Enhancement of Human Papillomavirus Type 18 Gene Expression in HeLa Cells by 12-O-Tetradecanoylphorbol-13-acetate, 3β,5α-Dihydroxycholestan-6-one, and Cholesterol

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Human papillomavirus type 18 (HPV18) is involved in the genesis of cervical cancer through expression of its viral oncoprotein in infected cells. A tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), was found to increase the level of HPV18 transcripts in an HPV18-harboring cervical cancer cell line, HeLa. A similar increase in HPV18 expression was also observed on treatment of the cells with an oxygenated cholesterol, 3β,5α-dihydroxycholestan-6-one (yakkastrone). The effects on HPV18 expression elicited by TPA and yakkastrone were repressed by a protein kinase inhibitor, staurosporine. Treatment of the cells with cholesterol under serum-free conditions also resulted in an apparent increase of HPV18 expression.

Keywords human papillomavirus type 18; HeLa cell; 12-O-tetradecanoylphorbol-13-acetate (TPA); oxygenated cholesterol; cholesterol

Several types of human papillomaviruses (HPV's), especially types 16 and 18 (HPV16 and HPV18, respectively), are associated with many cervical cancers, and etiological roles in the development of cervical cancer have been suggested. Transfection of HPV16/HPV18-DNA causes oncogenic transformation of normal rodent cell lines or immortalization of primary human keratinocytes in vitro. These potentials of HPV-DNA are attributed to the expression of viral early genes, E6 and E7. E6 and E7 are known to be expressed in vivo in HPV-infected cells of cervical neoplasias, and the products are considered to have roles in the maintenance of malignant phenotype of the cells. Their expression in the cells is regulated by both viral and cellular factors. HPV gene expression in the infected cells is thought to be regulated mainly through the upstream regulatory region (URR) of the HPV sequence, which contains viral early promoter and some consensus sequences for the binding of transcriptional regulatory factors such as AP-1, NF1, steroid receptors, and other viral or cellular regulatory factors.

We anticipated that modification of the viral gene expression by chemicals would influence on the proliferation of the infected cells. Retinoic acid has been reported to suppress growth of HPV-positive cells in vitro and to repress HPV expression. However, little is known concerning chemicals which affect HPV expression in a physiological situation.

12-O-Tetradecanoylphorbol-13-acetate (TPA) is a potent tumor promoter which is specific to the genesis of skin tumors and/or papilloma. Though a huge number of reports exist on TPA-induced changes in gene expression pattern, the effect of TPA on HPV gene expression has not been documented yet as far as we know. Therefore, we investigated the effect of TPA on HPV expression in an HPV18-infected cervical cancer cell line, HeLa S3. We also investigated the effects on HPV expression of cholesterol and its oxygenated derivative, 3β,5α-dihydroxycholestan-6-one (yakkastrone), the latter of which is expected to share at least some of the biological activities of TPA (vide infra).

MATERIALS AND METHODS

Cell Culture HeLa S3 cells were provided by the Japanese Cancer Research Resources Bank and maintained in Dulbecco’s modified Eagle’s MEM (DMEM) supplemented with 10% calf serum at 37°C in an atmosphere humidified with 5% CO2. For the treatment of the cells with TPA, yakkastrone, or cholesterol, the medium was changed to DMEM supplemented with 2.5% calf serum 24 h before the addition of the compounds.

Compounds TPA and cholesterol were commercial products (Sigma and Wako Japan, respectively). Yakkastrone was synthesized as described previously. The compounds were dissolved in ethanol, and 10 μl of the solution was added to 10 ml of medium in a culture dish. Staurosporine (Sigma) was dissolved in a mixture of ethanol and dimethyl sulfoxide (DMSO) (9:1), and added to the culture in the same manner as described above.

RNA Preparation and Northern Blot Analysis Total RNAs were extracted from the cultured cells by the method described by Chomczynski and Sacchi. Northern hybridization was performed as described previously. Total genome of HPV18 (7.9 kb) was kindly provided by the National Cancer Center Research Institute, and labeled with [α-32P]dCTP. The labeled DNA was used as a probe for the detection of HPV transcripts. For the internal control of the amounts of each RNA preparation subjected to gel electrophoresis, commercially supplied human β-actin oligonucleotide (40 mer, Oncogene Science) was used after endlabelling with [γ-32P]ATP (data not shown). The Northern blot experiments (effects of compounds on HPV18 expression) were basically reproducible, and a single set of experimental results is presented in the text.
RESULTS

Effect of TPA on HPV18 Expression in HeLa Cells
Total RNAs extracted from HeLa cells which were incubated in the presence or absence of TPA were subjected to Northern blot hybridization using HPV18 whole genome as a probe. HeLa cells cultured under the usual conditions were observed to express HPV18 as reported (Fig. 1a, lane 1, mRNAs of 1.6 kb and 3.4 kb).10 Treatment of the cells with 4—400 nM TPA for 1 h resulted in a 2—3 fold increase in HPV18 mRNA levels (Fig. 1a, lane 2—5). The increase of HPV18 expression started early (less than 30 min after addition of TPA), and continued for at least 3 h (Fig. 1b).

There are two AP-1 recognition sites (TRE sequences) within the URR of the HPV18 sequence and these sequences may be associated with the increase of HPV18 expression by TPA. c-fos, whose product is one of the constituents of AP-1, was induced to express transiently by the same TPA-treatment (data not shown).12 TPA has been regarded as an activator of protein kinase C (PKC), which in turn activates AP-1.11 Therefore, we investigated the effect of staurosporine, an inhibitor of protein kinases including PKC, on the TPA-induced increase of HPV-18 expression. As shown in Fig. 2 (lanes 5 and 6), staurosporine inhibited the TPA-induced increase of HPV18 expression, although staurosporine itself did not affect the level of HPV expression (lanes 1 and 2). This seemed to suggest that the TPA-induced increase of HPV18 expression is associated with PKC activation (vide infra).

Effect of Yakkasterone and Cholesterol on HPV18 Expression in HeLa Cells Next we investigated the effect of yakkasterone on HPV18 expression in HeLa cells to clarify whether activation of a protein kinase(s) is directly involved in the increase of HPV expression. Yakkasterone is a putative ligand specific to cytosolic-nuclear tumor promoter-specific binding protein (CN-TPBP), which we reported to be a nuclear receptor-like target molecule of TPA.17,18 As both yakkasterone and TPA strongly bind CN-TPBP in a mutually competitive manner, yakkasterone could be expected to share some of the biological activities of TPA, even though the former does not bind to or activate PKC.13,14

As shown in Fig. 1c, treatment of the cells with 100 nM yakkasterone for 1 h also increased the HPV18 expression. This increase was reproducible, though the magnitude of the increase was less than that in the case of TPA-treatment and no clear dose—response relationship was seen with yakkasterone. The yakkasterone-induced increase of HPV18 expression also became apparent at less than

Fig. 1. Enhancement of HPV18 Expression by TPA (a and b) and Yakkasterone (c and d)
(a): HeLa cells were treated for 1 h with TPA at the concentrations of 0.4 nM (lane 2), 4 nM (lane 3), 40 nM (lane 4), and 400 nM (lane 5). (c): HeLa cells were treated for 1 h with yakkasterone at the concentrations of 100 nM (lane 2) and 1 µM (lane 3). (b) and (d): HeLa cells were exposed to 40 nM TPA (b) or 100 nM yakkasterone (d) for 0 min (lane 1), 30 min (lane 2), 1 h (lane 3), and 3 h (lane 4). Lane 1 shows a control sample. Total RNAs (20 µg) were analyzed by Northern blot hybridization.
All three compounds apparently increased the level of HPV18 transcripts when applied for 1 h at such a low concentration as $10^{-7}$ M. Interestingly, cholesterol seemed to increase the HPV18 expression most strongly among the three compounds. Though cholesterol does not bind to PKC or CN-TPBP, this result suggests that cholesterol has intrinsic potential to increase HPV18 expression mediated through activation of a protein kinase(s).

**DISCUSSION**

TPA, yakkasterone and cholesterol all enhanced HPV18 expression in HeLa cells, though the activity of cholesterol was masked by the presence of serum in the medium. HPV18 gene should now be classified as one of the TPA-responsive/induced genes, which are associated with cell transformation, immortalization and/or malignancy. Enhancement of HPV18 expression by TPA might be one of the molecular bases for the tumor-promoting activity of TPA. If this were the case, our results might imply a relationship between cholesterol level (or the level of a putative cholesterol metabolite, yakkasterone) and tumor promotion leading to cervical cancer, skin tumor and/or papilloma.

It is difficult to interpret the effects elicited by the three examined compounds in terms of a common molecular mechanism: TPA binds to PKC and CN-TPBP, yakkasterone binds to CN-TPBP but not PKC, and cholesterol does not bind to PKC or CN-TPBP.\(^{13,14,17,18}\) Though staurosporine, a protein kinase inhibitor, inhibited the TPA-induced increase of HPV18 expression, staurosporine also inhibited the yakkasterone-induced effects. It is not clear whether CN-TPBP, a candidate TPA receptor with nuclear receptor-like features,\(^{17,18}\) is involved in the TPA/yakkasterone-induced increase of HPV18 expression, because cholesterol also increased HPV18 expression in a serum-free culture. These three compounds may act in different manners.

It is important from both clinical and biological view points to characterize compounds which increase or repress the HPV expression in HPV-infected cells such as cervical cancer cells, and which may regulate HPV expression. We have shown here that TPA, yakkasterone and cholesterol all increase HPV18 expression in HeLa cells exposed to them for a short time and at a low concentration. Although the mechanism of the effects elicited by these compounds remains to be resolved, the results represent novel and important information on HPV18 gene expression.

**REFERENCES**