

Human papillomavirus type 16 E7 protein sensitizes cervical keratinocytes to apoptosis and release of interleukin-1 α

Maite Iglesias^{1,4}, Katy Yen¹, Darci Gaiotti^{1,5}, Allan Hildesheim², Mark H Stoler³ and Craig D Woodworth^{1,5}

¹Laboratory of Biology and ²Environmental Epidemiology Branch, National Cancer Institute, Bethesda, Maryland 20892;

³Department of Pathology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22901, USA

Interleukin-1 α (IL-1 α) is a multifunctional cytokine that promotes inflammation, tissue remodeling and epithelial hyperplasia. Keratinocytes produce and sequester large amounts of biologically active IL-1 α which can be released after injury or infection. We show that high level expression of human papillomavirus (HPV) type 16 E6 and E7 oncoproteins enhanced release of IL-1 α from cultures of normal cervical keratinocytes (relative effectiveness E7 > E6/E7 > E6 > control). The amount of IL-1 α released was directly related to the ability of E7 or E6/E7 to stimulate apoptosis. E7 proteins that bound the retinoblastoma protein (Rb) strongly (HPV-16 and -18) induced more IL-1 α release than those that bound poorly (HPV-6 and an HPV-16 E7 24gly mutant). Furthermore, overexpression of the E2F-1 transcription factor, a downstream target of Rb, induced extensive apoptosis and IL-1 α release. Apoptosis and IL-1 α release in response to growth factor removal occurred in part through a p53-independent pathway as coexpression of E6 and downregulation of p53 did not prevent either response. Immunohistochemical analyses showed that IL-1 α was expressed by keratinocytes in normal cervical epithelia, low and high grade dysplasias, and cervical carcinomas. However, HPV-16 E6/E7 RNA expression and apoptosis increased in parallel in proliferating keratinocytes in severe dysplasias and carcinomas suggesting that IL-1 α release is associated with progression to high grade disease. Thus, high level expression of the HPV-16 E7 protein sensitizes keratinocytes to apoptosis which results in release of IL-1 α .

Keywords: apoptosis; cervical carcinoma; human papillomavirus; interleukin-1; keratinocyte

Introduction

Keratinocytes secrete a variety of cytokines that regulate growth, differentiation, and host response to infection. Interleukin-1 (IL-1) is particularly important as it promotes the inflammatory response by activating genes encoding multiple cytokines, receptors and adhesion molecules (reviewed in Dinarello, 1996).

Under normal conditions inflammation is a beneficial response as it stimulates host immunity and wound healing. However, inappropriate or continued release of IL-1 can also promote chronic inflammation, a risk factor for human cancer (Collins *et al.*, 1987; Correa, 1992). In this regard, IL-1 α induces expression of polypeptide growth factors that stimulate proliferation of normal keratinocytes and carcinoma cells through autocrine and paracrine pathways (Castrilli *et al.*, 1997; Li and Tang, 1997; Woodworth *et al.*, 1995). IL-1 α also mediates the hyperplastic and inflammatory effects of tumor promoters (Oberyszyn *et al.*, 1993), and contributes to production of mutagenic products including nitric oxide and oxygen radicals. Due to its pleiotropic effects on gene expression, synthesis and release of IL-1 are tightly regulated (reviewed in Kupper and Groves, 1995). Two IL-1 molecules exist: IL-1 α , which is active as both precursor and processed forms, and IL-1 β , which requires proteolytic cleavage. There are also two competitive inhibitors of IL-1 activity. IL-1 receptor antagonist (IL-1ra), a ligand which binds but does not stimulate the type 1 receptor (Eisenberg *et al.*, 1990) and IL-1 soluble receptor 2 (IL-1sr2), a nonsignaling receptor that is released from the membrane to sequester IL-1 (Colotta *et al.*, 1993). Although most cell types do not constitutively produce IL-1 α under normal conditions, keratinocytes are an exception (Ansel *et al.*, 1988; Wood *et al.*, 1996). These cells accumulate a large reservoir of biologically active intracellular IL-1 α that is available for local release after injury or infection.

Infection with a subset of 'high risk' HPVs is the major risk factor for development of cervical cancer (reviewed in zur Hausen, 1996). Two viral proteins, E6 and E7, are selectively retained and expressed in carcinoma cells and cooperate in immortalization of primary keratinocytes (Hawley-Nelson *et al.*, 1989). E6 binds to p53, promoting its degradation via ubiquitin mediated proteolysis (Scheffner *et al.*, 1990) and increasing genetic instability (White *et al.*, 1994) by over-riding cell cycle checkpoints for DNA repair. E7 binds to the retinoblastoma protein (Rb) (Munger *et al.*, 1989) causing dissociation of the E2F-1 transcription factor and activation of genes promoting DNA synthesis and cell proliferation (Zerfass *et al.*, 1995). However, E7 also sensitizes cells to apoptosis, a response that can be abrogated by E6 (Pan and Griep, 1995; Puthenveetil *et al.*, 1996). The biological effects of E6 and E7 depend on their level of expression. In low grade squamous intraepithelial lesions of the cervix (LSIL) production of E6/E7 RNAs is greatest in terminally differentiated keratinocytes undergoing viral DNA replication (Dürst *et al.*,

Correspondence: CD Woodworth

Current addresses: ⁴Laboratorio 1.7, Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid Spain;

⁵Laboratory of Cellular Carcinogenesis and Tumor Promotion, Building 37/Room 3B25, National Cancer Institute, Bethesda, MD, 20892

Received 12 December 1997; revised 14 April 1998; accepted 15 April 1998

1992; Stoler *et al.*, 1992). E6/E7 expression is low in proliferating keratinocytes in LSIL but often increases in high grade lesions (HSIL) and carcinomas (Dürst *et al.*, 1992; Stoler *et al.*, 1992). Increased expression in these lesions may be due to viral integration and disruption of HPV transcriptional control elements including the E2 gene and the long control region (zur Hausen, 1996).

The host's inflammatory and cell mediated immune responses serve important roles in determining whether HPV infections persist, regress or progress (reviewed in Frazer, 1996). Keratinocytes contribute directly to the host response by secreting immunoregulatory cytokines such as IL-1, that recruit and activate leukocytes at the site of infection (Dinarello, 1996). Keratinocyte cell lines immortalized by HPV-16 and cervical carcinoma-derived cell lines demonstrate altered expression of several immunoregulatory cytokines (Bryan *et al.*, 1995; Merrick *et al.*, 1996; Rösl *et al.* 1994; Woodworth and Simpson, 1993) suggesting that HPV gene products perturb cytokine production or secretion. The present work was undertaken to examine the effect of specific HPV oncoproteins on secretion of IL-1 α and other cytokines. Keratinocytes cultured from normal human ectocervix were infected with recombinant retroviruses encoding HPV-16 E6, E7 or E6/E7 genes and cytokine release into the culture medium was measured by ELISA. Retroviruses were used because they are efficient vectors for introducing and expressing genes in keratinocytes, therefore it was possible to examine effects of HPV proteins on cytokine release shortly after infection (6–7 days). Expression of E7 or E6/E7 promoted release of IL-1 α from cultured keratinocytes and the amount of release correlated directly with the ability of E7 to stimulate apoptosis. Analyses of cervical biopsies demonstrated that IL-1 α was produced by keratinocytes at all stages leading to malignancy. However, both E6/E7 RNA expression and apoptosis increased in parallel in HSIL and carcinomas suggesting that apoptosis and IL-1 α release are associated with progression to high grade disease.

Results

HPV-16 E7 protein sensitizes keratinocytes to release IL-1 α

Secondary cultures of cervical keratinocytes were infected with retroviruses encoding HPV-16 E6, E7 or E6/E7 in combination. Controls consisted of cells infected with retroviruses containing only vector sequences or mock-infected cultures. After selection for G-418 resistance, cells were subcultured and release of specific cytokines into the culture medium was measured by ELISA. Expression of HPV-16 E6, E7 or E6/E7 enhanced release of IL-1 α under several different culture conditions (Figure 1a). In most experiments the relative effectiveness of HPV proteins was E7>E6/E7>E6>vector although the absolute amount of IL-1 α release varied in cultures derived from different individuals. E7 stimulated IL-1 α production three- or 10-fold when keratinocytes were growing rapidly in complete keratinocyte-serum free

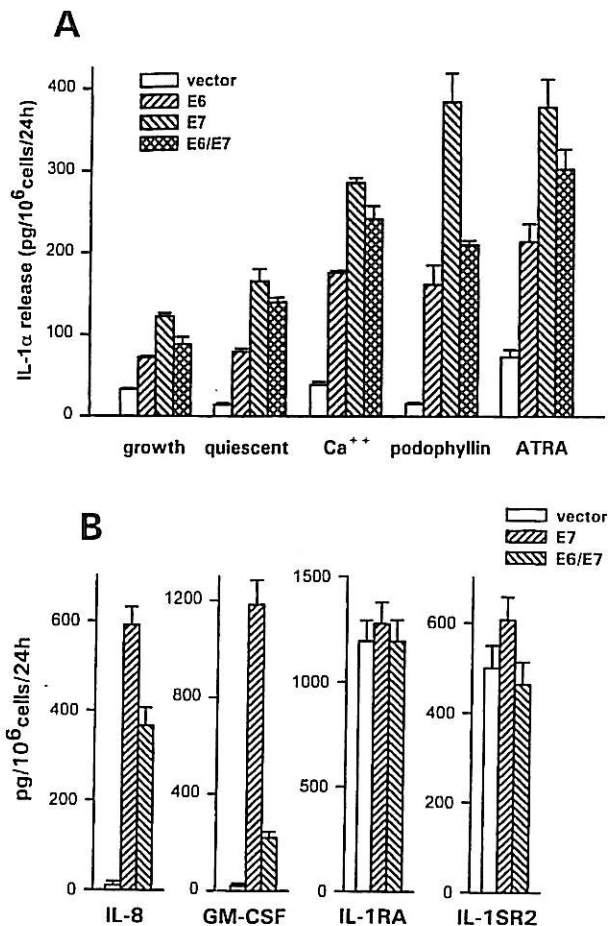


Figure 1 Influence of HPV-16 oncoproteins on release of IL-1 α (a) and other cytokines (b) from cultured cervical keratinocytes. (a) Modulation of IL-1 α release under various culture conditions. Cervical keratinocytes infected with HPV-16 retroviruses were maintained in complete K-SFM medium to induce rapid growth, basal medium lacking growth factors (insulin, epidermal growth factor, bovine pituitary extract, transferrin, hydrocortisone, and triiodothyronine) for 2 days to induce quiescence, basal medium containing a 10-fold increased calcium concentration (1.0 mM) for 2 days, or for 1 day in complete medium containing podophyllin (100 μ g/ml) or ATRA (1 μ M). Cytokine release into the culture medium was determined by ELISA. (b) Release of other cytokines including IL-8, GM-CSF, IL-1RA and IL-1SR2. Cervical keratinocytes were infected with retroviruses encoding either vector, E7 or E6/E7, and maintained for 2 days in basal K-SFM. Results in a. and b are from one representative experiment using cells from a single donor. Bars represent mean $\pm$ standard deviation of duplicate determinations on two culture dishes. All experiments were repeated at least three times.

medium (K-SFM) or made quiescent by depletion of growth factors, respectively. IL-1 α release was enhanced further when keratinocytes were stimulated to undergo differentiation by increasing the concentration of calcium in the medium from 0.1 to 1.0 mM. Two growth-inhibitory agents that are effective for treatment of HPV infections, podophyllin or all-trans-retinoic acid (ATRA) (Stone, 1995), stimulated a 10–20-fold increase in IL-1 α release from E7-expressing cells. E6 reproducibly increased IL-1 α release under different culture conditions although the effect was always lower than E7 or E6/E7.

Because papilloma cells proliferate in the upper layers of stratified squamous epithelia where paracrine growth factors provided by fibroblasts or serum may

be reduced, our studies focused on cells made quiescent by depletion of growth factors. In ten independent experiments using keratinocytes from different individuals E7 or E6/E7 significantly increased IL-1 α release (Dunn's test $P < 0.001$ and $P < 0.05$, respectively) relative to vector-only controls (Table 1).

Expression of E6 alone induced a lower level of IL-1 α release that was not statistically significant (26 ± 22 pg/ 10^6 cells/24 h). Although the relative effectiveness of HPV proteins in mediating IL-1 α release was similar in multiple experiments, the absolute level of release varied in cultures derived from different individuals leading to increased standard deviations. This was particularly evident for E6 alone and may explain in part why differences in IL-1 α release by this protein were not statistically significant. One consistent observation was that keratinocytes expressing E6 or E7 proteins continued to proliferate slowly in medium depleted of growth factors whereas vector-only or mock-infected cultures did not.

Release of other cytokines or components of the IL-1 regulatory network

Cervical keratinocytes maintained in basal K-SFM medium released low levels of IL-8 and granulocyte macrophage colony stimulating factor (GM-CSF), cytokines involved in stimulating chemotaxis of leukocytes or activation of Langerhan's cells, respectively. Expression of HPV-16 E7 or E6/E7 increased release of both cytokines in multiple experiments (Figure 1b and summarized in Table 1). In contrast, E7 or E6/E7 did not significantly stimulate release of two competitive inhibitors of IL-1 activity, IL-1ra or IL-1sr2 (Figure 1b and Table 1). Several other cytokines either were not expressed at detectable levels (IL-10, IL-12, and macrophage chemotactic protein-1) or produced at very low levels (IL-6, tumor necrosis factor- α) under these experimental conditions and E7 or E6/E7 did not significantly influence production. In summary, HPV-16 E7 and E6/E7 proteins enhanced release of IL-1 α IL-8 and GM-CSF from independently derived cultures of human keratinocytes.

Influence of HPV-16 E6 and E7 on IL-1 α gene expression

Keratinocytes maintained in basal K-SFM expressed a 2.4-kb IL-1 α RNA, and E7 increased steady state levels 2–5-fold in three independent experiments (Figure 2). When keratinocytes were cultured in medium containing 1.0 mM calcium baseline levels of IL-1 α RNA increased and expression of E6, E7, or E6/E7 stimulated levels further. To examine whether intracellular IL-1 α protein increased in parallel we performed ELISA on total cell lysates of infected keratinocytes. Cells expressing E6, E7 or E6/E7 reproducibly accumulated 1.5-, 2- and 2.9-fold more total intracellular IL-1 α than did vector-only controls, respectively. The values for mean intracellular IL-1 α levels (pg/ 10^6 cells) $\pm$ standard deviation in three experiments were: vector = 2438 ± 435 , E6 = 3653 ± 450 , E7 = 4825 ± 850 , E6/E7 = 7031 ± 771 . In sum-

mary, E7 or E6/E7 proteins stimulated small increases in IL-1 α RNA and protein indicating that alterations in post-translational regulation must be responsible for the 10-fold increase in release of IL-1 α .

Influence of HPV-16 E6 and E7 on apoptosis

IL-1 α does not contain a signal peptide and thus, is not released by the classic secretory pathway. One possible mechanism for IL-1 α release from the cell is leakage from the membrane after injury or apoptosis (Hogquist *et al.*, 1991). A characteristic feature of apoptosis is activation of intracellular nucleases that cleave cellular DNA into multiples of 180 bp (Montague and Cidlowski, 1996). Therefore, studies examined whether HPV-16 E6, E7 or E6/E7 proteins stimulated apoptosis in cultured cervical keratinocytes and whether the extent of apoptosis corresponded to the level of IL-1 α release. Keratinocytes expressing E7 or E6/E7 and maintained in basal medium demonstrated the characteristic ladder of fragmented DNA when analysed by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) (Figure 3a). Similar results were observed when cells were treated with podophyllin or ATRA. The level of DNA fragmentation increased with duration of exposure in culture (compare basal/1d and basal/3d)

Table 1. Summary of cytokine release from keratinocytes after infection with HPV-16 retroviruses

Cytokine	Vector	E7	E6/E7
IL-1 α (10)	8 ± 7^a	$82 \pm 50^*$	$69 \pm 41^*$
IL-8 (5)	61 ± 113	$388 \pm 276^*$	233 ± 151
GM-CSF (5)	28 ± 55	$360 \pm 449^*$	80 ± 102
IL-1 β (5)	4 ± 6	3 ± 5	4 ± 9
IL-1sr2 (5)	110 ± 200	236 ± 208	135 ± 174
IL-1ra (5)	950 ± 956	1862 ± 1894	1749 ± 1497

^aSecretion in pg/ 10^6 cells/24 h $\pm$ standard deviation from experiments in which cells were maintained in basal K-SFM 2 days prior to sampling. Values in parentheses indicate the number of independent experiments using cell cultures derived from different individuals. Asterisks indicate values that were significantly elevated ($P < 0.05$, Dunn's test) relative to vector controls

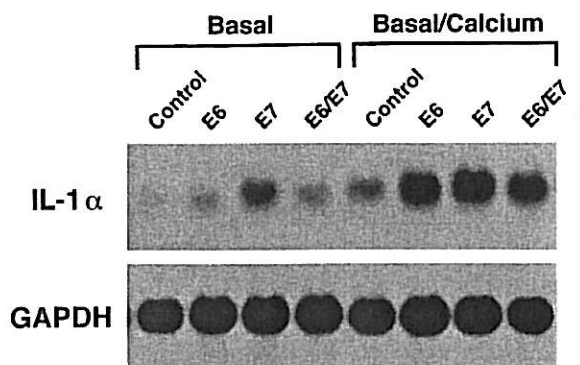


Figure 2 Induction of IL-1 α RNA expression in keratinocytes infected with HPV-16 retroviruses. Northern analysis of cervical keratinocytes that were either mock infected (control) or infected with retroviruses encoding specific HPV-16 genes. Cells were maintained for 2 days in basal K-SFM containing low calcium (0.1 mM) or high calcium (1.0 mM). RNA was hybridized to probes for either IL-1 α or glyceraldehyde phosphate dehydrogenase (GAPDH) to control for equal loading

and was enhanced by addition of calcium (1 mM), which activates cellular endonucleases (Montague and Cidlowski, 1996). Apoptosis also occurred in cells expressing E6 alone although it was observed after 2–3 days and thus, was delayed with respect to E7 or E6/E7. Apoptosis was low or undetectable in control cultures infected with vector-only retroviruses. The extent of DNA fragmentation after 48 h was measured by densitometric scanning of X-ray films

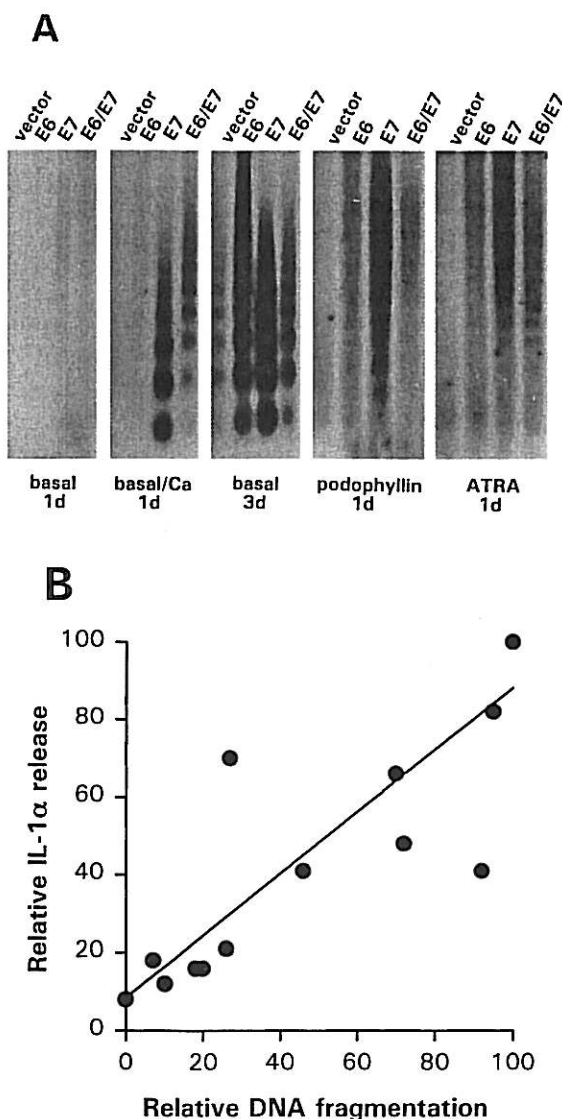


Figure 3 Stimulation of apoptosis in keratinocytes by HPV-16 proteins (a) and correlation between apoptosis and IL-1 α release (b). (a) Cervical keratinocytes were infected with retroviruses encoding HPV-16 genes and maintained for 1 day in basal medium, 1 day in basal medium containing 1.0 mM calcium, 3 days in basal medium, or for 1 day in complete K-SFM medium containing podophyllin (100 μ g/ml) or all transretinoic-acid (ATRA, 1 μ M). Fragmented DNA was labeled on free 3' ends by TUNEL, fractionated in agarose gels, and dried gels were exposed to X-ray film. (b) Correlation between relative DNA fragmentation and relative IL-1 α release. Values for relative DNA fragmentation were obtained by densitometric analyses of X-ray films from TUNEL experiments. Each experimental value was divided by the greatest value for that experiment (i.e., E7-infected keratinocytes) to obtain relative DNA fragmentation. Relative IL-1 α release was determined in the same manner from ELISA experiments. Results represent linear regression analysis from four independent experiments

and image analysis. In four experiments there was a significant positive correlation (Spearman correlation coefficient $r=0.79$, $P=0.0023$) between the relative extent of DNA fragmentation in each lane and the corresponding release of IL-1 α into the culture medium (Figure 3b).

A direct and quantitative method for measuring apoptosis consists of counting the percentage of cells in monolayer cultures undergoing DNA fragmentation as detected by TUNEL staining. Previous observations were verified using this method (Figure 4a). Apoptosis occurred in 0.2–1.0% of cells in control cultures (vector) maintained in basal medium in four independent experiments. However, expression of HPV-16 E7 stimulated apoptosis in 3–5% of keratinocytes. Expression of E6/E7 or E6 alone induced apoptosis in a lower percentage of cells. Most importantly, the percentage of apoptotic cells in each experimental group was directly related to the level of IL-1 α released into the culture medium (Figure 4a). Freeze-thaw lysis of five different keratinocyte cultures and quantitation by ELISA demonstrated that total intracellular IL-1 α was 2688 ± 678 pg/10⁶ cells. HPV-16 E7 stimulated keratinocytes to release approximately 80 pg of IL-1 α /10⁶ cells/24hr or about 4% of the total activity, consistent with 3–5% of cells undergoing apoptosis in these cultures (Figure 4a). In control experiments we determined that addition of recombinant IL-1 α (1 nM for 24 or 48 h) did not directly promote apoptosis of normal keratinocytes or cells expressing HPV-16 E6/E7 (data not shown). These results suggest that apoptosis is an important pathway for IL-1 α release from keratinocytes in response to HPV-16 E7.

Experiments were undertaken to discriminate between apoptosis and necrosis by double staining of viable cells with Hoechst 33258 (apoptotic) and propidium iodide (necrotic). Results indicated that necrotic cells were detected but that they composed a small minority of dying keratinocytes. E6, E7 or E6/E7 induced low levels of necrosis (0.3, 0.5 and 0.3%, respectively) but higher levels of apoptosis (3.1, 6.2 and 4.3%) when cells were maintained in basal medium.

Mechanism for induction of apoptosis and IL-1 α release by E7

The HPV-16 E7 protein binds to Rb and causes release of the transcription factor E2F-1, which in turn, stimulates cell cycle progression or apoptosis depending upon culture conditions (DeGregori *et al.*, 1997; Kowalik *et al.*, 1995; Shan and Lee, 1994). Our results showed that 'high risk' HPV-18 and -16 E7 proteins, which effectively bind Rb (Münger *et al.*, 1989), stimulated apoptosis and IL-1 α release (Figure 4a). In contrast, 'low risk' HPV-6 E7, which binds Rb ineffectively, did not. To confirm the importance of Rb binding we constructed recombinant retroviruses encoding a mutant HPV-16 E7 protein that contains a single amino acid substitution (cys to gly at position 24) and is defective in binding to Rb (Edmonds and Vousden, 1989). These retroviruses failed to stimulate either apoptosis or IL-1 α release (Figure 4a). Previous studies have shown that overexpression of E2F-1 stimulates apoptosis of fibroblasts (DeGregori *et al.*, 1997). We constructed retroviruses encoding E2F-1,

then used these to infect keratinocytes in order to directly examine whether increased apoptosis promoted release of IL-1 α . Expression of E2F-1 strongly inhibited keratinocyte proliferation and induced extensive apoptosis and IL-1 α release (Figure 4a). Western blot analyses confirmed that keratinocytes infected with retroviruses encoding the HPV-16 E7 p24Gly mutant or E2F-1 actually expressed the corresponding proteins (Figure 4b). Furthermore, the mutant E7 protein was stable and expressed at levels similar to the wild type E7.

Western analyses examined whether increased apoptosis in cells expressing HPV-16 E6, E7 or E6/E7 was associated with altered expression of pro-apoptotic cellular proteins such as p53 or Bax or anti-apoptotic proteins including Bcl-2 or Bcl-x_L. The most consistent finding was a strong downregulation of p53 in cells expressing E6 or E6/E7 (Figure 4c). Bax was increased twofold in cells expressing E7 alone, whereas Bcl-2 and Bcl-x were not significantly altered (data not shown).

Expression of IL-1 α in cervical biopsies

To examine the relevance of *in vitro* observations we compared expression of IL-1 α in normal cervical epithelia and in biopsies from progressive stages leading to cervical squamous neoplasia. Biopsy samples were selected from HPV-16-positive individuals so that we could also compare the level of apoptosis and HPV-16 E6/E7 RNA expression, as determined by *in situ* hybridization. IL-1 α was expressed in keratinocytes at all stages leading to malignancy (Figure 5). IL-1 α protein was detected in each layer of stratified epithelia and was low in the underlying connective tissue. Staining was strong in LSIL and HSIL but was variable in several squamous carcinomas; most stained strongly but others stained heterogeneously. To confirm that IL-1 α was expressed in carcinoma cells, six freshly isolated primary cervical carcinomas were enzymatically digested, tumor cells were maintained in culture, and the levels of intracellular IL-1 α were compared with cultures derived from normal cervix using ELISA (Figure 6). Intracellular levels of IL-1 α in cultured carcinoma cells varied from 800–4000 pg/10⁶ cells; five of six cell strains contained IL-1 α at levels similar to normal keratinocytes. Thus, most cervical carcinomas expressed significant amounts of IL-1 α both *in vitro* and *in vivo*.

Detection of apoptosis and HPV-16 RNA expression in cervical biopsies

Studies also examined the percentage of apoptotic cells within normal or HPV-16-infected epithelia using TUNEL (Table 2). Apoptosis was low or undetectable

Table 2. Percentage of apoptotic keratinocytes in normal, dysplastic and malignant cervical epithelia

Tissue	% apoptotic cells ^a
Normal cervix (n=9)	0.1 $\pm$ 0.1
Low grade dysplasia (n=15)	0.2 $\pm$ 0.3
High grade dysplasia (n=19)	0.6 $\pm$ 0.8
Carcinoma (n=13)	2.2 $\pm$ 1.6*

^aMeasured by TUNEL staining; examined 1000 cells/slide. Asterisk indicates value significantly different than normal cervix ($P < 0.001$, Dunn's test) and *n* represents the number of cases studied

in 15 biopsies from LSIL and in nine normal ectocervical epithelia. In LSIL apoptotic cells were

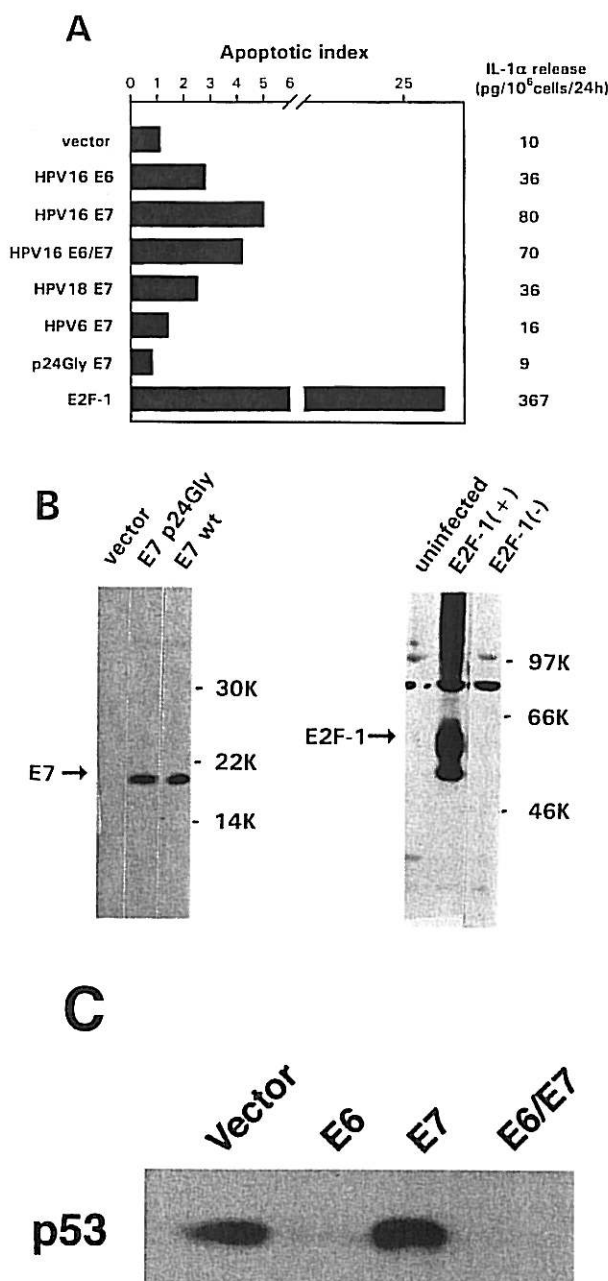


Figure 4 Correlation between IL-1 α release and percent apoptotic keratinocytes (apoptotic index) in response to specific HPV genes. (a) Cervical keratinocytes were infected with recombinant retroviruses encoding either vector alone or specific HPV-16, -18 or -6 genes. p24Gly is an HPV-16 E7 mutant defective in Rb binding. Cells were maintained in basal K-SFM for 1 day and IL-1 α release was measured by ELISA. Replicate cultures were analysed after 2 days for the percentage of cells undergoing apoptosis by TUNEL and counting 1000 cells (both attached and floating) from each experimental group. Results are from one representative experiment and experiments were repeated four times on independent cultures. (b) Western analyses of expression of HPV-16 E7 wild type (E7wt), E7 mutant (E7 p24Gly) or E2F-1 proteins in retrovirus-infected keratinocytes. E2F-1 (+) infected cultures exhibited numerous apoptotic cells, grew poorly, and overexpressed E2F-1 protein. E2F-1 (-) cultures contained few apoptotic cells, grew rapidly, and apparently lost the retroviral E2F-1 sequence. (c) Western analysis of p53 protein in keratinocytes infected with vector, HPV-16 E6, E7 or E6/E7 retroviruses and maintained for 1 day in basal KSF-M

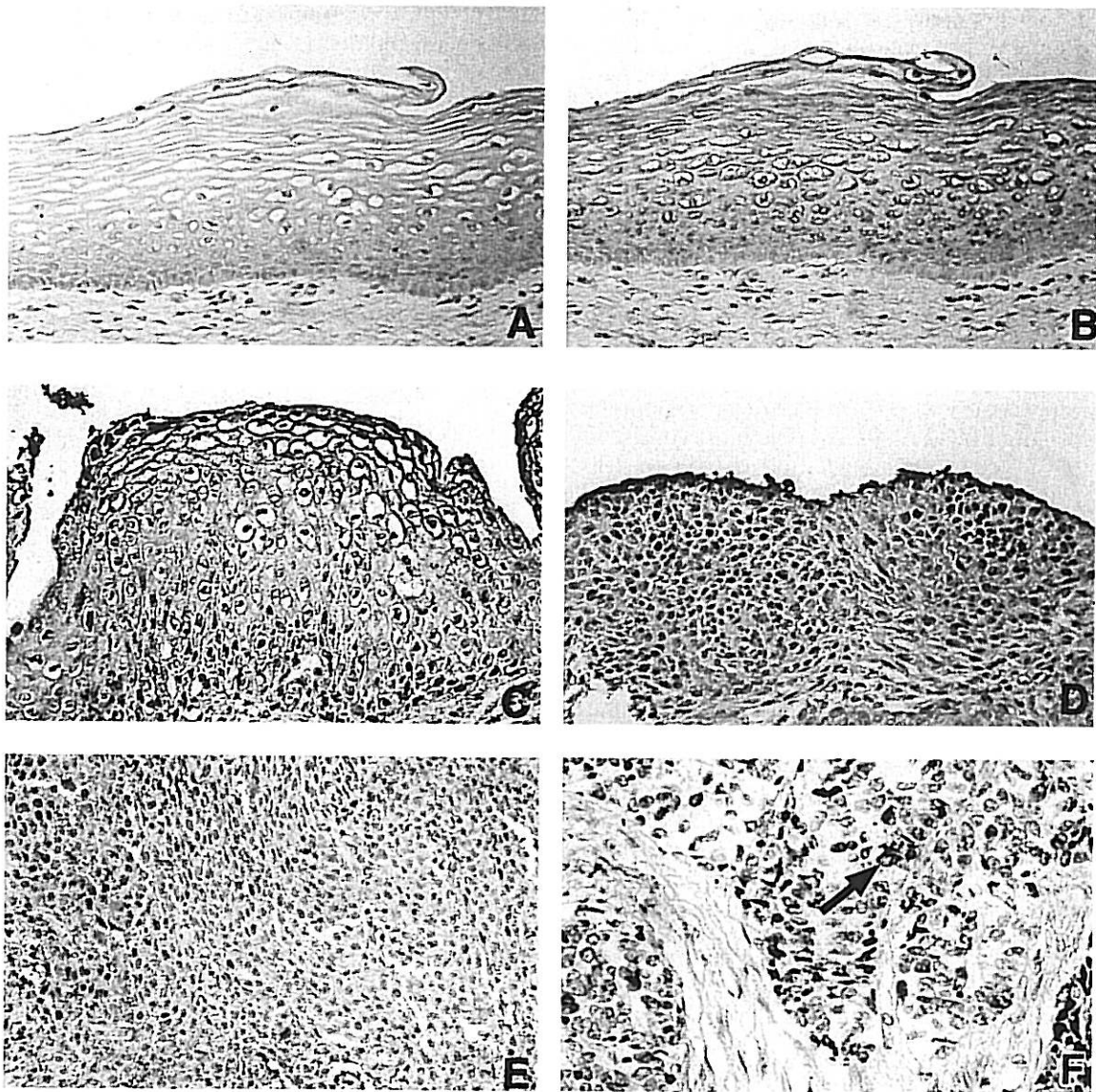


Figure 5 Immunohistochemical localization of IL-1 α and apoptosis in normal ectocervical epithelia, dysplasia and cervical carcinoma. Sections of normal ectocervical epithelium (a, b), LSIL (c), HSIL (d) and cervical carcinoma (e, f) were stained for IL-1 α (b, c, d, e) or for apoptosis by TUNEL (f). a is a control in which the primary antibody was omitted. The arrow in f indicates one of several apoptotic cells in the field

observed occasionally in the basal and suprabasal layers of proliferating cells but not in the superficial differentiated strata, suggesting that differentiating keratinocytes were not susceptible to apoptosis. Apoptosis occurred more frequently and was observed throughout the entire epithelium in 19 biopsies of HSIL. In 13 invasive cervical carcinomas the percentage of apoptotic keratinocytes increased significantly (approximately 20-fold) relative to LSIL or normal cervical epithelia (Figure 5f). Sections of HSIL and invasive cervical cancer showed evidence of inflammation. Inflammatory changes were variable in different specimens but were more prominent in invasive cervical cancers.

In situ hybridization analyses using probes for HPV-16 E6/E7 RNAs demonstrated a characteristic pattern of RNA expression in these lesions (Figure 7). In normal epithelia only minimal background signals were present. In biopsies of LSIL there was minimal E6/E7 hybridization in the parabasal zone but progressive induction occurred in suprabasal and subsurface regions where

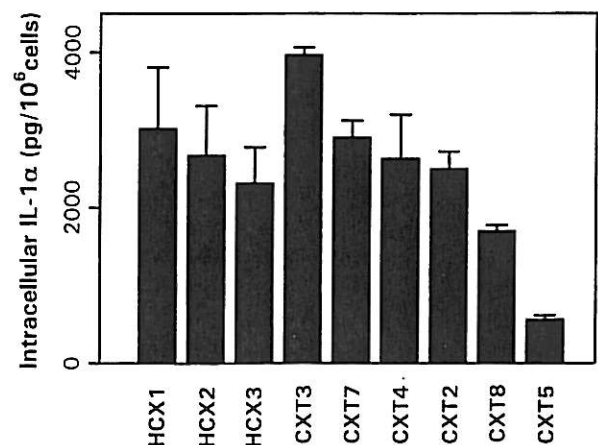


Figure 6 Comparison of intracellular levels of IL-1 α in normal cervical keratinocytes isolated from different individuals (HCX 1-3) and six early passage cultures (pass 3-5) derived from different primary cervical carcinomas (CXT2-8). Error bars represent the mean $\pm$ standard deviation

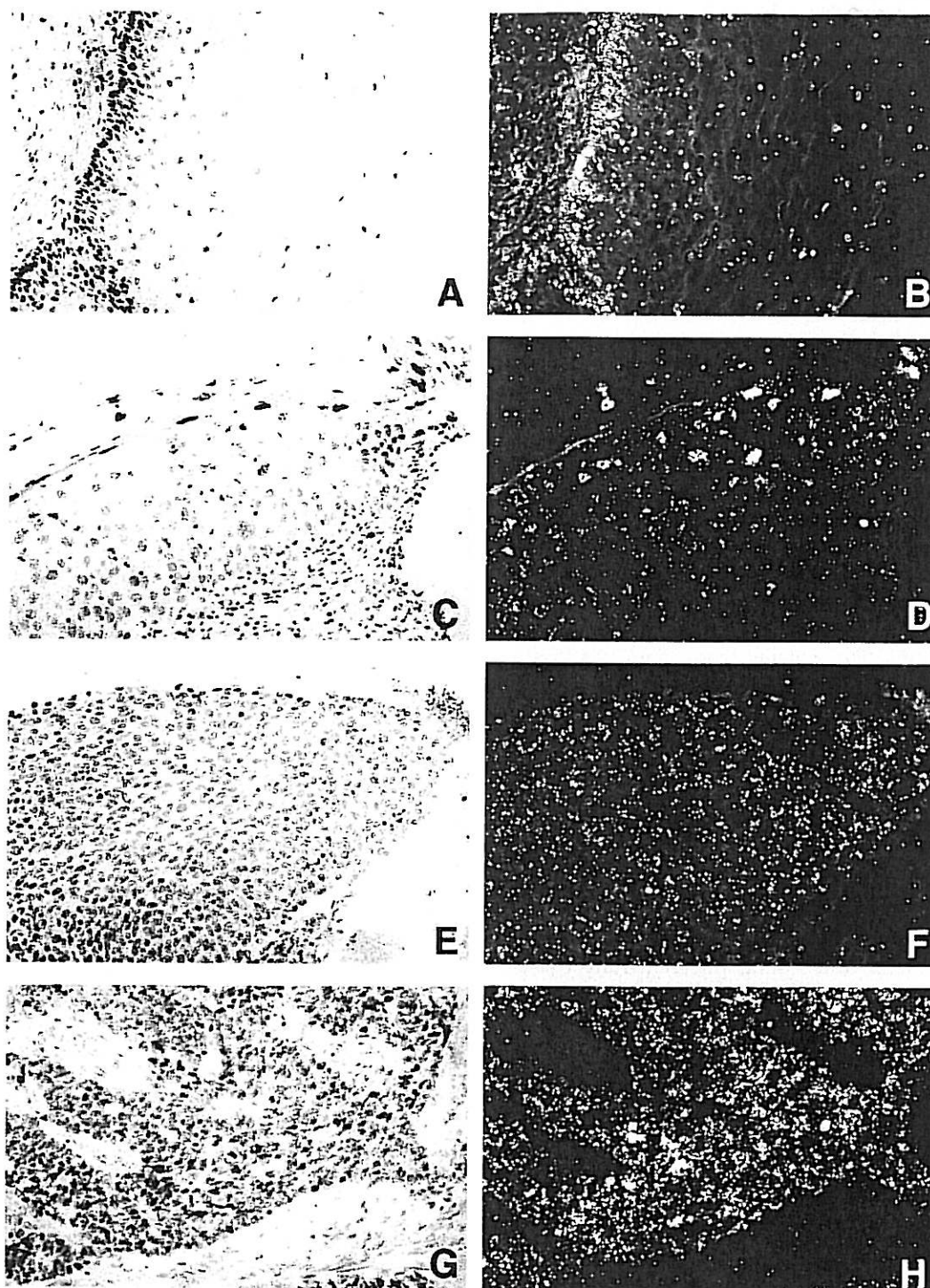


Figure 7 *In situ* hybridization analyses of HPV-16 E6/E7 RNA expression in cervical biopsies. Representative examples of normal cervical epithelium (a, b), LSIL (c, d), HSIL (e, f), and invasive carcinoma (g, h) examined by brightfield illumination for morphology (a, c, e, g) or darkfield illumination to localize silver grains corresponding to HPV-16 E6/E7 RNA expression (b, d, f, h)

viral DNA replication and productive infection occurs in terminally differentiating keratinocytes. In contrast, biopsies from HSIL expressed HPV-16 E6/E7 RNAs diffusely throughout the epithelial thickness at levels equal to or greater than that seen in the parabasal zone of LSIL. Invasive cervical carcinomas usually expressed E6/E7 RNAs at a very high level throughout the tumor. In summary, these results indicate that apoptosis and E6/E7 RNA expression increased with increasing lesion grade in proliferating keratinocytes.

Discussion

Keratinocytes produce high levels of biologically active IL-1 α , a multifunctional cytokine that stimulates inflammation, wound healing and epithelial hyperplasia. Under normal conditions intracellular IL-1 α is not released from proliferating keratinocytes in stratified squamous epithelia or *in vitro*. Our results demonstrate that high-level expression of HPV-16 E7 or E6/E7 proteins significantly stimulated release of IL-1 α from

cultured cervical keratinocytes. The direct correlation between the magnitude of IL-1 α release and the extent of apoptosis measured by TUNEL staining suggests that IL-1 α release was mediated by the ability of E7 to sensitize cells to apoptosis. This was supported by our observation that induction of apoptosis by overexpression of E2F-1 directly induced IL-1 α release. Current results are consistent with observations that the HPV-16 E7 or E6/E7 proteins enhance apoptosis in a variety of cell types (Kim *et al.*, 1997; Pan and Griep, 1995; Puthenveettil *et al.*, 1996; Rorke and Jacobberger, 1995) and that apoptosis of macrophages results in release of biologically active IL-1 α (Hogquist *et al.*, 1991). *In vitro* results using retrovirus-infected keratinocytes were consistent with observations on cervical biopsies. IL-1 α was produced by keratinocytes in the cervical mucosa at all stages leading to malignancy but HPV-16 E6/E7 RNA expression and apoptosis increased in parallel in high grade lesions and carcinomas. Thus, high level expression of HPV-16 E7 or E6/E7 genes may enhance apoptosis of cervical keratinocytes which in turn leads to release of IL-1 α .

The relative ability of HPV proteins to stimulate apoptosis and IL-1 α release was E7 > E6/E7 > E6 > vector control and thus, appeared to be related to expression of E7. E7 proteins from 'high risk' HPVs stimulate cell cycle progression by binding to Rb which in turn facilitates release of E2F-1 and stimulation of cellular genes required for DNA synthesis (DeGregori *et al.*, 1997; Kowalik *et al.*, 1995). However, increased activity of E2F-1 can also sensitize cells to apoptosis when mitogens or serum are reduced (DeGregori *et al.*, 1997; Kowalik *et al.*, 1995; Shan and Lee, 1994). Our *in vitro* results suggest that E7 sensitizes cervical keratinocytes to IL-1 α release through a similar pathway. E7 proteins from 'high risk' HPV-16 and -18 that bind Rb strongly induced IL-1 α release, whereas the 'low risk' HPV-6 E7 protein, which binds poorly (Münger *et al.*, 1989) did not. This association was confirmed by our finding that an HPV-16 E7 mutant that fails to bind Rb also failed to induce IL-1 α release, and that overexpression of E2F-1, a downstream effector of Rb, induced extensive IL-1 α release and apoptosis. Thus, 'high risk' but not 'low risk' E7 proteins sensitize keratinocytes to apoptosis and IL-1 α release.

Although both E7 and E2F-1 stimulated apoptosis and IL-1 α release they had opposite effects on cell proliferation. E7 stimulated cell growth whereas E2F-1 did not. Overexpression of E2F-1 stimulates G1 progression, but it also promotes apoptosis if it is not downregulated in S phase (Krek *et al.*, 1995). Thus, apoptosis and IL-1 α release in response to E7 may be due to failure to completely downregulate E2F-1 activity in a subpopulation of cells rather than to the overall increased proliferation of these cultures. The increased growth rate of cells expressing E7 does not explain their increased IL-1 release because rapidly growing cells release less than cells made quiescent by growth factor deprivation or treatment with podophyllin (Figure 1).

HPV-16 E6 also induced a lower level of apoptosis and IL-1 α release. E6 is considered an anti-apoptotic protein (Pan and Griep, 1995; Puthenveettil *et al.*, 1996) as it binds to and promotes degradation of p53 (Scheffner *et al.*, 1990). In our experiments using growth factor deficient medium E6 strongly decreased p53 levels

but only partially inhibited E7-mediated apoptosis. Thus, apoptosis of cervical keratinocytes under these conditions can occur through a p53-independent pathway as reported for lens epithelial cells (Pan and Griep, 1995). Apoptosis induced by other agents may be mediated by different pathways which may differ in their requirements for p53. Previous studies have shown that high level expression of HPV-16 E6 sensitizes cultured human fibroblasts, immortalized breast epithelial cells, or cervical carcinoma cell lines to apoptosis (Wahl *et al.*, 1996; Xu *et al.*, 1995; Brown *et al.*, 1997). Our *in vitro* results show that E6 promotes a low level of apoptosis in normal human cervical keratinocytes, the natural target of HPV infection.

Observations on biopsies of normal, HPV-infected, and malignant cervical epithelia led to three conclusions. Firstly, IL-1 α was expressed by keratinocytes at all stages leading to cervical cancer. Secondly, apoptosis occurred primarily in HSIL and carcinomas. Taken together these observations imply that release of IL-1 α from apoptotic keratinocytes is a feature of progression to high grade disease. Due to the large amount of intracellular IL-1 α it was not possible to directly measure release by discriminating between intra- and extracellular IL-1 α in tissue sections using immunohistochemistry. Thirdly, the levels of HPV-16 E6/E7 RNA expression in proliferating keratinocytes increased with increasing lesion grade and thus, paralleled the observed increase in apoptosis. Thus, *in vitro* experiments demonstrating apoptosis and IL-1 α release from keratinocytes expressing high levels of E6 and E7 genes from retroviral vectors may be biologically relevant and representative of HSIL or carcinoma where E6 and E7 genes are integrated and transcriptionally upregulated (Dürst *et al.*, 1992; Stoler *et al.*, 1992). Previous studies have shown that apoptosis is increased in HSIL and cervical carcinoma (Isacson *et al.*, 1996; Shoji *et al.*, 1996) and correlates with increased cellular proliferation rather than with 'low or high risk' HPV types (Isacson *et al.*, 1996). Our results confirm these observations and extend them by suggesting that the extent of apoptosis is related to levels of HPV RNA expression. Because apoptosis occurs rapidly, within several hours *in situ* (Bursch *et al.*, 1990) and fragmented epithelial cells are quickly phagocytized by neighboring cells, a relatively low incidence of histological signs of apoptosis might still indicate a significant rate of cell loss and IL-1 α release (Bursch *et al.*, 1990).

Release of IL-1 α may influence the pathogenesis of HPV infection or progression to cervical cancer. In LSIL both HPV-16 E6/E7 expression and apoptosis occur at a relatively low level implying that release of IL-1 α is not a major factor. This is consistent with the observation that LSIL is not normally associated with an inflammatory response. E6/E7 RNA expression and apoptosis increase in HSIL and particularly, in cervical carcinomas which in turn may lead to enhanced release of IL-1 α . This is consistent with observations that inflammation increases in HSIL and invasive cervical cancers (Riethdorf *et al.*, 1996). In the presence of an effective host response against HPV, IL-1 α release may contribute to papilloma regression by stimulating inflammation and immunity. In this regard, high level expression of IL-1 α in HPV-16 transformed keratinocytes using recombinant

retroviruses decreases their tumorigenicity in nude mice (Merrick *et al.*, 1996). Our results indicate that podophyllin or ATRA enhanced apoptosis and IL-1 α release from keratinocytes expressing HPV-16 E6/E7 genes, thus, these compounds might enhance inflammation and the host's immune response to infected cells. Alternately, in the absence of an effective host response to HPV, continued release of IL-1 α may contribute to malignant conversion or tumor progression. IL-1 α stimulates mitogenesis of HPV-immortalized cervical keratinocytes and cervical carcinoma cell lines (Castrilli *et al.*, 1997; Woodworth *et al.*, 1995), and promotes release of mutagenic products including nitric oxide and reactive oxygen species. IL-1 also contributes to tumor progression in animal models by stimulating angiogenesis and tumor metastasis (Fan *et al.*, 1993; Vidal-Vanaclocha *et al.*, 1994). Studies are in progress to examine whether enhanced expression of IL-1 α and chronic inflammation contribute to cervical carcinogenesis.

Materials and methods

Cell culture

Samples of cervix were obtained after hysterectomy for nonmalignant disease including fibroids or endometriosis. Keratinocytes were isolated using a two-step enzymatic digestion with dispase followed by trypsin as described previously (Woodworth and Simpson, 1993). Cultures were maintained in K-SFM medium (Life Technologies Inc., Gaithersburg, MD) prior to infection with retroviruses. In some experiments cultures were maintained in basal K-SFM lacking growth factors (transferrin, epidermal growth factor, insulin, bovine pituitary extract, hydrocortisone and triiodothyronine) to induce quiescence. Podophyllin and ATRA were purchased from Sigma Chemical Co (St. Louis, MO).

Construction of retrovirus vectors and infection of keratinocytes

Retroviruses containing the HPV-16 p24gly E7 mutant (Edmonds and Vousden, 1989) which binds Rb poorly were constructed by digesting p24gly E7 DNA with *Bam*HI and insertion into the *Bam*HI site of the retroviral vector pLXSN (Miller and Rosman, 1989). A retrovirus expressing E2F-1 was constructed by *Bam*HI digestion of pcmvE2F-1 DNA (Helin *et al.*, 1993) and insertion into the *Bam*HI site of pLXSN. Plasmids were transfected into the Psi 2 ecotropic packaging cell line, supernatants were collected after 48 h and used to infect the PA317 amphotropic packaging line in order to generate multiple clones of virus-producer lines. Secondary cultures of normal foreskin or cervical keratinocytes were infected with high-titer amphotropic retroviruses containing HPV-16 E6, E7 or E6/E7 plus the neomycin resistance gene (Halbert *et al.*, 1992), or with retroviruses encoding HPV-6 E7 (Halbert *et al.*, 1992), -18 E7, -16E7gly, or E2F-1 as described (Woodworth *et al.*, 1992). Cells were subcultured 24 h after infection and selected for growth in K-SFM containing G-418 (200 μ g/ml for 2 days). Typically, 40–80% of keratinocytes acquired G-418 resistance. After selection in G418, cultures were split and used for ELISA or analyses of gene expression within 3–4 days.

ELISA assays

Cytokine release from keratinocyte cultures was measured using commercially available kits for IL-1 α (Immunotech, Marseille, France), mature IL- β , IL-1ra, IL-1sr2, IL-6, IL-

8, granulocyte-macrophage colony stimulating factor, tumor necrosis factor- α , IL-10, IL-12, and macrophage chemotactic protein (all from R&D Systems Minneapolis, MN) as described (Woodworth and Simpson, 1993). Briefly, when cells were 50–60% confluent, cultures were rinsed with PBS and 5 ml of medium was added per 100 mm dish. After 24 h the conditioned medium was collected, supplemented with protease inhibitors including AEBSF (10 μ g/ml), aprotinin and leupeptin (2 μ g/ml each), centrifuged to remove cells and debris, and frozen at -70°C for 1–6 weeks prior to analysis. After removing the medium each culture was trypsinized and cell number was determined using a Coulter counter. To measure total cellular IL-1 α each culture was frozen and thawed three times in basal medium, lysates were supplemented with protease inhibitors, centrifuged at 2000 g for 5 min and stored at -70°C prior to ELISA.

RNA analyses

Monolayer cultures were lysed with TRIzol Reagent (Life Technologies Inc.), extracted with chloroform and RNA was precipitated by addition of isopropyl alcohol. Total cellular RNA (10 μ g/lane) was fractionated on 1.4% agarose gels containing formaldehyde, transferred to nylon membranes and hybridized to DNA probes labeled to high specific activity ($>1 \times 10^9$ c.p.m./ μ g DNA) by the random primer method. Probes included a cDNA encoding human IL-1 α (1.6 kb *Eco*RI/*Pst*I fragment) or mouse glyceraldehyde phosphate dehydrogenase.

Protein analyses

Western analyses were performed as described (Woodworth *et al.*, 1996). Primary antibodies included rabbit anti-human Bax, Bcl-x, or E2F-1, mouse anti-human p53 or Bcl-2 (all from Santa Cruz Biotechnology, Santa Cruz, CA), or mouse monoclonal antibody to HPV-16 E7 (Zymed Laboratories Inc., South San Francisco, CA). Normal rabbit or mouse IgG was substituted for the primary antibodies to control for nonspecific binding. Primary antibody binding was visualized using the Western Light Plus Chemiluminescent Detection System (Tropix Inc. Bedford, MA) as described (Woodworth *et al.*, 1996).

For immunohistochemical detection of IL-1 α formalin-fixed sections of human cervical biopsies were incubated for 10 min in 1% hydrogen peroxide to eliminate endogenous peroxidase activity, treated for 30 min with 10% normal goat serum to block nonspecific binding, and incubated with dilutions (1–10 μ g protein/ml) of rabbit anti-human IL-1 α (Genzyme, Cambridge, MA) in 1% goat serum at 37°C for 1 h. Primary antibody binding was visualized using the Vectastain kit (Vector Laboratories, Burlingame, CA) and sections were lightly counterstained with Harris hematoxylin. Controls consisted of omitting the primary antibody or substituting an equal concentration of rabbit IgG (3–10 μ g/ml).

Apoptosis assays

Measurement of DNA fragmentation was performed using TUNEL as described (Rösl, 1992). Cells were lysed in buffer (5 mM Tris pH 8, 10 mM EDTA, 0.5% Triton X-100), treated sequentially with 100 μ g/ml RNase for 60 min at 37°C , 200 μ g/ml proteinase K for 50 min at 50°C , and extracted gently two times with an equal volume of phenol-chloroform-isoamyl alcohol. DNA was precipitated and resuspended in reaction buffer (10 mM Tris pH 7.5, 5 mM MgCl_2) containing 5 U Klenow polymerase and 5 μ Ci [^{32}P]dCTP for 10 min at room

temp. The reaction was terminated by addition of EDTA and the DNA precipitated to remove unincorporated deoxynucleotides, and fractionated on 1.8% agarose gels. Gels were dried overnight by blotting with 3M Whatman paper and exposed to X-ray film. Hybridization signals were measured using a flatbed scanner, relative intensities were compared quantitatively using NIH Image software. Apoptosis and necrosis in monolayers of viable keratinocytes were also measured by costaining cells with Hoechst 33258 and propidium iodide (Sigma Chemical Co) for 5 min at a concentration of 3 and 10 $\mu\text{g/ml}$, respectively. Excitation wavelengths were 365 and 530–585 nm and emission wavelengths examined were 615 and 420 nm for Hoechst and propidium iodide, respectively. Cells with condensed and fragmented nuclei were scored as apoptotic whereas cells with intact nuclei that stained with propidium iodide were scored as necrotic.

The percentage of apoptotic cells in cultures of keratinocytes or in sections of cervical epithelia was determined by the TUNEL method using a commercially available kit (ApopTag, Oncor Inc., Gaithersburg, MD). Culture supernates were centrifuged at 2000 g for 5 min to collect nonadherent apoptotic cells and these were combined with trypsinized cell suspensions. Cells were treated with 3% neutral-buffered formalin for 5 min, rinsed with PBS, and allowed to dry on sialinized slides for 30 min at 37°C. Slides were treated with 70% ethanol for 5 min prior to detection of apoptotic cells. To examine apoptosis *in vivo* formalin-fixed paraffin-embedded sections of cervical epithelium were pretreated with 25 ng/ml proteinase K for 15 min and exposed to 3% hydrogen peroxide to inactivate endogenous peroxidase activity. After staining for TUNEL according to the manufacturer's instructions, slides were lightly counterstained using methyl green. Positive controls for apoptosis consisted of involuting rat mammary epithelium and negative controls consisted of omitting the enzyme TdT. In most sections 1000 cells were counted to estimate the percentage of apoptotic cells.

References

- Ansel JC, Luger TA, Lowy D, Perry P, Roop DR and Mountz JD. (1988). *J. Immunol.*, **140**, 2274–2278.
- Brown J, Higo H, McKalip A and Herman B. (1997). *J. Cell Biochem.*, **66**, 245–255.
- Bryan D, Sexton CJ, Williams D, Leigh IM and McKay IA. (1995). *Cell Growth & Differ.*, **6**, 1245–1250.
- Bursch W, Paffe S, Putz B, Barthel G, and Shulte-Hermann, R. (1990). *Carcinogenesis*, **11**, 847–853.
- Castrilli G, Tatone D, Diodoro MG, Rosini S, Piantelli M, and Musiani P. (1997). *Br. J. Cancer*, **75**, 855–859.
- Collins RH Jr, Feldman M, and Fordtran JS. (1987). *N. Engl. J. Med.*, **316**, 1654–1658.
- Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, Sironi M, Giri JG, Dower SK, Sims JE, and Mantovani A. (1993). *Science*, **261**, 472–475.
- Correa P. (1992). *Cancer Res.*, **52**, 6735–6740.
- DeGregori J, Leone G, Miron A, Jakoi L, and Nevins JR. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 7245–7250.
- Dinarelli CA. (1996). *Blood*, **87**, 2095–2147.
- Dürst M, Glitz D, Schneider A, and zur Hausen H. (1992). *Virology*, **189**, 132–140.
- Edmonds C, and Vousden KH. (1989). *J. Virol.*, **63**, 2650–2656.
- Eisenberg SP, Evans RJ, Arend WP, Verderber E, Brewer MT, Hannum CH, and Thompson RC. (1990). *Nature*, **343**, 341–346.
- Fan TP, Hu DE, Guard S, Gresham GA and Watling KJ. (1993). *Br. J. Pharmacol.*, **110**, 43–49.
- Frazer IH. (1996). *Curr. Opin. Immunol.*, **8**, 484–491.
- Halbert CL, Demers GW and Galloway DA. (1992). *J. Virol.*, **66**, 2125–2134.
- Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR and Schiller JT. (1989). *EMBO J.*, **8**, 3905–3910.
- Helin K, Harlow E and Fattaey A. (1993). *Mol. Cell Biol.*, **13**, 6501–6508.
- Hogquist KA, Nett MA, Unanue ER and Chaplin DD. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 8485–8489.
- Isacson C, Kessis TD, Hedrick L and Cho KR. (1996). *Cancer Res.*, **56**, 669–674.
- Kim CY, Tsai MH, Osmanian C, Graeber TG, Lee JE, Giffard RG, DiPaolo JA, Peehl DM and Giaccia AJ. (1997). *Cancer Res.*, **57**, 4200–4204.
- Kowalik TF, DeGregori J, Schwarz JK and Nevins JR. (1995). *J. Virol.*, **69**, 2491–2500.
- Krek W, Xu G and Livingston DM. (1995). *Cell*, **83**, 1149–1158.
- Kupper TS and Groves RW. (1995). *J. Invest. Dermatol.*, **105**, 62S–66S.
- Li DQ and Tseng SC. (1997). *J. Cell. Physiol.*, **172**, 361–372.
- Merrick DT, Winberg G and McDougall JK. (1996). *Cell Growth & Differ.*, **7**, 1661–1669.
- Miller AD and Rosman GJ. (1989). *Biotechniques*, **7**, 980–988.
- Montague JW, and Cidlowski JA. (1996). *Experientia*, **52**, 957–962.
- Münger K, Werness BA, Dyson N, Phelps WC, Harlow E and Howley PM. (1989). *EMBO J.*, **8**, 4099–4105.

In situ hybridization

In situ hybridization to detect HPV-16 E6/E7 RNAs was performed as described (Stoler *et al.*, 1992). Controls included the use of sense probes without prior heat denaturation of DNA to ascertain that signals resulted solely from RNA, as well as pretreatment of selected specimens with RNase prior to hybridization. The normal histologic elements within the sections served as controls for nonspecific trapping of probes.

Statistical analyses

The Kruskal–Wallis non parametric ANOVA test was used to compare overall differences in cytokine release *in vitro* or apoptotic indices in cervical biopsies. Two by two comparisons were performed using Dunn's test. The correlation coefficient for measurements of apoptosis (DNA ladder) and associated IL-1 α release was calculated by subtracting vector response from experimental values (E6, E7, E6/E7) then using those standardized values to calculate the non parametric Spearman correlation coefficient and *P* value.

Acknowledgements

We thank Adam Glick, Mark Schiffman and Stuart Yuspa for their critical comments. We also are grateful to Denise Galloway for supplying retrovirus producer cell lines, Karen Vousden for providing the HPV-16 E7 24gly mutant, Kristian Helin for the E2F-1 cDNA, Masaaki Yamada for the IL-1 α cDNA, and Patricia Goggins for help in preparation of the manuscript. Human cervical tissue was supplied by the Cooperative Human Tissue Network.

- Oberyszyn TM, Sabourin CLK, Bijur GN, Oberyszyn AS, Boros LG and Robertson FM. (1993). *Mol. Carcinogen.*, **7**, 238–248.
- Pan H and Griep AE. (1995). *Genes & Dev.*, **9**, 2157–2169.
- Puthenveetil JA, Frederickson SM and Reznikoff CA. (1996). *Oncogene*, **13**, 1123–1131.
- Riethdorf L, Riethdorf S, Gützlaß K, Prall F and Löning T. (1996). *Am. J. Pathol.*, **149**, 1469–1476.
- Rösl F, Lengert M, Albrecht J, Kleine K, Zawatzky R, Schraven B and zur Hausen H. (1994). *J. Virol.*, **68**, 2142–2150.
- Rösl F. (1992). *Nucl. Acids Res.*, **20**, 5243.
- Rorke EA and Jacobberger JW. (1995). *Exp. Cell Res.*, **216**, 65–72.
- Scheffner M, Werness BA, Huibregtse JM, Levine AJ and Howley PM. (1990). *Cell*, **63**, 1129–1136.
- Shan B and Lee W-H. (1994). *Mol. Cell. Biol.*, **14**, 8166–8173.
- Shoji Y, Saegusa M, Takano Y, Ohbu M and Okayasu I. (1996). *J. Clin. Pathol.*, **49**, 134–138.
- Stoler MH, Rhodes CR, Whitbeck A, Wolinsky SM, Chow LT and Broker TR. (1992). *Human Pathol.*, **23**, 117–128.
- Stone KM. (1995). *Clin. Infect. Dis.*, **20**, 591–597.
- Vidal-Vanaclocha F, Amézaga D, Asumendi A, Kaplanski G and Dinarello DA. (1994). *Cancer Res.*, **54**, 2667–2672.
- Wahl AF, Donaldson KL, Fairchild C, Lee FYF, Foster SA, Demers GW and Galloway DA. (1996). *Nature Med.*, **2**, 72–79.
- White AE, Livanos EM and Tlsty TD. (1994). *Genes & Dev.*, **8**, 666–677.
- Wood LC, Elias PM, Calhoun C, Tsai JC, Grunfeld C and Feingold KR. (1996). *J. Invest. Dermatol.*, **106**, 397–403.
- Woodworth CD, Cheng S, Simpson S, Hamacher L, Chow LT, Broker TR and DiPaolo JA. (1992). *Oncogene*, **7**, 619–626.
- Woodworth CD, Chung J, McMullin E, Plowman GD, Simpson S and Iglesias M. (1996). *Cell Growth & Differ.*, **7**, 811–820.
- Woodworth CD, McMullin E, Iglesias M and Plowman GD. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 2840–2844.
- Woodworth CD, and Simpson S. (1993). *Am. J. Pathol.*, **142**, 1544–1555.
- Xu C, Meikrantz W, Schlegel R and Sager R. (1995). *Proc. Nat. Acad. Sci. USA*, **92**, 7829–7833.
- Zerfass K, Schulze A, Spitkovsky D, Friedman V, Henglein B and Jansen-Dürr P. (1995). *J. Virol.*, **69**, 6389–6399.
- zur Hausen H. (1996). *Biochem. Biophys. Acta.*, **1288**, F55–F78.