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Low p53 Level in Immortal, Non-tumorigenic Oral Keratinocytes Harboring HPV-16 DNA

Sheng-Lin Li, Myong Soo Kim, Henry M. Cherrick and No-Hee Park

The p53 protein level was determined in normal oral keratinocytes and two non-tumorigenic, immortal oral keratinocyte lines harboring human papillomavirus-16 (HPV-16)DNA. The p53 mRNA level in the immortal cells was higher than the normal counterpart, but the p53 protein level was notably lower in the immortalised cells. The half-life of p53 protein in the normal and immortal cells was <1 h, and the p53 cDNA sequence of these cells showed no mutation. The immortal cells transcribed a high amount of E6/E7 mRNA encoded by HPV-16, but normal cells did not. These observations suggest that the immortal keratinocytes may translate normal level of wild-type p53 protein, and the low p53 level in these cells may be due to the enhanced degradation of the protein by HPV-16 E6 protein.
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INTRODUCTION

CANCER CAN arise from an inactivation of tumour suppressor genes [1]. Among tumour suppressor genes, the p53 gene has been most extensively studied. The p53 gene was initially considered as an oncogene, because several studies have described that transfection of primary cells with plasmids expressing p53 immortalises them and converts immortalised cells to tumorigenic cells in concert with activated *ras* [2, 3]. Further, p53 expression vectors enhance carcinogenicity of weak tumorigenic cell lines and increase the metastatic potential of cancer cells [4]. Such observations indicate that p53 is an oncogene similar to the *myc* gene. Interestingly, the plasmids employed in such studies encode mutant p53 protein [5]. The p53, a nuclear phosphoprotein, appears to be a negative regulator of cell proliferation and the p53 gene seems to function as a tumour suppressor gene [6]. Mutations in p53 result in not only loss of the suppressor activity associated with the gene product, but also induce expression of mutant p53 protein which may well be intrinsically oncogenic. In fact, mutations of the p53 gene are present in most cancer subtypes, including human lung cancinomas [7], colon carcinomas [8], hepatocellular carcinomas [9], cervical cancer cells [10], bladder cancer [11], and oesophageal cancers [12]. The protein expressed from the mutant p53 gene has been implicated in growth deregulation and malignant progression of these cancers.

Human papillomavirus (HPV) infection is closely associated with the development of female genital epithelial cancers: Over 90% of cervical cancer biopsy specimens contain HPV DNA [13]. Of the more than 60 genotypes of HPV, type 16 (HPV-16) and type 18 (HPV-18) are most frequently associated with malignant genital lesions [14]. Similarly, HPV infection is also closely linked to benign and malignant oral lesions [15]. Recent studies show that up to 40–50% of oral cancer biopsy specimens contain the viral DNA (Personal communication with Dr Edward Shillitoe, University of Texas, Houston, Texas).

Inasmuch as the oral mucosal epithelium resembles the female genital tract and is continuously challenged by innumerable environmental influences, close association between HPV infection and oral malignancies is not surprising. Further evidence of HPV's role in carcinogenesis derives from its *in vitro* transforming capacity. Transformation of human skin fibroblasts and keratinocytes was established with cloned HPV-16 and HPV-18 DNA transfection [16, 17]. More recently, our laboratory transformed normal oral keratinocytes—one of the major *in vivo* target cells for HPV infection—with cloned HPV-16 and -18 DNA [18, 19]. These transformed cells are immortal, contain HPV DNA integrated into the cellular genome, and express viral mRNAs, but they are non-tumorigenic in nude mice.

Several studies have shown that HPV-16 and HPV-18 E6 proteins complex and promote the degradation of cellular p53 protein *in vitro* [20, 21], indicating a close association between the function of p53 protein and HPV-associated tumorigenesis. Confirmation of this observation in an *in vivo* system is absolutely necessary for further understanding the association of HPV infection and p53 protein in oral carcinogenesis. We thus investigated the p53 protein level, p53 transcription, and the sequence of p53 cDNA from normal human oral keratinocytes (NHOK) and two immortal, non-tumorigenic human oral keratinocytes lines (HOK-16A and HOK-16B) harboring HPV-16 DNA [18]. The study shows that the p53 mRNA level is higher in the immortalised keratinocytes compared with the normal counterpart, whereas the p53 protein level was notably lower in the immortal cells. The half-life of p53 in the normal and immortal cells is <1 h. The p53 cDNA sequence of these cells showed no mutation. However, only the immortal oral keratinocytes contained viral E6/E7 mRNAs encoded by HPV-16. These data indicate that the immortal keratinocytes may translate normal level of wild-type p53 protein, and the low p53 level in these cells may due to the enhanced degradation of the protein by HPV-16 E6 protein.

MATERIALS AND METHODS

Cells

Normal human oral keratinocytes (NHOK) were prepared as described previously [18]. To separate the epithelium from

Correspondence to No-Hee Park.
The authors are at the UCLA School of Dentistry, 43-033 CHS, 10833 Le Conte Avenue, Los Angeles, California 90024, U.S.A.
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the underlying submucosa, excised retromolar tissue from the oral cavity was treated with collagenase (type II; 1.0 mg/ml, Millipore Corp.) and dispase (grade II; 2.4 mg/ml, Boehringer-Mannheim) for 60 min at 37°C. Separated epithelial sheets were then dissociated into single cells by incubation in trypsin with agitation at 37°C for 30 min. The cells were washed with phosphate buffered saline (PBS), resuspended with keratinocyte growth medium (KGM; supplemented with pituitary extract, Clonetics Corp., San Diego, California), and plated on plastic at a density of 5×10^4 cells per 28 cm^2 (60 mm Petri dish). The confluent primary cells were subcultured to generate the tertiary cells that were employed in the present study.

Two human oral keratinocytes lines (HOK-16A and HOK-16B) immortalised by transfecting the primary cells with recombinant HPV-16 DNA were cultured in the keratinocytes growth medium (KGM, Clonetics, San Diego, California) [18]. The HOS cell line (American Type Culture Collection, Rockville, Maryland), derived from a human osteosarcoma and expressing mutant p53 protein [22], was grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% bovine serum.

Northern blot hybridisation analysis

Cytoplasmic poly(A^+)RNA was extracted from cells by using standard procedures to determine the transcription of p53, HPV-16 E6/E7, and β-actin gene [18]. The 1.2 kbp fragment (nucleotides 24-880, 3357-3820) representing the major early HPV-16 message including E6/E7 genes (from Dr J. Doniger, Georgetown University, Washington, DC), p53 cDNA (from Dr E. Harlow, Massachusetts General Hospital Cancer Center, Charlestown, Massachusetts), and the human β-actin gene (from Dr L. Kedes; Stanford University, Palo Alto, California) were used as probes for the northern analysis. These probes were labelled with ^{32}P by using the multiprime labeling system (Amersham Corp., Arlington Heights, Illinois), and the specific radioactivities of labelled probes were always higher than 5×10^8 cpm/μg of DNA.

Five micrograms of poly(A^+)RNA were denatured and run in 1.2% formaldehyde agarose gel with marker RNAs (9.5, 7.5, 4.4, 2.4, 1.4, and 0.24-kilobase (kb) RNA ladder, Bethesda Res. Lab., Gaithersberg, Maryland). The RNA was transferred to a nylon filter (Amersham Corp., Arlington Heights, Illinois) and were crosslinked by exposing the filter to ultraviolet light for 5 min. The filter was hybridised to ^{32}P-labelled probe at 42°C for 24 h in 50% formamide/10% dextran sulphate/0.5% sodium dodecyl sulphate (SDS)/5× SSPE (0.15 mol/l NaCl/0.01 mol/l Na_2HPO_4/0.001 mol/l EDTA)/5× Denhardt's solution/denatured salmon sperm DNA (20 μg/ml). After the hybridisation the filter was washed in 5× SSPE for 15 min at 42°C, in 1× SSPE/0.1% SDS for 30 min at 42°C, and in 0.1X SSPE/0.1% SDS for 30 min at 42°C. The filter was then autoradiographed on SB-5 X-ray film (Eastman Kodak, Rochester, New York) for 12 h at −70°C. After exposure the probe was stripped from the filter for rehybridisation.

Immunoprecipitation analysis

Cells were cultured in 60 mm Petri dishes to 80% confluency using keratinocyte growth medium (KGM, Clonetics, San Diego, California) and incubated in methoinine-free KGM containing [^{35}S]methoinine (3.7 GBq/dish, ICN Biomedicals, Costa Mesa, California) for 2 h. After labeling the cells were washed with phosphate buffered saline (PBS) five times. The washed cells were then harvested with lysis buffer or incubated for 0, 1, 3, or 5 additional hours in KGM to determine the decay rate ($t_{1/2}$) of p53 protein before harvesting. For harvesting, 0.5 ml of ice cold lysis buffer solution (10 mmol/l dibasic sodium phosphate/0.9% NaCl/1% Triton X-100/0.5% sodium deoxycholate/0.1% SDS/0.2% sodium azide/0.004% sodium fluoride, pH 7.25) were added to cell monolayer, and the cells were incubated at 4°C for 10 min. The cells were then disrupted by repeated aspiration through a 21 gauge needle and transferred to a 1.5 ml centrifuge tube. Disrupted cells were pelleted by centrifugation at 13 000 rpm at 4°C for 15 min. The supernatant was transferred to a new tube and stored at −70°C before immunoprecipitation.

The frozen cellular extract was melted on ice and centrifuged again at 13 000 rpm for 15 min at 4°C, and the supernatant was transferred to a 1.5 ml microcentrifuge tube containing 80 μl of protein-G agarose and 10 μg of p53 monoclonal antibody (PAb-2, Oncogene Sciences, Manhasset, New York). The mixtures were then incubated at 4°C on a rocking platform for 4 h. The immunoprecipitate was collected by centrifugation at 2500 rpm for 15 min at 4°C. The supernatant was discarded by washing four times with the cell lysis buffer. After the final wash, the pellet was resuspended in 20 μl of electrophoresis sample buffer (mixture of 1.0 ml of glycerol, 0.5 ml β-mercaptoethanol, 3.0 ml of 10% SDS, 1.25 ml of 1.0 mol/l Tris–HCl buffer, 2.0 ml of 0.1% bromophenol blue and 0.6 g of solid urea in distilled water to final volume of 10 ml). The samples were then analysed by electrophoresis in 10% polyacrylamide gels. The gel was fixed in acetic acid and methanol, dried, and exposed overnight with XAR-5 X-ray film (Eastman Kodak). The intensity of p53 bands was analysed using a densitometer (Hoeffer Scientific Instruments, San Francisco, California).

Polymerase chain reaction (PCR) analysis, cloning, and DNA sequencing

To determine p53 gene mutations, reverse transcription-polymerase chain reaction (RT-PCR) was carried out in a DNA thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, Connecticut) using 100 ng of poly(A^+)RNA extracted from cells in a total volume of 100 μl containing 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 3 mmol/l $MgCl_2$, 0.01% gelatin, 200 μmol/l each of the four deoxyribonucleotide triphosphates, and 100 pmol/l of primers. After an initial denaturation of poly(A^+)RNA at 94°C for 2 min, 2 units of avian myeloblastosis virus (AMV) reverse transcriptase were added and reverse transcription proceeded at 42°C for 45 min. The cDNA product was then amplified by the addition of 2.5 units of recombinant *Taq* DNA polymerase. The sense primer for PCR amplification extended from nucleotide (nt) 296 to nt 315 and the antisense primer extended from nt 1015 to nt 996 (sense primer: 5′−CCCAGAAAACCTACCAGGGC-3′; antisense primer: 5′-CGAAGCGCTCACGCCCACGG-3′). Each cycle of amplification was consisted of 1 min denaturation at 94°C followed by 2 min annealing (58°C) and 3 min extension (72°C) steps. A total of 30 cycles were run with a final extension step at 72°C for 7 min with additional *Taq* DNA polymerase and nucleotides to generate homogeneous blunt ends of amplified sequences. Amplified cDNA was ligated to pCR1000 vector by using TA™ Cloning Kit (Invitrogen, San Diego, California) under the conditions recommended by the manufacturer.

The nucleotide sequence of the cloned DNA was determined by the primer extension method. 3–5 μg aliquots of double-stranded plasmid DNA were denatured in 0.2 mol/l NaOH, 0.2 mmol/l EDTA at 37°C for 30 min. The denatured DNA was neutralised with 0.1 volume of 3 mol/l sodium acetate (pH 5.2) and the DNA precipitated with 3 volumes of ethanol, and then the mixture was centrifuged. The DNA pellet was redissolved in 7 μl of distilled water, 2 μl of Sequenase (T7 DNA polymerase, USB Corporation, Cleveland, Ohio) reaction buffer, and 1 μl of 0.1 μl of T7 or T3 primer (20 pmol) followed by an incubation at 65°C for 2 min for annealing. To the annealed mixture, 2 μl of Sequenase and 0.5 μl [^{35}S]α-dATP (370 mlq/ml, 37 Bq/mmol/l; Amersham Corp., Arlington Heights, Illinois) were added. After incubation at room temperature for 5 min, 3.5 μl of the mixtures were added to each of four different tubes, each containing 2.5 μl of the appropriate termination mixtures. After 5 min incubation at 37°C, the reaction was stopped with 95% formamide-dye, samples were heated to 95°C for 2 min, and 1.8 μl of each sample were loaded onto a 7% acrylamide urea sequencing gel. Samples were run at a constant voltage for 4.5 h. The gel was fixed in acetic acid and methanol, dried, and exposed for 2 days to XAR-5 X-ray film. The DNA sequence was read manually.

RESULTS

Analysis of p53 in NHOK and the immortalised cell lines

Figure 1 shows that 2.7-kb mRNA is the transcript of p53 gene in NHOK, HOK-16A, and HOK-16B cell lines. The amounts of 2.7-kb p53 mRNA expression in two immortal cell lines were similar to each other. However, the level of p53 mRNA in the immortal cells was notably higher than that in NHOK, though the amounts of β-actin mRNAs were similar in the immortal cells and NHOK. Many reports have indicated that the point mutation of p53 gene at highly conserved region (exons 4–9) increases the p53 level in cancer cells possibly through an overexpression [23] or an accumulation of mutant p53 having prolonged half-life [6]. The PCR amplification of the cDNA spanning the conserved p53 region (codons 117–309) was carried out to analyse the nucleotide sequences. No p53 mutations were found from the amplified region of p53 cDNA originated from NHOK and cell lines HOK-16A and HOK-16B.

The p53 protein levels in NHOK, HOS cell line, and the immortal cell lines were examined by immunoprecipitation analysis. The HOS cells expressing excessive mutant p53 protein were included in this experiment as a positive control. The amount of p53 levels was measured by analysing the intensity of p53 bands and expressed relative to the p53 level determined in NHOK. Though the immortalised cells contained higher amount of p53 mRNAs, these cells had lower amount of p53 protein (0.38–0.44 times lower) compared with NHOK (Fig. 2 and Table 1).

The half-life of p53 in NHOK, HOS, and the immortal keratinocytes was determined by using pulse labelling and immunoprecipitation (Table 1 and Figs 3–6). Newly

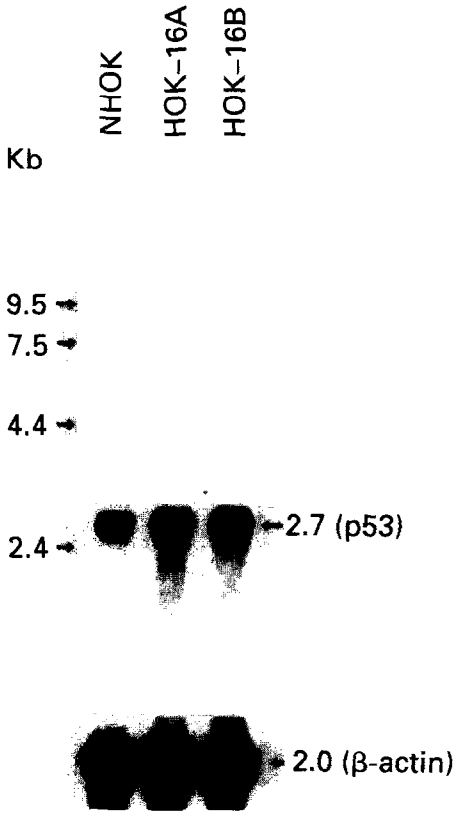

Fig. 1. Autoradiogram of the northern blot hybridisation of poly (A$^+$)RNA from NHOK, HOK-16A, and HOK-16B cell lines to ^{32}P-labelled p53 cDNA. The probe was stripped from the filter, and the cellular poly(A$^+$)RNA was again hybridised to ^{32}P-labelled human β-actin gene and ^{32}P-labelled HPV-16 DNA.

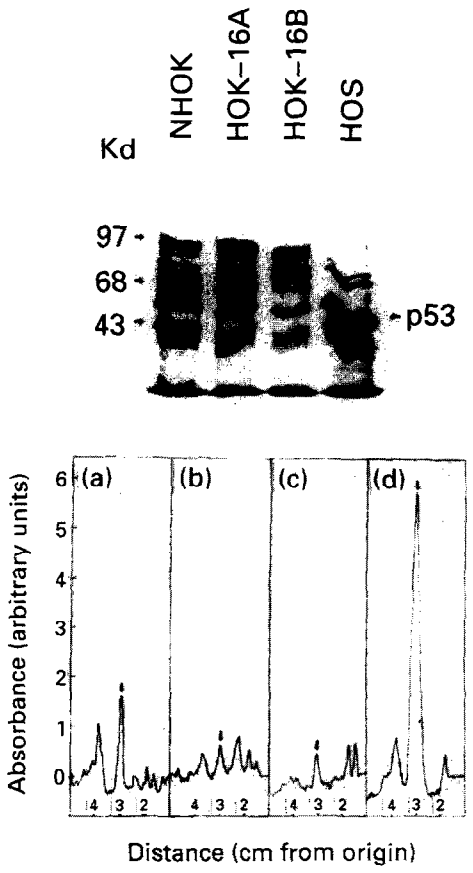

Fig. 2. (a) Autoradiographic images of immunoprecipitated and electrophoretically separated p53 protein from HOS, NHOK, HOK-16A, and HOK-16B cell lines. The cells were labelled with [^{35}S]methionine for 2 h, and the p53 protein was immunoprecipitated for analysis. (b) Densitometric analysis of p53 protein (arrow).

Table 1. Analysis of p53 and presence of HPV-16 nucleic acids in immortalised cell lines

Type of cells	Relative p53 mRNA level	Relative p53 level	p53 gene mutation	Half life of p53 (min)	Presence of HPV-16 DNA	Presence of HPV-16 E6/E7 mRNA
NHOK	1.0	1.0	Negative	51	Negative	Negative
HOK-16A	1.5	0.44	Negative	54	Positive*	Positive
HOK-16B	1.5	0.38	Negative	59	Positive*	Positive
HOS	N/O	3.5	Positive†	330	N/O	N/O

*Both the HOK-16A and HOK-16B cell lines contain HPV-16 DNA (7.9 kbp) as integrated form [18].
†Previously published [22].
N/O = Not observed.

synthesised total cellular proteins were labelled with [^{35}S]methionine for 2 h, and the cells were incubated in [^{35}S] methionine-free medium for 0, 1, 3, and 5 h before immunoprecipitation of p53 protein to calculate the half-life of the protein. Inasmuch as the HOS cell line was known to express mutant p53 having prolonged half life, it was expected the p53 half-life of HOS cells would be longer than 3 h, whereas the half-life of wild-type p53 would be 30–60 min as reported earlier [22]. The half-life of p53 translated from the HOS cells was 5.5 h (Table 1, Fig. 3), while the half-lives of p53 translated from NHOK, HOK-16A, and HOK-16B cell lines were 51, 54, and 60 min, respectively (Table 1, Fig. 4–6), indicating that the p53 proteins translated from NHOK, HOK-16A, and HOK-16B cell lines were wild-type protein.

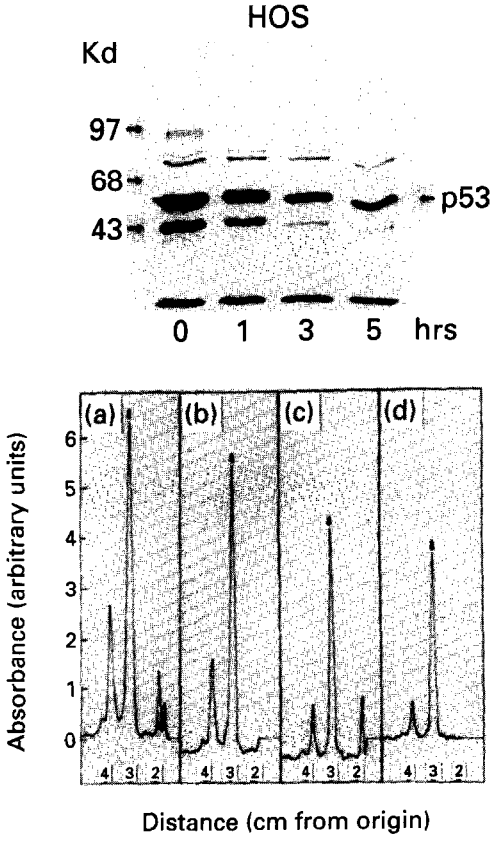

Fig. 3. Autoradiographic images of immunoprecipitated and electrophoretically separated p53 protein form the HOS cell line. The cells were labelled with [^{35}S]methionine for 2 h and incubated in [^{35}S]methionine-free medium for 0, 1, 3, and 5 h to determine the half life of p53 protein in the cells.

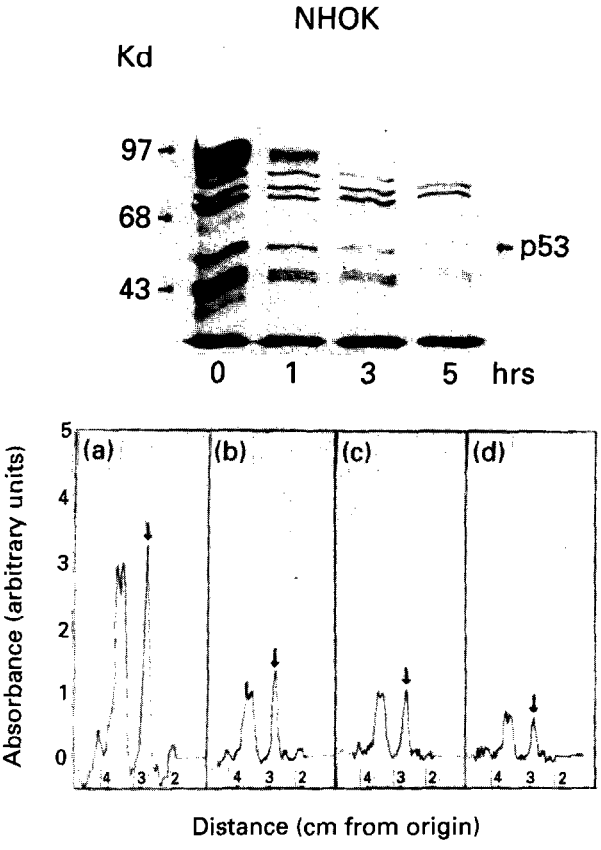

Fig. 4. Autoradiographic images of immunoprecipitated and electrophoretically separated p53 protein from NHOK. The cells were labelled with [^{35}S]methionine for 2 h and incubated in [^{35}S]methionine-free medium for 0, 1, 3, and 5 h to determine the half life of p53 protein in the cells.

Determination of the expression of HPV-16 E6/E7

Northern blot hybridisation analysis using the probe containing HPV-16 E6/E7 gene showed that both HOK-16A and HOK-16B cell lines expressed viral messages. An abundant 1.6 kb HPV-16 E6/E7 mRNA was detected from these cell lines, but not from NHOK (Fig. 7).

DISCUSSION

The molecular biological events underlying the development of oral cancer are unknown. Several studies have shown the amplification and/or overexpression of c-*erb*B-1/epidermal growth factor receptors (EGFR) [24], c-*myc* [24], and c-*bcl*-1 [25] in oral cancer tissue. Amplification and overexpression of c-*erb*B-1/EGFR gene has also been demonstrated in several oral epidermoid cancer cell lines [26], and can be induced by

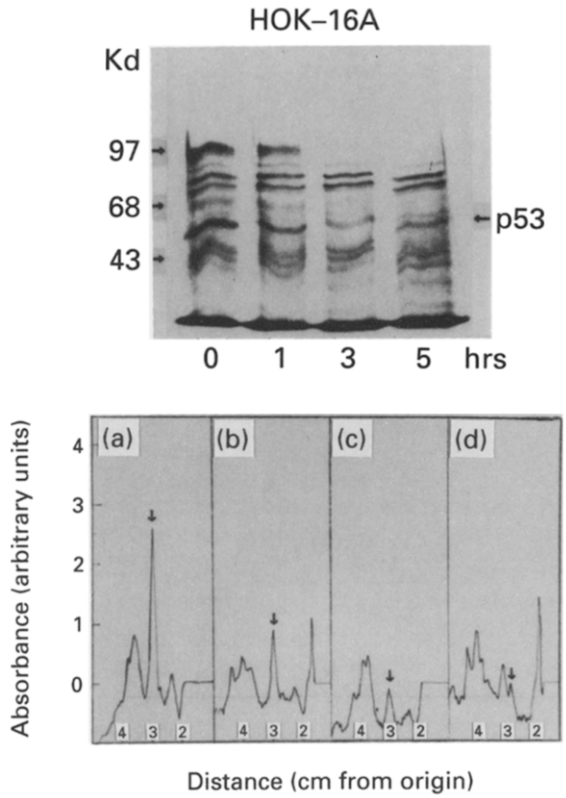

Fig. 5. Autoradiographic images of immunoprecipitated and electrophoretically separated p53 protein from HOK-16A. The cells were labelled with [^{35}S]methionine for 2 h and incubated in [^{35}S]methionine-free medium for 0, 1, 3, and 5 h to determine the half life of p53 protein in the cells.

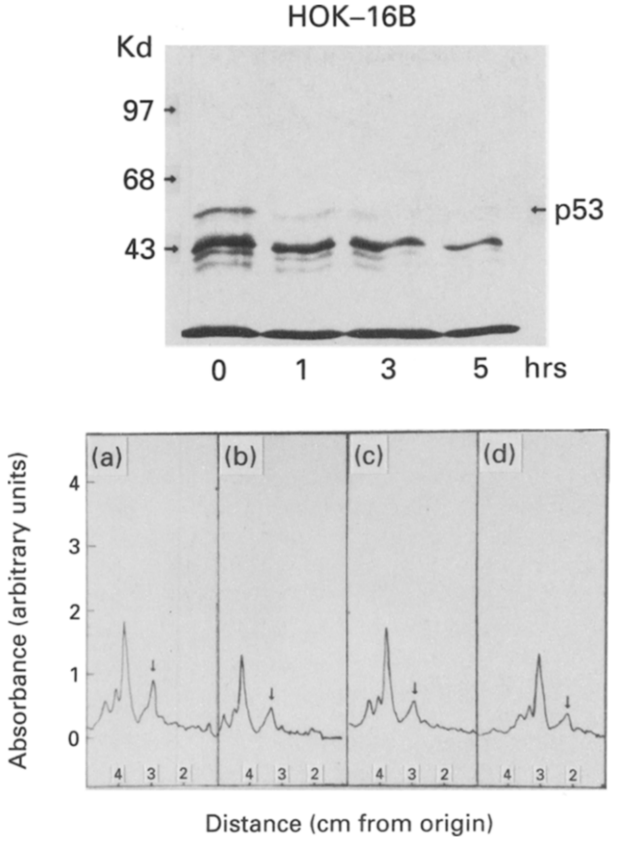

Fig. 6. Autoradiographic images of immunoprecipitated and electrophoretically separated p53 protein from HOK-16B. The cells were labelled with [^{35}S]methionine for 2 h and incubated in [^{35}S]methionine-free medium for 0, 1, 3, and 5 h to determine the half life of p53 protein in the cells.

application of chemical carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA) in hamster cheek pouch epithelium [27]. More recently, the loss of tumour suppressor gene function was reported in oral squamous carcinoma cells derived from hamster cheek pouch induced by DMBA [28]. These studies indicate that inactivation of tumour suppressor gene function, along with dominant activation of cellular proto-oncogenes, involves in oral carcinogenesis. Among the tumour suppressor genes, the p53 gene could be most closely associated with the development of oral cancer because (1) the 'high risk' HPV (HPV-16 and HPV-18) DNA is present in a high percentage of oral cancer tissue and (2) the E6 protein encoded by 'high risk' HPV can enhance the degradation of wild-type p53 protein *in vitro* [21].

Our present data show that immortal oral keratinocytes harboring HPV-16 DNA transcribe notably higher amount of p53 mRNA than the normal counterpart, suggesting that the immortal oral keratinoctyes could produce higher amount of p53 protein compared with NHOK. However, the immunoprecipitation data show that the immortal cells contain lower p53 level compared with the normal counterpart. These data suggest that the p53 protein may be normally translated in the immortal cells, but the protein may be more easily degraded in these cells than in NHOK, or that the p53 translation process may be less efficient in the immortal cells compared with NHOK. Though it is not understood, if the former assumption is true, the immortal oral keratinocytes may possess certain substance that directly destroy or enhances the degradation of the p53 protein. This presumption may be, in part, correct because of the presence of HPV-16 E6/E7 mRNA in the immortal cells, but not in NHOK. Inasmuch as the HPV-16 E6 protein complexes and promotes the degradation of wild-type p53 protein *in vitro* [20, 21], the low p53 protein level in the immortal cells may be due to the promoted degradation of the protein by HPV-16 E6.

Inasmuch as the 'high risk' HPV E6 protein binds and enhances the degradation of the wild-type p53, not the mutant p53, the p53 protein translated from the immortal cells is assumed to be the wild-type. In fact, the nucleotide sequence of highly conserved region of p53 gene shows no mutation, and the half-life of the p53 in the immortal cells and NHOK is shorter than 1 h, whereas the half-life of mutant p53 from HOS cell line is 5.5 h. Such observations suggest that the p53 expressed from the immortal cells is, in fact, wild-type. Inspite of these observations, it is possible that mutations have occurred outside the conserved region, and that some mutations do not result in expression of a more stable p53 protein. Our results are similar to previous report showing low wild-type p53 protein level in cervical cancer cell lines harboring HPV-16 or HPV-18 DNA compared with the normal counterpart [10]. The reason why the immortal oral keratinocytes contain a significantly higher amount of p53 mRNA compared with the normal cells is presently unknown, but we assume that it is due to a compensation response of the cells to overcome the enhanced degradation of p53.

Many studies suggest that the wild-type p53 protein functions as a tumour suppressor substance. Mutant p53 cooperates with an activated *ras* gene to transform rodent cells

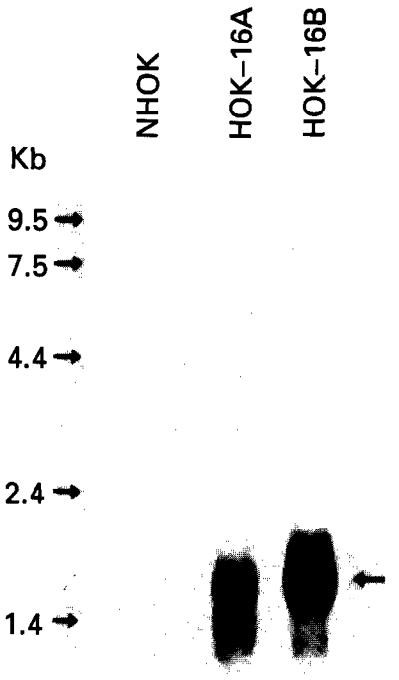

Fig. 7. Autoradiogram of the northern blot hybridisation of poly (A^+)RNA from NHOK, HOK-16A, and HOK-16B cell lines to ^{32}P-labelled HPV-16 DNA. The arrow indicates 1.6-kb HPV-16 E6/E7 mRNA.

[3], whereas wild-type p53 does not induce the transformation in cooperation with activated *ras* gene. Moreover, the wild-type p53 inhibits the cotransforming activity of *ras* with either mutant p53, c-*myc*, or E1A in rodent cells [29]. It seems that wild type p53 is an indispensable negative growth regulator of cells, and the loss of the function of p53 resulting from mutations, gene rearrangement, or destruction may result in the loss of suppressive growth control mechanism. Therefore, the low wild-type p53 protein level in the immortal cells may be, in part, responsible for the immortalisation of these cell lines.

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