Tumor Res. **28**, 63—76 (1993)

Effect of Retinoic Acid on the Growth and the Expression of the Human Papillomavirus 16 E6 and E7 Genes of the Cervical Carcinoma Cell Lines

Tomohisa FURUHATA[1], Toshiharu YAMASHITA[1], Koichi HIRATA[2], Kenji SUGAWARA[3] and Kei FUJINAGA[1]

[1] *Department of Molecular Biology, Cancer Research Institute,*
[2] *Department of Surgery (Section 1), Sapporo Medical University School of Medicine, South 1, West 17, Chuo-ku, Sapporo 060 and*
[3] *Department of Microbiology, Faculty of Medicine, Kyoto University, Konoe-cho, Yoshida, Sakyo-ku, Kyoto 606, Japan*

ABSTRACT

Human papillomavirus type 16 (HPV16) is involved in the development of cervical carcinoma and its viral gene products E6 and E7 are believed to be essential for the carcinogenic process. We analyzed the effect of retinoic acid (RA) on the growth and/or HPV16 E6 and E7 gene expression in the HPV16-containing cervical carcinoma cell lines SiHa, CaSki and QG-U and the HPV-free cell lines C33A and EBC-1. RA (10^{-6} M) suppressed the growth of SiHa cells by more than 90% and of QG-U cells by about 40%; however, the growth of CaSki, C33A and EBC-1 cells was not affected by RA. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was applied for investigation of the relationship between RA and the level of HPV16 E6/E7 mRNA. As a result, the E6/E7 mRNA level in SiHa and QG-U was reduced by RA by about 60% and 75%, respectively, while RA had no obvious effect on the E6 and E7 gene expression in CaSki cells. The chloramphenicol acetyltransferase (CAT) assay showed that transcription driven by HPV16 non-coding region (NCR) was suppressed by RA by about 75%. These results suggest that at least a part of the growth suppression of SiHa and QG-U by RA is mediated by suppression of E6 and E7 expression.

Key words: Retinoic acid, Cervical carcinoma cell line, Growth suppression, Human papillomavirus type 16 (HPV16), E6/E7 gene expression.

INTRODUCTION

The HPV group comprises more than 60 different virus types that infect epithelial cells and induce hyperproliferative lesions. Some types, such as HPV16 and HPV18, present a high risk for malignant conversions. These are believed to be involved in the pathogenesis of anogenital cancer, particularly in cervical carcinoma. HPV DNA is found in close to 90% of such tumors and HPV16 is the most frequent type among genital HPVs which are isolated from cervical carcinomas (1). The oncogenic potential of HPV16 has been attributed to two early genes, E6 and E7. These genes are expressed in cervical carcinomas and carcinoma derived cell lines containing HPV16 DNA (2, 3, 4). E6 and E7 genes together effectively induce immortalization of human epithelial cells (5, 6, 7, 8, 9); thus, they seem to play important roles in the development of tumors and also in the maintenance of tumor phenotypes (5, 10). Transcription of these genes is regulated by the non-coding region (NCR) that contains the early E6 and E7 promoter and enhancer elements with binding sites for various transcription factors (11).

Retinoids regulate the differentiation of epithelial cells and affect the proliferation and differentiation of preneoplastic and neoplastic cells (12). Retinoic acid (RA) is one of the biologically active retinoids and has been shown to be a potent inhibitor of the *in vitro* growth of various tumor cell lines (13, 14). Cellular responses to RA are mediated by nuclear retinoic acid receptors (RARs), including subtypes RARα, β, and γ (15, 16, 17, 18) and retinoids X receptors (RXRs) (19). RAR and RXR are transcription factors and belong to the superfamily of nuclear hormone receptors (20). The RARα gene is localized to chromosome 17q21.1, which is in close proximity to the translocation breakpoint t (15;17) (q22-q11-22) found consistently in acute promyelocytic leukemia (APL) (21). In clinical trials, RA has led to a high frequency of complete remission in APL patients (22).

It is reported that RA suppresses the growth of HPV18-carrying cervical carcinoma cell line HeLa (23) and human keratinocyte cell lines immortalized by HPV16 (24). However, it is not clear whether or not RA also inhibits the growth of HPV 16 positive and negative cell lines derived from cervical carcinoma. Here, we investigated the effect of RA on the growth and/or HPV16 E6 and E7 gene expression in the HPV16-containing cell lines SiHa, CaSki and QG-U and the HPV-free cell lines C33A and EBC-1.

MATERIALS AND METHODS

Cell culture and DNA transfection

Cervical carcinoma cell lines SiHa (25, 26), CaSki (26, 27), C33A (28) and HeLa (29, 30) and lung squamous carcinoma cell line EBC-1 (31) were obtained from the Japanese Cancer Research Resources Bank. Cervical carcinoma cell line QG-U (32) was kindly provided by Dr. B. Shimizu (Chiba Univ., Chiba, Japan). Cells were cultured in Dulbecco modified Eagle medium (DME) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Bio-product, MD, U. S. A.), 30 μg/m*l* of penicillin G potassium (Banyu, Tokyo, Japan) and 40 μg/m*l* of streptomycin (Meiji Seika, Tokyo, Japan). For the assay of plating efficiency, cells were seeded at densities of 2×10^5 cells per 100 mm dish, cultured for 24 hours and inoculated with pSV2hygro (5 μg) and either RARβ plasmid or pGEM-3Zf(+) (Promega, MD, U. S. A.) (20 μg) by a modification of the calcium phosphate coprecipitation procedure (33). Cells were cultured for 24 hours, trypsinized and split into four dishes. Cells were then cultured in the media containing 150 μg/m*l* (SiHa) or 50 μg/m*l* (CaSki, QG-U, C33A and EBC-1) of hygromycin B (Calbiochem, CA, U. S. A.). Ten days after transfection, hygromycin B resistant-colonies were fixed with methanol and stained with Giemsa so that the colonies could be scored.

Plasmids

The plasmid pSG5-RARβ (Wt-RARβ), which contains human RARβ cDNA under the cytomegalovirus immediate early promoter (17), was kindly provided by Dr. P. Chambon (Institute de Chimie Biologique, Strasbourg, France). The pSG5-RARβ*dl* (Mt-RARβ) is a plasmid containing an inert RARβ cDNA which was constructed by removing the *Kpn*I fragment, sequences of DNA binding domain of RARβ cDNA, from pSG5-RARβ. The NCR of HPV16 was cloned upstream of chloramphenicol acetyltransferase (CAT) gene and the recombinant plasmid p16NCR-CAT was obtained. This was constructed as follows. The *Bgl*II-*Bam*HI fragment of the pCAT promoter vector (Promega) which contains SV40 promoter, CAT gene and SV40 poly (A) signal sequences was isolated and re-cloned in the *Bam*HI site of pHSG299 (34). The resulting plasmid was linealized with *Kpn*I, blunted with T4 DNA polymerase, and re-digested with *Pst*I. Then, the fragment containing the CAT gene and control sequences was isolated and inserted downstream (*Pst*I and blunt-ended *Hind*III sites) of the cottontail rabbit papillomavirus (CRPV) NCR sequences (639 base pairs [bp]) cloned in the *Xba*I and *Pst*I sites of pUC19, thus yielding the pSCAT0. The pSNC was constructed from pSCAT0 by deleting the CRPV NCR sequence; pCAT0 was digested with *Pst*I and *Sma*I, and the fragment containing the CAT gene and control sequences was blunted with T4 DNA polymerase and self-ligated. The HPV16 NCR (*Hae*II 7428 nucleotide position [np] to *Hpa*II 7457 np) was iso-

lated from pHPV16M (35), blunted with Klenow enzyme and inserted into the *Sma*I site of pGEM-3Zf(+) (Promega). Then, the HPV16 NCR fragment was excized with *Xba*I and *Sac*I, and re-inserted downstream of SV40 promoter-CAT sequence of pSNC (*Xba*I and *Sac*I sites), yielding the plasmid p16NCR-CAT. The pSV2hygro is a plasmid carrying a hygromycin resistant gene (36).

RNA isolation and semi-quantitative RNA analysis

Total RNA was isolated from each of the cell lines by the method using acid guanidinium thiocyanate-phenol chloroform of Chomcyski and Sacchi (37). For the cDNA synthesis, reverse transcription was carried out at 42℃ for 30 min in 30 μl of a reaction mixture containing 5 μg of total cellular RNA, 2 μg of oligo dT (Boehringer Mannheim, GmbH, Germany), 55 units of RNase inhibitor (TaKaRa, Kyoto, Japan), 1 mM of each dNTP, 50 mM Tris-HCl (pH 8.3), 100 mM KCl, 10 mM $MgCl_2$, and 10 mM DTT, and 50 units of reverse transcriptase (TaKaRa). PCR was performed according to the method of Saiki *et al* (38) with a slight modification (39).

Semi-quantitative analysis of mRNA by RT-PCR was performed essentially by the method of T. Kinoshita *et al* (40). One-twentieth of the cDNA product was amplified in 50 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (W/V) gelatine, 0.2 mM of dNTP, 1 μl of 25 μM primer, 10 μCi of α-^{32}P-dCTP and 2.5 units of Taq polymerase (TaKaRa). Samples were overlaid with 30 μl of mineral oil and denatured for 10 min at 94℃. The reaction mixture was then subjected to 33 cycles of amplification in a DNA thermal cycler (Cetus, CT, U. S. A.). Each PCR cycle included 1 min at 94℃, 2 min at 55℃ and 2 min at 72℃. Primers used to amplify the HPV16 E6/E7 cDNA sequence were pE7-F and pE7-R (41), and primers to amplify the β-actin cDNA sequence were BA2 and BA3 (40).

The expected sizes of the amplified products were 356 bp for HPV16 E6/E7 and 218 bp for β-actin. Amplification was started with the primers for the HPV16 E6/E7 mRNA sequence. After the first 6 cycles, 1 μl of BA2 and BA3 (25 μM each) was added to the reaction mixture, and 27 additional cycles of amplification were carried out. During 19-33 cycles, 3 μl aliquots of samples were removed every 2 or 3 cycles. The 3 μl serial samples of reaction mixture were electrophoresed in Tris-borate buffer on a 2% agarose gel. Then, gels were dried and exposed to an imaging plate (Fuji Film, Tokyo, Japan) for 60 min, and the amplified products were evaluated with a laser image analyzer (Fujix BAS 2000, Fuji Film) by measuring the intensity of photo-stimulated luminescence (PSL) of the bands.

The PSL values of each band were plotted on a semilogarithmic scale

against the sampling cycle number to draw amplification curves. The difference in cycle number between the logarithmic phases of the amplification curves of HPV16 E6/E7 and β-actin in each sample was defined as Δcycle. The relative amount of HPV16 E6/E7 mRNA to β-actin mRNA was determined as $2^{\Delta cycle}$.

CAT assay

For the CAT assay, HeLa cells were seeded at 5×10^6 cells per 100 mm dish and transfected with 8 μg of the reporter CAT plasmid p16NCR-CAT and 8 μg of either Wt-RARβ or Mt-RARβ plasmid. Cell extracts were made 48 hours after transfection and CAT activity was measured as described (42). Briefly, the cell extracts were incubated with ^{14}C-chloramphenicol and 4 mM acetyl CoA (Pharmacia, Uppsala, Sweden) in 250 mM Tris-HCl (pH 7.8) at 37℃ for 30 min. After analysis by ascending thin layer chromatography, the conversion rates were determined with the laser image analyzer.

RESULTS

Effect of retinoic acid treatment on the growth of cervical carcinoma cell lines

The effect of RA on the growth of cervical carcinoma cell lines was determined by the assay of the plating efficiency of cells which acquired hygromysin B resistancy. RA (10^{-6}M) suppressed the growth of SiHa and QG-U cells by over 90% and about 40%, respectively, while the number of colonies of C33A and CaSki cells were not affected by RA (Fig. 1, 2). The growth of EBC-1 cell line, which is free of HPV as well as C33A, was not suppressed by RA in this assay (data not shown). SiHa and QG-U cells contain a few copies of HPV16 DNA per cell (26, 32), while CaSki cells, about 600 copies per cell (26). It is not possible here to say whether RAR protein is not functional in CaSki cells or is simply not present in sufficient quantity for RA to take effect. Therefore, we tested the plating efficiency of CaSki cells after transfection of pSV2hygro with the RARβ plasmid. However, the growth of CaSki cells was not suppressed by RA in this condition (Fig. 1).

Effect of retinoic acid treatment on the HPV16 E6/E7 mRNA level

We have addressed the question whether RA-mediated growth suppression is associated with changes of HPV16 E6 and E7 gene expression. The influence of RA on the expression of HPV16 E6 and E7 genes was examined. Northern blot analysis was performed using RNA extracted from cells cultured in the presence of RA for 0 to 48 hours. A total quantity of 20 μg of poly (A) RNA was used, but the RNA band hybridized to a HPV16 E6/E7 probe was very faint in SiHa cells (data not shown). Next, we performed a semi-quantitative analysis of

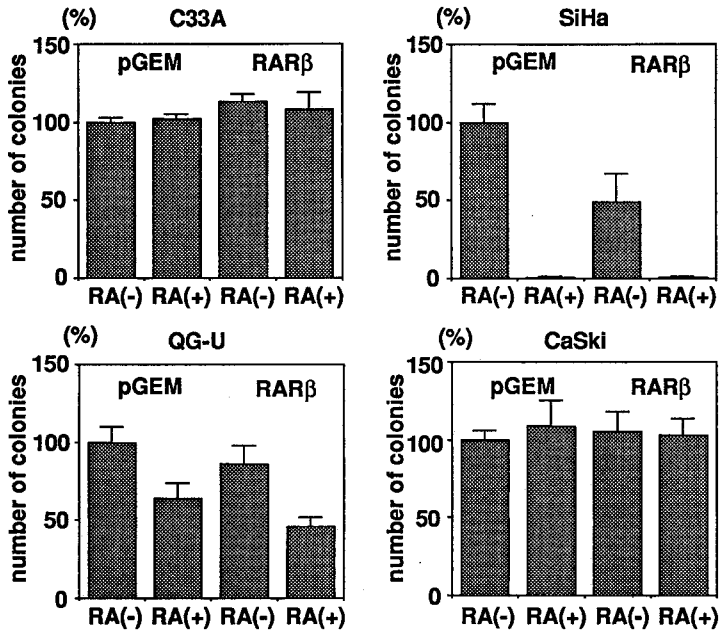

Fig. 1 Plating efficiencies of drug-resistant clones of cervical carcinoma cell lines C33A, SiHa, CaSki and QG-U. Cells were seeded at 2×10^5 cells per 100 mm dish and transfected with pSV2hygro and pGEM-3Zf(+) or pSV2hygro and pSG5-RARβ. Cells were cultured in the DME containing 5% FBS and Hygromycin B with or without RA (10^{-6}M). The number of drug-resistant colonies was scored ten days after transfection. Each value represents the mean and SE of at least two separate experiments. The number of colonies which was transfected with pGEM-3Zf(+) and pSV2hygro in the absence of RA was normalized to 100%.

viral mRNA by RT-PCR (Fig. 3). cDNA synthesized from SiHa, QG-U and CaSki cells were used as substrates from which HPV16 E6/E7 and β-actin sequences were co-amplified by mixed primer pairs specific for each of them. Aliquots of PCR solution were removed at every other cycle from the 19th on and electrophoresed in an agarose gel and the amounts of amplified DNA were quantified as described in Materials and Methods. For example, as shown in Fig. 3A and C, HPV16 E6/E7 and β-actin cDNA sequences were amplified simultaneously from SiHa cDNA obtained without RA. HPV16 E6/E7 cDNA was amplified 1.5 cycles behind β-actin in the presence of RA (Fig. 3B and D), indicating that the molar ratio of evolved HPV16 E6/E7 mRNA to β-actin mRNA was $2^{-1.5}$.

A summary of the results using SiHa, QG-U and CaSki cells is shown in Fig. 4. The level of E6/E7 mRNA was reduced by about 60% and 50% in SiHa cells

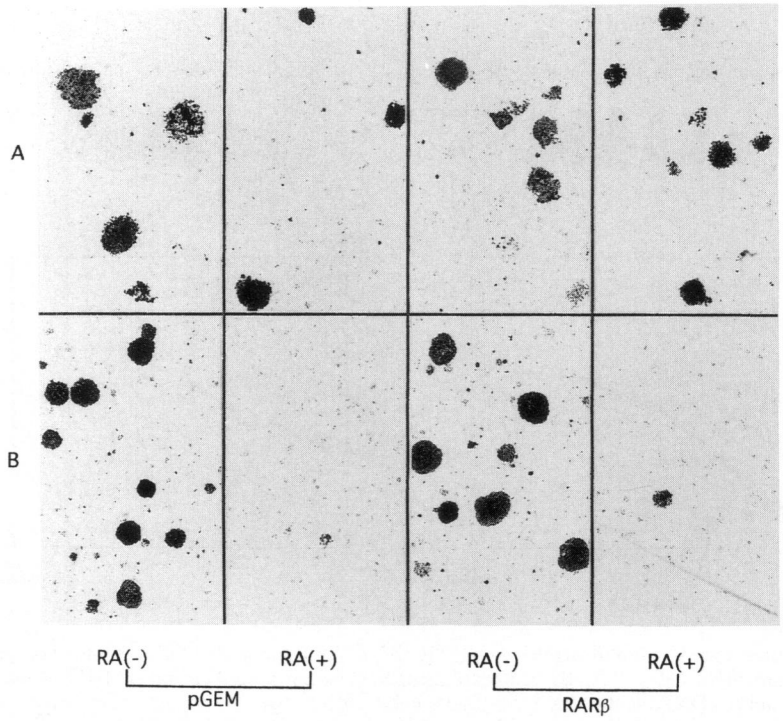

Fig. 2 Representative dishes of cervical carcinoma cell lines transfected with pSV2hygro and pSG5-RARβ. Cells were transfected with pSV2hygro and pGEM-3Zf(+) or pSV2hygro and pSG5-RARβ, split and cultured in media containing Hygromycin B with or without RA (10^{-6}M). Cells were fixed by methanol and stained with Giemsa ten days after transfection. (A) C33A, (B) SiHa (magnification: 10×).

and by about 60% and 75% in QG-U cells 24 and 48 hours after culture, respectively, in the presence of RA. However, the decrease of E6/E7 transcript was not observed in CaSki cells cultured in RA-containing media for 24 to 48 hours. (Fig. 4). These results suggest that the growth suppression of SiHa and QG-U cells by RA is mediated by down-regulation of HPV16 E6/E7 mRNA.

Effect of retinoic acid treatment on transcription of HPV16 RNA

In order to study the effect of RA on transcription of HPV16 RNA, we performed a CAT assay in HeLa cells (Fig. 5A). When HeLa cells were cotransfected with reporter plasmid p16NCR-CAT, which contains a regulatory region, a non-coding region (NCR) of HPV16 upstream of CAT gene and Wt-RARβ plasmid DNA, CAT activity was reduced about 75% in the presence of RA. In case of transfection of p16NCR-CAT along with Mt-RARβ plasmid, CAT activ-

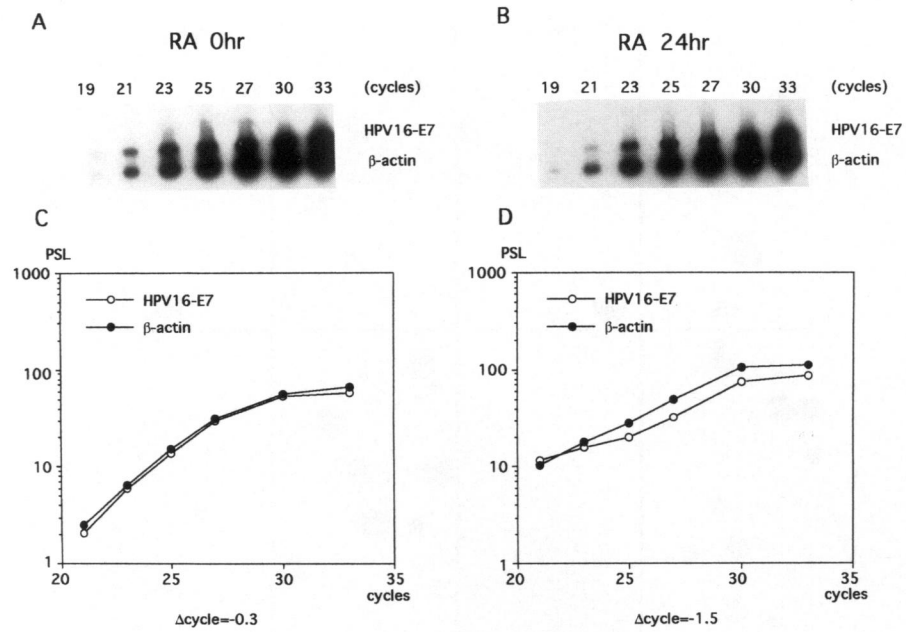

Fig. 3 Simultaneous amplification of HPV16 E6/E7 and β-actin cDNA sequences prepared from SiHa cells. (A, B) Autoradiographs showing amplification of HPV16 E6/E7 and β-actin cDNA sequences from SiHa cells. RNA was extracted from SiHa cells cultured in the media with RA (10^{-6}M) for 0 hours (A) and 24 hours (B). (C, D) Amplification curves of HPV16 E6/E7 and β-actin cDNA sequences. Gels which were used in the autoradiography (A, B) were exposed to an imaging plate, and the PSL value of each band was measured with the laser image analyzer and plotted on a semilogarithmic scale against cycle number.

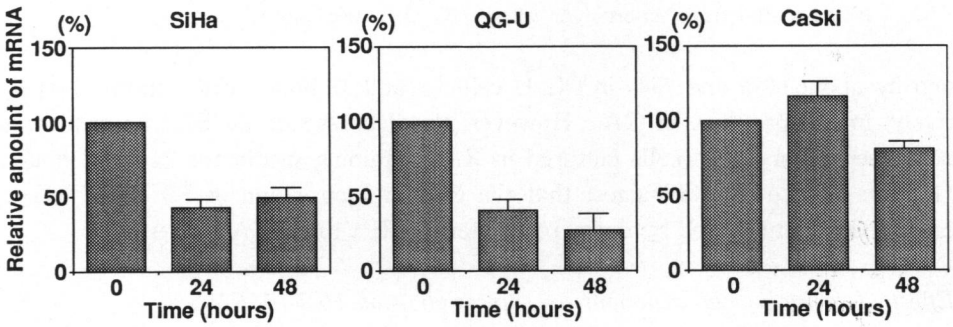

Fig. 4 Relative amount of HPV16 E6/E7 mRNA sequence determined by RT-PCR. RNA was extracted from each cell line cultured in the media with RA (10^{-6}M) for 0, 24 and 48 hours. The relative amount of HPV16 E6/E7 to β-actin mRNA was estimated using RT-PCR as described in Materials and Methods. The relative amount of HPV16 E6/E7 mRNA in each cell line cultured in the media with RA (10^{-6}M) for 0 hours was normalized to 100%. Each value represented the mean and SE of at least two separate experiments.

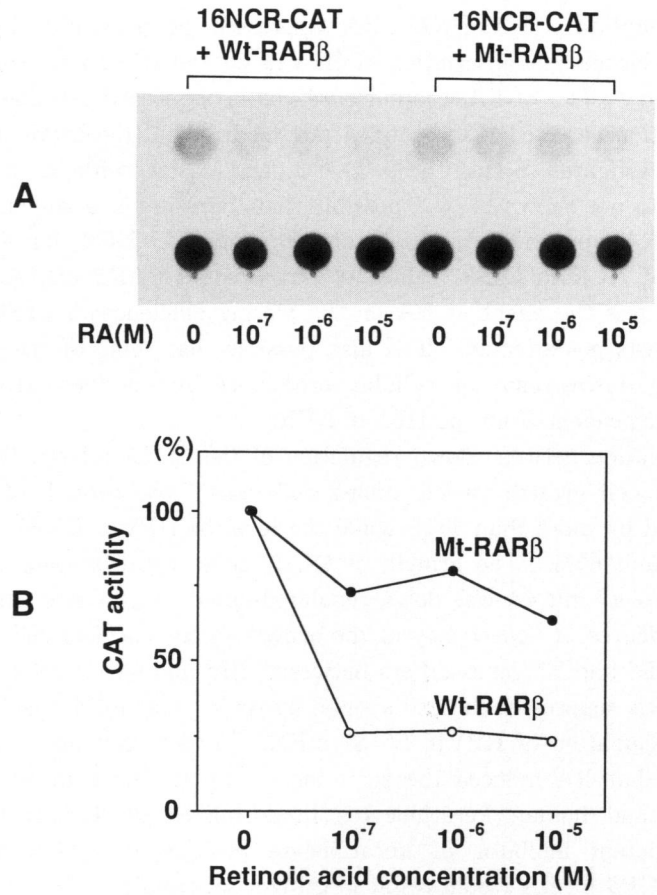

Fig. 5 The effect of RA on the regulatory region (NCR) of HPV16 E6 and E7 transcription. (A) Autoradiograph of CAT assay in HeLa cells transfected with p16NCR-CAT and Wt-RARβ or p16NCR-CAT and Mt-RARβ plasmids in the presence or absence of RA. The experiment was carried out as described in Materials and Methods. (B) The CAT activity was measured by a laser image analyzer, and the CAT value in the absence of RA was normalized to 100%. Values were averages of double determinations.

ity was reduced only about 25% (Fig. 5B). This result suggests that the promoter activity of HPV16 NCR is reduced by RA on condition that sufficient RARβ exists in the cell.

DISCUSSION

Here, we examined the effect of RA on the growth of cervical carcinoma cel

lines which contained HPV16 DNA. RA suppressed the growth of SiHa and QG-U cells and reduced the transcription of HPV16 E6 and E7 genes. However, this growth-inhibiting effect of RA was not observed in the CaSki cell line. Northern blot hybridization detected RARα and β mRNA in all of the cervical carcinoma cell lines investigated, SiHa, CaSki, QG-U and C33A, without a significant difference (data not shown). It is possible that there is not a sufficient quantity of functional RAR protein, which acts on 600 copies of NCR, for RA to affect the function of NCR in CaSki cells. We transfected RARβ cDNA along with pSV2hygro to test the effect of RA on the plating efficiency of CaSki cells, but their growth was not affected. It is also possible that some of the viral copies are integrated downstream of cellular promoters and actively transcribed in CaSki cells independent from the HPV16 NCR.

The correlation between down-regulation of the E6/E7 mRNA level and the suppression of cell growth varied among cell lines. The growth of SiHa cells was suppressed by more than 90%, while the level of HPV16 E6/E7 mRNA was reduced by about 60%. The growth of QG-U cells were decreased only about 40%, though E6/E7 mRNA was down-regulated about 75% by RA. It is conceivable that the degree of dependency of the growth of the cervical cell lines on the expression of E6 and E7 genes differs between SiHa and QG-U cells. Also, it is possible that RA suppresses the cell growth by some other mechanism in addition to the down-regulation of HPV16 E6/E7 mRNA in these cell lines. Recently, it was reported that RA induced the secretion of latent TGFβ in normal human keratinocytes and human keratinocytes immortalized by HPV16 (43). Since TGFβ is a potent inhibitor of keratinocyte proliferation (44, 45, 46), induced secretion of TGFβ by RA would result in growth suppression.

E6 and E7 genes of HPV 16 are always expressed in HPV16 positive cervical carcinomas (5). Moreover, the inhibition of E6 and E7 gene expression by antisense RNA results in the growth suppression of cervical carcinoma cell lines which contain HPV DNA (10, 11). This study has shown that reduction of NCR activity by RAR can repress the growth of cancer cells containing HPV DNA. By using CAT assay, we and others (23) showed that RA repressed the E6/E7 transcription from the HPV16 NCR. Although the mechanism of the suppression is not well understood, it is suggested that the transcription factor AP-1 is involved in the RA-mediated NCR suppression (23). A few other factors, NF-IL6 (47), Oct-1 (48) and TGFβ (49) have been shown to repress NCR activity and E6/E7 transcription. We would like to continue further analysis of the effects of RAR in cooperation with other factors on the activity of NCR and cell growth.

ACKNOWLEDGEMENTS

We thank Dr. P. Chambon for providing us the RAR cDNA plasmids and Drs. H. Shirasawa and B. Shimizu (Chiba Univ.) for QG-U cell line. We also thank Drs. H. Morioka and E. Ohtsuka (Hokkaido Univ., Sapporo, Japan) for helping us to use the laser image analyzer.

REFERENCES

1. zur HAUSEN H. Viruses in human cancers. Science 1991, 254: 1167-1173.
2. BAKER CC, PHELPS WC, LINDGREN V, BRAUN MJ, GONDA MA, HOWLEY PM. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J Virol 1987, 61: 962-971.
3. CHOO KB, PAN CC, HAN SH. Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: Preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. Virology 1987, 161: 259-261.
4. SCHWARZ E, FREESE UK, GISSMANN L, MAYER W, ROGGENBUCK B, STREMLAU A, zur HAUSEN H. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature 1985, 314: 111-114.
5. von KNEBEL DOEBERITZ M, OLTERSDORF T, SCHWARZ E, GISSMANN L. Correlation of modified human papilloma virus early gene expression with altered growth properties in C4-1 cervical carcinoma cells. Cancer Res 1988, 48: 3780-3786.
6. BARBOSA MS, SCHLEGEL R. The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage *in vitro* transformation of human keratinocytes. Oncogene 1989, 4: 1529-1532.
7. HAWLEY-NELSON P, VOUSDEN KH, HUBBERT NL, LOWY DR, SCHILLER JT. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J 1989, 8: 3905-3910.
8. MÜNGER K, PHELPS WC, BUBB V, HOWLEY PM, SCHLEGEL R. The E6 and E7 genes of human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol 1989, 63: 4417-4421.
9. HUDSON JB, BEDELL MA, MCCANCE DJ, LAIMINS LA. Immortalization and altered differentiation of human keratinocytes *in vitro* by the E6 and E7 open reading frames of human papillomavirus type 18. J Virol 1990, 64: 519-526.
10. WATANABE S, KANDA T, YOSHIIKE K. Growth dependence of human papillomavirus 16 DNA-positive cervical cancer cell lines and human papillomavirus 16-transformed human and rat cells on the viral oncoproteins. Jpn J Cancer Res 1993, 84: 1043-1049.
11. CHONG T, CHAN WK, BERNARD HU. Transcriptional activation of human papillomavirus 16 by nuclear factor 1, API, steroid receptors and a possibly novel transcription factor, PVF: a model for the composition of genital papillomavirus enhancers. Nucleic Acids Res 1990, 18: 465-470.
12. SPORN MB, ROBERTS AB. Role of retinoids in differentiation and carcinogenesis. Cancer Res 1983, 43: 3034-3040.

13. JETTEN AM, KIM JS, SACKS PG, REARICK JI, LOTAN D, HONG WK, LOTAN R. Inhibition of growth and squamous-cell differentiation markers in cultured human head and neck squamous carcinoma cells by β-all-trans retinoic acid. Int J Cancer 1990, 45: 195-202.
14. PODDAR S, HONG WK, THACHER SM, LOTAN R. Retinoic acid suppression of squamous differentiation in human head-and-neck squamous carcinoma cells. Int J Cancer 1991, 48: 239-247.
15. GIGUERE V, ONG ES, SEGUI P, EVANS RM. Identification of a receptor for the morphogen retinoic acid. Nature 1987, 330: 624-629.
16. PETKOVICH M, BRAND NJ, KRUST A, CHAMBON P. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 1987, 330: 444-450.
17. BRAND N, PETKOVICH M, KRUST A, CHAMBON P, de Thé H, MARCHIO A, Tiollais P, Dejean A. Identification of a second human retinoic acid receptor. Nature 1988, 332: 850-853.
18. KRUST A, KASTNER P, PETKOVICH M, ZELENT A, CHAMBON P. A third human retinoic acid receptor, hRAR-γ. Proc Natl Acad Sci USA 1989, 86: 5310-5314.
19. MANGELSDORF DJ, ONG ES, DYCK JA, EVANS RM. Nuclear receptor that identifies a novel retinoic acid response pathway. Nature 1990, 345: 224-229.
20. EVANS RM. The steroid and thyroid hormone receptor superfamily. Science 1988, 240: 889-895.
21. de Thé H, CHOMIENNE C, LANOTTE M, DEGOS L, DEJEAN A. The t(15;17) translocation of acute promyelocytic leukemia fuses the retinoic acid receptor α gene to a novel transcribed locus. Nature 1990, 347: 558-561.
22. MENG-ER BH, YU-CHEN Y, SHU-RONG C, JIN-REN C, JIA-XIANG L, LIN Z, LONG-JUN G, ZHEN-YI W. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood 1988, 72: 567-572.
23. BARTSCH D, BOYE B, BAUST C, zur HAUSEN H, SCHWARZ E. Retinoic acid-mediated repression of human papillomavirus 18 transcription and different ligand regulation of the retinoic acid receptor β gene in non-tumorigenic and tumorigenic HeLa hybrid cells. EMBO J 1992, 11: 2283-2291.
24. PIRISI L, BATOVA A, JENKINS GR, HODAM JR, CREEK KE. Increased sensitivity of human keratinocytes immortalized by human papillomavirus type 16 DNA to growth control by retinoids. Cancer Res 1992, 52: 187-193.
25. FRIEDL F,KIMURA I, OSATO T, ITO Y. Studies on a new human cell line (SiHa) derived from carcinoma of uterus. I. Its establishment and morphology. Proc Exp Biol Med 1970, 125: 543-545.
26. YEE C, KRISHNAN-HEWLETT I, BAKER CC, SCHLEGEL R, HOWLEY PM. Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. Am J Pathol 1985, 119: 361-366.
27. PATTILLO RA, HUSSA RO, STORY MT, RUCKERT ACF, SHALABY MR, MATTINGLY RF. Tumor antigen and human chorionic gonadotropin in CaSki cells: A new epidermoid cervical cancer cell line. Science 1977, 196: 1456-1458.
28. AUERSPERG N. Long-term cultivation of hypodiploid human tumor cells. J Nat Cancer Inst 1964, 32: 135-163.

29. GEY GO, COFFMAN WD, KUBICEK MT. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res 1952, 12: 264-265.
30. SCHNEIDER-GÄDICKE A, SCHWARZ E, Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J 1986, 5: 2285-2292.
31. IMANISHI K, YAMAGUCHI K, SUZUKI M, HONDA S, YANAIHARA N, ABE K. Production of transforming growth factor-α in human tumor cell lines. Br J Cancer 1989, 59: 761-765.
32. SHIRASAWA H, TOMITA Y, SEKIYA S, TAKAMIZAWA H, SHIMIZU B. Integration and transcription of human papillomavirus type 16 and 18 sequences in cell lines derived from cervical carcinomas. J Gen Virol 1987, 68: 583-591.
33. GRAHAM FL, van der EB AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 1973, 52: 456-467.
34. TAKESHITA S, SATO M, TOBA M, MASAHASHI W, HASHIMOTO-GOTOH T. High-copy-number and low-copy number plasmid vectors for *lacZ*α-complementation and chloramphenicol- or kanamycin-resistance selection. Gene 1987, 61: 63-74.
35. NISHIKAWA T, YAMASHITA T, YAMADA T, KOBAYASHI H, OHKAWARA A, FUJINAGA K. Tumorigenic transformation of primary rat embryonal fibroblasts by human papillomavirus type 8 E7 gene in collaboration with the activated H-*ras* gene. Jpn J Cancer Res 1991, 82: 1340-1343.
36. KASTER KR, BURGETT SG, NAGARAJA RAO R, INGOLIA TD. Analysis of a bacterial hygromycin B resistance gene by transcriptional and translational fusions and by DNA sequencing. Nucleic Acids Res 1983, 11: 6895-6911.
37. CHOMCZYNSKI P, SACCHI N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162: 156-159.
38. SAIKI RK, SCHARF S, FALOONA F, MULLIS KB, HORN GT, ERLICH HA, ARNHEIM N. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985, 230: 1350-1354.
39. SHIMADA M, FUKUSHIMA M, MUKAI H, KATO I, NISHIKAWA A, FUJINAGA K. Amplification and specific detection of transforming gene region of human papillomavirus 16, 18 and 33 in cervical carcinoma by means of the polymerase chain reaction. Jpn J Cancer Res 1990, 81: 1-5.
40. KINOSHITA T, IMAMURA J, NAGAI H, SIMOTOHNO K. Quantification of gene expression over a wide range by the polymerase chain reaction. Anal Biochem 1992, 206: 231-235.
41. FUJINAGA Y, OKAZAWA K, OHASHI Y, YAMAKAWA Y, FUKUSHIMA M, KATO I, FUJINAGA K. Human papillomavirus type 16 E7 gene sequence in human cervical carcinoma analysed by polymerase chain reaction and direct sequencing. Tumor Res 1990, 25: 85-91.
42. GORMAN C, MOFFAT N, HOWARD BH. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 1982, 2: 1044-1051.
43. BATOVA A, DANIELPOUR D, PIRISI L, CREEK KE. Retinoic acid induces secretion of latent transforming growth factor beta 1 and beta 2 in normal and human papillomavirus type 16-immortalized human keratinocytes. Cell Growth Differ 1992, 3: 763-772.
44. SHIPLEY GD, PITTELKOW MR, WILLE JJ, SCOT RE, MOSES HL. Reversible inhibition of normal human prokeratinocyte proliferation by type β transforming growth factor-growth

inhibitor in serum-free medium. Cancer Res 1986, 46 2068-2071.
45. COFFEY RJ, SIPES NJ, BASCOM CC, GRAVES-DEAL R, PENNINGTON CY, WEISSMAN BE, MOSES HL. Growth modulation of mouse keratinocytes by transforming growth factors. Cancer Res 1988, 48: 1596-1602.
46. REISS M, SARTORELLI AC. Regulation of growth and differentiation of human keratinocytes by type β transforming growth factor and epidermal growth factor. Cancer Res 1987, 47: 6705-6709.
47. KYO S, INOUE M, NISHIO Y, NAKANISHI K, AKIRA S, INOUE H, YUTSUDO M, TANIZAWA O, HAKURA, A. NF-IL6 represses early gene expression of human papillomavirus type 16 through binding to the noncoding region. J Virol 1993, 67: 1058-1066.
48. MORRIS, P. J., DENT CL, RING CJ, LATCHMAN DS. The octamer binding site in the HPV16 regulatory region produces opposite effects on gene expression in cervical and non-cervical cells. Nucleic Acids Res 1993, 21: 1019-1023.
49. WOODWORTH CD, NOTALIO V, DIPAOLO JA. Transforming growth factors beta 1 and 2 transcriptionally regulate human papillomavirus (HPV) type 16 early gene expression in HPV-immortalized human genital epithelial cells. J Virol 1990, 64: 4767-4775.