential to interact with the pRB family of pocket proteins. The mutation c27g in 18E7 disrupts the LXCXE motif, which is known to be essential to bind pRB and related pocket proteins [8,41], and thus this mutation was shown to abrogate the capacity of E7 to bind pRB and related pocket proteins [42,43]. The low-risk type E7 has less affinity for pRB than the high-risk type, and the residue immediately upstream of LXCXE can be used to distinguish high- and low-risk type E7s as shown by the sequence alignment analysis; an aspartic acid residue at this position in the high-risk E7 proteins is the key to this binding. The mutation d24g decreased the binding capacity of 18E7 to that of a low-risk type [44]. When these mutant E7s were co-expressed with E2 in HeLa cells, E7c27g was almost completely defective in its ability to rescue E2-mediated growth inhibition, whereas E7d24g retained an intermediate level of activity (see Fig. 6A). These results indicated that the ability of E7s to rescue HeLa cells from the E2-mediated growth inhibition correlated with their capacity to bind pRB, suggesting that the inactivation of pRB-related functions by E7 was critical to the maintenance of the growth potential of HeLa cells.

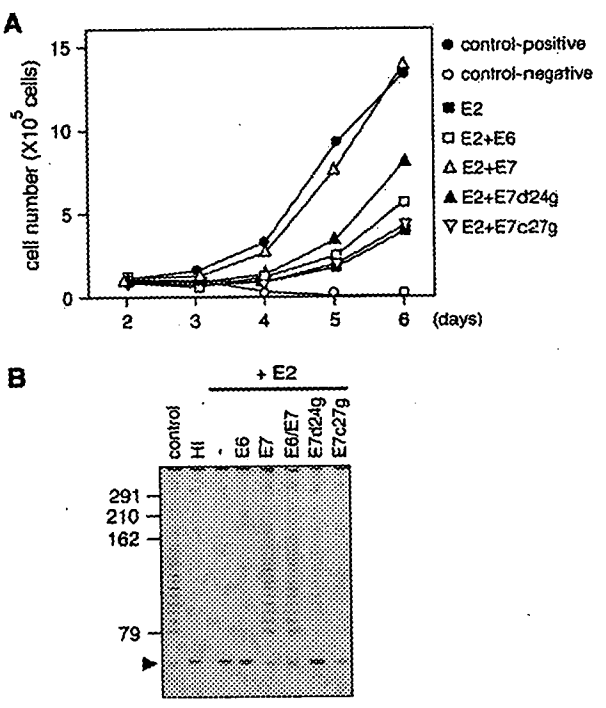

Fig. 3. Growth inhibition by E2 was rescued by E7 expression. (A) HeLa cells were transfected with the E2 expression plasmid in combination with the E6 or E7 expression plasmid. The pPUR selection plasmid was co-transfected and the cells were maintained with puromycin-based selection as described in Fig. 1A. Positive and negative controls were prepared as described in Fig. 1A. (B) The telomerase activity in the cells shown in (A) was analyzed 3 days after transfection. Positive and negative controls (control and HI) were prepared as described in Fig. 2A. DNA size markers are indicated at the left.

In parallel experiments, the intracellular telomerase activity was monitored. As shown in Fig. 3B, E7 expression was also required for maintaining telomerase activity in HeLa cells. The analysis of E7 mutants indicated that the pRB-binding capacity of E7 was also involved in the maintenance of telomerase activity. It was reported that high-risk type HPV E6 contributed to telomerase activity in primary keratinocytes [6,7], which is involved in the immortalization process. Notably, E6 expression was not required for the maintenance of telomerase activity in HeLa cells (Fig. 3B). Taken together, these results suggested that high-level expression of telomerase was associated with the growth potential of HeLa cells, and the effect was mediated by E7 through the inactivation of a pRB-related pathway.

3.3. Effect of E7 on the transcriptional activity of hTERT promoter

Telomerase activity is mainly defined by the expression of human telomerase reverse transcriptase (hTERT), an enzymatic subunit of telomerase [30,45–47]. In order to examine whether the regulation of telomerase activity by E7 was mediated by the modulation of hTERT promoter activity, we employed reporter plasmids, which contained a luciferase reporter gene under the control of a hTERT promoter (Fig. 4A). p3328-GL3 contains the entire hTERT promoter, whereas p181-GL3 contains the core promoter [31]. The expression of E2 significantly suppressed the activity of the hTERT promoter in both reporters. The promoter activity was rescued by the complementation of wild-type E7 expression in the E2-expressing cells, and partially rescued by the E7d24g mutant. Complementation of E7c27g failed to rescue the promoter activity. This result suggested that the inactivation of pRB by E7 contributed to the regulation of hTERT promoter activity. E6 co-expression could slightly upregulate the promoter activity and E6/E7 complementation rescued the hTERT promoter activity more efficiently than the effect of E7. Although the effect of E6 on the hTERT promoter appeared to be additive to the E7 activity in HeLa cells, it might reflect the function of E6 in primary keratinocytes as reported previously [7]. In order to investigate whether the effect of E7 expression on the hTERT promoter is specific to HPV-positive cancer cells, we performed the reporter assay with HPV-negative cell lines, CV1 and 293T. Unlike in the HeLa cells, the expression of E2, E6 and E7 did not affect significantly the hTERT promoter activity in these cell lines (Fig. 4B). Thus the regulation of the hTERT promoter by E7 function seemed to be specific to HeLa cells, or HPV-positive cancer cells, where the cell proliferation is supported by HPV oncoprotein expression.

The hTERT promoter region contains three E box elements and it has been reported that c-Myc contributes to the regulation of the promoter activity by binding to the E-box elements

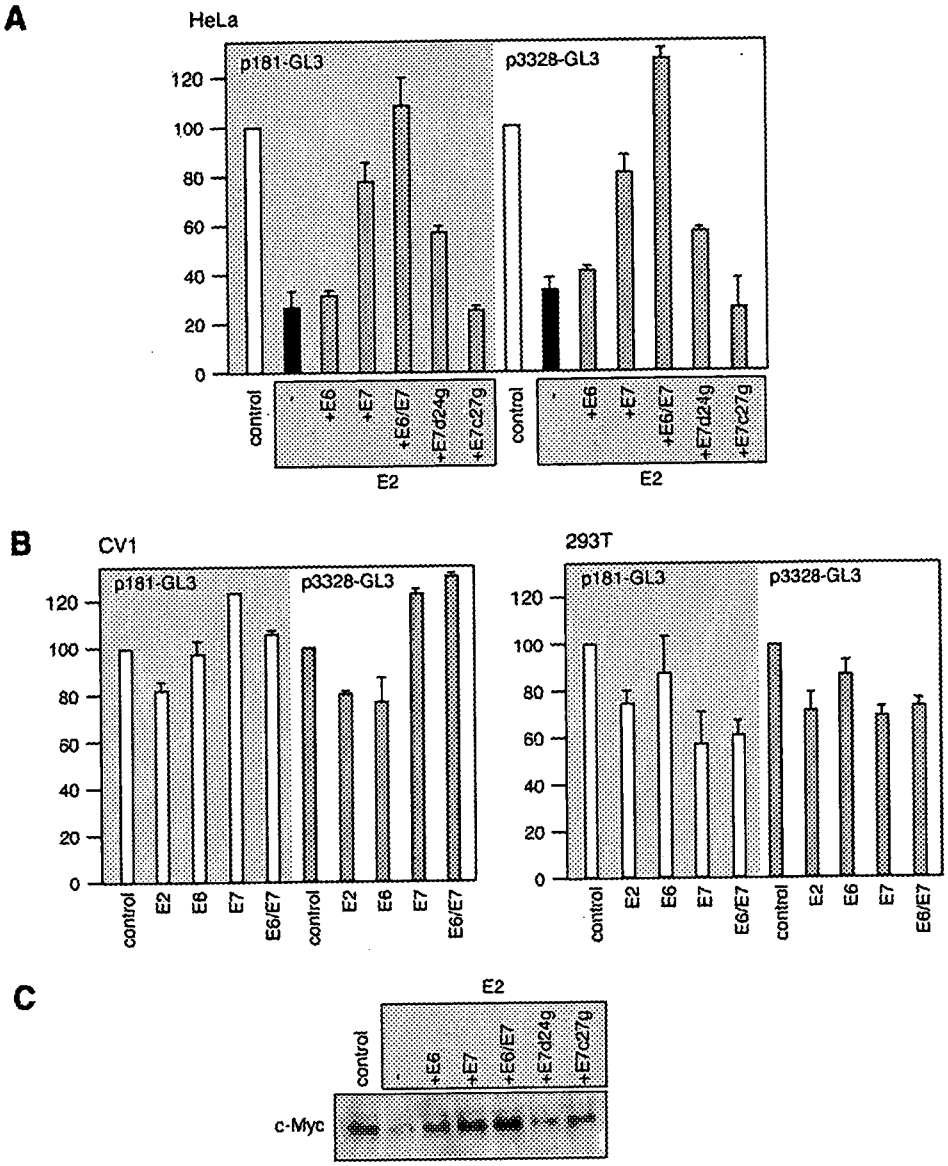

Fig. 4. hTERT promoter activity was repressed by E2 expression in HeLa cells. (A) E2 (2.0 μg) and E6/E7 (2.0 μg) expression plasmids were transfected into HeLa cells with either the hTERT reporter plasmid, p181-GL3 or p3328-GL3. The control sample was obtained by transfection of pCMV4 with the hTERT reporter plasmid. To normalize the transfection efficiency, pSVβ was co-transfected as an internal control. (B) The same analyses shown in (A) were performed with CV1 or 293T cells as indicated. (C) HeLa cells were transfected with expression plasmids in the indicated combinations and c-Myc expression in the cells was monitored by immunoblot analysis using a specific antibody.

[48]. In order to analyze whether c-Myc was involved in the E7-mediated hTERT regulation in HeLa cells, we examined the protein expression level of c-Myc. As reported previously [25,27,49], E2 expression reduced c-Myc expression in HeLa cells, and the complementation of E7 expression could rescue the c-Myc expression to the control level, indicating that E7 was involved in this function. The mutant E7s failed to rescue the c-Myc expression, suggesting that the inactivation of the pRB pathway was involved in this function. On the other hand, E6 could only slightly upregulate c-Myc expression (Fig. 4C). The correlation between the c-Myc expression level and the hTERT promoter activity observed here (Fig. 4A,C) suggested that the promoter activity was regulated mainly through c-Myc expression.

3.4. Complementation of telomerase activity partially rescued E2-mediated growth inhibition of HeLa cells

The results described above indicated that E7 was critical for the maintenance of HeLa cell proliferation and also required for upregulation of telomerase activity, suggesting a functional linkage between cell viability and telomerase activity. As the mean length of the telomere region is close to the threshold triggering senescence in HeLa cells [36,50], it is possible that the upregulation of telomerase activity helps cells to escape the induction of senescence. In order to investigate the contribution of telomerase activity to the proliferation of HeLa cells, we performed a hTERT complementation analysis in E2-expressing HeLa cells. When the hTERT expression

vector was introduced into HeLa cells with E2, the telomerase activity increased even higher than the control level (Fig. 5A). However, the effect of hTERT expression on the E2-mediated growth inhibition was only partial: it rescued the cells from growth inhibition significantly, but not completely (Fig. 5A,B), indicating that the other function(s) of E7 in addition to telomerase activation was required for the HeLa cell proliferation. The result of the SA-βGal assay also showed that hTERT complementation alone was not sufficient to prevent the cells from undergoing E2-mediated senescence (data not shown), which also supported this possibility.

3.5. Involvement of p21Cip1 expression in E2-mediated growth inhibition and senescence

p21Cip1 cdk inhibitor is known to function in the induction of "replicative senescence" [51,52]. Regarding HPV-positive cells, p21Cip1 expression was reported to induce

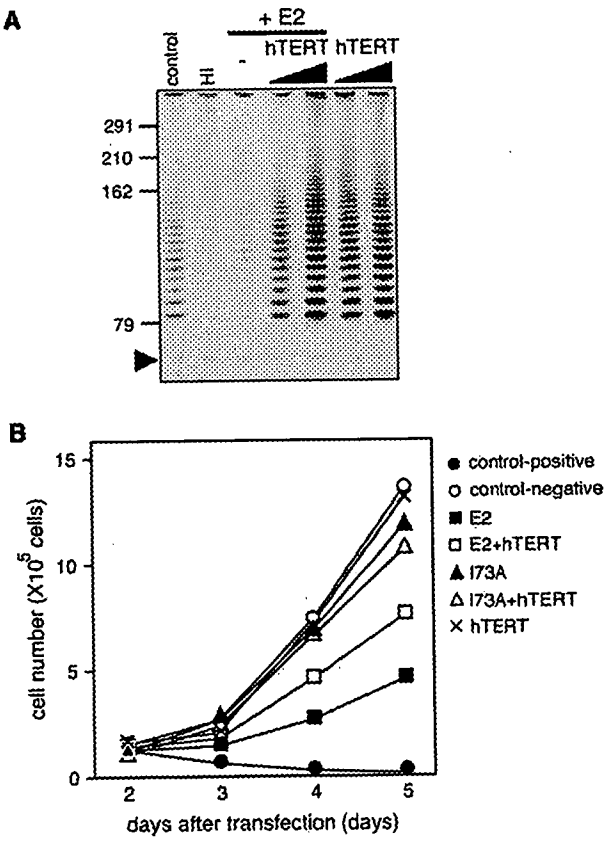

Fig. 5. hTERT expression could partially rescue E2-mediated growth inhibition. (A) The hTERT expression plasmid (0 μg, 0.5 μg, or 2.0 μg) with or without the E2 expression plasmid (2.0 μg) was transfected into HeLa cells and the telomerase activity was analyzed. Positive and heat inactivated controls (control and HI) were prepared as described in Fig. 2A. The sizes of DNA size markers are indicated at the left. (B) The proliferation of HeLa cells transfected with the E2 (wild type or I73A) expression plasmid plus or minus the hTERT expression plasmid was determined as described in Fig. 1A. The E2 expression plasmid (2.0 μg) and hTERT expression plasmid (0.5 μg) were transfected into HeLa cells. The positive control was the HeLa cells transfected with pPUR and pCMV4. The negative control was the HeLa cells transfected with pCMV4 alone.

senescence-like growth inhibition in HeLa cells [26]. In this report, we described that E7 expression was required for the prevention of senescence in HeLa cells. E7 is known to interact and inactivate p21Cip1 in addition to pRB [10,53], which raised the possibility that the reactivation of p21Cip1 caused by downregulation of E7 triggered cellular senescence.

In order to examine this possibility, we analyzed the expression of p21Cip1 as well as p53 and pRB in HeLa cells transfected with various combinations of expression vectors as indicated in Fig. 6A. E2 expression in HeLa cells induced the accumulation of p53 and hypophosphorylated pRB, which was considered a consequence of the E2-mediated suppression of E6/E7 expression (Fig. 6A, lane 2). Co-expression of E6 with E2 reduced the p53 protein level through E6/E6AP-mediated degradation [3]. The p21Cip1 protein level was increased by E2 expression (lane 2) and this effect was counteracted by the co-expression of E6, but not by E7 (lanes 3 and 4, respectively), suggesting that p21Cip1 expression was controlled primarily by p53. Taken that E6 expression could not rescue the E2-mediated growth inhibition and that E7 could rescue it in spite of the high level of p21Cip1 expression, there was no correlation between the p21Cip1 level and the growth potential of the cells under these experimental conditions.

The effects of the E7 mutants, E7d24g and E7c27g, on pRB expression were confirmed to be correlated with their potential to bind to pRB; when transduced with E2, E7c27g, which is defective in binding to pRB, showed a high level of hypophosphorylated pRB (lane 7), while E7d24g, which retains a weakened capacity for pRB binding, showed a more hyperphosphorylated form of pRB (lane 6). Taken together with the result of the growth suppression assay (Fig. 3A), there was a correlation between the accumulation of hypophosphorylated pRB and the inhibitory effect on cell proliferation, indicating that the reactivation of pRB-related function played a pivotal role in the growth inhibition of HeLa cells.

Although p21Cip1 expression might not be essential for the E2-mediated growth inhibition, overexpression of p21Cip1 induced growth inhibition (Fig. 6B) and senescence (data not shown) as did E2 expression. p21Cip1 is a cdk inhibitor and therefore the overexpression was expected to affect the phosphorylation status of pRB. However, HeLa cells with p21Cip1 overexpression showed no difference in the profile of pRB expression compared with the control cells (Fig. 6A, lane 10), which might be due to the E7-mediated degradation of pRB. Contrary to the E2-mediated growth inhibition, p21Cip1 overexpression did not modify either the telomerase activity (data not shown) or hTERT promoter activity (Fig. 6C). These observations suggested that the growth inhibitory effects by E2 and p21Cip1 came through different molecular pathways.

4. Discussion

A unique feature of HPV-associated cancer is that the cancer cells maintain the expression of viral oncoproteins, E6 and E7 [13–15]. Among a variety of functions reported for E6 and E7, the inactivation of tumor suppressor gene products, p53 and pRB, plays a key role in cancer development [54].

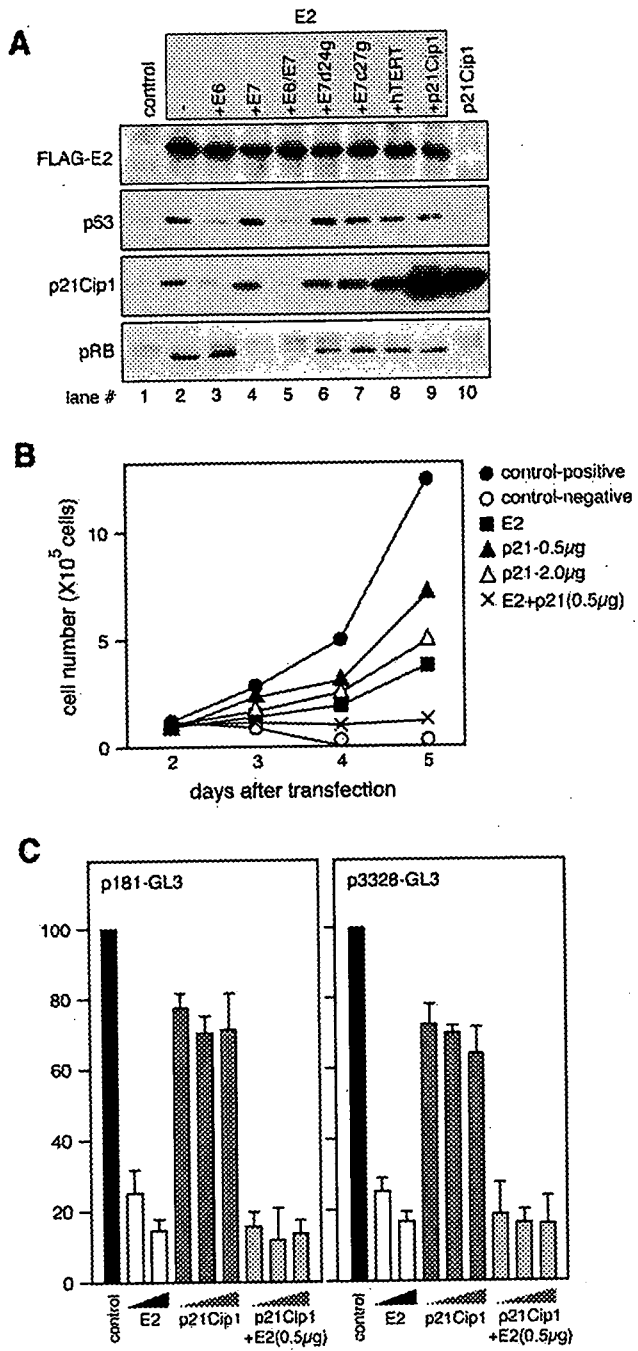

Fig. 6. p21Cip1 expression was not involved in E2-mediated growth inhibition. (A) Protein expression analysis of the HeLa cells expressing E2 alone or in combination with E6 and/or E7, E7 mutants, hTERT, or p21Cip1. E2 was detected with anti-FLAG mAb (M5). p53, pRB, and p21Cip1 were detected using specific antibodies. Please note that E7-mediated pRB degradation caused the reduction in the total amount of pRB. (B) Growth potential of the HeLa cells transfected with E2 and p21Cip1. The positive control was the HeLa cells transfected with pPUR and pCMV4. The negative control was the HeLa cells transfected with pCMV4. HeLa cells were transfected with 16E2 (2.0 μg) and p21Cip1 (0.5 or 2.0 μg) and analyzed as described in Fig. 1A. (C) Effect of p21Cip1 expression on the hTERT promoter. E2 (0.5 μg and 2.0 μg) and p21Cip1 (0.2 μg, 0.5 μg, and 2.0 μg) expression plasmids were transfected into HeLa cells with the hTERT reporter plasmid. Three days after transfection, the luciferase activity in the cell extract was measured as described in Fig. 4. The control sample was obtained with pCMV4 transfection. To normalize the transfection efficiency, pSVβ was cotransfected as internal control.

Inactivation of both tumor suppression pathways disturbs the cell cycle regulatory mechanisms and suppresses the induction of apoptosis, which renders the cells tolerant to the accumulation of mutations and allows the mutant cells to survive. However, the continuous expression of E6/E7 seems dispensable for the viability of well-developed cancer cells, because the cells usually accumulate various mutations during the cancer progression, which might enable the cells to survive independently of the viral gene functions. Still, the fact that most cervical cancer cells maintain E6/E7 expression indicates the requirement of these gene functions for the viability of those cells. Such a possibility is also supported by the reports that the inhibition of E6/E7 expression by either E2 expression, antisense or RNAi causes growth inhibition in HPV-positive cancer cells [18,19,55–57].

4.1. Different contribution of E6 and E7 expression to E2-mediated growth inhibition

In this study, we analyzed the molecular mechanism of the E2-mediated growth inhibition of HPV-positive cancer cells in order to examine the individual contribution of E6 or E7 to the proliferation of cancer cells. The results showed that E2 expression in the HeLa cells caused senescence and a reduction in telomerase activity concomitantly, both of which effects could be revoked by the reintroduction of E7. Analysis of the pRB-binding capacity of E7 mutants revealed that the E7 function to inactivate the pRB pathway was crucial for the maintenance of HeLa cell proliferation and high levels of telomerase activity. This result indicated that a pRB-related pathway might prevent transformation by inducing senescence under physiological conditions. It was also reported that an inappropriate growth stimulus or the irregular activation of a signal pathway induces a response in precancerous cells, which triggers "premature senescence" through a monitoring machinery integrating a pRB pathway [58,59].

Our results indicated that E6 was not required for the growth potential of HeLa cells. On the other hand, there was a report that E6 expression contributed to the growth potential of HeLa cells [27], even though the contribution was minor as compared with that of E7. The discrepancy may be caused by different experimental conditions. For example, they used BPV-1 E2 introduced into a SV40-based recombinant virus while we employed HPV16 E2 inserted in a CMV-based expression plasmid. We used a standard strain of HeLa cells (ATCC no. *CCL-2*) while they used a sub-population of HeLa cells that respond efficiently to the E2-expression. These differences in the experimental setting might cause the different cellular response both in quality and in quantity. Although we did not observe any significant role for E6 in the growth potential of HeLa cells, it was still possible that the inactivation of p53 by E6 was required for long-term survival of cancer cells and for escape from apoptosis induced by the host surveillance machinery in vivo. Wells et al. demonstrated the involvement of E6 in HeLa cell viability by using a long-term colony reduction assay, which supports the possibility that E6 functions in the long-term survival of HeLa cells. E6

is reported to have many biological functions [60], and further investigation will be required to clarify the role of E6 in cancer cells.

4.2. Regulation of hTERT promoter activity by E7

It was a novel finding that inactivation of the pRB pathway by E7 contributed to the activation of the hTERT promoter in HeLa cells. As the pRB tumor suppressor functions mainly through the regulation of the E2F/DP family of transcription factors, E2F/DP was considered to be a reasonable candidate for the regulator of the hTERT promoter. However, it was reported that E2F-binding sites in hTERT promoter had a negative effect on the promoter activity in tumor cells [61,62], suggesting that some other factor was involved in the regulation of hTERT promoter activity through the pRB pathway.

As c-Myc was reported to be involved in the promoter activity by binding to E-box elements [63,64], we examined the expression of c-Myc as another candidate for the regulator of the hTERT promoter and found a correlation between the expression level of c-Myc and hTERT promoter activity. Because *c-myc* is a major target of the E2F/DP family, c-Myc expression could be upregulated with a disruption of the pRB pathway. c-Myc is also reported to contribute to the E6-mediated activation of the hTERT promoter [65,66]. However, there were controversial reports regarding the involvement of c-Myc in the regulation of the hTERT promoter [25,67,68], and further analysis is necessary to clarify the regulatory mechanism through viral oncoproteins.

We found no apparent involvement of E6 in the regulation of hTERT expression in HeLa cells. In contrast, E6 is involved in the upregulation of hTERT promoter activity in primary human keratinocytes [7], for which the E6–E6AP function was reported to be essential [69,70]. Taken together, the regulatory mechanism of hTERT expression might be switched in the course of cancer progression.

4.3. Involvement of telomerase activity in the proliferation of HeLa cells

We found that telomerase activity in HeLa cells was suppressed by E2-expression through modulation of the E7-pRB pathway. Goodwin et al., reported a reduction in telomerase activity in HeLa cells with E2 expression, concluding that the weak telomerase activity did not trigger the senescence of the cells [24]. Our result also indicated that the E2-mediated growth inhibition was not absolutely dependent on the reduction in telomerase activity, because complementation of the activity in the E2-expressing HeLa cells only partially rescued the growth potential (Fig. 5B). The growth inhibition by E2 could be observed as early as 2 days after transfection and senescence-like cells appeared on the same day. Therefore, it is hard to believe that the downregulation of telomerase activity caused telomere shortening and induced senescence in this short period. Goodwin et al. also suggested that telomere length was not a factor in the growth potential of HeLa cells [23,24].

Although a high level of telomerase activity is observed in HeLa cells, the mean length of the telomere region is shortened to about 3.7 kb [50], which is considered close to the threshold needed to trigger "replicative senescence" in primary cells [36]. It is possible that a telomerase-containing complex associates with the shortened telomere region to prevent it from being recognized by the monitoring machinery. The E2 expression decreases the expression of telomerase and the monitoring machinery senses the signal of such a shortened telomeric region and triggers the program of senescence induction.

Telomerase activity was reported to be involved in the maintenance of the telomere structure as well as the control of telomere length. In normal human cells, low-level expression of telomerase activity contributes to the maintenance of the 3′ single-stranded telomeric overhang, and such a function is involved in cell proliferation and the prevention of premature senescence [71]. It is also evidence supporting the partial requirement of telomerase activity for HeLa cell viability as shown in Fig. 5B.

4.4. Mechanisms of p21Cip1- and E2-mediated growth inhibition

The accumulation of p21Cip1 has been known to be involved in senescence [51,52], and Wells et al., reported that the accumulation of p21Cip1 contributed to the growth inhibition and senescence of E2-expressing HeLa cells [26]. We could confirm that p21Cip1 induction by E2 expression is likely to be a consequence of re-activation of the p53 pathway by E6 downregulation. However, the p21Cip1 induction did not appear to be directly involved in the growth inhibition, as the HeLa cells co-expressing E2 and E7 showed high-level expression of p21Cip1 (Fig. 6A) but still retained their growth potential as shown in Fig. 3A. E6 expression appeared not to be essential for the growth potential of HeLa cells as shown in Fig. 3A. We observed that p21Cip1 overexpression caused growth inhibition and senescence of HeLa cells (Fig. 6B), which seemed to function via a different pathway to the E2-mediated growth inhibition. It is also possible that a factor other than the pRB pathway is involved in this mechanism because the pRB pathway is inactivated by E7.

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ORIGINAL ARTICLE

Molecular mechanisms of hyperplasia induction by human papillomavirus E7

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Infections of human papillomavirus (HPV) induce a variety of benign tumors, such as warts and condylomas. During the process of aberrant cell proliferation, genetic mutations are accumulated in the cells, from which malignant tumor cells arise. The viral oncoproteins E6 and E7 are known to help disrupt the cell cycle checkpoint machinery and accelerate chromosomal instability, events which are critical in malignant conversion. However, the mechanisms involved in the hyperplasia caused by HPV infection have remained unknown. We analysed the effects of regulatory genes of HPV18, a typical high-risk-type HPV, on the formation of the epithelial organ by using an organotypic culture system, and found that E7 had potent activity to induce hyperplasia, to which the disruption of the pRb pathway was well correlated. However, analysis with the E7 variants indicated that other pocket proteins are also involved in the activity.
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Keywords: HPV; E7; hyperplasia; pRb; p130; pocket proteins

Introduction

Human papillomavirus (HPV) is a small virus containing an approximately 8 kb double-stranded DNA genome (Howley and Lowy, 2001). Human papillomavirus is a pathogenic virus, which infects basal layer cells in squamous epithelia and induces a variety of benign tumors. Human papillomaviruses are known to show tissue tropism, and are categorized as cutaneous or mucosal. The mucosal HPVs are grouped into high- and low-risk types in accordance with their relationship with cancers. The high-risk HPVs are associated with various mucosal epithelial cancers, such as cervical cancer and pharyngeal carcinoma. Most cervical cancers are associated with an HPV infection, which is therefore considered a major risk factor for cancer development (zur Hausen, 1996). Although cutaneous HPVs are rarely associated with cancers, HPV5 and HPV8 can be classified into the high-risk group because of their relationship with the development of epidermodysplasia verruciformis, a rare skin tumor associated with inherent factors and immune-suppressive conditions (Majewski and Jablonska, 2003; Pfister, 2003).

Human papillomavirus infections induce hyperproliferation, the development of dysplasia, immortalization, the accumulation of genetic mutations in cells and, eventually, malignant phenotypes. Thus, the tumorigenesis induced by HPV infections is an excellent model for multistage cancer development (Boutwell, 1989). The viral oncoproteins E6 and E7 are mainly responsible for the accumulation of genetic mutations and immortalization (Münger *et al.*, 2004), given that (i) they bind to and inactivate p53 and pRb, respectively, which have important roles in cell cycle checkpoints (Dyson *et al.*, 1989; Scheffner *et al.*, 1990, 1993; Huibregtse *et al.*, 1991, 1993); (ii) they are reported to disrupt the processes for eliminating aberrant cells, including apoptosis and senescence (Pan and Griep, 1995; Helt *et al.*, 2002; Iwasa *et al.*, 2003); (iii) E7 is reported to actively promote chromosomal abnormality by inducing the aberrant duplication of centrosomes (Duensing *et al.*, 2001a, b; Duensing and Münger, 2003) and (iv) E6 is critical in the immortalization of cells by reactivating hTERT expression, which abolishes the limitation on the cell's lifespan in corporation with the shutdown of the pRb pathway by E7 (Klingelhutz *et al.*, 1996; Kiyono *et al.*, 1998). The importance of E6 and E7 in the development of cancer is also indicated by the finding that their expression is maintained in most HPV-positive cancers (Baker *et al.*, 1987; Choo *et al.*, 1987; Jeon and Lambert, 1995) and that the viability of cancerous cells is reduced by a suppression of their expression (Dowhanick *et al.*, 1995; Desaintes *et al.*, 1997; Nishimura *et al.*, 2000). Thus, these functions of E6 and E7 render the infected cells tolerant of various genetic mutations, and, consequently, some of the cells obtain malignant phenotypes, although the responsible cellular genes remain unknown.

The acceleration of cellular proliferation through inflammatory responses or organ regeneration is important in cancer development, because it promotes the accumulation of genetic alterations, creating large pools of variant cells, and thus allows cells having malignant

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properties to emerge. The hyperplasia induced by HPV infection is also considered responsible for the viral oncogenesis, although the molecular mechanism involved has not been fully understood. Here, we analysed the effects of regulatory genes of high-risk HPV on the formation of the epithelial organ by using an organotypic culture system (raft culture). The raft culture system can reproduce epithelial morphogenesis *in vitro* and is reported to support the HPV replication cycle (Dollard *et al.*, 1992; Meyers *et al.*, 1992; Frattini *et al.*, 1996), indicating that the system is suitable for the analysis of HPV gene function in hyperplasia. Among the regulatory genes studied here, E7 showed strong activity to induce hyperplasia. Although the involvement of E7 function in hyperplasia formation has already been described in several reports (Blanton *et al.*, 1992; Halbert *et al.*, 1992; Cheng *et al.*, 1995), the index for hyperplasia induction was mostly restricted to the expression of proliferative cell nuclear antigen (PCNA) in the suprabasal layer of epidermis. In contrast to them, our assay model could clearly recapture thickly developed hyperplasia, which makes it possible to analyse the mechanism for hyperplasia induction in detail. The analysis with the E7 variants revealed that multiple functions of E7, including the inactivation of pRb, contributed to the activity.

Results

Introduction of human papillomavirus regulatory genes in human keratinocytes

Human papillomavirus infection induces various benign hyperproliferative lesions at the infected locus. By using a raft culture system, keratinocytes containing high-risk-type HPV DNA were reported to organize hyperplasia-like structure (McCance *et al.*, 1988; Steenbergen *et al.*, 1998), although the molecular mechanism(s) responsible for the hyperproliferation has not been fully understood. In order to identify the responsible factor(s), each of the regulatory genes of HPV18 was introduced into primary human foreskin keratinocytes (HFKs) using a retrovirus-mediated gene transfer, and their effects on cell proliferation were monitored. The growth potential and morphology of the control cells infected with the empty vector (LXSN) were similar to those of the mock-infected cells (data not shown), indicating that the gene transfer procedure did not affect the cellular properties. The growth potential of the cells expressing E6, E7 or both did not differ significantly from the control under the monolayer culture conditions (Figure 1). We observed reduced growth potential in the E4-expressing cells, which showed flattened and enlarged shapes, suggesting that the E4 expression induced G2/M cell cycle arrest (Davy *et al.*, 2002; Nakahara *et al.*, 2002). With the morphological analysis, apoptotic cells appeared in the E5-expressing cell population, which resulted in a retardation of growth as observed with E4-expressing cells (Figure 1). Under the conditions of monolayer culture, the gene responsible for the HPV-induced hyperplasia could not be identified.

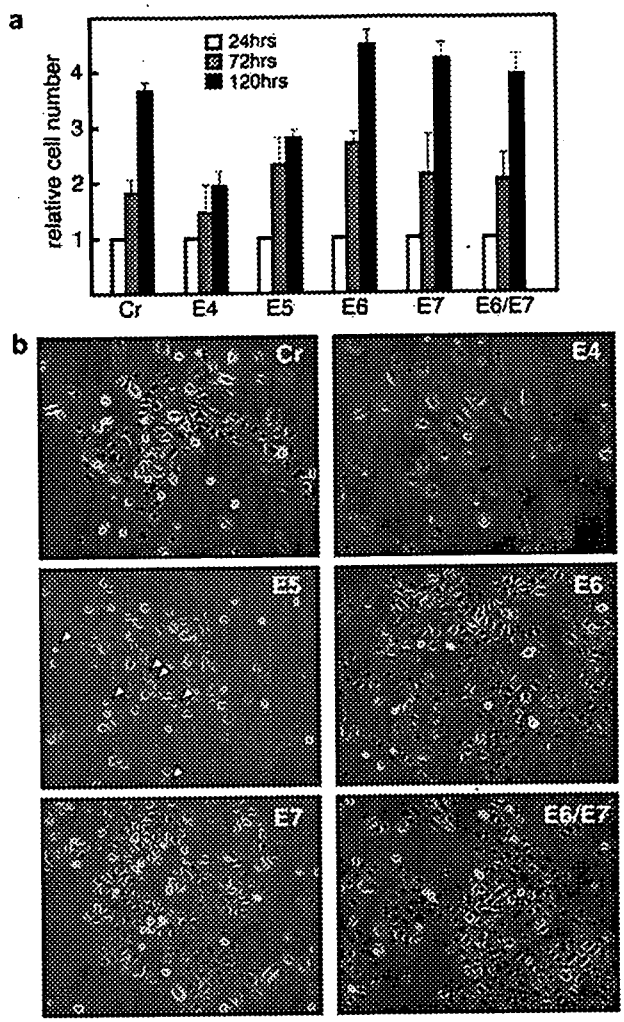

Figure 1 Effects of HPV18 regulatory gene expression in the monolayer culture. (**a**) The effects on cell proliferation. Human foreskin keratinocytes expressing each HPV gene were seeded in multiple 6-cm plastic dishes at a density of 1×10^5/dish. At 24, 72 and 120 h after seeding, the cells in the dishes were counted, and the relative cell numbers (that after 24 h transfection was set as 1) are indicated as a bar chart. The control cells (Cr) used in this and the following experiments were those infected with empty retroviral vector derived from pLXSN. (**b**) Morphology of the HFKs expressing each *HPV18* gene. Phase-contrast images of the cells were taken at 120 h after the seeding in (**a**). The apoptotic-like cells found in the E5-expressing population are indicated with white arrowheads.

Potential of human papillomavirus regulatory genes to induce hyperplasia in the raft culture system

Next, the effects of HPV regulatory genes on the epithelial morphogenesis were analysed by using a raft culture with the keratinocytes expressing the genes. There was no significant difference in the epithelial structure of the raft cultures constructed with the cells infected with the empty vector and the mock-infected cells (data not shown). Among the genes tested here, E7 expression induced a very strong hyperplasia as shown in Figure 2. The E7-induced hyperplasia was reported with transgenic mouse model (Herber *et al.*, 1996; Gulliver *et al.*, 1997), in which clearly thickened

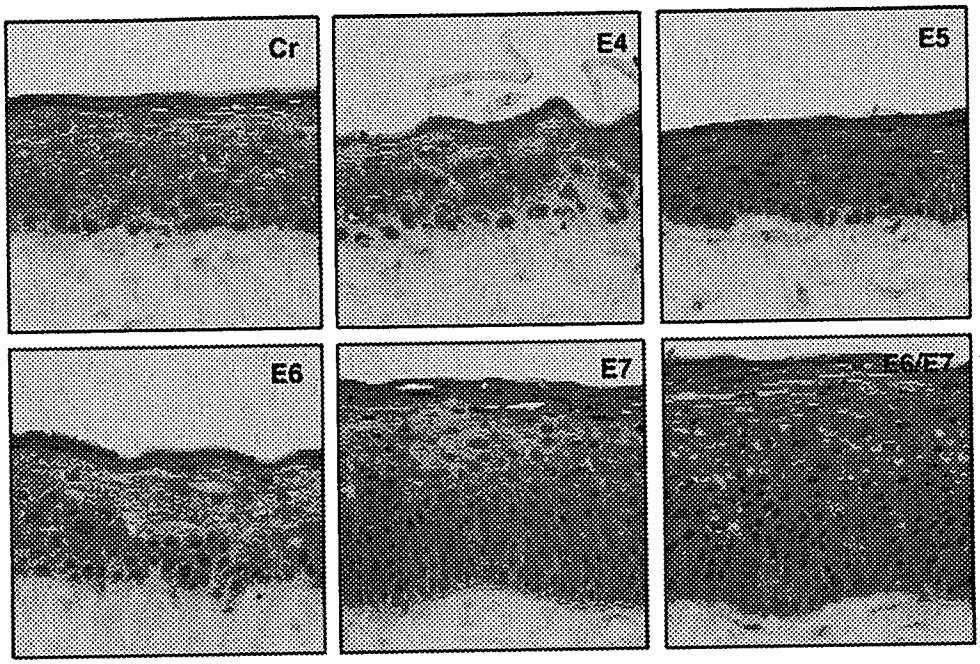

Figure 2 Ability of the HPV regulatory genes to induce hyperplasia. The activity for hyperplasia was analysed with the organotypic raft culture. The HFKs expressing each *HPV18* gene were used to construct the raft culture. The cryosection (7 μm) was fixed with 4% paraformaldehyde and stained with hematoxylin and eosin.

epidermal hyperplasia could be observed. Although the E7-mediated aberrant DNA synthesis in suprabasal layer of epidermis has been reported with raft culture system (Blanton *et al*., 1992; Halbert *et al*., 1992; Cheng *et al*., 1995), those models could capture limited extent of hyperplasia found in the transgenic mouse model. Our assay system makes it possible to analyse the molecular mechanism for hyperplasia formation observed in mouse model under tissue culture condition.

Although E6 was reported to have a potential for hyperproliferation in mouse models (Song *et al*., 1999; Nguyen *et al*., 2003), it had minimal effect on the epithelial structure in this assay. The function of E6 was confirmed by the analysis of p53 expression (Figure 3a and b), in which E6 could reduce the p53 protein level in both the monolayer and raft cultures. E7 significantly upregulated p53 expression, and such effect was counteracted in the cells expressing both E6 and E7, possibly as a result of E6/E6AP-mediated degradation of p53 (Scheffner *et al*., 1990, 1993). The coexpression of E6 and E7 induced a hyperplasia in the raft culture similar to E7 expression, indicating that the modification of p53 level did not affect the hyperplasia formation.

E4 and E5 expression caused a significant reduction in the thickness of the epidermal layer in the raft culture (Figure 2), which could be considered a result of the growth-inhibitory effects by those genes as shown in Figure 1.

Contribution of the inactivation of pRb to hyperplasia

The results described above indicated that the high-risk type E7 had strong activity to induce hyperplasia. It is

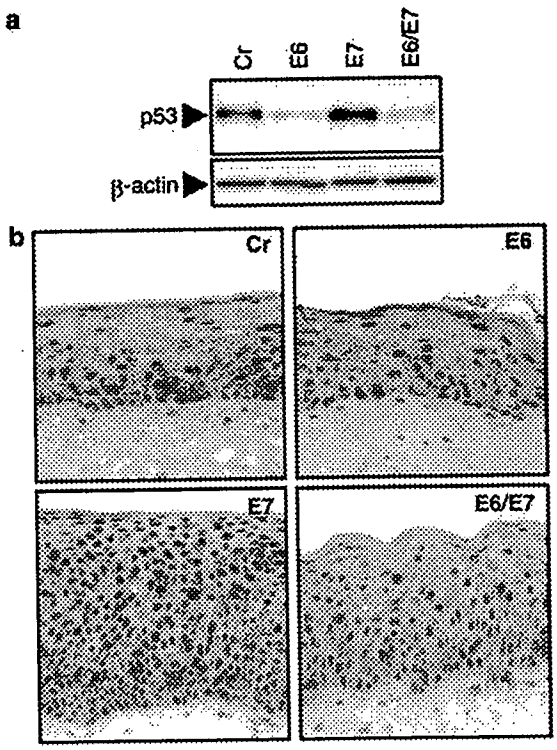

Figure 3 Effect of p53 expression on hyperplasia. (**a**) p53 expression in the HFKs expressing E6 and/or E7 genes was analysed by immunoblotting with a p53-specific monoclonal antibody. The position of p53 is indicated with an arrowhead. β-Actin immunoblot is shown as loading control. (**b**) Immunohistochemical analysis of p53 expression in the raft culture. The p53 expression appears as brownish precipitates of DAB substrates. The immunohistochemical samples were counterstained with hematoxylin.

known that E7 interacts with pRb, one of the major tumor suppressor gene products, and inactivates it (Dyson *et al.*, 1989). With transgenic mouse model, the interaction between E7 and pRb was reported to be critical for induction of epidermal hyperplasia (Gulliver *et al.*, 1997). In order to investigate the relationship between the activities for inducing hyperplasia and inactivating pRb, the effects of a mutant HPV18 E7 (18E7) and a low-risk type E7, HPV11 E7 (11E7), on the formation of the epithelial structure were examined. An E7d24g mutant of 18E7 has a substitution at the adjacent amino acid to the binding motif to the pocket protein family, LXCXE (Dyson *et al.*, 1992; Lee *et al.*, 1998). Aspartic acid and glycine at this position are unique to high- and low-risk E7s, respectively (Figure 4a). The d24g mutation, a substitution of aspartic acid at the position with glycine, converted the high-risk type 18E7 into a low-risk type (Heck *et al.*, 1992).

Each E7 was introduced into HFKs by retrovirus-mediated gene transfer, and then the cells were analysed under the monolayer conditions. These E7s did not exhibit any significant effect on cell proliferation and morphology (data not shown). Wild-type 18E7 reduced the pRb protein level. In contrast, such activity was apparently diminished with E7d24g and 11E7 (Figure 4a).

Next, the raft culture system was constructed with the E7-expressing cells. Although the wild-type 18E7 showed strong activity to induce hyperplasia, such activity was significantly reduced with the mutant and low-risk E7s, suggesting that the inactivation of pRb was critical to the epithelial hyperproliferation (Figure 4b). The inhibitory effect of the wild-type 18E7 on pRb expression shown in Figure 4a could be observed also in the raft culture, and the mutant and low-risk type E7s lost such an effect. The epithelial hyperproliferation induced by pRb inactivation was reported previously with conditional gene disruption in mice (Balsitis *et al.*, 2003). Although the inactivation of pRb could be considered critical in the hyperplasia formation, there remained a possibility that some other activity of E7 was also involved, because the hyperplasia could be observed morphologically with the mutant and low-risk-type E7s, even though their effects were weak in comparison with the wild type. The hyperplasia induced by E7d24g and 11E7 was confirmed by the ectopic DNA synthesis in the upper layer of the epidermis (Figure 4b, BrdU).

Involvement of other pocket proteins in hyperplasia

E7 is known to interact with other pocket proteins in addition to pRb, and 18E7 and 11E7 showed a similar binding affinity for p107 and p130 with *in vitro* binding experiments (data not shown). The effect of E7 on the expression of p107 and p130 was monitored with the monolayer cultures (Figure 5a). Although the p107 expression was not modified, the p130 level was clearly reduced by 18E7. Interestingly, the inhibitory effect on p130 expression was also observed with E7d24g and

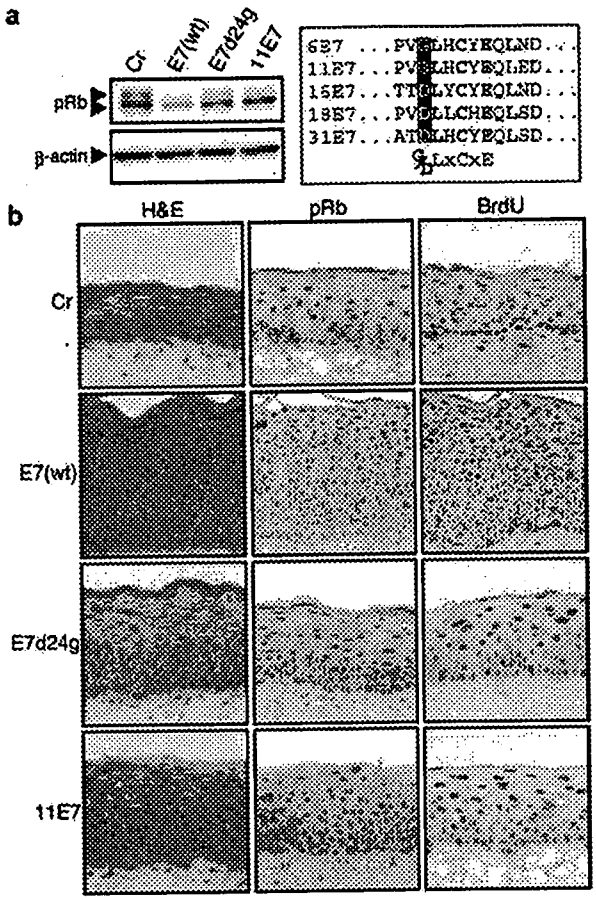

Figure 4 Involvement of pRb repression by E7 in the hyperplasia. (a) pRb expression in the HFKs expressing wild-type E7 (E7(wt)), mutant E7 (E7d24g) and low-risk type E7 (11E7). Arrowheads indicate the positions of pRb. The signal of slow migration represents the hyperphosphorylated form of pRb. β-Actin immunoblot is shown as loading control. The position of risk type-specific amino acid and the pocket protein-binding motif (LxCxE) are shown in an inset. (b) Hematoxylin & eosin (H&E) staining and the immunohistochemical analysis of pRb expression (pRb) and BrdU incorporation (BrdU). The immunohistochemical samples were counterstained with hematoxylin.

11E7. It was reported that p130 was associated with G0 exit of the cell division cycle and cellular differentiation process (Lipinski and Jacks, 1999; Classon and Dyson, 2001). Consistent with the reports, the nuclear expression of p130 was upregulated as the basal cells moved up to the surface layer of the raft culture (Figure 5b, Cr). In contrast, the p130 level was apparently suppressed in the 18E7-expressing raft culture, expression of which could be detected only at the suprasurface. Such suppression was also observed with E7d24g and 11E7, indicating that the suppression of p130 expression by E7 might contribute to the hyperplasia formation by disturbing the differentiation program.

Effect of RNAi-mediated suppression of pocket protein expression on epithelial morphogenesis

The results shown above indicate a possibility that the inactivation of both pRb and p130 by E7 is involved in

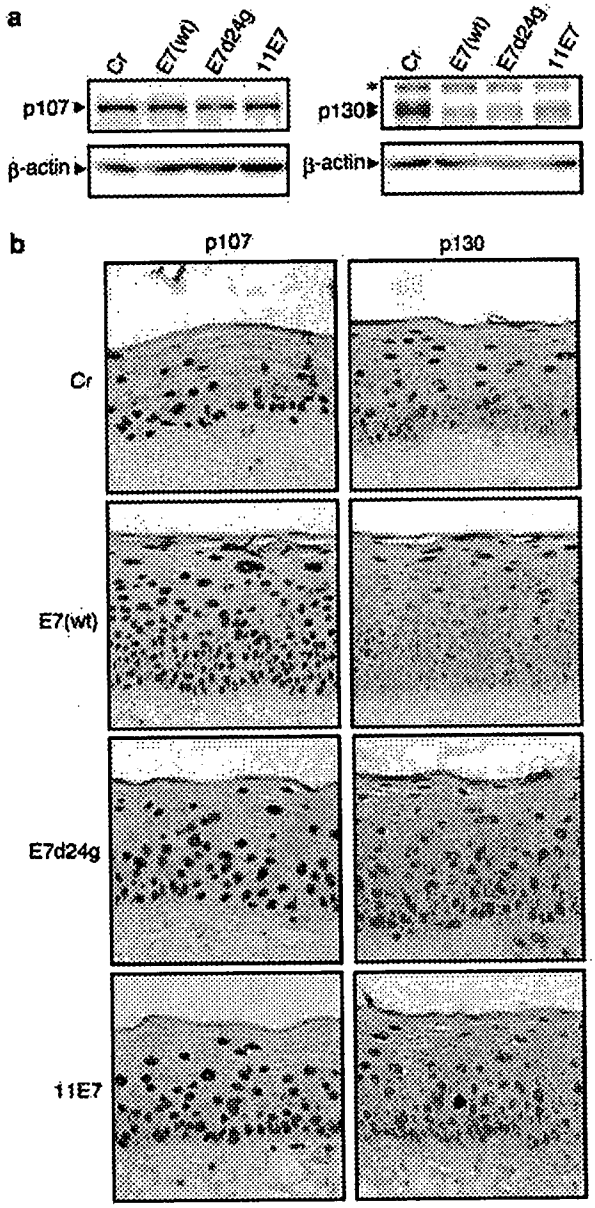

Figure 5 Modulation of p107 and p130 expression in the raft culture. (a) p107 and p130 expression in the HFKs expressing E7(wt), E7d24g, and 11E7. The positions of p107 and p130 are indicated by arrowheads. The signal of slow migration presumably represents the hyperphosphorylated form of p130. The signals indicated with an asterisk are nonspecific. β-Actin immunoblot is shown as loading control. (b) The immunohistochemical analysis of p107 and p130 expression in the raft culture. The immunohistochemical samples were counterstained with hematoxylin.

the hyperplasia formation. However, we could not exclude the possibility that some other function of E7 might be a major responsible factor for the hyperplasia formation, because E7 is known to associate with a variety of cellular events other than the interaction with pocket proteins (Münger *et al.*, 2001). To obtain direct evidence of the involvement of pRb and/or p130 in the hyperplasia formation, we employed RNAi-mediated gene suppression for the pocket proteins.

Three shRNAs were designed for each pocket protein, and those shRNA-expression retroviral vectors were introduced into HFKs. About half of the shRNAs could effectively suppress the pocket protein expression (Figure 6a). The knockdown of pRb expression did not significantly affect the expression of p130, indicating that the suppression of p130 by E7 shown in Figure 5 was not a secondary effect of the downregulation of pRb. The suppression of pocket protein expression did not affect significantly the cell viability and morphology under the monolayer culture condition (data not shown). The effect of the suppression of pocket protein expression on epithelial morphogenesis was analysed by constructing the raft culture (Figure 6b). The pRb suppression induced apparent hyperplasia and ectopic DNA synthesis in upper layer of epidermis. The p130 suppression also induced hyperplasia and ectopic DNA synthesis, even though its effect was relatively mild as compared with the pRb suppression. These results support the hypothesis that wild-type 18E7 induces hyperplasia mainly through the pRb inactivation and that E7d24g and 11E7 cause mild hyperplasia through p130 inactivation. Interestingly, the cells that introduced p107-shRNA displayed thinner epidermal layer and weaker DNA synthesis than the control (Figure 6b, p107-sh1), suggesting that p107 is involved in the growth potential of epithelial cells.

Discussion

The hyperproliferation induced by HPV infection is considered an essential process in the development of cancer. Wide varieties of mutant cells arise from the enlarged cell population, among which a cell having a malignant phenotype can be selected as cancerous. In this report, we proved that E7, one of the oncoproteins of HPV, had strong activity for hyperplasia by using the raft culture system, and indicated that the inactivation of pRb by E7 mainly contributed to that activity. It was also suggested that the downregulation of p130 expression and/or JNK activation were involved in the hyperplasia. Although there were several reports that described the E7-induced hyperplasia using raft culture system (Blanton *et al.*, 1992; Halbert *et al.*, 1992; Cheng *et al.*, 1995), the hyperplasia reported in those reports was moderate as compared with that observed by using transgenic mouse system (Herber *et al.*, 1996; Gulliver *et al.*, 1997; Balsitis *et al.*, 2003). Our assay system well captured the epidermal hyperplasia observed in the mouse model, and it allows to analyse the function of E7 in hyperplasia formation in detail. It is difficult to identify the key factor that determines the difference between our raft culture and the others, because the culture consists of many variable elements, including the condition of primary cells.

The association between pRb and E7 induces the degradation of pRb through ubiquitin-dependent proteolysis (Boyer *et al.*, 1996; Jones and Münger, 1997; Gonzalez *et al.*, 2001), which causes the release of the

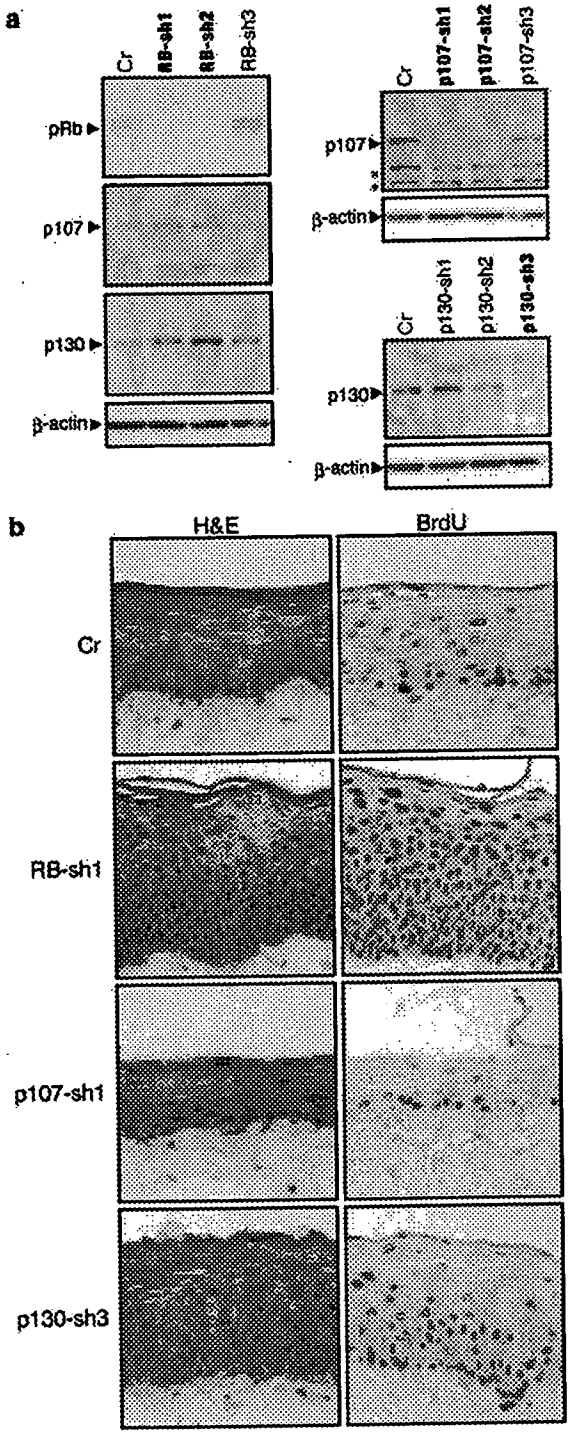

Figure 6 Effects of gene suppression of pocket proteins in the raft culture. (**a**) Effects of shRNA expression in HFKs. The positions corresponding to pRb, p107, and p130 are indicated by arrowheads. The signals indicated with an asterisk are non-specific. The shRNAs that effectively suppress the target gene products are shown with bold typefaces. β-Actin immunoblot is shown as loading control. (**b**) The H&E staining and the immunohistochemical analysis of BrdU incorporation (BrdU). Note that the results with RB-sh2 and p107-sh2 were similar to those with RB-sh1 and p107-sh1, respectively. The immunohistochemical samples were counterstained with hematoxylin.

activated form of E2Fs, resulting in an acceleration of the cell cycle (Chellappan *et al.*, 1992). However, as shown in Figure 1, the E7 expression did not affect significantly the proliferation of the HFKs at low-passage numbers under the monolayer culture conditions, while it induced an apparent hyperproliferation in the raft culture (Figure 2). It is known that the viability of normal HFKs is reduced and the cells enter a replicative senescence at higher numbers of passages, under which conditions the E7 expression is able to suppress the growth arrest and the acceleration of the cell cycle by E7 becomes apparent. The normal keratinocytes of epithelium lose the potential to proliferate and enter the differentiation program when they leave the basal layer. The inactivation of pRb by E7 might allow the cells to retain their proliferative potential after leaving the basal layer, causing the hyperplastic structure in the raft culture. The involvement of pRb–E7 interaction in hyperplasia formation was also reported previously (Gulliver *et al.*, 1997; Chien *et al.*, 2000). However, the pRb expression pattern was not correlated with the cellular proliferation in the raft culture of normal HFKs (Figure 4b, Cr), suggesting that the function of pRb in cell proliferation may not be critical to epithelial development. In addition to its regulatory effect on cell proliferation through its association with E2Fs, pRb is reported to have a role in the differentiation process, which is independent of E2Fs (Lipinski and Jacks, 1999; Ruzinova and Benezra, 2003) and the disruption of which by E7 might be a key mechanism behind the hyperplasia. The importance of pRb in the normal development of epithelium is indicated by a report that a conditional gene targeting experiment caused keratinocyte hyperproliferation in the epithelia of the mouse (Balsitis *et al.*, 2003). It will be necessary to investigate the regulatory mechanism of the growth and differentiation of epithelial cells mediated by pRb function.

The result that the wild-type 18E7 induced the strongest hyperplasia in the raft culture indicated the importance of pRb's inactivation in epithelial hyperplasia (Figure 4b). However, the E7s in which such activity is attenuated, E7d24g and 11E7, still had significant activity to induce hyperplasia, indicating that some other activity of E7 is involved. This was supported by the observation in the report mentioned above, in which the E7-induced augmentation of hyperplasia was observed in pRb-deleted tissues (Balsitis *et al.*, 2003). The hyperplasia induction by low-risk-type E7 was previously reported (Cheng *et al.*, 1995), although there was a controversial paper (Halbert *et al.*, 1992). It is well recognized that E7 can associate with the other pocket proteins, p107 and p130. The latter is considered to play a role in the transition into the G0 phase of the cell cycle and the induction of cellular differentiation (Graña *et al.*, 1998; Lipinski and Jacks, 1999; Classon and Dyson, 2001; Classon and Harlow, 2002). Consistent with this, p130 was detected in the nucleus in the differentiated layer in the raft culture with the normal HFKs. The results shown in this report that the wild-type E7 expression as well as the mutant and low-risk

type E7s could suppress the p130 expression raised the possibility that the modulation of p130 function by E7 contributes to the aberrant differentiation and hyperplasia. The possibility was strengthened by the gene suppression experiments using shRNAs (Figure 6), in which the involvement of pRb and p130 in the epidermal organization could be observed in the raft culture.

The action mechanism of E7 in the hyperplasia found in this report is illustrated in Figure 7. While E7 expression accelerates the cell cycle through the inactivation of pRb, it inhibits the induction of differentiation through downregulation of p130, making the cells replicative and undifferentiated in the upper layer of the epidermis. It may be worth of note that the downregulation of p107 reduced the potential of cell proliferation at the basal cell layer. Taking into account the fact that E7 affects the expressions of pRb and p130 but not of p107 seems to be rational, because the DNA synthesis potential of the epithelial cells should be maintained for the HPV replication. Although p107 has been known to interact with the inhibitory E2Fs, E2F4 and E2F5, its role in cell proliferation and differentiation remains unclear. Our model system might reveal a novel biological activity of p107. Our result indicates that 18E7 could suppress the expression of pRb and p130, but not of p107. In contrast, reported that 16E7 inhibited the expression of all pocket proteins. Although we did not investigate the factor(s) involved in this discrepancy, it might result from the difference of the cells used in the experiments. Recently, reported the effect of high- and low-risk HPV E7 on the expression of pRb family member, the result shown in which is consistent with ours with regard to low-risk E7 (Zhang *et al*., 2005).

It has been reported that many cellular factors interact with E7 and that some pRb-independent function of E7 is associated with the transformation activity (Münger *et al*., 2001). E7 is known to associate with p21 and p27 independently of the E7–pocket protein interaction, which might modulate the cell proliferation and differentiation program. It was also indicated previously that E7 expression modified the downstream pathway of the TNFα receptor (TNFR) in HFKs and a human fibroblast cell line (Basile *et al*., 2001; Thompsom *et al*., 2001), which is critical in the regulation of cell proliferation and apoptosis through the modification of NFκB activity and the activation of MAPKs, including JNK. With regard to the epidermal hyperplasia, there was a report that the activation of JNK in RelA$^{-/-}$ epidermis of mouse was causative for the hyperproliferation of keratinocytes (Zhang *et al*., 2004). We have a preliminary result that 18E7 expression suppresses NFκB signaling upon the activation of TNFR pathway, suggesting a possibility that the E7 expression causes similar effect on epidermal organization, as observed with RelA knockout mice. It is necessary to take into account the various biological activities of E7 for full understanding of the mechanisms of the E7-mediated hyperplasia formation.

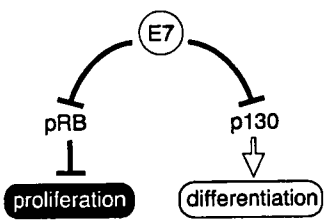

Figure 7 A model for E7-mediated hyperplasia. E7 accelerates the cell proliferation through the inactivation of pRb. E7-mediated downregulation of p130 suppresses the cell differentiation program. The inactivation of pRb might be also involved in the attenuation of cell differentiation (see Discussion).

This report indicates the possibility that E7 is critical to the hyperplasia in HPV-infected lesions. The result showed that the high-risk-type E7 had stronger potential to induce hyperplasia than the low-risk-type E7 and the difference might be ascribable to their activities to inactivate pRb. However, it is considered that the hyperplasia by the low-risk HPV infection is more severe than that caused by the high-risk type, as seen in condylomas and warts, suggesting that the HPV-induced hyperplasia is mediated also by other gene functions. E6 was reported to activate the proliferation of epithelial cells, for which the PDZ-binding motif at the C-terminus was critical (Nguyen *et al*., 2003). Although some aberrant DNA synthesis could be observed with the E6-expressing raft culture (data not shown), the E6 expression did not significantly induce hyperplasia as shown in Figure 2, indicating that the contribution of E6 to the hyperplasia might be trivial. E5 is a major transforming gene in the bovine papillomavirus, which activates cellular proliferation by inducing PDGF receptor and/or EGF receptor activation (Howley and Lowy, 2001). As shown in Figure 1, 18E5 induced an apoptotic response in the cells, resulting in the suppression of cell viability. The activity was, however, cell-type dependent; 18E5 occasionally induced senescence-like growth arrest with other cell types (data not shown), indicating that the cellular protective response might be triggered for escaping from the aberrant stimulus induced by E5. Recently, hyperplasia induction by HPV16 E5 was reported with transgenic mouse model (Genther Williams *et al*., 2005), indicating a possibility that such activity of E5 can be reproduced with the raft culture system. On co-expression with E6 or E7, the growth acceleration by E5 might become evident by avoiding apoptosis and senescence. The assay system used in this report seems to be suitable for analysis of HPV-induced hyperplasia in depth, and it will be interesting to analyse the effects of various combinations of HPV genes on hyperplasia.

Materials and methods

Construction of plasmid DNAs

DNA fragments containing each regulatory gene of HPV18 were isolated by a polymerase chain reaction (PCR) with a full-length HPV18 clone (GenBank Accession number X05015) as the template, and then transferred into the vector

pLXSN (BD Biosciences Clontech, Palo Alto, CA, USA). The *E7* gene of HPV11 (11E7) was also obtained by PCR with a full-length HPV11 clone (GenBank, M14119). The mutation d24g, designed to substitute a codon for aspartic acid (GAC) with that for glycine (GGG) at position 24 in 18E7, was introduced by PCR-mediated site-directed mutagenesis (Cormack, 1987). At the 5′-terminus of each gene, a Kazak consensus sequence was introduced by PCR to optimize translation efficiency. The retroviral packaging plasmid, pCL10A1 (IMGENEX Corp., San Diego, CA, USA), and green fluorescence protein (GFP)-expression plasmid, pGreen-Lantern-1 (Invitrogen Corp., Carlsbad, CA, USA), were obtained commercially.

Cell culture and transfection

Human foreskin fibroblasts (HFFs) and HFKs were purchased commercially (KURABO Industries, Ltd, Osaka, Japan). 293T cells and HFFs were maintained with Dulbecco's-modified MEM (DMEM) supplemented with 10% fetal bovine serum (FBS), and HFKs were maintained with serum-free keratinocyte growth medium (KGM) (EpiLife-KG2, KURABO Industries, Ltd, Osaka, Japan) in 5% CO_2 at 37°C. 293T cells were transfected by a standard $CaPO_4$ co-precipitation method (Sambrool *et al.*, 1989b). The transfection efficiency was monitored using GFP, which was expressed from the co-transfected GFP-expression plasmid, pGreenLantern-1.

Production and infection of retroviral vector

The pLXSN-based gene expression plasmid (3.5 μg), pCL10A1 (1 μg), pGreenLantern-1 (0.5 μg) and herring sperm DNA (5 μg) were introduced into 293T cells by transfection. At 2 days after the transfection, the culture medium was replaced with fresh KGM and the retroviral vector was collected in the medium for 5 h (hrs). The vector-containing KGM was clarified through a 0.45-μm-pore filter and used to infect the HFKs. For the infection, the old KGM was removed and the virus-containing KGM was overlaid on the HFKs. The cells were maintained for 6 h in CO_2 incubator, and then the medium was changed to fresh KGM. At 24 h after infection, the cells were spread at a low density. G418 (80 μg/ml) was added to the medium at 48 h after infection for 2 days, after which the cells were maintained in 40 μg/ml G418/KGM. Using this selection procedure, the mock-infected cells were eliminated at 4 days post-infection. Usually, the multiplicity of infection was more than one.

Organotypic raft culture system

The organotypic raft culture system was constructed as described previously (Tsunenaga *et al.*, 1994). For preparation of the dermal equivalent, one part of type I collagen (Cell Matrix type I-P, Nitta Gelatin Co. Ltd, Osaka, Japan) and two parts of growth medium containing HFFs (1×10^6 cells) were mixed, while cooling, poured into a 6-cm dish, and then the cells were maintained in 5% CO_2 incubator at 37°C until the collagen gel became contracted to 2 cm diameter. The gel was soaked in fresh KGM for several hours, and then transferred in a trans-well insert (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The insert was put in a 6-well plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA), and fresh KGM was filled both in the bottom and insert. Human foreskin keratinocytes (1×10^6 cells) were overlaid on the gel (day 0). At day 1, the medium was changed to a mixture of KGM and DMEM growth medium (KGM:DMEM = 1:1), and 24 h later the medium was changed to the same mixture, adjusting the $CaCl_2$ concentration to 1.8 mM. At day 3, the surface of the collagen gel was exposed to air, and then the medium in the bottom was changed to fresh medium every day. Multilayered cultures of keratinocytes were obtained at day 10. For BrdU incorporation, 50 μg/ml BrdU (Sigma-Aldrich Co., St Louis, MO, USA) was added in the medium 6 h before harvest.

Immunoblot analysis

Total cell lysate of HFKs was obtained with a triple-detergent buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecylsulfate (SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate (Sambrool *et al.*, 1989a) supplemented with a protease inhibitor cocktail (0.5 mM PMSF, 0.15 μM aprotinin, 1 μM E-64, 1 μM leupeptin, 0.5 μM EDTA) (Nakarai Tesuque KK, Kyoto, Japan) and 1 mM dithiothreitol. Equal amounts of cell lysate (5 μg protein) were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and the gel was blotted to a PVDF membrane (Hypond-P, Amersham Biosciences UK Limited, Little Chalfont, UK). The equalities of the loaded amounts were confirmed with the anti-β-actin immunoblot (clone AC-15) (Sigma-Aldrich Co., St Louis, CO, USA) (data not shown). Horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Biosciences UK Limited, Little Chalfont, UK) and a luminal reagent (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were purchased commercially.

Immunohistochemical analysis

The specimens of raft culture were embedded in OCT compound (Sakura Finetechnical Co., Ltd, Tokyo, Japan), and thin sections (7 μm) were obtained by cryo-sectioning (Cryo-Star HM560M, MICROM Laborgeräte GmbH, Walldorf, Germany). The sections were transferred onto slide glasses, dried, fixed with 4% paraformaldehyde, and then treated with the target retrieval solution (Target Retrieval Solution, Dako Japan KK, Kyoto, Japan). The endogenous peroxidase activity was quenched with 0.3% H_2O_2/MeOH after the target retrieval process. Antigen detection was performed with the TSA-biotin system (Perkin-Elmer, Boston, MA, USA) following the manufacturer's instructions. Chromogenic detection of HRP was performed with metal-enhanced diaminobenzidine (DAB) substrate (Roche Diagnostics GmbH, Mannheim, Germany). Antibodies for p53 (Ab-6) (Oncogene Research Products, San Diego, CA, USA), pRb (BD Biosciences Pharmingen, San Diego, CA, USA), BrdU (clone 2B-1)(MBL, Nagoya, Japan), P107 (C-18), p130 (C-20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were purchased commercially. The HRP-conjugated secondary antibodies were also commercially available (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

RNAi-mediated gene suppression

The designed sequences of shRNA for the pocket proteins are the following. pRb-sh1: GAT ACT AGA TCG TGT CAG ATT CAA GAG ATC TGA CAT GAT CTG GTA TCT TTT TTA TCG AT; pRb-sh2: GTT GAT AAT GCT ATG TCA ATT CAA GAG ATT GAC ATA GCA TTA TCA ACT TTT TTA TCG AT; pRb-sh3: GGT TCG ACT ACG TGT GTA ATT CAA GAG ATT ACA CGC GTA GTT GAA CCT TTT TTA TCG AT; p107-sh1: ATC GTT CCT TCT TCG AGC ATT CAA GAG ATG CTT GAA GAA GGA GCG ATT TTT TTA TCG AT; p107-sh2: AGG GTG TCA TGG AGG GCA ATT CAA GAG ATT GCC TTC CAT GAT ACC CTT TTT TTA TCG AT; p107-sh3: GGG GGT CTT CGA GGA GAT ATT CAA GAG ATA TCT CTT CGA AGA CTC CCT TTT TTA TCG AT; p130-sh1: GCC AGC GGT AGT AAT

GGA CCT TCA AGA GAG GTC TAT TAC TAC TGC TGG CTT TTT TAT CGA T; p130-sh2: ATA CCC TCG AGA GGG GCA ATT CAA GAG ATT GCT CCT CTT GAG GGT ATT TTT TTA TCG AT; p130-sh3: GGT TCG ACA ACG TGT GTA ATT CAA GAG ATT ACA CGC GTA GTT GAA CCT TTT TTA TCG AT. For each shRNA, top and bottom oligonucleotides were synthesized and the annealed oligonucleotides were inserted into pSIREN-RetroQ vector by following the manufacturer's protocol (BD Biosciences Clontech, Palo Alto, CA, USA). The shRNA-expressing retrovirus vectors were produced as described in the section *Production and infection of retroviral vector*.

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