Interleukin 1α and tumor necrosis factor α stimulate autocrine amphiregulin expression and proliferation of human papillomavirus-immortalized and carcinoma-derived cervical epithelial cells

(keratinocyte/epidermal growth factor/proinflammatory cytokines/cervical cancer)

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ABSTRACT **Infection with multiple sexually transmitted agents has been associated with inflammation of the cervix and an increased risk of cervical cancer in women infected with human papillomaviruses (HPVs). Two proinflammatory cytokines, interleukin 1α (IL-1α) and tumor necrosis factor α (TNF-α), inhibited proliferation of normal epithelial cells cultured from human cervix. In contrast, both cytokines significantly stimulated proliferation of cervical cell lines (5 of 7) immortalized by transfection with HPV-16 or -18 DNAs or lines derived from cervical carcinomas (7 of 11). Stimulation was dose dependent from 0.01 to 1.0 nM and was blocked by specific inhibitors, such as the IL-1 receptor antagonist or the TNF type 1 or 2 soluble receptors. Growth stimulation by IL-1α or TNF-α was accompanied by a 6- to 10-fold increase in RNA encoding amphiregulin, an epidermal growth factor (EGF) receptor ligand. Recombinant human amphiregulin (0.1 nM) was as effective as IL-1α or TNF-α in promoting proliferation. Monoclonal antibodies that blocked signal transduction by the EGF receptor or that neutralized amphiregulin activity prevented mitogenic stimulation by IL-1α or TNF-α. These studies indicate that IL-1α and TNF-α stimulate proliferation of immortal and malignant cervical epithelial cells by an EGF receptor-dependent pathway requiring autocrine stimulation by amphiregulin. Furthermore, they suggest that chronic inflammation and release of proinflammatory cytokines might provide a selective growth advantage for abnormal cervical cells *in vivo*.**

Interleukin 1α (IL-1α) and tumor necrosis factor α (TNF-α) are distinct cytokines that share several biological functions. Both cytokines promote the inflammatory response and regulate aspects of cellular immunity and, thus, are important in host defense against infection (reviewed in refs. 1 and 2). A wide variety of cells produce IL-1α or TNF-α; however, expression is tightly regulated. Usually, these cytokines are not produced constitutively, but expression is activated in response to infection or injury (1, 2). IL-1α and TNF-α are produced at high levels by macrophages and monocytes, and both are expressed by epithelial cells that are common targets of infection (3–5). Both cytokines exert pleiotropic effects on cell growth and differentiation because of their ability to regulate expression of genes encoding growth factors, cellular receptors, and other cytokines (1, 2).

Cervical cancer is the second leading cause of cancer deaths in women worldwide (6). Recent epidemiologic and experimental evidence strongly implicates infection with a subset of human papillomaviruses (HPVs) as etiologically important in this disease (reviewed in ref. 7). HPV infections occur frequently in sexually active individuals; however, only a minority of infected women actually develop cervical cancer (7). Thus, additional environmental and/or hereditary factors are involved in malignant progression. A number of other sexually transmitted diseases frequently accompany infection with HPV (8, 9) and cause acute or chronic inflammation within the cervical mucosa (10). Although no strong evidence supports the involvement of any particular agent other than HPV, the coexistence of multiple sexually transmitted infections may be a risk factor for cervical cancer in women with HPV (8, 9), possibly due to stimulation of inflammation and tissue damage. Chronic inflammation contributes to the pathogenesis of several different types of cancer (11, 12). In the uterine cervix, inflammation and cell injury cause release of proinflammatory cytokines, which in turn might regulate growth of HPV-infected cells. The present studies examined whether specific proinflammatory cytokines acted as growth factors for cultured cervical epithelial cells. The response of cervical cells at progressive stages of malignant transformation was compared by examining normal cervical cells, 7 nontumorigenic cell lines immortalized by transfection with HPV DNAs (13), and 11 cell lines derived from cervical carcinomas.

MATERIALS AND METHODS

Cell Cultures. Cultures of human ectocervical and endocervical epithelial cells were established from cervical tissue obtained after hysterectomy due to fibroids or endometriosis (13). HPV-immortalized cell lines were derived as described (13) and were maintained in MCDB153-LB medium (14). Cervical carcinoma-derived cell lines, including SiHa, CaSki, HT-3, C-33A, C-41, and ME-180 (American Type Culture Collection) and QGU and QGH (15), were maintained in a 50/50 mixture of Ham's F-12 nutrient medium/Dulbecco's modified Eagle's medium (DMEM) containing 0.4 μg of hydrocortisone per ml and 5% fetal bovine serum. Other cervical carcinoma lines, CXT-1, CXT-2, and CXT-3, were derived and maintained in MCDB153-LB (unpublished data). All carcinoma lines contained HPV DNA with the exception of CXT-3, which has not been fully characterized, and HT-3 and C-33A, which are HPV negative.

Cell Growth Assay. Cells were inoculated into six-well plates at clonal density (500–2000 cells per well). After 18 hr, cultures were rinsed with phosphate-buffered saline to remove growth factors and then maintained in basal medium for 48 hr to arrest cell growth. Basal media were MCDB153-LB without specific supplements [insulin, epidermal growth factor (EGF), bovine

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Abbreviations: IL-1α, interleukin 1α; TNF-α, tumor necrosis factor α; EGF, epidermal growth factor; HPV, human papillomavirus; BPE, bovine pituitary extract; TGF-α, α type transforming growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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pituitary extract (BPE), transferrin, hydrocortisone, and triiodothyronine] or F-12/DMEM without serum. In some experiments, sodium heparin (10 μg/ml) (Sigma) was added to facilitate growth arrest (16). Each cytokine (R & D Systems) was tested in either complete culture medium or in medium lacking specific growth factors. These included MCDB153-LB lacking EGF and BPE or F-12/DMEM lacking fetal bovine serum. Some cell growth assays were performed in the presence of specific antibodies, including a goat neutralizing polyclonal antibody to human type α transforming growth factor (TGF-α) (R & D Systems), a mouse neutralizing monoclonal antibody to the human EGF receptor (clone LA-1; Upstate Biotechnology, Lake Placid, NY), or a mixture of three mouse IgG1 neutralizing monoclonal antibodies to amphiregulin (clones 4.14.18, 12.38.4, and 6R1C2.4; G.D.P., unpublished results). The activities of recombinant IL-1α and TNF-α were 1.6×10^5 and 1.1×10^5 units per μg of protein, respectively. Cytokines were added in fresh medium every 2 days, and the total number of cells in duplicate wells was measured after 8–10 days with a Coulter Counter. Each experiment was repeated three to five times, and the overall mean values for each experimental group were compared using the *t* test at a value of $P \leq 0.01$.

RNA Analysis. RNA analyses were performed as described (13). Recombinant plasmids containing cDNAs for human TGF-α (17), human amphiregulin (18), rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (19), or the complete HPV-16 genome were labeled with [^{32}P]dCTP using the random primer reaction and hybridized at high stringency as described (13). The intensity of hybridization signals was measured with a scanning laser densitometer. All values were normalized to the intensity of the GAPDH signal.

RESULTS

Differential Regulation of Cell Growth by IL-1α and TNF-α. Two proinflammatory cytokines, IL-1α and TNF-α, were tested for their ability to regulate proliferation of normal cervical cells, a HPV-16-immortalized cell line (CX16-2) that is nontumorigenic in nude mice, and a cervical carcinoma-derived line (CXT-1). Both cytokines slightly inhibited rapid cell proliferation that occurred in MCDB153-LB medium supplemented with EGF and BPE (data not shown). Similar results have been reported (20–22). However, when assays were performed in MCDB153-LB lacking EGF and BPE, both cytokines inhibited proliferation of normal cervical cells but significantly stimulated division of the HPV-immortalized and carcinoma-derived cell lines. When immortal or carcinoma cells were maintained in medium lacking EGF and BPE, they formed small, slowly growing colonies that consisted of closely associated, flattened cells (Fig. 1*A*). Parallel cultures treated with IL-1α or TNF-α contained round proliferating cells that were motile and formed large, diffuse colonies (Fig. 1 *B* and *C*). Cultures exposed to IL-1α or TNF-α resembled those treated with TGF-α (Fig. 1*D*). Normal cervical cells also grew slowly as small colonies in medium lacking EGF and BPE, but treatment with IL-1α or TNF-α induced cell flattening (data not shown) and growth inhibition. Thus, IL-1α and TNF-α selectively stimulated proliferation of immortal and malignant cervical cells in the absence of EGF and BPE.

To test whether growth stimulation by IL-1α or TNF-α was a common response of immortal or carcinoma cells, 18 different cell lines were examined. These were derived by transfection and immortalization of normal cervical epithelial cells with high-risk HPV DNAs (7 lines) or isolated from spontaneous cervical carcinomas (11 lines). IL-1α or TNF-α stimulated proliferation of 5 of 7 immortal lines and 7 of 11 lines derived from cervical carcinomas (Fig. 2). In contrast, IL-1α and TNF-α inhibited proliferation reproducibly in 12 of 12 cell cultures derived from normal ectocervical or endocervical epithelia (Fig. 2). Thus, stimulation of proliferation by IL-1α and TNF-α was a common response in most immortal and malignant cervical cell lines.

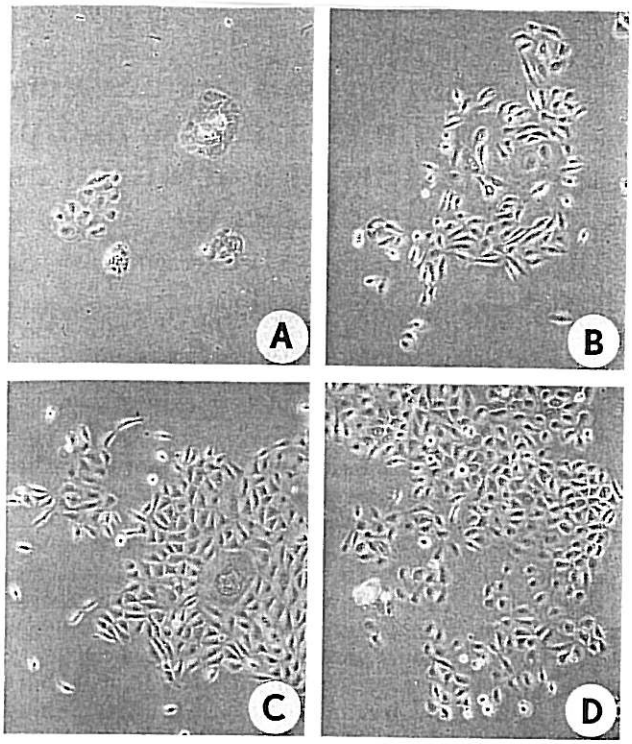

FIG. 1. Phase-contrast micrographs of HPV-16-immortalized cervical cells maintained for 7 days in MCDB153-LB medium lacking EGF and BPE (*A*) or in the same medium supplemented with 0.1 nM IL-1α (*B*), TNF-α (*C*), or TGF-α (*D*).

Specificity of IL-1α and TNF-α Effects on Proliferation. To determine whether the opposing effects on growth of normal and immortal cells were due to differential sensitivity to cytokines, dose–response analyses were performed. IL-1α and TNF-α stimulated proliferation of a HPV-16-immortalized cell line (CX16-2) over a broad range of concentrations (10 pM to 1.0 nM; Fig. 3*A*). Similar results were obtained with other HPV-immortalized and carcinoma lines; however, two tumor lines (SiHa and ME-180) had increased sensitivity to TNF-α and were inhibited by intermediate doses (≥0.5 nM; data not shown). In contrast, IL-1α and TNF-α did not stimulate proliferation of normal endocervical or ectocervical cells at any concentration tested, but both inhibited proliferation of normal cultures over a wide range of concentrations (Fig. 3*A*). Thus, the differential effects of cytokines on growth of normal and immortal cells resulted from inherent cell differences and not from dose effects.

Studies with competitive inhibitors were undertaken to determine whether growth stimulation was a specific response to each cytokine. Cell proliferation induced by IL-1α was inhibited significantly by a 20-fold excess of IL-1 receptor antagonist, which competes for binding to the IL-1 receptor but does not activate signal transduction (Fig. 3*B*). A 20-fold excess of type 1 or 2 soluble TNF receptors had no significant effect. Growth stimulation by TNF-α was blocked by excess soluble types 1 and 2 TNF receptors but not by the IL-1 receptor antagonist (Fig. 3*B*). Therefore, growth stimulation was a specific response to exogenous IL-1α or TNF-α.

Mechanism of Growth Stimulation. IL-1α and TNF-α stimulate proliferation of a wide variety of cell types via induction of specific growth factors or their receptors (1, 2, 23–26). Because IL-1α and TNF-α stimulated proliferation of immortal cervical cells only when EGF and BPE were removed from the culture medium, Northern blot analyses were performed to

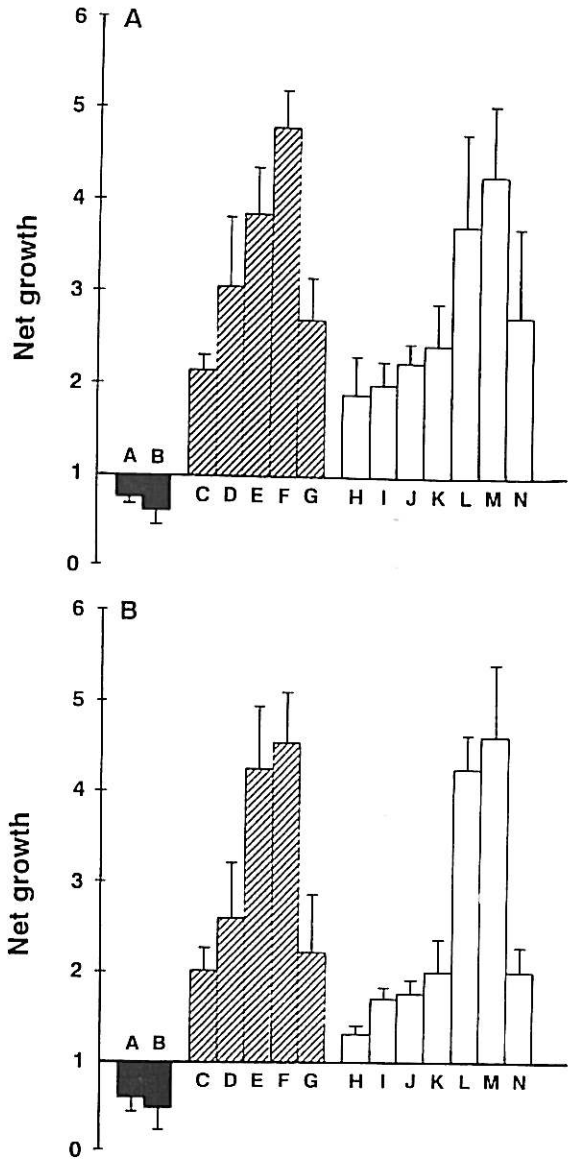

FIG. 2. Growth modulation by IL-1α (*A*) and TNF-α (*B*) in normal, HPV-immortalized, and carcinoma-derived cervical cell lines. Normal cells (solid bars) from ectocervix (A) or endocervix (B); five HPV-immortalized cervical cell lines (hatched bars), including CX16-7 (C), CX16-10 (D), CX16-2 (E), CX18-1 (F), and CX16-5 (G); and seven carcinoma-derived lines (open bars), including CaSki (H), C-41 (I), ME-180 (J), HT-3 (K), CXT-1 (L), CXT-3 (M), and SiHa (N), were maintained in medium lacking growth factors (MCDB153-LB lacking EGF and BPE or F-12/MEM without serum) but supplemented with 0.1 nM IL-1α (*A*) or TNF-α (*B*). Net growth represents cell number in the presence of each cytokine divided by cell number in the absence of cytokine. Values represent the mean of three to five independent experiments ± SE.

determine whether either cytokine stimulated expression of EGF receptor ligands. Cultured cervical cells expressed RNAs for two EGF receptor ligands, amphiregulin, and TGF-α. Steady-state levels of both RNAs were reduced in the immortal line compared to normal cells (Fig. 4). Treatment with IL-1α or TNF-α for 7 days induced amphiregulin RNA 6- to 10-fold in immortal cells but decreased levels slightly (11% and 40%) in the normal cells. In contrast, both cytokines stimulated TGF-α RNA only minimally in both types of cells. In addition, IL-1α and TNF-α induced a decrease (45% and 60%) in RNAs encoding the HPV-16 E6 and E7 oncoproteins. Hybridization to a cDNA encoding GAPDH, a housekeeping gene, indicated that each lane contained equivalent amounts of RNA.

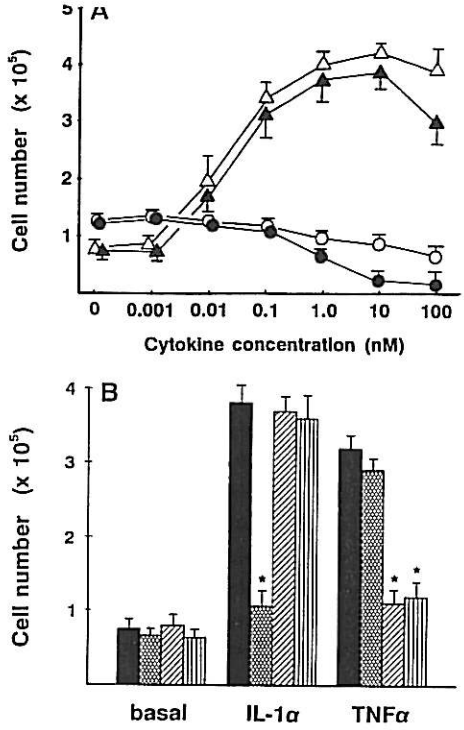

FIG. 3. Dose–response (*A*) and specificity (*B*) of IL-1α- and TNF-α-mediated cell proliferation. (*A*) Normal ectocervical cells (circles) and a HPV-16-immortalized cell line, CX16-2 (triangles), were maintained for 10 days in MCDB153-LB without EGF and BPE but containing various concentrations of IL-1α (open symbols) or TNF-α (solid symbols). (*B*) Specificity of stimulation of CX16-2 cells by IL-1α or TNF-α was measured in the absence (solid bars) or presence of a 20-fold excess of competitive inhibitors, including the IL-1 receptor antagonist (cross-hatched bars) and the TNF-binding proteins type 1 (hatched bars) or type 2 (vertically striped bars). Values represent the mean of three independent experiments ± SE. Asterisks indicate values that were significantly different from untreated cultures ($P \leq 0.01$; *t* test).

To directly examine the mitogenic activity of these two growth factors on cervical cells, several HPV-immortalized or carcinoma-derived cell lines were treated with various con-

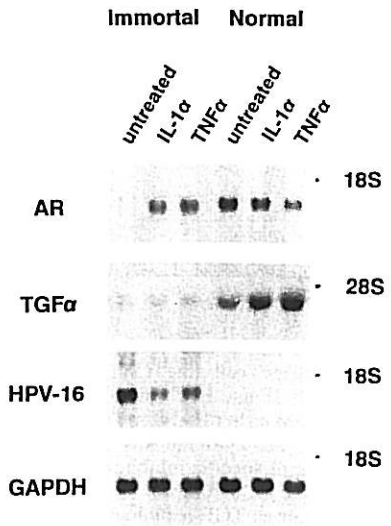

FIG. 4. Northern blot analyses of cervical cells treated with cytokines. The HPV-16-immortalized cell line CX16-2 and normal ectocervical cells were maintained for 7 days in MCDB153-LB medium lacking EGF and BPE (untreated) or in the same medium supplemented with 0.1 nM IL-1α or TNF-α. AR, amphiregulin.

Table 1. Growth stimulation of immortal cervical cells by TGF-α or amphiregulin

Growth factor	Growth factor concentration, nM				
	0.0	0.001	0.01	0.1	1.0
TGF-α	0.1	0.3	2.6	6.8	12.0
Amphiregulin	0.1	0.1	0.2	3.4	8.9

Values represent mean cell number ($\times 10^{-5}$) in duplicate wells of six-well cluster dishes. Results are shown from one representative experiment in which quiescent cultures of CX16-2 cells were exposed to each factor for 10 days in MCDB153-LB lacking EGF and BPE.

centrations of recombinant amphiregulin or TGF-α. Results using one representative cell line, CX16-2, are shown in Table 1. Both growth factors stimulated proliferation in the absence of EGF and BPE; however, TGF-α was effective at a 10-fold lower dose than amphiregulin. Similar results were observed with five other immortal or carcinoma cell lines (data not shown).

To determine whether autocrine induction of amphiregulin or TGF-α contributed to growth stimulation by IL-1α or TNF-α, studies were undertaken with specific inhibitors of the EGF receptor-signaling pathway (Fig. 5). Stimulation by IL-1α or TNF-α was assessed in HPV-immortalized cells in both the presence and absence of (*i*) a neutralizing monoclonal antibody to the EGF receptor; (*ii*) a neutralizing polyclonal antibody to TGF-α; (*iii*) heparan sulfate, a glycosaminoglycan that binds and sequesters specific EGF receptor ligands including amphiregulin (18); and (*iv*) a mixture of three monoclonal antibodies, each known to bind to amphiregulin at distinct regions and to neutralize activity. In three independent experiments, growth stimulation by IL-1α and TNF-α was completely blocked by antibodies to the EGF receptor (Fig. 5, hatched bars). Therefore, IL-1α and TNF-α mitogenesis is dependent on EGF receptor signal transduction. However, a monoclonal antibody that neutralized TGF-α activity (cross-hatched bars) did not significantly inhibit IL-1α- or TNF-α-mediated growth. This antibody did significantly inhibit proliferation induced by exogenous TGF-α, indicating that the antibody was active. In contrast, treatment with heparan sulfate (3 μg/ml; vertically striped bars) inhibited IL-1α or TNF-α growth stimulation by 50% and 60%, respectively. This suggested that autocrine production of heparin-binding EGF receptor ligands, such as amphiregulin, contributed to growth stimulation. To directly test the importance of amphiregulin, a mixture of three monoclonal antibodies that neutralized amphiregulin activity was examined (open bars). These pooled antibodies inhibited IL-1α- and TNF-α-induced growth by 95% and 85%, respectively. As expected, the mixture of antibodies did not appreciably influence TGF-α-mediated proliferation. Thus, they were not simply cytotoxic. These experiments provide evidence that growth stimulation by IL-1α and TNF-α is mediated by an EGF receptor-dependent pathway and is dependent on autocrine stimulation by amphiregulin.

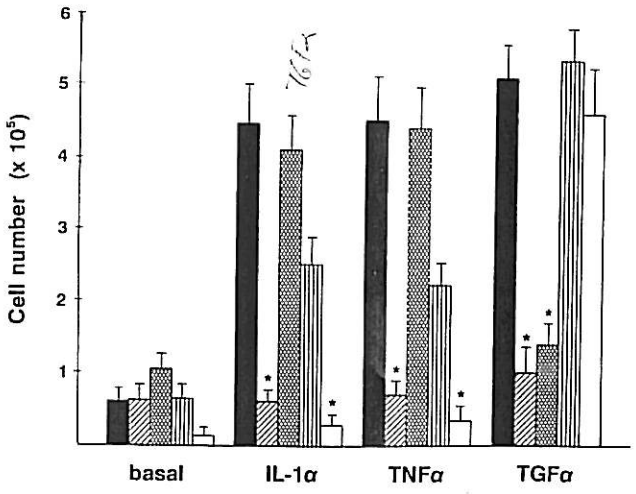

FIG. 5. Inhibition of IL-1α- and TNF-α-mediated proliferation. The HPV-16-immortalized cell line CX16-2 was maintained in MCDB153-LB lacking EGF and BPE (basal) or in the same medium supplemented with 0.1 nM either IL-1α or TNF-α or 0.01 nM TGF-α. Growth stimulation by each cytokine was examined in the absence (solid bars) or presence of specific inhibitors, including an antibody that blocks binding to the EGF receptor (hatched bars; 0.25 μg/ml), an antibody that neutralizes TGF-α biologic activity (cross-hatched bars; 1.0 μg/ml), heparan sulfate (vertically striped bars; 3 μg/ml), or a mixture of 3 antibodies that bind to amphiregulin and neutralize activity (open bars; each at 2.5 μg/ml). Results represent the mean of three to six experiments ± SE. Asterisks identify values that were significantly reduced relative to cells grown in the absence of inhibitors ($P \leq 0.01$; *t* test).

DISCUSSION

The proinflammatory cytokines, IL-1α and TNF-α, are secreted by macrophages infiltrating tissue in response to injury or infection (1, 2) and are released by cervical cells comprising the epithelium (3, 5). The current results demonstrate that these two cytokines have opposite effects on growth of normal vs. immortal cervical cells. Both IL-1α and TNF-α inhibited proliferation of cells derived from normal ectocervical or endocervical epithelia. In contrast, both cytokines significantly stimulated proliferation of most cervical cell lines that were immortalized with HPV-16 or -18 DNAs or derived from cervical carcinomas. These observations indicate that IL-1α and TNF-α are mitogenic for immortal and malignant cervical epithelial cells *in vitro* and suggest that chronic inflammation and release of cytokines might contribute to cervical carcinogenesis by providing a selective growth advantage to these cells *in vivo*. The latter is relevant because infection with multiple sexually transmitted agents has been reported to be a risk factor for progression to high-grade cervical neoplasia in women with HPV infection (8, 9).

Amphiregulin is a member of a large family of polypeptide growth factors that bind and activate the EGF receptor (reviewed in ref. 27). Amphiregulin was originally purified from MCF-7 breast carcinoma cells (28), but it has also been detected in normal tissues, including ovary, placenta, colon, and epidermis (18). Our results implicate amphiregulin as an important autocrine factor that mediates growth stimulation of HPV-immortalized cervical cells by IL-1α or TNF-α. This conclusion is based on several observations: (*i*) both IL-1α and TNF-α induced amphiregulin RNA expression in cultured cervical cells; (*ii*) recombinant amphiregulin stimulated growth of these cells as effectively as IL-1α or TNF-α; (*iii*) a monoclonal antibody that blocks EGF receptor signal transduction completely prevented growth stimulation by IL-1α or TNF-α; and (*iv*) a mixture of monoclonal antibodies that neutralize amphiregulin activity inhibited IL-1α- or TNF-α-mediated proliferation by 95% or 85%, respectively. Autocrine amphiregulin expression is important in supporting autonomous growth of cultured epidermal keratinocytes (18) as well as colon and breast carcinoma cell lines (29, 30). Furthermore, amphiregulin is often overexpressed in malignant colon or mammary tissue relative to the normal epithelia (31, 32), suggesting that altered regulation of this growth factor may contribute to malignant development. Our results demonstrate that two proinflammatory cytokines, IL-1α and TNF-α, stimulate proliferation of cervical cells via autocrine induction of amphiregulin.

These results demonstrate that transfection and immortalization of cervical epithelial cells with HPV-16 or -18 DNA induces sensitivity to growth stimulation by IL-1α and TNF-α.

However, growth stimulation by these cytokines is not limited to cells containing HPV DNA. Previous studies have shown that several proinflammatory cytokines (IL-1α, IL-6, or TNF-α) stimulate proliferation of carcinoma cell lines derived from several different tissues (24, 33, 34) including cervix (35). Thus, proinflammatory cytokines might act as paracrine or autocrine growth factors in promoting malignant progression. Our results extend these observations to nontumorigenic immortal cervical cells and, thus, provide evidence that these cytokines might contribute to an early stage of malignant development. The current results also demonstrate that the biological effects of IL-1α and TNF-α are influenced by the presence of other growth factors. TNF-α (1–5 nM) has previously been shown to inhibit growth of several HPV-containing epithelial cell lines *in vitro* (20, 22). Our experiments with similar media supplemented with growth factors (EGF or serum) are consistent with these reports and extend previous work by showing that IL-1α and TNF-α stimulate growth only in the absence of EGF. This is consistent with our observation that IL-1α and TNF-α induce proliferation via an EGF receptor-dependent pathway.

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