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Effects of Dexamethasone and Epidermal Growth Factor on Activity of Viral Promoter p97 and Expression of HPV-16 E6/E7 in Human Oral Keratinocytes Transformed with HPV-16 and Benzo(a)pyrene

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Primary human oral keratinocytes were previously transformed by transfection with cloned human papillomavirus type 16 (HPV-16) DNA and subsequent exposure to benzo(a)pyrene, and an oral cancer cell line, CTHOK-16B-BaP, was established. To determine the effects of dexamethasone and epidermal growth factor (EGF) on cell proliferation, the expression of HPV-16 E6/E7 and several proto-oncogenes, and activity of HPV-16 E6/E7 promoter, p97, the CTHOK-16B-BaP cells were exposed to either dexamethasone and EGF alone or together. After incubation for 3 days, the degrees of both cell proliferation and the expression of HPV-16 E6/E7, EGF receptor (EGFR), c-myc, and c-fos genes was determined. Dexamethasone and EGF, when added alone or together in the culture media, increased cell proliferation. Although dexamethasone did not affect the transcriptional levels of HPV-16 E6/E7, EGFR, or c-myc in the cells, it down-regulated c-fos mRNA expression. On the other hand, EGF, alone or in conjunction with dexamethasone, down-regulated the transcriptional levels of HPV-16 E6/E7, c-myc, and c-fos genes, but it had little effect on the level of EGFR transcription. These results suggest that the increased proliferation of CTHOK-16B-BaP cells in the presence of dexamethasone and/or EGF may not depend on the extent of expression of such genes as HPV-16 E6/E7, EGFR, c-myc, and c-fos. In addition, the HPV-16 E6/E7 mRNA level was not changed and reduced by treatment with dexamethasone and EGF, respectively. Unexpectedly, however, dexamethasone and EGF enhanced the activity of the viral promoter p97 in CTHOK-16B-BaP line as analyzed by transient expression assays using the chloramphenicol acetyltransferase gene as a reporter. It appears that dominant regulatory mechanisms presumably depending on the chromosomal integration site are able to override the response of the viral promoter to dexamethasone and EGF. Another EGF-responsive element (7454-7643 nt) which acts as an enhancer may be located within the HPV-16 LCR portion.

Key words : *Dexamethasone, EGF, human oral keratinocytes, viral promoter p97, HPV-16 E6/E7*

Introduction

Human papillomavirus (HPV) infection is closely linked to benign and malignant oral lesions (Kellokoski *et al.*, 1992) as well as female genital epithelial cancers (zur Hausen, 1986). Among the known HPV types, type 16 (HPV-16) and type 18 (HPV-18) are most frequently associated with malignant oral lesions (Woods *et al.*, 1993). Recent studies show that the role of HPV in carcinogenesis derives from its *in vitro* transforming capacity by means of E6 and E7 early gene products (von Knebel Doeberitz *et al.*, 1988; Dürst *et al.*, 1989). Transfection of normal human epithelial cells with either cloned HPV-16 or HPV-18 DNA induces immortalization of these cells in culture (Woodworth *et al.*, 1988; Park *et al.*, 1991). These immortalized cells contain integrated viral DNA expressing various HPV messages including E6/E7 mRNAs. Although chronic propagation of HPV-immortalized human skin keratinocytes can lead to a malignant phenotype (Hurlin *et al.*, 1991), HPV-immortalized epithelial cells are non-tumorigenic in nude mice (Woodworth *et al.*, 1988; Park *et al.*, 1991). Therefore, HPV infection by itself is not sufficient for neoplastic conversion, and various environmental factors are implicated in the malignant progression of most HPV-infected cells (Dürst *et al.*, 1989; Park *et al.*, 1992).

Epidemiological studies indicate that women with dysplasia who take the steroid contraceptives are at increased risk for developing carcinoma *in situ* of the cervix (Stern *et al.*, 1977; Vessey *et al.*, 1983). In addition, a glucocorticoid-responsive element (GRE) is identified within the long control region (LCR) of HPV-16 DNA, and glucocorticoid significantly activates the viral promoter in cervical carcinoma cells (Gloss *et al.*, 1987). Since the ep-

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ithelium of oral mucosa is histologically similar to that of the female genital tract, the role of glucocorticoid in oral carcinogenesis can be anticipated. The growth responses of transformed cells, when treated with glucocorticoids, range from inhibition to augmentation of the growth *in vitro*. It seems that a balance among many different factors, including cell types, *in vitro* growth conditions, presence of glucocorticoid receptors, the specific transforming agents, and the potential hormonal influence on expression of that agent, dictates the response of specific cells (O'Banion *et al.*, 1992).

Epidermal growth factor (EGF) modulates cell proliferation and differentiation in various cells or cell lines, and is necessary for the multiplication of cultured human epidermal keratinocytes (Rheinwald and Green, 1977; Barrandon and Green, 1987). The mitogenic effect of EGF is believed to be mediated through the ligand-dependent tyrosine kinase activity of the EGF receptor (EGFR) (Carpenter, 1987). Signal transduction may be an ongoing process after EGF binds to EGFRs (Schlessinger, 1986), subsequently resulting in positive and negative regulations of specific genes leading to cell proliferation and differentiation (Yasumoto *et al.*, 1991). It has been shown that EGF has controversial effects on cell proliferation depending on the cell lines of squamous cell carcinoma of the head and neck (SCCHN) (Weber *et al.*, 1988; Weichselbaum *et al.*, 1989). The effect of EGF on proliferation of SCCHN cells is complex and may depend on a variety of factors including the number of EGF receptors expressed and their affinity for growth factor (Grandis and Tweardy, 1992). HPV-16 contains the EGF-responsive element which has a predominant silencer activity, and EGF elicits negative regulation of HPV-16 E6/E7 at the mRNA level in the HPV-16-immortalized human keratinocyte cell line (Yasumoto *et al.*, 1991).

We previously transformed human oral keratinocytes by transfection with recombinant HPV-16 DNA and subsequent exposure to benzo(a)pyrene, and established an oral cancer cell line, CTHOK-16B-BaP. This cell line contained HPV-16 DNA as an integrated form, expressed numerous viral genes, proliferated well in DMEM containing a physiological level of calcium, and acquired neoplastic properties. In the present study, we determined the effects of dexamethasone, a synthetic glucocorticoid, and EGF on cell proliferation, the expression of HPV-16 E6/E7, EGFR, c-*myc*, and c-*fos* genes, and the activity of HPV-16 E6/E7 promoter p97 in CTHOK-16B-BaP line.

Materials and Methods

Cells and culture condition

The CTHOK-16B-BaP cells, human oral keratinocytes transformed by transfection with cloned HPV-16 DNA and subsequent exposure to benzo(a)-pyrene, were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.4 μg/ml hydrocortisone at 37℃ in a humidified atmosphere of 5% CO_2 in air.

Determination of cell proliferation rate

Confluent cell monolayers in 100-mm Petri dishes were trypsinized, suspended in medium, and counted with a hemacytometer. The cells were suspended in DMEM supplemented with 10% FBS and 0.4 μg/ml hydrocortisone and 2×10^5 cells were plated onto 60-mm Petri dishes. The plated dishes were divided into two sets: One set received ethanol (final conc. 0.01%) and either 1×10^{-7} M dexamethasone or 50 ng/ml EGF, the second set received both 1×10^{-7} M dexamethasone and 50 ng/ml EGF. After incubation for 3 days, the cells were harvested and viable cells were counted with a hemacytometer. There were four cultures in each group.

Northern analysis

To determine the transcription of HPV-16 E6/E7, EGFR, c-*myc*, c-*fos*, and β-actin genes, cytoplasmic poly(A$^+$)RNAs were extracted from cells using standard procedures. Probes used for Northern analysis were as follows: 570-bp fragment (nucleotides 198-767) representing the major early HPV-16 message including E6/E7 genes, human EGFR cDNA (ATCC, Rockville, MD), v-*myc* cDNA (ATCC), v-*fos* cDNA (ATCC), and human β-actin cDNA (from Dr. L. Kedes, Stanford University, Palo Alto, CA). All were labeled with [^{32}P]dCTP (Amersham Corp., Arlington Heights, IL) by megaprime labeling (Amersham Corp.). Specific radioactivities of labeled probes were always higher than 5×10^8 cpm/μg of DNA. Five μg of poly(A$^+$) RNAs were denatured and run on a 1.2% formaldehyde agarose gel with marker RNAs (9.5 kb,

6.2 kb, 3.9 kb, 2.8 kb, 1.9 kb, 0.9 kb, 0.6 kb, and 0.4 kb RNA marker, International Biotechnologies, Inc., New Haven, CT). The RNAs were transferred onto nylon filters (Amersham Corp.) and crosslinked with ultraviolet light for 5 min. The filters were hybridized to [^{32}P]-labeled probe at 42℃ for 24 h in 50% formamide/10% dextran sulfate/5X SSPE (0.15 M NaCl, 0.01 M Na_2HPO_4, and 0.001 M EDTA)/5X Denhardt's solution/ denatured salmon sperm DNA (20 μg/ml). Filters were washed twice in 5X SSPE for 15 min at 42℃, then in 1X SSPE/0.1% SDS for 30 min at 42℃, and finally in 0.1X SSPE/0.1% SDS for 15 min at room temperature. Filters were then autoradiographed on Hyperfilm-MP (Amersham Corp.) for 24 h at -70℃. After exposure, the probe was stripped off the filter for rehybridization to the next radiolabeled probe.

Construction of plasmid DNAs

Chloramphenicol acetyltransferase (CAT) plasmids containing the 5' deletion of the HPV-16 LCR were constructed so that CAT expression occurred from the HPV-16 transcriptional promoter. Briefly, each CAT plasmid contains LCR portions cleaved by various restriction enzymes which create 5' ends as follow: *Pst*I [7,003 nucleotides (nt)], *Eco*RI (7,454 nt), *Rsa*I (7,643 nt), and *Hae*III (7,767 nt) for CAT plasmids pHPV16-LCR11, pHPV16-LCR21, pHPV16-LCR31, and pHPV16-LCR41, respectively. The 3' end of each 5'-deleted segment is common at an *Ava*II site (113 nt). The generated cohesive termini were filled by Klenow fragment and a *Hin*dIII linker [d(pCAAGCTTG)] was attached to the blunt-ended DNA fragments. The resulting DNA fragments were cleaved by *Hin*dIII, and cloned into pCAT-Basic plasmid (Promega Corp., Madison, WI) which digested by *Hin*dIII and dephosphorylated by bacterial alkaline phosphatase. The, plasmid DNAs were prepared by alkaline lysis method and purified by precipitation with polyethylene glycol (Sambrook *et al.*, 1989).

Polybrene-induced DNA transfection

Confluent cell monolayers in 100-mm Petri dishes were trypsinized and counted. The cells were suspended in DMEM supplemented with 10% FBS and 0.4 μg/ml hydrocortisone, and 2×10^5 cells were plated onto 60-mm Petri dishes. When the cultures reached 80% confluency, they were transfected with pHPV16-LCR11, pHPV16-LCR21, pHPV16-LCR31, and pHPV16-LCR41 using polybrene (Aldrich Chemical Co., Milwaukee, WI) as described previously (Jiang *et al.*, 1991). pCAT-Control plasmid and pCAT-Basic plasmid (Promega Corp.) were used as a positive control and a negative control, respectively. For each 60-mm dish, the cells were exposed to 1 ml of medium containing CAT plasmid (5 μg), pSV-β-Galactosidase reporter plasmid (5 μg; Promega Corp.), and polybrene (10 μg) for 6 h, and shocked by exposure to 2 ml of culture medium containing 30% DMSO for 3 min at room temperature. The cells were then washed twice with PBS, re-fed with fresh medium, and incubated for an additional 12 h. The transfected cells were exposed to 1×10^{-7}M dexamethasone or 50 ng/ml EGF for 48 h. After washing with the PBS, the cells were lysed with 1X reporter lysis buffer (Promega Corp.), and harvested by scraping. Cell debris was removed by centrifugation. An aliquot of the supernatant was saved for β-galactosidase assay. The remainder of the supernatant was heated at 65℃ for 10 min, clarified by centrifugation, and stored at -20℃ until used in the CAT assay. Protein content was measured by the method of Lowry *et al.* (1951).

Determination of the expression of reporter gene constructs (CAT gene) containing LCR sites

The activity of CAT was assayed in reaction mixture composed of 0.05 μCi [^{14}C]chloramphenicol, 3.5 μg n-butyryl CoA, and 82 μl of the cell extract, in a final volume of 100 μl. The enzymatic reaction was allowed for 4 h in a water bath adjusted to 37℃. After extraction of the n-butyrylated chloramphenicol with mixed xylenes, the radioactivity was determined with a liquid scintillation counter. The CAT activity for a 1 h-reaction is calculated according to the following formula:

$$\text{cpm}\left(\begin{matrix}\text{sample}-\\ \text{background}\end{matrix}\right)\times\frac{1000}{\mu\text{g of protein in }82\,\mu\text{l}}\times\frac{1\text{ h}}{4\text{ h}}$$

The activity of β-galactosidase was assayed in reaction mixture composed of 150 μl of 2X assay buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 1.33 mg/ml O-nitrophenyl-β-D-galactopyranoside) and 150 μl of the cell extract, in a final volume of 300 μl. The enzymatic reaction was allowed for 24 h in a

water bath adjusted to 37℃, and stopped by adding 500 μl of 1 M sodium carbonate. The formation of O-nitrophenyl was monitored at 420 nm. The β-galactosidase activity was calculated according to the following formula:

$$A_{420} \times 1000 \times \frac{1000}{\mu g \text{ of protein in } 82\,\mu l} \times \frac{1\text{ h}}{24\text{ h}}$$

In Fig. 5B the values were normalized for transfection efficiency by calculating the ratio of CAT activity to β-galactosidase activity in each transfected dish, and represented as a value relative to the activity derived from pHPV-LCR11.

Statistical analysis

Data were analyzed by Student's t-test for their statistical significance.

Results

Effects of dexamethasone and EGF on cell proliferation

The effects of dexamethasone and EGF on the proliferation of CTHOK-16B-BaP line were investigated. Dexamethasone and EGF, when added individually to the media, increased cell proliferation by 36% and 34%, respectively; when added together, the agents increased the cell proliferation by 45%. However, there was no synergistic effect of dexamethasone and EGF on cell proliferation (Fig. 1).

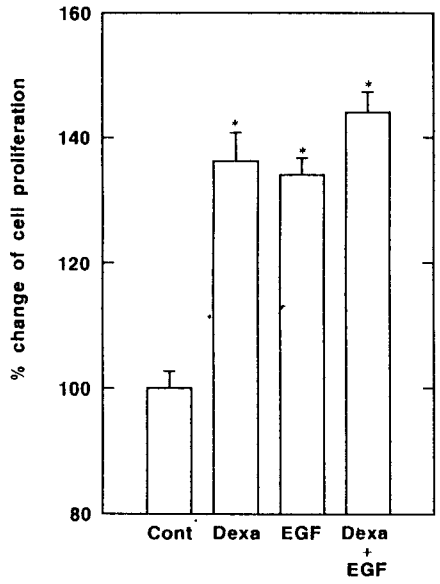

Fig. 1. Effects of dexamethasone and EGF, alone or together, on cell proliferation. Cells were plated at 2×10^5 density per dish and grown in DMEM supplemented with 10% FBS and 0.4 μg/ml hydrocortisone for 24 h. The cells were treated as described in the section of "Materials and Methods". The values are means±S.E. of quadruplicate cultures and expressed as the percent change of control cell proliferation. *Significantly different from the control, $P < 0.01$.

Effects of dexamethasone and EGF on the expression of HPV-16 E6/E7, EGFR, c-*myc*, and c-*fos*

Northern analysis using the probe containing 570 bp HPV-16 E6/E7 gene fragment (nt 198-767) showed that CTHOK-16B-BaP cells as well as the cells treated with either dexamethasone and EGF alone or together expressed the 1.9- and 1.6-kb HPV-16 E6/E7 mRNAs (Fig. 2). Dexamethasone

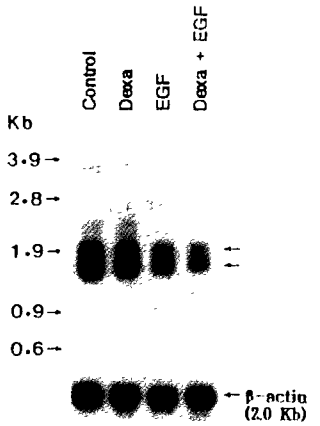

Fig. 2. Northern blot hybridization of cellular polyadenylated RNAs to [^{32}P]-labeled 0.57-kbp HPV-16 E6/E7 DNA fragment. The RNAs were prepared as described in the section of "Materials and Methods". Kb, kilobase.

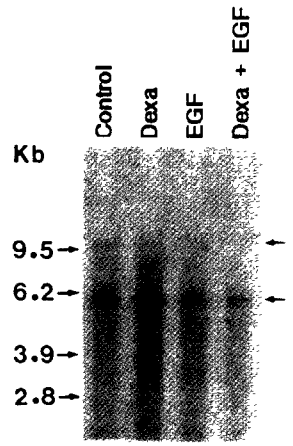

Fig. 3. Northern blot hybridization of cellular polyadenylated RNAs to [^{32}P]-labeled 2.4-kbp EGFR cDNA insert of pE7. The RNAs were prepared as described in the section of "Materials and Methods". Kb, kilobase.

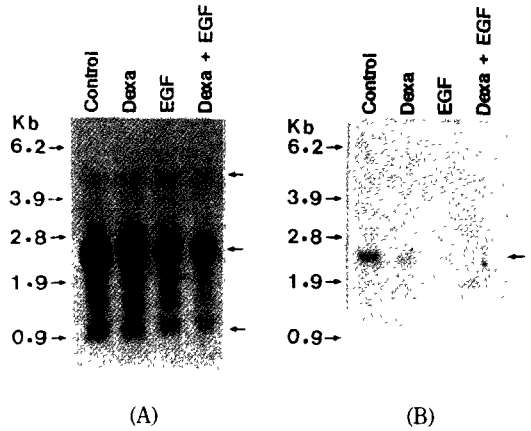

Fig. 4. Northern blot hybridization of cellular polyadenylated RNAs to [^{32}P]-labeled 1.5 kbp v-*myc* DNA (A) and to [^{32}P]-labeled 1.0 kbp *v-fos* DNA (B). The RNAs were prepared as described in the section of "Materials and Methods". Kb, kilobase.

had no effect on the expression of HPV-16 E6/E7 gene, but EGF alone or in conjunction with dexamethasone substantially down-regulated the level of HPV-16 E6/E7 transcripts in CTHOK-16B-BaP line. Fig. 3 shows that 10- and 5.6-kb messages are the major transcripts of EGFR gene from control cells and cells treated with dexamethasone and EGF either individually or together. However, dexamethasone and EGF, individually or together, had no effect on the level of EGFR transcripts in CTHOK-16B-BaP line. The c-*myc* transcripts with size of 5.2-, 2.4-, and 1.1-kb were expressed from control cells and cells treated with dexamethasone and EGF either individually or together (Fig. 4A). Among these transcripts, the 2.4-kb mRNA is the major transcript of c-*myc* gene in these cells. The amount of 2.4-kb c-*myc* messages from the cells treated with EGF alone, or in conjunction with dexamethasone, was slightly decreased compared to the control. C-*fos* transcripts with size 2.2-kb were expressed from control cells and cells treated with dexamethasone and EGF either individually or together, but the amount of transcripts was substantially lower in cells treated with dexamethasone and EGF either individually or together than in control cells (Fig. 4B). All cells expressed 2.0-kb β-actin mRNAs in a similar manner, indicating that all cells were metabolically active.

Effects of dexamethasone and EGF on the transcriptional activity of the HPV-16 LCR as determined by using various CAT plasmids containing the 5'-deleted LCR

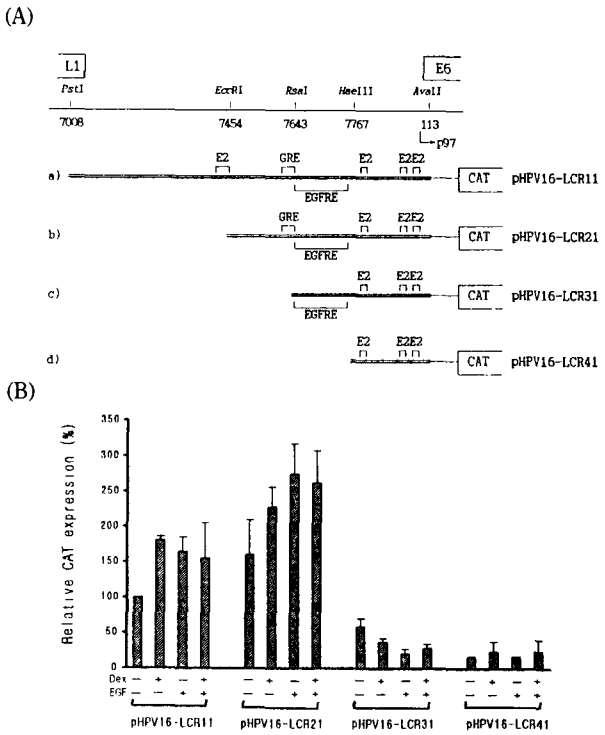

Fig. 5. Effects of dexamethasone and EGF on the activity of viral promoter p97. A) Functional maps of deletion mutants from 5'-side of the LCR ligated in front of the CAT gene. The locations of the known protein binding sites are as follows: E2, E2-responsive element; GRE, glucocorticoid-responsive element; EGFRE, EGF-responsive element. p97, transcriptional start site of HPV-16 E6/E7. B) Relative CAT expression of the various CAT plasmids containing the 5'-deleted LCR in CTHOK-16B-BaP line. The value of CAT activity was normalized with that of β-galactosidase activity and the data were represented as the value relative to the activity derived from pHPV16-LCR11.

To investigate whether the effects of dexamethasone and EGF, individually or together, on the transcriptional activity of the HPV-16 E6/E7 promoter are linked to the HPV-16 LCR function, various CAT plasmids containing the 5'-deleted LCR were used to determine the CAT activity (Fig. 5A). Dexamethasone and EGF, individually or together, enhanced CAT activity expressed from pHPV16-LCR-11 plasmid, which contained whole HPV-16 LCR portion. We then attempted to identify dexamethasone- and EGF-responsive HPV-16 elements. To do so, we made CAT constructs carrying the 5'-deleted LCR (Fig. 5A). These CAT plasmids were transfected into CTHOK-16B-BaP cells to assess dexamethasone- and EGF-responsive elements. The results demonstrated that dexamethasone and EGF positively regulated CAT expression in pHPV16-LCR-21-transfected cells, while EGF alone or in conjunction with dexamethasone, negatively regulated

in pHPV16-LCR31-transfected cells (Fig. 5B).

Discussion

Growth responses of transformed cells, when treated with glucocorticoids or EGF, range from inhibition to augmentation of growth *in vitro*. Interestingly, EGF inhibits the growth and colony formation of squamous cell carcinoma cell lines of the skin, oral cavity, and esophagus at doses that are mitogenic in many other cells, however, it does not inhibit and in some cases slightly stimulates the growth of other tumor cells, such as adenocarcinomas of the stomach, cervix, breast, and sarcomas (Kamata *et al.*, 1986). Our data showed that EGF or dexamethasone stimulated cell proliferation of CTHOK-16B-BaP cells which were human oral keratinocytes transformed by transfection with cloned HPV-16 DNA and subsequent exposure to benzo(a)pyrene.

Recent studies have demonstrated that transcription of the major early HPV-16 E6/E7 genes appears to be largely dependent on regulatory elements in the LCR of the HPV genome (Yasumoto *et al.*, 1991). Among the known regulatory elements, GRE and EGF-responsive element are located in the upstream of viral promoter, p97. The former acts as an enhancer and the latter as a silencer in human keratinocyte cell lines (Gloss *et al.*, 1987; Chan *et al.*, 1989; Yasumoto *et al.*, 1991). Furthermore, inasmuch as HPV-16 E6/E7 proteins may be related to the proliferation properties of HPV-infected epithelial cells (Li *et al.*, 1992), the HPV-16 E6/E7 mRNA levels were studied. The amounts of HPV-16 E6/E7 transcripts were similar to that from the control counterpart and reduced by treatment with dexamethasone and EGF, respectively. Unexpectedly, however, dexamethasone and EGF enhanced the activity of the viral promoter p97 in CTHOK-16B-BaP line as analyzed by transient expression assays. The reason for this unexpected results is largely unknown but it may be due to cis-acting mechanisms in the CTHOK-16B-BaP cells. They might be evoked by direct or indirect dexamethasone- or EGF-sensitive regulatory elements within the adjacent sequences of the viral LCR and presumably involved cellular elements at the site of chromosomal integration or they might be due to the usage of cellular instead of viral promoters for E6/E7 transcription. This is supported by the report that dexamethasone leads to different response of the transcriptional rate of HPV-18 E6/E7 genes in four cervical cancer cell lines depending on the chromosomal integration site of the viral DNA (von Knebel Doeberitz *et al.*, 1991).

It is shown that EGF stimulates cell proliferation, but down-regulates the expression of HPV-16 E6/E7 and concomitantly enhances c-*myc* expression in HPV-16-immortalized human epidermal keratinocyte line (Yasumoto *et al.*, 1991). In the present study, EGF also stimulated cell proliferation of CTHOK-16B-BaP line, but it down-regulated c-*myc* mRNA expression. The reason for this difference is not clear, but it may be due to the differences between the two cell lines. The results suggest that another EGF-responsive element (7454-7643 nt) which acts as an enhancer is located within the HPV-16 LCR portion. Three nuclear factor-1 (NF-1/CTF)-recognition sequences and two activator protein-1 (AP-1)-sites are located in the putative EGF-responsive enhancer region. Therefore, it remains to be shown which element actually contribution to the transcriptional stimulating activity in CTHOK-16B-BaP line when treated with EGF.

Amplification or overexpression of EGFR was observed in both tissues and cell lines derived from oral squamous cell carcinomas (Todd *et al.*, 1989; Sherer *et al.*, 1990; Leonard *et al.*, 1991). These data indicate that overexpression of EGFR can participate in cell proliferation of oral squamous carcinoma cells. However, our data showed that EGFR cannot contribute to the increased cell proliferation. The present results demonstrate that overexpression of EGFR is not necessary for maintenance of transformed phenotypes or cell proliferation of CTHOK-16B-BaP line. Myc and Fos proteins are activated by growth factor stimulation. They may function as transcriptional activator and participate in control of cell proliferation. Our data indicated that c-*myc* and c-*fos* could not contribute to the increased cell proliferation of EGF- and/or dexamethasone-treated CTHOK-16B-BaP line. The reason for the down-regulation of the transcriptional level of c-*myc* and c-*fos* gene is presently unknown. Our results showed that the effect of EGF in conjunction with dexamethasone on cell proliferation and expression of HPV-16 E6/E7, EGFR, c-*myc*, and c-*fos* were similar to that of EGF alone. These data suggest that the effect of 50 ng/ml of EGF is dominant over that of 1×10^{-7} M of dexamethasone.

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