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Expression of Cellular Genes in HPV16-Immortalized and Cigarette Smoke Condensate-Transformed Human Endocervical Cells

Xiaolong Yang,[1] Yoshifumi Nakao,[1,2] Mary M. Pater,[†] Shou-Ching Tang,[1,3] and Alan Pater[1]*

[1]Division of Basic Medical Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada
[2]Department of Obstetrics and Gynecology, Saga Medical School, Saga, Japan
[3]Newfoundland Cancer Treatment and Research Foundation, Dr. H. Bliss Murphy Cancer Center, St. John's, NF, Canada

Abstract We studied the molecular mechanism of successive multistep cervical carcinogenic progression with our previously established in vitro model system. This system was composed of primary human endocervical cells (HEN), two lines of HEN immortalized by HPV16 and their counterparts subsequently malignantly transformed by cigarette smoke condensate (CSC). The expression was examined of diverse cellular genes associated with oncogenesis and senescence, especially for cervical cancer. Consistent results were seen for the pairs of immortalized and malignantly transformed lines. Immortalization of HEN by HPV16 resulted in enhanced expression of H-*ras*, c-*myc*, B-*myb*, p53, $p16^{INK4}$ and PCNA mRNA; enhanced expression of p16 and PCNA proteins; decreased expression of WAF1/p21/Cip1/Sid1 and fibronectin mRNA; and decreased p53 protein. On the other hand, the CSC-transformed counterparts of HPV16-immortalized cells had up-regulated levels of B-*myb*, p53 and WAF1 mRNA and p53 protein. Our results indicate that the differential activation or inactivation of multiple cellular genes is important for the immortalization, as well as the transformation, of human cervical cells. Further, we suggest that our in vitro model system is useful for investigating the molecular mechanism of multistep cervical carcinogenesis. J. Cell. Biochem. 66: 309–321, 1997.

Key words: oncogenes; tumor suppressors; human papillomavirus type 16; smoking cofactor; immortalization; tumorigenesis; mRNA; proteins; oncogenesis; senescence

Cervical cancer is the second most common cancer among women in the world [Parkin et al., 1993]. In the last 10 years, compelling epidemiological and experimental evidence proved that human papillomaviruses (HPVs), most frequently HPV16 and HPV18, are the major cause of this cancer [for reviews, see zur Hausen, 1991; zur Hausen and de Villiers, 1994]. HPVs are detected and transcriptionally active in most premalignant cervical intraepithelial neoplasias (CINs) and cervical carcinomas. Therefore, HPVs have been considered the most important agent in the initiation and maintenance of the cervical malignant phenotype. The interval between virus infection and cervical cancer is long and only a low percentage of infected individuals eventually develop cancer [zur Hausen, 1982]. Thus, HPV infection was suggested to be necessary but not sufficient for causing cervical cancer. Other factors, such as cigarette smoking and dysregulated expression of oncogenes and tumor suppressor genes, are required for full malignant transformation [Herrington, 1995]. However, the mechanism of this multistep, multifactor carcinogenesis towards full malignant conversion of human cervical cells is unclear.

Experimental investigation of the mechanism of multistage cervical carcinogenesis was facilitated by several in vitro systems. Transfection of high-risk HPV16 and HPV18 into hu-

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*Correspondence to: Alan Pater, Memorial University of Newfoundland, Faculty of Medicine, Division of Basic Medical Sciences, HSC, Room 5329, 300 Prince Philip Drive, St. John's, Newfoundland, Canada, A1B 3V6.
E-mail: apater@morgan.ucs.mun.ca

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man foreskin and ectocervical keratinocytes and endocervical epithelial cells caused immortalization [Durst et al., 1987b; Woodworth et al., 1990; Tsutsumi et al., 1992]. The immortalized cells contained integrated HPV DNA, expressed viral transcripts and were defective in terminal differentiation, mimicking the phenotypes of mild CIN I or severe CIN III found in women [Woodworth et al., 1990; Sun et al., 1992]. However, the immortalized cells failed to form tumors in nude mice [DiPaolo et al., 1989; Tsutsumi et al., 1992; Yang et al., 1996a]. Further, transfection of HPV-immortalized cells with H-*ras* or v-*fos* oncogenes, and treatment of HPV-immortalized cells with benzo(a)pyrene, nitrosomethylurea or CSC chemical carcinogens, caused malignant transformation [DiPaolo et al., 1989; Li et al., 1992; Garrett et al., 1993; Pei et al., 1993; Park et al., 1995; Yang et al., 1996a; Nakao et al., 1996]. Grown in organotypic raft culture, the transformed cells reconstructed a malignant phenotype resembling carcinomas in situ [DiPaolo et al., 1989; Park et al., 1995; Yang et al., 1996a; Nakao et al., 1996]. Most earlier studies of the molecular mechanism of human cervical cell transformation used established tumor cell lines. Since most tumor cell lines acquire changes during decades in culture, these cells inadequately represent the in vivo condition. Therefore, an in vitro system representing different stages of carcinogenesis of primary cells would be more relevant to probe the molecular mechanism of cervical oncogenesis. However, few such in vitro systems are available to date.

We previously established an in vitro system in which primary human endocervical cells (HEN) were immortalized by HPV16 and subsequently transformed by treatment with CSC. The system mimics the multistage oncogenesis of cervical cells in situ [Tsutsumi et al., 1992; Yang et al., 1996a]. The immortalized cells, HEN-16 and HEN-16-2, contained integrated HPV16 DNA and formed lesions resembling severe CIN III in the in vivo implantation system and the in vitro raft organotypic culture system [Sun et al., 1992; Shindoh et al., 1995]. However, neither line displayed anchorage-independent growth or formed tumors in nude mice. The CSC-transformed cells, HEN-16T and HEN-16-2T, displayed more severe CIN III in raft lesions, anchorage-independent growth, faster in media containing serum and tumorigenicity, compared with their immortalized counterparts [Yang et al., 1996a]. Comparison also showed that the pattern of mRNA expression of HPV16 was the same in all four cell types [Sarma et al., 1996]. Thus, the main cause of transformation was apparently not the altered expression of HPV oncogenes, but possibly that of cellular genes. The purpose of this study was to determine whether alterations of those important cellular genes that are commonly involved in senescence, immortalization or carcinogenesis play roles in endocervical cell immortalization by HPV16 and tumorigenic transformation by CSC. Therefore, the human endocervical cells of our unique in vitro system were analyzed for the expression patterns of diverse cellular genes associated with oncogenesis generally and cervical oncogenesis particularly.

MATERIALS AND METHODS

Cell Culture

HEN, HEN-16 and HEN-16-2 were established and propagated, as described previously [Tsutsumi et al., 1992; Sun et al., 1992]. CSC-transformed cells, HEN-16T and HEN-16-2T, were established by treatment of HPV16-immortalized cells with CSC [Yang et al., 1996a]. All the cells were maintained in serum-free media for keratinocytes (GIBCO/BRL, Bethesda, MD). The growth rates of all cell types were similar in this culture condition [Yang et al., 1996a].

DNA Probes and Antibodies

Ki-*ras* cDNA probe was purchased from Oncor (Gaithersburg, MD). PCNA 40-mer probe was from Oncogene Sciences (Cambridge, MA). Mouse anti-human PCNA (PC10) and anti-human p53 (Ab-2) monoclonal antibodies (MAbs) were purchased from DAKO Corp. (Carpinteria, CA) and Oncogene Sciences, respectively. Santa Cruz Biotechnology (Santa Cruz, CA) was the supplier of anti-human p16 polyclonal antibody.

Northern Blot Analysis

Total RNA was extracted from cells at 70% confluence using the CsCl gradient centrifugation method [Sambrook et al., 1989]. Polyadenylated RNA was isolated from the total RNA using the Quickprep mRNA Purification Kit (Pharmacia-LKB Biotech Inc., Quebec, Canada). For Northern blot, 20 µg of total RNA or 3 µg polyadenylated RNA was separated in denaturing 1% agarose gels and transferred to BioTrace

HP membranes (Gelman Sciences, Ann Arbor, MI). The blots were prehybridized in hybridization buffer [1% nonfat dry milk, 0.5 M NaH_2PO_4, 7% sodium dodecyl sulfate (SDS), pH 7.2] for 2–3 h and then hybridized with cDNA probes, which had been ^{32}P-labelled with RediPrime Labelling Kit (Amersham), in hybridization buffer at 65°C overnight. The hybridized blots were washed twice with 2 × sodium saline citrate (SSC), 0.1% SDS at room temperature for 15 min and twice with 0.1 × SSC, 0.1% SDS at 42–65°C for 10 min. After washing, the membranes were exposed to Kodak XAR film for 1–5 days. γ-actin was used as an internal control. Every experiment was repeated at least twice.

Western Blot Analysis

Proteins were extracted from 10^7 cells by lysis in 1 ml ice-cold extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) for 30 min and centrifuged at 14,000 rpm for 10 min. The DC Protein Assay Kit (Bio-Rad, Hercules, CA) was used to quantify the protein. Ten micrograms of protein was fractionated by 8–15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using standard protocols. Stained protein markers (Amersham) were included in each gel as molecular weight standards. The proteins were subsequently transferred to Hybond enhanced chemilumiscence (ECL) nitrocellulose membrane (Amersham) under semi-dry conditions. Immunodetection was done using the ECL system (Amersham), according to the manufacture's instructions.

RT-PCR Assay

One microgram of total RNA was treated with RNase-free DNase I and then reverse transcribed to cDNA in 1 × RT buffer, 0.5 mM dNTP, 10 mM DTT, 40 units RNase ribonuclease inhibitor (Promega, Madison, WI), and 1 unit Superscript reverse transcriptase (GIBCO/BRL) at 37°C for 1 h. One tenth of the RT product was further used for the PCR amplification using a Hybraid thermal reactor (Bio/CAN, Mississauga, Canada). The conditions for PCR were: 1 × PCR buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% (w/v) gelatin], 0.5 mM dNTP, 1.5 mM $MgCl_2$, 0.2 μM each of sense and antisense primers, and 2 units Taq polymerase (Promega) in 50 μl. The primers used for PCR are listed in Table I. The PCR program for the DCC gene was: 94°C for 3 min for the first cycle; 94°C for 1 min, 56°C for 2 min and 72°C for 2 min for 35 cycles; and 72°C for 7 min for the last cycle.

PCR-Single Strand Conformational Polymorphism (SSCP) Analysis

PCR-SSCP analyses of H-*ras* and Ki-*ras* mutations were as described [Suzuki et al., 1990] with some modifications. Briefly, approximately 0.1 μg genomic DNA was used to amplify the exon 1 and exon 2 regions of H-*ras* and Ki-*ras* in 10 μl containing 1 × PCR buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% (w/v) gelatin], 50 μM each of the four dNTPs, 1.5 mM $MgCl_2$, 0.5 μM each of sense and antisense primers, 1 μCi α-^{33}P-dCTP (3,000 Ci/mmol, DuPont) and 0.4 units Taq DNA polymerase (Promega). Primers used in PCR are listed in Table I. The conditions for PCR were 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min. PCR products were diluted fivefold in loading buffer [95% formamide, 0.02 M EDTA (pH 8.0), 0.05% bromophenol blue and 0.05% xylene cyanol], denatured at 90°C for 5 min and separated in a 6% polyacrylamide gel at 4°C without glycerol. The gels were dried and exposed to Kodak XAR film at −80°C for 12–72 h.

TABLE I. Description of Primers Used in RT-PCR and PCR-SSCP Analyses of Cellular Genes

Genes	Exons	Strand	Sequences
H-*ras*	Exon 1	Sense	5′-CAGGCCCCTGAGGAGCGATG-3′
		Antisense	5′-TTCGTCCACAAAATGGTTCT-3′
	Exon 2	Sense	5′-TCCTGCAGGATTCCTACCGG-3′
		Antisense	5′-GGTTCACCTGTACTGGTGGA-3′
Ki-*ras*	Exon 1	Sense	5′-GGCCTGCTGAAAATGACTGA-3′
		Antisense	5′-GTCCTGCACCAGTAATATGC-3′
	Exon 2	Sense	5′-TTCCTACAGGAAGCAAGTAG-3′
		Antisense	5′-CACAAAGAAAGCCCTCCCCA-3′
DCC	Exon 22–23	Sense	5′-AACAGAGGATTCAGCCAAT-3′
		Antisense	5′-AGCAGCTAAACTTTGACATT-3′

In Situ Hybridization Assay

The BioNick Labelling System and in situ Hybridization and Detection System (GIBCO-BRL) were used for labelling nonradioactive fibronectin (FN) probes and detection for in situ hybridization. In brief, endocervical cells grown on slides were first fixed in fresh 4% paraformaldehyde at 22°C for 30 min, immersed in 50% ethanol for 2 min, rinsed with 1 × PBS, and then incubated in prewarmed 40 µg/ml proteinase K in 1 × PBS at 37°C for deproteinization. The slides were subsequently dehydrated through a graded ethanol series, hybridized with denatured biotin-labelled probe in 1 × hybridization buffer (2 × SSC, 0.1 M sodium phosphate, pH 6.5, 1 × Denhart's solution), 10% dextran sulphate at 42°C overnight. After hybridization, the slides were washed twice with 0.2 × SSC, 0.1% SDS for 15 min; once with buffer 1 (0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$, and 0.05% Triton X-100); and blocked in blocking buffer [3% (w/v) bovine serum albumin in buffer 1] for 1 h. The FN signals were detected using nitroblue tetrazolium (NBT) and 4-bromo-5-chloro-3-indolyl phosphate (BCIP).

RESULTS

Oncogene Expression in Endocervical Cells

To study the important role reported for the transactivation of oncogenes in the pathways for transformation from primary cervical cells, four oncogenes were examined. We used HEN primary cells, HEN-16 and HEN-16-2 HPV16-immortalized cells, and HEN-16T and HEN-16-2T CSC-transformed cells (Fig. 1). Compared with primary cells and the actin control and quantified by densitometric analysis; the transcript levels were significantly higher for H-*ras*, unchanged for Ki-*ras*, significantly higher for c-*myc* and substantially higher for B-*myb* in immortalized cells and transformed cells (Fig. 1, A–D, respectively; Table II). Transformation by CSC resulted in further enhanced B-*myb* mRNA expression (Fig. 1D). Since oncogenic and transcription-activating oncogene mutations were previously detected [Suzuki et al., 1990], we similarly used PCR-SSCP analysis,

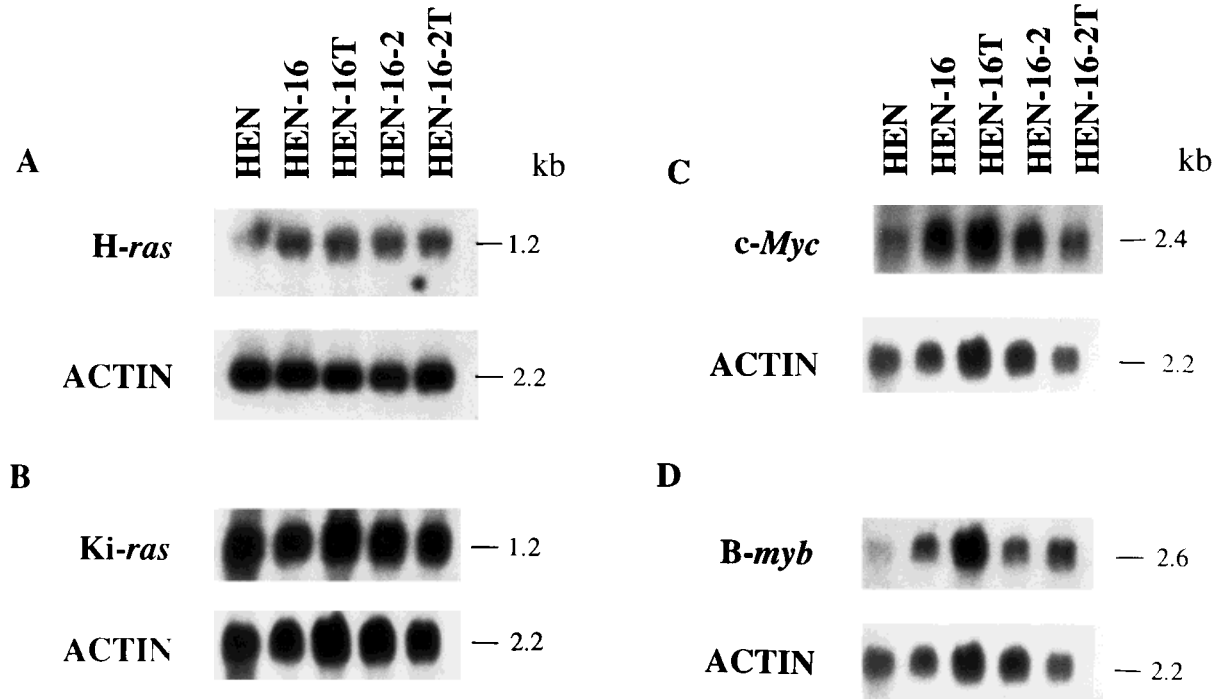

Fig. 1. Northern blot analyses of H-*ras*, Ki-*ras*, c-*myc* and B-*myb* oncogene mRNA expression in the five types of endocervical cells. Total RNA (20 µg) for Ki-*ras*, c-*myc*, and B-*myb*, or 3 µg poly(A)+ RNA for H-*ras* was used. The RNA was size-fractionated in 1.0% formaldehyde agarose gels, transferred to nylon membranes and hybridized with ^{32}P-labelled cDNA probes for H-*ras* (**A**), Ki-*ras* (**B**), c-*myc* (**C**) and B-*myb* (**D**). After the membranes were stripped of ^{32}P label, the same membranes were hybridized with ^{32}P-labelled human γ-actin cDNA probe (ACTIN). The sizes in kb of the mRNAs are indicated on the right.

TABLE II. Effect of HPV16-Immortalization and CSC-Transformation on mRNA and Protein Expression of Endocervical Cellular Genes

Cells	H-*ras*	c-*myc*	B-*myb*	p53	WAF1	$p16^{INK4}$	PCNA	FN
HEN	+/ND	+/ND	−/ND	−/++	++/ND	−/−	−/−	++/ND
HEN-16	++/ND	++/ND	+/ND	+/−	−/ND	++/++	++/++	+/ND
HEN-16T	++/ND	++/ND	++/ND	++/+	++/ND	++/++	++/++	−/ND
HEN-16-2	++/ND	++/ND	+/ND	+/−	−/ND	++/++	++/++	−/ND
HEN-16-2T	++/ND	++/ND	++/ND	++/+	+/ND	++/++	++/++	−/ND

The table summarizes the expression (mRNA/protein) at increasing levels, which are indicated by −, +, ++. ND, not determined.

the test for mutation of H-*ras* or Ki-*ras* DNA in both HPV-immortalized and both CSC-transformed cell lines. However, other mechanisms may be involved, since neither exon 1 nor exon 2 regions of H-*ras* or Ki-*ras* were mutated in any immortalized or CSC-transformed cells (Table I; data not shown).

Expression of p53 and Its Downstream Effector Genes

Expression of p53 and its downstream effector genes was assayed, since they are involved in many important facets of cell growth related to carcinogenesis [see Zambetti and Levine, 1993 for review]. The p53 mRNA level was low in HEN, but increased dramatically in HEN-16 and HEN-16-2. The level of p53 transcripts further increased after transformation by CSC to form HEN-16T and HEN-16-2T, especially relative to the actin control (Fig. 2A). The expression of p53 protein was highest in HEN, decreased significantly after immortalization and was an intermediate level after transformation by CSC (Fig. 2B). The RNA and protein assays were repeated and consistent three times. In addition, densitometric analysis of the Western blot data of the expression of p53 protein confirmed the effects. The expression of WAF1 mRNA was markedly diminished after immortalization of HEN and the level was restored after transformation for HEN-16T and partially restored for HEN-16-2T (Fig. 3). Up-regulation of GADD45 and MDM2 mRNA was observed only in HEN-16-2T.

Tumor Suppressor Gene Expression and Status

While the expression of p16 tumor suppressor gene was not detected in HEN by either Northern blot or immunoblot analyses, dramatic increases were seen in both analyses for HEN-16 and HEN16-2; however, transformation of these HPV16-immortalized cells did not result in any changes in p16 mRNA or protein levels (Fig. 4A and B). PCR-SSCP analysis of exon 1 and exon 2 indicated that p16 is not mutated in any of the cells in the in vitro system (data not shown).

In our system, the mRNA of deleted in colon cancer (DCC) tumor suppressor was undetectable in Northern blots (data not shown). RT-PCR analysis was used for immortalized or CSC-transformed cells to examine the region of DCC most frequently deleted during oncogenesis. Although not associated with cervical cancer, DCC was deleted in some chemically transformed cervical cell lines [Klingelhutz et al., 1993]. The expected 400 bp fragment containing exons 22 and 23 of DCC was detected for all five cell types (data not shown). The results in our system indicated that DCC deletion was not a common phenomenon in malignant progression induced by HPV16 or cigarette smoke in vitro, and may not be in vivo.

Proliferating Cell Nuclear Antigen and Fibronectin Expression

Marked up-regulation of mRNA and protein levels for PCNA was observed in all four HPV-immortalized and CSC-transformed cell types. However, the increased levels were the same in the immortalized cells and transformed cells (Fig. 5A and B). In contrast to the PCNA immortalization result, the fibronectin (FN) mRNA was substantially reduced (Fig. 6A). In situ hybridization assays of FN in monolayer cultures confirmed that FN was expressed and mostly in the cytoplasm, as expected. The expression pattern was heterogeneous and significantly higher in HEN than in HEN-16, HEN-16T, HEN-16-2 or HEN-16-2T (Fig. 6Ba–e, respectively).

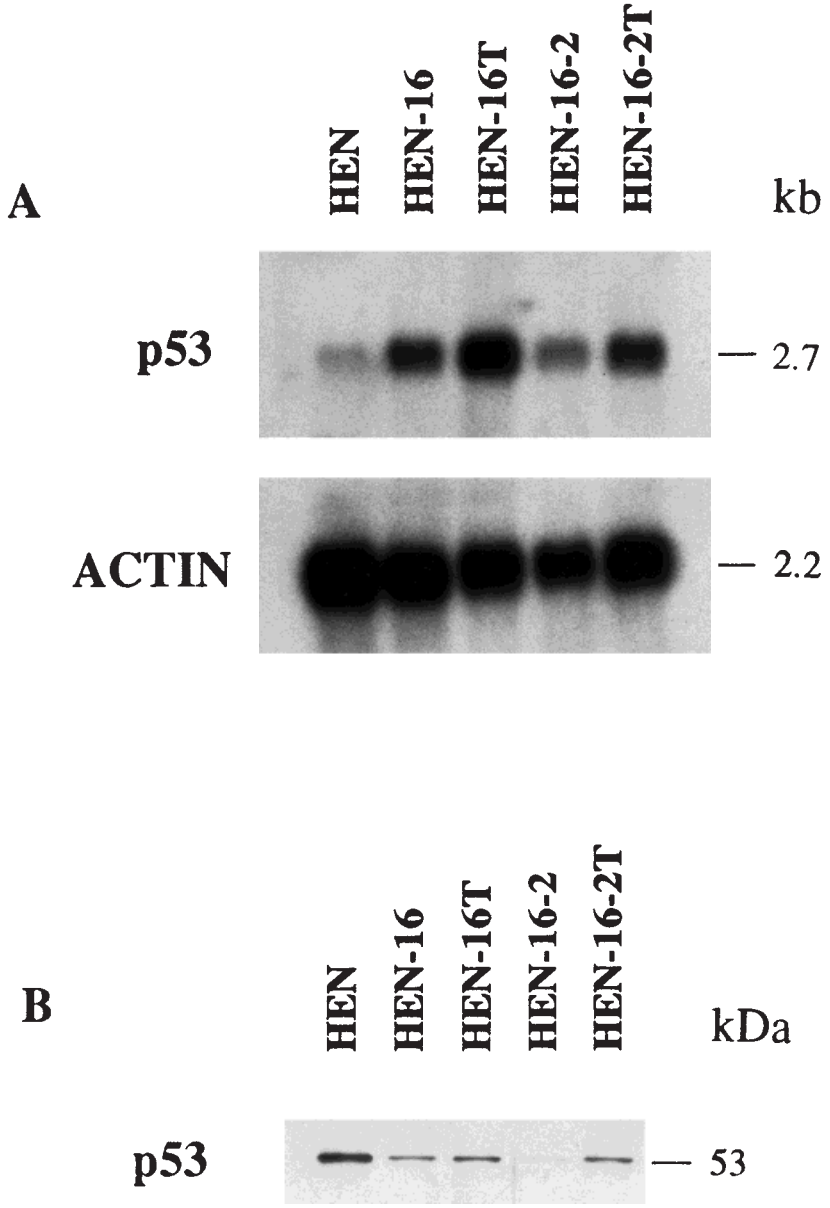

Fig. 2. Northern blot mRNA and Western blot protein analyses of p53 in the five types of endocervical cells. **A:** Northern blot analysis. Poly(A)+ RNA (3 µg) were used. Labels and conditions are as in Figure 1. **B:** Immunoblot protein analysis. Protein (10 µg) was separated by SDS-PAGE, transferred to an ECL-Hybond nitrocellulose membrane and sequentially incubated with monoclonal mouse anti-human p53 MAb at 1:100 dilution and mouse anti-IgG at 1:1,500 dilution. The molecular weight is indicated in kDa on the right.

DISCUSSION

The oncogenes chosen for this study were of interest for the following reasons. First, enhanced and more frequent expression of H-*ras* and c-*myc* was found in some transformed cell lines and in tissues representing oncogenic progression in situ [Durst et al., 1987a; Crook et al., 1990; Li et al., 1992; Shin et al., 1994]. Second, transfection of the H-*ras* oncogene into HPV-immortalized cells induced tumorigenicity [DiPaolo et al., 1989]. Third, amplification or point mutation of c-*myc* and H-*ras* oncogenes was detected in cervical carcinomas [Ocadiz et al., 1987; Sagae et al., 1989]. Consistent with the earlier results in situ, increased expression of H-*ras* and c-*myc* was also seen in our HPV-immortalized cells. However, no change was found after malignant CSC-transformation of

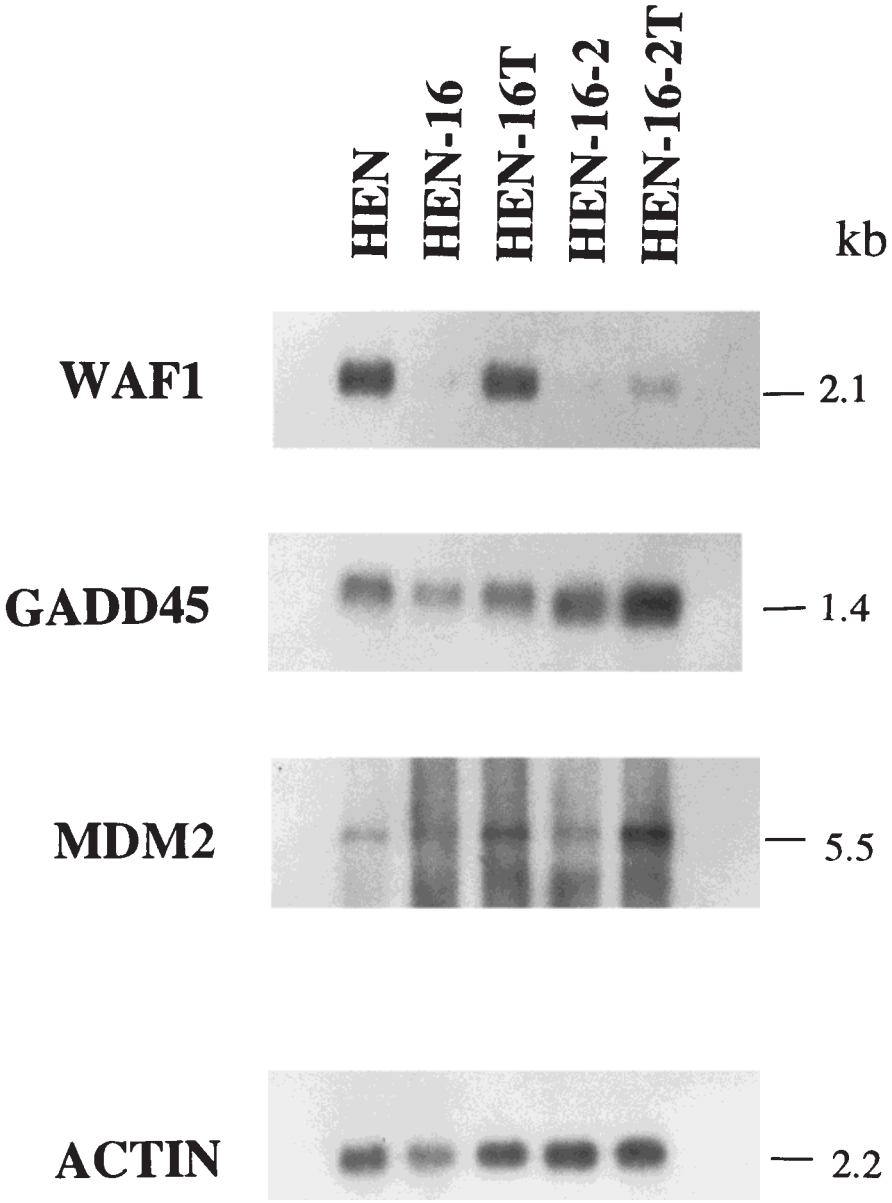

Fig. 3. Northern blot analyses of mRNA expression of p53 downstream effector genes, WAF1, GADD45 and MDM-2, in the five types of endocervical cells. Labels and conditions are as in Figure 1.

the immortalized cells (Table II, Fig. 1). This indicated that these oncogenes are important during early stage(s) and continued high-level, but not higher-level, expression may be involved in later stage(s) of carcinogenesis.

This study is the first to show that B-*myb* oncogene deregulation by HPV and CSC may be involved in the pathways leading to the transformation of primary cervical cells. B-*myb* was up-regulated by the HPV16 E7 oncoprotein in 3T3 fibroblasts [Lam et al., 1994]. Thus, B-myb transactivation in HPV16-immortalized endocervical cells and enhanced expression in CSC-transformed cells (Fig. 1, Table II) may be caused by E7 expression in both lines [Sarma et al., 1996]. In noncervical cells, elevated expression of B-*myb* bypassed p53-induced WAF1-mediated G_1 arrest [Lin et al., 1994]. Thus, activation of B-*myb* by HPV E7 may allow cells to bypass the normal cell cycle arrest mediated by tumor suppressor genes, and lead to immortalization. In addition, the higher levels of B-*myb* mRNA after CSC-tumorigenesis suggest a role of B-*myb* in transformation.

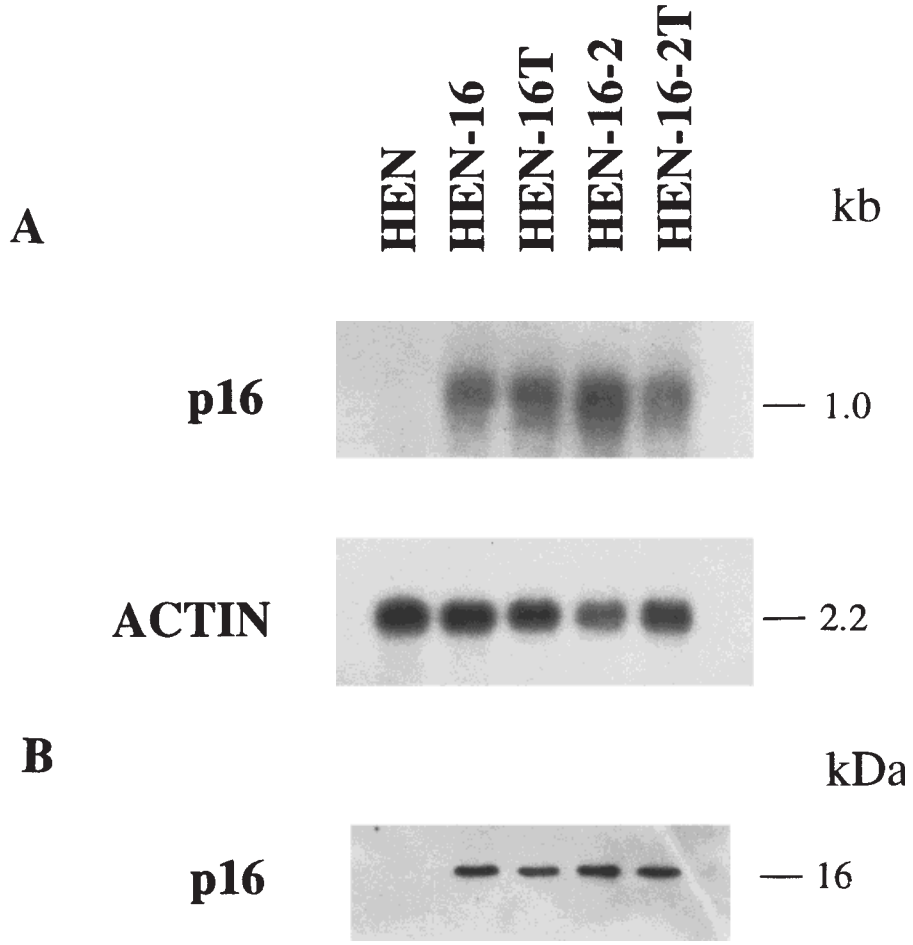

Fig. 4. Northern blot mRNA and Western blot protein analyses of p16 expression in the five types of endocervical cells. Conditions and labels are as in Figure 2.

The p53 tumor suppressor is usually inactivated by oncogenic type HPV E6-mediated degradation in cervical carcinoma cells [Werness et al., 1990; Scheffner et al., 1994]. However, little is known of the role of p53 downstream effector genes in cervical cell oncogenesis. For example, whether the inactivation of p53 is an early or late event in cervical carcinogenesis is uncertain. The cellular WAF1, GADD45 and MDM2 genes are directly up-regulated by p53 [Kastan et al., 1992; Oliner et al., 1992; El-Deiry et al., 1993; Harper et al., 1993; Chen et al., 1994]. Changes in expression in this study were parallel for p53 mRNA and protein and WAF1 mRNA, as expected, but not for GADD45 or MDM2. This suggests p53 selectively acts via WAF1. For immortalization, the results were consistent with most previous studies [reviewed by Scheffner et al., 1994], but the p53 mRNA and protein were significantly increased in CSC-transformed cells (Table II). The increase was unexpected, since the expression of HPV16 mRNA was the same in HPV16-immortalized and CSC-transformed cells [Sarma et al., 1996]. Increased levels of p53 protein were observed only for HPV-negative cervical carcinoma cell lines containing p53 mutations [Scheffner et al., 1991]. We did not assay p53 mutations in the CSC-transformed cells. WAF1-transactivating p53 protein was recently found in HPV-positive cervical cancer cell lines. However, only HPV-negative lines contained mutant p53 instead of HPV [Butz et al., 1995]. Wild type p53 status is expected due to our finding parallel levels of p53 protein and WAF1 mRNA. Increased p53 protein levels were also observed by others in chemically transformed oral keratinocytes [Shin et al., 1994]. Although the mechanism by which p53 protein is increased during transformation is unknown, our data support

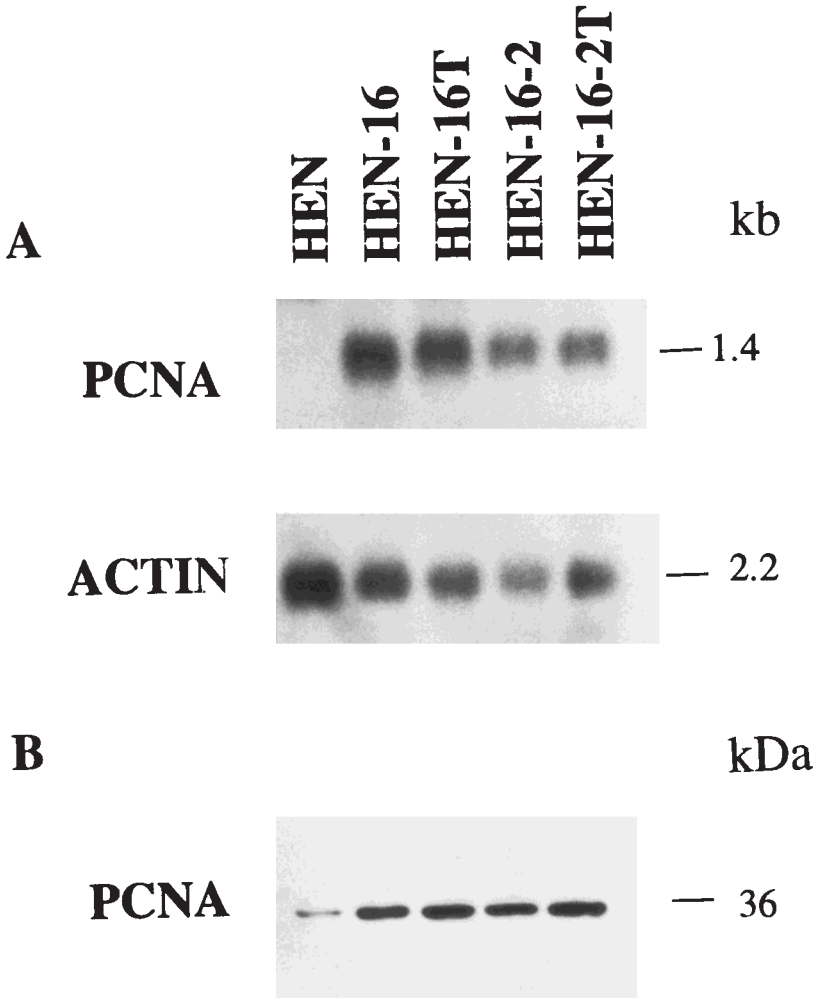

Fig. 5. Northern blot mRNA and Western blot protein analyses of PCNA expression in the five types of endocervical cells. Conditions and labels are as in Figure 2. Mouse anti-human PCNA MAb at 1:500 dilution and mouse anti-IgG at 1:1500 dilution were used.

the hypothesis that p53 degradation via HPV E6 is not involved in malignant carcinogenesis [Peacock et al., 1995]. Thus, the present results help clarify the confounding roles of p53 in immortalization and malignant transformation of cervical cells.

PCNA/cyclin is involved in DNA replication and repair and was overexpressed in many types of malignancies and CINs [Hall et al., 1990; Demeter et al., 1994; Karakitsos et al., 1994; Zeng et al., 1994]. We found that PCNA mRNA and protein were also increased in immortalized and transformed endocervical cells, but neither was different between HPV16-immortalized cells and CSC-transformed cells. Therefore, activation of PCNA is important for immortalization of human cervical cells. The mechanism by which PCNA is involved in immortalization is unclear. However, p53 suppressed the transcriptional activity of PCNA indirectly [Subler et al., 1992]. Since HPV16 E6 inactivates p53, PCNA may be activated in HPV-immortalized cells.

p16 is a tumor suppressor gene having cell cycle-associated expression and containing frequent point mutations or deletions in various noncervical primary human tumors and tumor cell lines [Nobori et al., 1994; Kamb et al., 1994; Brenner and Aldas, 1995; Cairns et al., 1995]. Increased expression of p16 during G_1 results from inactivation of pRB, since the inhibition of the cell cycle and suppression of tumor cells by p16 require functional pRB [Aagaard et al., 1995; Lukas et al., 1995; Medema et al., 1995]. p16 protein expression was higher in both pairs of HPV-16 immortalized and CSC-transformed

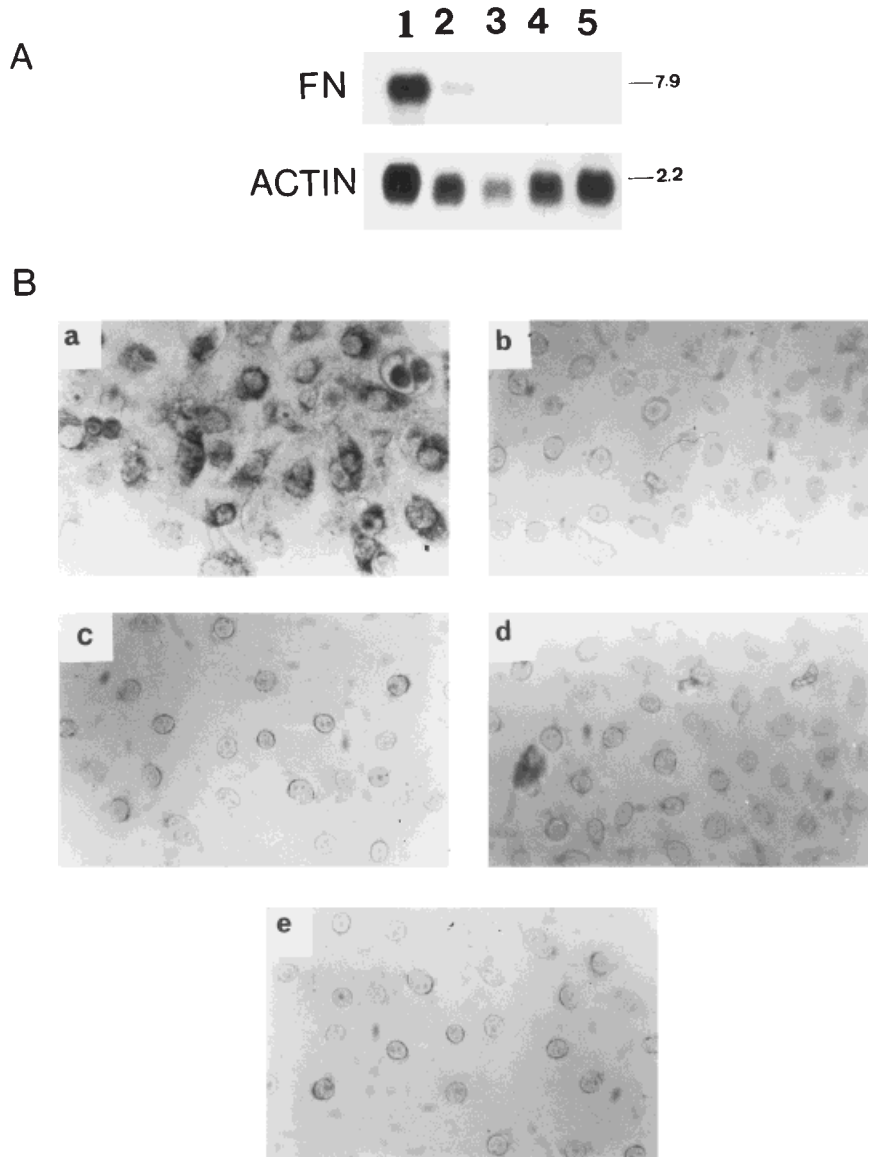

Fig. 6. Northern blot and in situ hybridization analyses of fibronectin (FN) expression in the five types of endocervical cells. **A:** Northern blot mRNA analysis for HEN, HEN-16, HEN-16T, HEN-16-2, HEN-16-2T, lanes 1–5, respectively. Conditions and labels are as in Figure 2A. **B:** In situ hybridization analysis of FN mRNA in cultured cells. Nonradioactive detection was for HEN, HEN-16, HEN-16T, HEN-16-2, HEN-16-2T, a–e, respectively.

HEN cell lines, since the expression of pRb was significantly decreased and pRb was inactivated by HPV16 E7 in these cells [Scheffner et al., 1991; data not shown]. Our unique in vitro system gave results consistent with previous reports. However, in distinction from noncervical systems, no p16 deletions or mutations were detected in our HPV16-immortalized or CSC-transformed tumor cell lines; or cervical premalignant and malignant clinical specimens [Nakao et al., 1997]. Further, the present results indicate that dysregulation of p16 expression occurs in an early, but not late, stage of progression towards cervical transformation.

Senescence is a process in which cells become unresponsive to all proliferative stimuli and is regarded as the growth arrest that blocks immortalization. Previous evidence implicated four dominant genes or complementation groups in senescence, indicating that the gene functions are essential for immortalization [Pereira-Smith and Smith, 1988]. FN is an established marker of cellular senescence, as evidenced by the significantly increased FN mRNA and pro-

tein towards the end of cellular proliferative ability in tissue culture [Kumazaki et al., 1991; Khandjian et al., 1992]. However, FN mRNA levels were low in SV40-transformed cells [Khandjian et al., 1992]. Consistently, expression of FN mRNA in both assays was diminished in both matching HPV-immortalized cell line pairs (Fig. 6). Noda et al. [1994] found that the expression of WAF1 increased significantly in senescent cells. In our study, similar results support the role of WAF1 in senescence that was reported previously [El-Deiry et al., 1993]. A relatively low level of WAF1 was found in HPV16-immortalized and CSC-transformed cells, compared with that in normal HEN (Fig. 3). Thus, the down-regulation of senescence-promoting genes, such as WAF1, is probably important in the immortalization of primary endocervical cells by HPV16.

Cellular genes not examined in this study might also be important for immortalization, tumorigenicity and understanding the molecular mechanism of the multistep carcinogenesis from primary cervical cells. For example, chromosome 11 alterations were frequent in cervical neoplasia and microcell chromosome 11 transfer suppressed HeLa cell tumorigenic expression [Saxon et al., 1986; Mullokandov et al., 1996]. Thus, malignant transformation of cervical cells may require one or more tumor suppressor gene(s) in chromosome 11. In addition, we used mRNA differential display to identify novel cDNAs; one gene was activated and another was suppressed after immortalization [Yang et al., 1996b]. Furthermore, our unique in vitro system consists of HPV16 and cigarette smoke as factors for the initiation and malignant progression of endocervical cells. The next interesting experiment is to test the implications of the results in vivo, using tissues from women who are or are not infected by HPV16 or smokers.

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