Interleukin-6 and Interleukin-6 Soluble Receptor Regulate Proliferation of Normal, Human Papillomavirus-Immortalized, and Carcinoma-Derived Cervical Cells *in Vitro*

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A variety of sexually transmitted diseases frequently accompany infection with human papillomavirus and stimulate inflammation of the cervical mucosa. Inflammation and cell injury cause release of proinflammatory cytokines, which in turn might regulate growth of human papillomavirus-infected cells. This study compared the interaction of the proinflammatory cytokine, interleukin-6 (IL-6), and its soluble receptor with normal ecto- and endocervical cells, human papillomavirus-immortalized ectocervical cells, and squamous carcinoma-derived cell lines. Proliferation of normal cervical cells was enhanced by IL-6 but inhibited by its soluble receptor. However, both IL-6 and its soluble receptor significantly stimulated growth of the three immortal and four cervical carcinoma-derived cell lines analyzed. Stimulation by IL-6 was dose dependent and was blocked by an antibody that neutralized IL-6 activity. IL-6-mediated proliferation was accompanied by increased expression of RNAs encoding transforming growth factor-α and amphiregulin, two epidermal growth factor receptor ligands. Furthermore, growth stimulation by IL-6 was significantly inhibited by antibodies that either blocked signal transduction by the epidermal growth factor receptor or that neutralized transforming growth factor-α or amphiregulin activity. Thus, IL-6 stimulates proliferation of human papillomavirus-immortalized cervical cells via an epidermal growth factor receptor-dependent pathway involving autocrine stimulation by transforming growth factor-α and amphiregulin. (Am J Pathol 1995, 146:944–952)

Interleukin 6 (IL-6) serves an important function in the host response to infection as it participates in regulation of the immune response,[1,2] hematopoiesis,[3,4] and the acute phase reaction.[5] IL-6 is expressed by a wide variety of different cell types, including keratinocytes of the uterine cervix.[6] IL-6 gene expression is stimulated by virus infection[7,8] and exposure to proinflammatory cytokines such as IL-1α or tumor necrosis factor-α (TNF-α).[8–11] Furthermore, the IL-6 gene contains regulatory elements that stimulate transcription in response to serum[12] and inhibit expression in the presence of glucocorticoids.[13]

As expected from the pleiotropic functions of IL-6, the receptor is expressed by various cells including lymphocytes, hepatocytes, and keratinocytes (reviewed by Taga and Kishimoto[14]). The IL-6 receptor is a heterodimeric membrane glycoprotein consisting of a low-affinity, ligand-binding peptide, IL-6R, and a signal-transducing peptide, gp130, which does not bind IL-6. Together these form a high affinity receptor. A soluble form of the ligand-binding domain, IL-6sR, is released from the cell surface by proteolytic cleavage of the membrane receptor.[15] IL-6sR has been observed in normal urine[16]; however, increased IL-6sR levels are detected in culture medium conditioned by the growth of a human myeloma cell line,[17] cellfree supernatants of phorbol myristate acetate-activated peripheral blood mononuclear cells,[18] and human immunodeficiency virus-seropositive blood donors,[18] suggesting a role for this receptor in the pathogenesis of these diseases.

A subset of human papillomaviruses (HPVs) are associated with the majority of cervical cancer.[19]

However, additional factors must contribute to pathogenesis because only a minority of HPV infections results in persistent lesions or progress to malignancy. Cervical cancer frequently develops in close association with chronic inflammation due to infection with a variety of other sexually transmitted agents. Furthermore, epidemiological studies suggest that the presence of multiple sexually transmitted diseases represents a risk factor for progression to high grade cervical malignancy in women with HPV infection.[20,21] One explanation is that cervicitis may cause tissue damage and release of proinflammatory cytokines that may in turn stimulate growth of HPV-immortalized epithelial cells.

The current study examines the production of and response to IL-6 and IL-6sR in cultures of human cervical epithelial cells. The response at different stages leading to malignancy was compared by examining normal cells, three nontumorigenic cell lines immortalized by transfection with HPV DNAs,[22] and four cell lines derived from cervical carcinomas. Results indicate that IL-6 stimulates growth of normal, immortal, and malignant cervical cells and that IL-6 mediated proliferation is dependent upon autocrine stimulation by two epidermal growth factor (EGF)-like growth factors, amphiregulin and transforming growth factor-α (TGF-α).

Material and Methods

Cell Culture

Cultures of human ectocervical and endocervical cells were established from tissues obtained after hysterectomy due to fibroids or endometriosis.[6] Fibroblast cultures were derived from cervical connective tissue according to methods of Pirisi.[23] The HPV-immortalized cell lines were obtained by transfection of normal primary cervical cell cultures with recombinant HPV-16 or -18 DNA, as described.[22] Both normal cells and HPV-immortalized cell lines were maintained in serum-free MCDB153-LB medium.[23] Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Cervical carcinoma-derived cell lines, SiHa, HT-3, and ME-180 (American Type Culture Collection, Rockville, MD) were maintained in a 50/50 mixture of Hams F12 nutrient medium/DMEM (F12/DMEM) containing 0.4 µg/ml hydrocortisone and 5% fetal bovine serum. The CXT-1 cervical carcinoma cell line was derived and maintained in MCDB153-LB medium (unpublished data).

Immunoassay for IL-6 and IL-6sR

Levels of IL-6 and IL-6sR in conditioned culture media were measured by enzyme-linked immunosorbent assays (R & D Systems, Minneapolis, MN). Secondary cultures of ectocervical epithelial cells, secondary and tertiary cultures of cervical stromal fibroblasts, HPV-immortalized cell lines, and the CXT-1 carcinoma-derived cell line were maintained in monolayer culture in the appropriate medium. Because IL-6 expression is positively regulated by serum[12] and negatively regulated by glucocorticoids,[13] assays were performed in basal medium lacking these factors. Basal media were MCDB153-LB lacking insulin, EGF, bovine pituitary extract (BPE), transferrin, hydrocortisone, triiodothyronine (for normal epithelial cells, HPV-immortalized cell lines, and the CXT-1 carcinoma line), and DMEM without serum (used for fibroblasts). When cultures grown in complete media became 60 to 90% confluent, they were washed once with phosphate-buffered saline and switched to basal media for 24 hours. After 24 hours, fresh basal media (5 ml) was added and cells were cultured for an additional 24 hours in the presence or absence of 1.0 nmol/L recombinant human IL-1α or TNFα (specific activities of 1.6×10^5 and 1.1×10^5 U/mg protein, respectively; R & D Systems). Samples of conditioned media were collected, supplemented with proteinase inhibitors, including 0.2 mmol/L 4-(2-aminoethyl) benzenesulfonylfluoride hydrochloride and 2 mg/ml leupeptin, and centrifuged for 10 minutes at $2000 \times g$ to remove cell debris. Aliquots of 0.5 ml were stored at −80 C for 24 to 48 hours before use.

Cell Growth Assay

Cells were seeded at clonal density (1×10^3 to 2×10^3 cells/well) in 6-well cluster dishes (Costar, Cambridge, MA) containing complete medium. After 18 hours cultures were rinsed with phosphate-buffered saline and shifted to basal media (as described above) for 48 hours to arrest cell growth. In some experiments basal media were supplemented with 10 µg/ml heparin sulfate (Sigma Chemical Co., St. Louis, MO) to facilitate growth arrest.[24] To measure growth stimulation, either recombinant human IL-6 (specific activity, 7×10^3 U/mg protein) or IL-6sR (both from R & D Systems) was added to either 2 ml of complete media or media lacking specific growth factors (MCDB153-LB lacking EGF and BPE or F12/DMEM lacking fetal bovine serum). Media were changed every 2 days and after 8 to 11 days the total number of cells was determined with a Coulter counter. Specificity of growth stimulation by IL-6 was assessed with

a goat polyclonal antibody that neutralized human IL-6 (R & D Systems). Normal goat immunoglobulin (Ig) G (R & D Systems) served as the negative control. In some experiments specific inhibitors of the EGF receptor signaling pathway were used including a mouse monoclonal antibody that blocks signaling by the human EGF receptor (clone LA-1, Upstate Biotechnology, Lake Placid, NY), a polyclonal goat antibody that neutralizes human TGF-α (R & D Systems), and a mixture of three mouse IgG, monoclonal antibodies that bind amphiregulin at different sites and neutralize biological activity (clones 4.14.18, 12.38.4, and 6RIC2.4; G. Plowman, unpublished results).

RNA Analyses

Monolayer cultures were washed in sterile phosphate-buffered saline, and cells were lysed in guanidine thiocyanate. RNA was purified by centrifugation through cesium trifluoroacetate (Pharmacia Biotech, Piscataway, NJ), and 10-µg samples of total cellular RNA were separated on 1.2% formaldehyde agarose gels and transferred to GeneScreen nylon membranes (DuPont NEN Research Products, Boston, MA). Filters were prehybridized for 2 hours at 42 C in Hybrisol I (Oncor, Gaithersburg, MD), containing 50% formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate SDS, 6X standard saline citrate SSC, and blocking agents, and hybridized for 20 hours in the same solution containing [^{32}P] dCTP-labeled randomly primed probes (specific activity, 1.2×10^9 cpm/µg DNA). Probes included cDNAs encoding human amphiregulin,[25] human TGF-α,[26] rat glyceraldehyde phosphate dehydrogenase,[27] and the complete HPV-16 genome. Filters were washed twice (30 minutes each) at room temperature in 2X SSC and 0.1% SDS, twice at 42 C in 0.1X SSC and 0.1% SDS, and then exposed to Kodak X OMAT AR film.

Results

Differential Production of IL-6 and IL-6sR

The current experiments were designed to quantitatively compare release of IL-6 and IL-6sR into the medium by cultures of human fibroblasts or ectocervical epithelial cells. Furthermore, the effect of immortalization with HPV on secretion was assessed. Both constitutive production of IL-6 and IL-6sR and their inducibility by two proinflammatory cytokines, IL-1α and TNF-α, were measured.

Cervical stromal fibroblasts maintained in basal DMEM constitutively secreted into the culture medium significant amounts of IL-6 (Figure 1A). In contrast, ectocervical epithelial cells secreted lower levels, and production was detectable in only three of five samples. This difference was statistically significant ($P \leq 0.05$, *t*-test). Differences in production of IL-6 by fibroblasts and epithelial cells were enhanced when cells were stimulated by either IL1α or TNF-α, two proinflammatory cytokines that induce IL-6 expression.[8–11] Fibroblasts grown in medium supplemented with IL-1α or TNF-α secreted 2150 and 1200 pg/10^6 cells/24 hours IL-6, respectively (an increase of 200- and 120-fold over base line secretion). IL-1α and TNF-α also enhanced secretion of IL-6 by cultures of epithelial cells although the increase (9- and 1.5-fold, respectively) was significantly less than observed with fibroblasts.

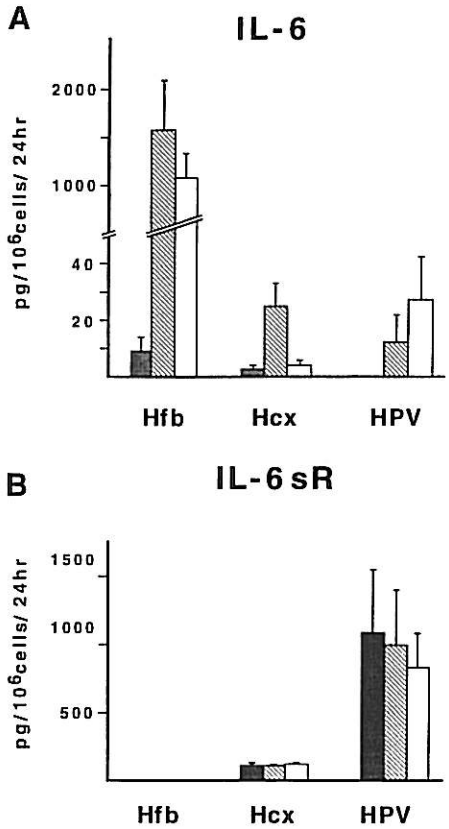

Figure 1. *Enzyme-linked immunosorbent assay analyses of IL-6* (A) *and IL-6sR* (B) *secretion by monolayer cultures of human cervical cells. Human fibroblasts (Hfb), normal ectocervical epithelial cells (Hcx) and HPV-immortalized cell lines (HPV) were maintained in basal medium (cross-batched bars) or were stimulated by addition of 1 nmol/L IL-1α (diagonally striped bars) or 1 nmol/L TNF-α (open bars). Values represent the mean ± standard error of three to five different experiments, each done with cultures of fibroblasts or epithelial cells derived from different individuals. The means for immortal lines represent the average of three different cell lines, each examined in three different experiments.*

Infection with HPV may activate IL-6 gene expression in keratinocytes.[7] To determine whether immortalization and expression of HPV genes stimulated IL-6 secretion, three cervical cell lines immortalized

by recombinant HPV-16 (CX16-2, CX16-10) or HPV-18 (CX18-1) DNAs were examined. Constitutive release of IL-6 was not detected in the conditioned medium in any of these cell lines (Figure 1A). However, all cell lines released IL-6 after stimulation with IL-1α or TNF-α. A cervical carcinoma-derived cell line CXT-1, containing integrated HPV-16 DNA, also secreted IL-6 after treatment with IL-1α or TNF-α; levels were similar to the immortal cell lines CX16-10 and CX18-1 (data not shown).

The presence of the soluble form of the IL-6 receptor in culture supernatants was also analyzed (Figure 1B). Normal ectocervical cells produced IL-6sR; however, the three different HPV-immortalized cell lines released significantly greater amounts. The tumor line CXT-1 also released high levels of IL-6sR (1950 pg/10^6 cells/24 hours). In contrast, IL-6sR was not detected in conditioned medium from fibroblast cultures (Figure 1B). Furthermore, neither IL-1α nor TNF-α augmented production of IL-6sR in any cell type examined (Figure 1B). These results demonstrate that cultures of fibroblasts, cervical epithelial cells, and HPV-immortalized cells differed significantly in production of IL-6 and IL-6sR.

Effects of IL-6 and IL-6sR on Cell Proliferation

The ability of IL-6 and IL-6sR to stimulate cell proliferation was evaluated with epithelial cells derived from normal ecto- or endocervix, three cell lines immortalized with HPV DNA, and four cervical carcinoma-derived cell lines. The effects of IL-6 and IL-6sR on cell growth were examined in both complete culture medium supplemented with growth factors and in medium lacking specific factors (MCDB153-LB lacking EGF and BPE or F12/DMEM without serum). When cervical cells were maintained in complete medium they grew rapidly, and neither IL-6 nor IL-6sR significantly stimulated or inhibited cell proliferation in any line (data not shown). In contrast, when cells were maintained in medium lacking exogenous growth factors, IL-6 significantly stimulated proliferation of each of the normal, immortal, and carcinoma-derived cell cultures (Figure 2A). However, growth stimulation was consistently higher in the immortal cell lines than in normal epithelial cells (2- to 4.5-fold). IL-6 also enhanced growth significantly in three of four carcinoma cell lines compared with normal ectocervical cells ($P \leq 0.05$, *t*-test).

In contrast to IL-6, which stimulated growth of all epithelial cultures, IL-6sR had a differential effect on the normal *versus* the immortal or malignant cells. IL-

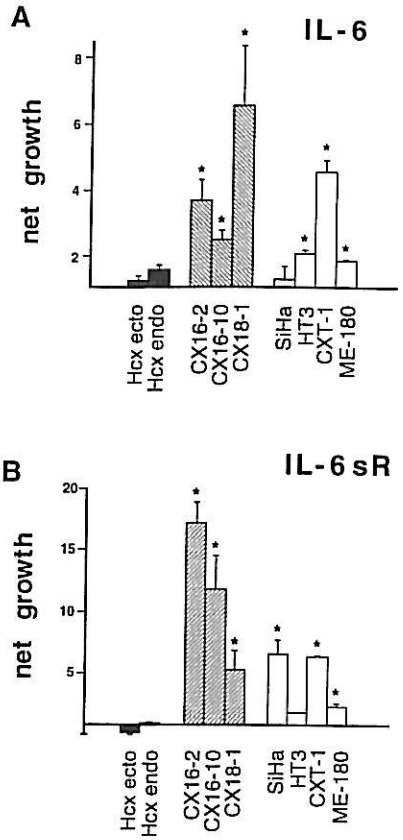

Figure 2. *Regulation of cell proliferation by IL-6* (A) *and IL-6sR* (B). *Normal ectocervical (Hcx ecto) and endocervical (Hcx endo) epithelial cells (cross-hatched bars), three HPV-immortalized cervical cell lines (diagonally striped bars), and four carcinoma-derived lines (open bars) were treated with IL-6 (0.1 nmol/L) or IL-6sR (1 nmol/L) for 8 or 11 days, respectively, as described (see Materials and Methods). IL-6sR assays were slightly longer as growth stimulation was slightly less. Net growth represents cell number in the presence of each factor divided by cell number in its absence. Values represent the mean of three to five independent experiments ± standard error. Asterisks indicate values that were significantly different from normal ectocervical cell cultures ($P \leq 0.05$, t-test).*

6sR promoted growth of immortalized and carcinoma-derived cell lines (Figure 2B); however, it consistently failed to stimulate cultures derived from normal ectocervical or endocervical epithelia (Figure 2B). In fact, growth inhibition was observed in four of five strains of normal ectocervical cells analyzed. Thus, growth stimulation by IL-6 and IL6sR was a common response in several HPV-immortalized and cervical carcinoma cell lines.

The CX16-2 immortal line was particularly sensitive to growth stimulation by both factors; therefore, subsequent work focused on this cell line. CX16-2 cells maintained in medium lacking exogenous growth factors formed small, compact colonies that grew slowly and progressively (Figure 3A). Treatment with IL-6 caused cells to become rounded (Figure 3B) and to divide rapidly. In addition, IL-6 induced motility resulting in formation of large diffuse colonies that filled

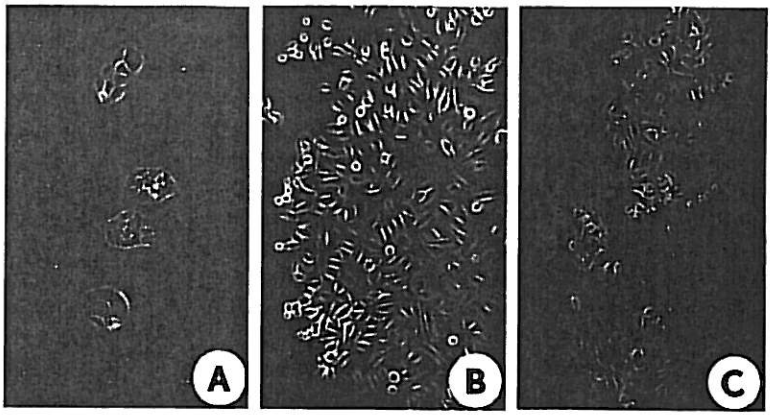

Figure 3. *Phase-contrast microscopy of a HPV-immortalized ectocervical cell line (CX16-2) maintained in the absence of growth factors (A) or in the presence of 0.1 nmol/L IL-6 (B) or 1.0 nmol/L IL-6sR (C) for 5 days.*

the 100-mm culture dish after 3 to 5 days. IL-6sR also stimulated proliferation of HPV-immortalized cells. Approximately 50% of colonies grew progressively after treatment with IL-6sR for 5 days (Figure 3C). However, after 10 days all cells were dividing.

Dose-Response Analysis and Specificity of IL-6-Mediated Proliferation

To determine whether growth stimulation by IL-6 and IL-6sR was dose dependent and whether the response was specific, additional studies were undertaken. Both IL-6 and IL-6sR stimulated growth of CX16-2 cells in a dose-dependent manner (Figure 4). Both factors induced growth at a concentration as low as 0.03 nmol/L. For comparison, TGF-α, a potent mitogen for cervical cells, stimulated proliferation at 0.001 nmol/L.

To confirm that growth stimulation by IL-6 was a specific response and not due to contaminating proteins, growth assays were performed in the presence of a specific antibody that neutralized IL-6 activity. Growth stimulation by IL-6 was completely blocked by a neutralizing goat polyclonal antibody to human IL-6 (Figure 5). Inhibition was dose dependent (data not shown) and maximal at the highest concentration of antibody used, 3 µg/ml. In addition, growth inhibition was not observed when purified goat anti-human IgG was added to the medium, confirming the specificity of IL-6 (Figure 5).

Mechanism of Cell Growth Stimulation by IL-6

When cervical cells were cultured in complete medium, they grew rapidly, and addition of IL-6 did not enhance proliferation (data not shown). Growth stimulation by IL-6 was observed only in medium lacking

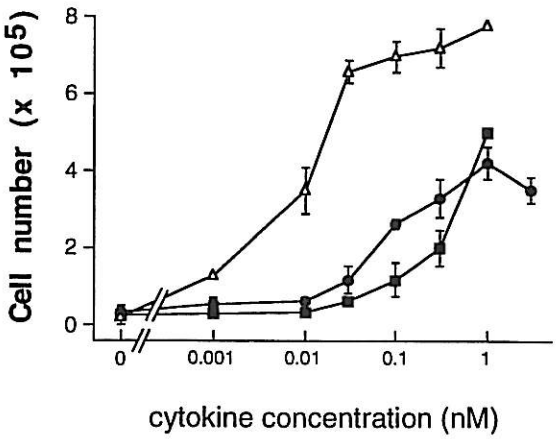

Figure 4. *Dose-response analyses of IL-6 and IL-6sR on cell proliferation. The HPV-immortalized cell line, CX16-2, was maintained in MCDB153-LB medium lacking EGF and BPE in the presence of the indicated concentrations of IL-6 (●), IL-6sR (■), or TGF-α (▽) as positive control. Each point represents the mean of three independent experiments ± standard error.*

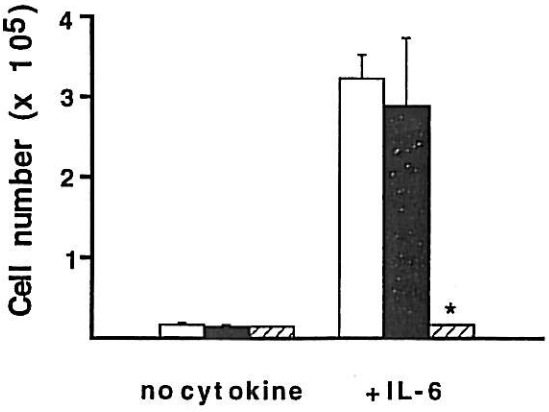

Figure 5. *Inhibition of IL-6-mediated growth by neutralizing antibodies to IL-6. The HPV-immortalized cell line, CX16-2, was cultured in MCDB153-LB medium lacking EGF and BPE. Cells were maintained in medium without antibodies (open bars), in the presence of normal IgG (3 µg/ml; black bars), or with goat polyclonal anti-human IL-6 IgG (3 µg/ml; diagonally striped bars). Values represent the mean of three independent experiments ± standard error. The asterisk identifies a value that was significantly reduced relative to cells grown in the absence of antibodies ($P \leq 0.005$, t-test).*

EGF. Therefore, studies were undertaken to examine whether IL-6 stimulated proliferation by increasing expression of specific EGF-like growth factors. Northern analyses indicated that the HPV-16-immortalized cell line, CX16-2, expressed low levels of RNAs encoding two EGF receptor ligands, TGF-α and amphiregulin (Figure 6). When CX16-2 cells were incubated with IL-6 for various intervals (2, 4, 8, 24, 48, and 72 hours), an increase in TGF-α RNA was first detected after 24 hours. Furthermore, induction of TGF-α occurred before increased cell division, which was observed between 48 and 72 hours (data not shown). In contrast, induction of amphiregulin RNA was first detected at 72 hours (Figure 6). IL-6 did not alter expression of RNAs encoding the HPV-16 E6 and E7 oncoproteins (data not shown). Hybridization to a housekeeping gene, glyceraldehyde phosphate dehydrogenase, indicated that each lane contained equivalent amounts of RNA. These results suggest that induction of TGF-α and amphiregulin contributed either directly or indirectly to growth stimulation by IL-6.

To test whether autocrine production of TGF-α or amphiregulin directly contributed to mitogenic stimulation by IL-6, studies were undertaken with specific inhibitors of the EGF receptor signaling pathway (Figure 7). CX16-2 cells were treated with IL-6 for 10 days in the presence or absence of specific inhibitors including 1), an anti-EGF receptor monoclonal antibody that prevents signal transduction by the receptor,[28]; 2), a polyclonal antibody that neutralizes activity of TGF-α; 3), heparin sulfate, a sulfated glycosaminoglycan that binds to specific EGF receptor ligands, including amphiregulin, and inhibits mitogenic activity[24]; and 4), a mixture of three monoclonal antibodies, each known to bind to amphiregulin at distinct sites resulting in neutralization of biological activity (G. Plowman, unpublished results). In three independent experiments, growth stimulation by IL-6 was significantly inhibited by the anti-EGF receptor antibody (bars with horizontal stripes). This indicates that IL-6 stimulates growth of the immortalized cells indirectly through activation of the EGF receptor. Furthermore, neutralizing antibodies to TGF-α inhibited IL-6 mitogenesis by approximately 35% (black bars). These antibodies completely inhibited growth stimulation by exogenous TGF-α, indicating their effectiveness. In contrast, cell growth due to IL-6 was dramatically inhibited after treatment with heparin sulfate (1 µg/ml; open bars). This suggests that autocrine production of heparin-binding EGF receptor ligands such as amphiregulin represented an important factor in IL-6-stimulated growth. To directly test this hypothesis, growth assays were performed in the presence of neutralizing antibodies to amphiregulin. These antibodies inhibited IL-6-induced growth by 95% (spotted bars). However, they did not influence TGF-α-induced growth, indicating that they were not simply cytotoxic. These results provide direct evidence that IL-6-stimulated growth is mediated via an EGF receptor-dependent pathway that involves autocrine stimulation by both TGF-α and amphiregulin.

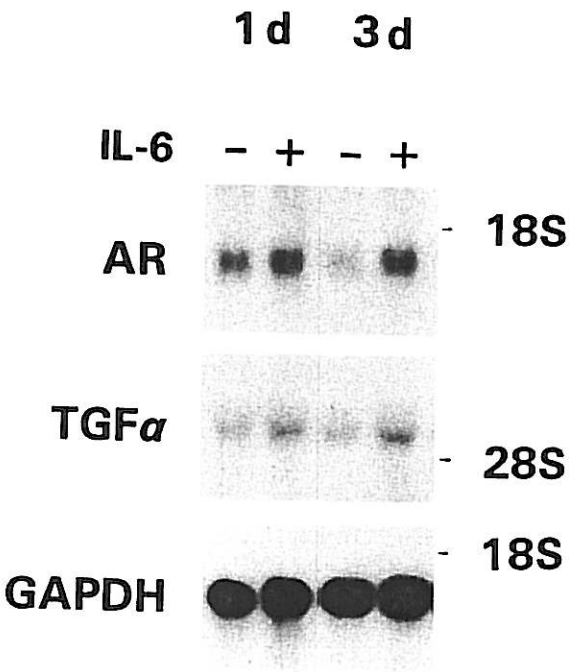

Figure 6. *Northern analysis of immortal cervical cells treated with IL-6. A HPV-immortalized line (CX16-2) was maintained in MCDB153-LB medium lacking EGF and BPE either in the absence (−) or presence (+) of 1 nmol/L IL-6 for 24 or 72 hours. Positions of the 28 and 18S ribosomal RNAs are shown at the right. AR, amphiregulin; GAPDH, glyceraldehyde phosphate dehydrogenase.*

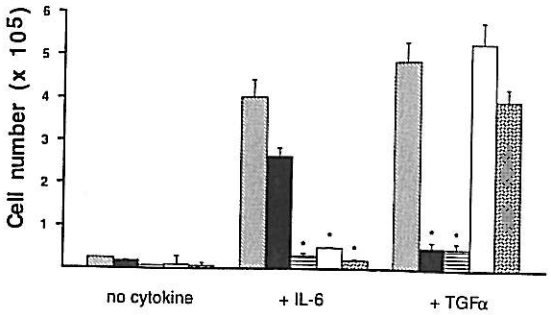

Figure 7. *Inhibition of IL-6- or TGF-α-induced mitogenesis. The HPV-immortalized cell line, CX16-2, was maintained in MCDB153-LB medium lacking EGF and BPE in the absence (diagonally striped bars) or presence of specific inhibitors, including 1 µg/ml of a polyclonal neutralizing antibody to TGF-α (black bars), 1 µg/ml of a monoclonal antibody preventing signal transduction by the EGF receptor (horizontal stripes), 1 µg/ml heparin sulfate (open bars), 3 µg/ml of a mixture of three monoclonal antibodies that neutralize amphiregulin activity (spotted bars). Results represent the mean of three independent experiments ± standard error. Asterisks indicate values that were significantly reduced compared with cells incubated in the absence of inhibitors ($P \leq 0.01$, t-test).*

Discussion

The epithelium of the uterine cervix is exposed to a variety of infectious agents that cause tissue damage and inflammation. The present studies demonstrate that IL-6, an important mediator of the inflammatory response, is secreted by both epithelial cells and fibroblasts isolated from the cervical mucosa. Furthermore, IL-6 stimulates proliferation of cells derived from normal cervical epithelia, cell lines immortalized with HPV-16 or HPV-18 DNAs, and lines derived from cervical carcinomas. Previous studies have shown that IL-6 enhances proliferation of carcinoma lines derived from several different organs,[29–31] including the cervix.[32] The present results extend these observations to normal cervical epithelial cells and nontumorigenic HPV-immortalized cell lines. Thus, *in vitro* experiments demonstrate that IL-6 is mitogenic for cervical cells at different stages leading to malignancy and thus might promote growth *in vivo* when produced in response to inflammation or tissue damage. The relevance of this fact is that infection with multiple sexually transmitted agents stimulates cervicitis and may represent a risk factor for progression to high grade neoplasia in women with HPV infection.[20,21]

In contrast to IL-6, IL-6sR regulated cell growth differentially in normal, compared with abnormal, cervical cells. IL-6sR significantly stimulated proliferation of three HPV-immortalized cell lines and four carcinoma-derived cell lines but inhibited epithelial cells derived from normal ectocervix. IL-6sR was also released in significantly greater amounts (ninefold) by HPV-immortalized and carcinoma-derived lines than by normal cells. Therefore, increased production of and responsiveness to IL-6sR is a property of both premalignant HPV-immortalized cells and carcinoma lines, implying that IL-6sR provides a selective growth advantage for these cells. Although soluble forms of murine IL-6R potentiate IL-6 activity,[15,33] the present results are the first to demonstrate that IL-6sR stimulates proliferation of HPV-immortalized cervical cells in the absence of detectable IL-6.

Amphiregulin and TGF-α are members of a family of polypeptide growth factors that bind to and activate the EGF receptor. TGF-α secreted by keratinocytes stimulates cell proliferation.[34–36] Amphiregulin is produced by normal colon and epidermal epithelia[24,25] and is often overexpressed in carcinomas of the breast and colon[37,38] or in human mammary cell lines transformed by an activated *ras* gene.[39] Amphiregulin is a major autocrine growth factor for keratinocytes.[40] The current results provide direct evidence that mitogenic stimulation by IL-6 is mediated by an EGF receptor-dependent pathway involving autocrine stimulation by TGF-α and amphiregulin. First, the mitogenic effect of IL-6 on HPV-immortalized cervical cells is inhibited by a monoclonal antibody that blocks EGF receptor signal transduction.[28] This is consistent with our observations and those obtained with epidermal keratinocytes[41,42] that growth stimulation by IL-6 is apparent only in the absence of exogenous EGF. Secondly, IL-6 stimulates expression of both TGF-α and amphiregulin RNAs. Most importantly, monoclonal antibodies that neutralized the activity of either TGF-α or amphiregulin inhibited IL-6-mediated growth stimulation. Of particular interest, antibodies to amphiregulin inhibited mitogenesis by 95% even though IL-6 stimulated amphiregulin RNA only after 3 days. In contrast IL-6 stimulated TGF-α RNA before increased cell division (as early as 24 hours); however, a neutralizing antibody to TGF-α only partially inhibited proliferation. Whether IL-6 alters regulation of amphiregulin expression by additional post-transcriptional or translational mechanisms that might result in accumulation of the growth factor before mitogenesis is unclear.

Cervical fibroblasts constitutively secreted IL-6 in significantly greater amounts than normal ectocervical epithelial cells. This difference was enhanced after stimulation of IL-6 expression by two proinflammatory cytokines, IL-1α or TNF-α. These observations suggest that fibroblasts are a significant source of IL-6 production in the cervix and that they stimulate epithelial cells in a paracrine manner. Constitutive production of IL-6 was not detected in cervical cells immortalized with HPV-16 or HPV-18 DNAs or in the CXT-1 tumor line. These results are in contrast to other reports that cervical carcinoma-derived cell lines[6,32] and a HPV-harboring line derived from vulvar bowenoid papules[7] constitutively secrete IL-6. One possible explanation for these differences is that constitutive IL-6 expression was induced by serum in the culture medium. The current experiments were performed in serum-free medium. Another possibility is that the HPV-immortalized cells and CXT-1 cell line used in the current experiments produced abundant IL-6sR that might interact with secreted IL-6 and interfere with the enzyme-linked immunosorbent assay. The mechanism responsible for shedding of the IL-6sR as well as the biological function of these soluble receptors is unclear. However, overexpression of IL-6sR renders cells more susceptible to mitogenic stimulation by IL-6.[43] Our results indicate that high levels of IL-6sR are released by HPV-immortalized and carcinoma-derived cervical cells, suggesting that IL-6sR may be a marker associated with an early stage of malignant development. Previous studies

have suggested that increased production of IL-6sR is a marker for myeloma and myelomonocytic cells.[17]

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References

1. Smyth MJ, Ortaldo JR, Bere W, Yagita H, Okumura K, Young HA: IL-2 and IL-6 synergize to augment the pore-forming protein gene expression and cytotoxic potential of human peripheral blood T cells. J Immunol 1990, 145:1159–1166
2. Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, Kashiwamura S, Nakajima K, Koyama K, Iwamatu A, Tsunasawa S, Sakiyama F, Matsui H, Takahara Y, Taniguchi T, Kishimoto T: Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature 1986, 324:73–76
3. Bruno E, Hoffman R: Effect of interleukin 6 on *in vitro* human megakaryocytopoiesis: its interaction with other cytokines. Exp Hematol 1989, 17:1038–1043
4. Ogawa M, Clark SC: Synergistic interaction between interleukin-6 and interleukin-3 in support of stem cell proliferation in culture. Blood 1988, 14:329–337
5. Baumann H, Isseroff H, Latimer JJ, Jahreis GP: Phorbol ester modulates interleukin-6 and interleukin-1 regulated expression of acute phase plasma proteins in hepatoma cells. J Biol Chem 1989, 265:17390–17396
6. Woodworth CD, Simpson S: Comparative lymphokine secretion by cultured normal human cervical keratinocytes, papillomavirus-immortalized, and carcinoma cell lines. Am J Pathol 1993, 142:1544–1555
7. Malejczyk J, Malejczyk M, Urbanski A, Köck A, Jablonska S, Orth G, Luger TA: Constitutive release of IL-6 by human papillomavirus type 16 (HPV16)-harboring keratinocytes: a mechanism augmenting the NK-cell-mediated lysis of HPV-bearing neoplastic cells. Cell Immunol 1991, 136:155–164
8. Partridge M, Chantry D, Turner M, Feldmann M: Production of interleukin-1 and interleukin-6 by human keratinocytes and squamous cell carcinoma cell lines. J Invest Dermatol 1991, 96:771–776
9. Van Damme J, Opdenekker G: Interaction of interferons with skin reactive cytokines: from interleukin-1 to interleukin-8. J Invest Dermatol 1990, 95:90S–93S
10. Yoshizaki K, Nishimoto N, Matsumoto K, Tagoh H, Taga T, Deguchi Y, Kuritani T, Hirano T, Hashimoto K, Okada N, Kishimoto T: Interleukin-6 and expression of its receptor on epidermal keratinocytes. Cytokine 1990, 2:381–387
11. Zhang Y, Lin J-X, Vilček J: Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a κB-like sequence. Mol Cell Biol 1990, 10:3818–3823
12. Ray A, Sassone-Corsi P, Sehgal PB: A multiple cytokine and second messenger-responsive element in the enhancer of the human interleukin-6 gene: similarities with c-*fos* gene regulation. Mol Cell Biol 1989, 9:5537–5547
13. Zander B, Walz G, Wieder KJ, Strom TB: Evidence that glucocorticoids block expression of the human interleukin-6 gene by accessory cells. Transplantation 1990, 49:183–185
14. Taga T, Kishimoto T: IL-6 receptor. Cellular and Molecular Mechanisms of Inflammation. Edited by CG Cochrane and MA Gimbrone. New York, Academic Press, 1990, pp 219–243
15. Müllberg J, Schooltink H, Stoyan T, Günther M, Graeve L, Buse G, Mackiewicz A, Heinrich PC, Rose-John S: The soluble interleukin-6 receptor is generated by shedding. Eur J Immunol 1993, 23:473–480
16. Novik D, Engelmann H, Wallach D, Rubinstein M: Soluble cytokine receptors are present in normal human urine. J Exp Med 1989, 170:1409–1414
17. Nakajima T, Yamamoto S, Cheng M, Yasukawa K, Hirano T, Kishimoto T, Tokunaga T, Honda M: Soluble interleukin-6 receptor is released from receptor-bearing cell lines *in vitro*. Jpn J Cancer Res 1992, 83: 373–378
18. Honda M, Yamamoto S, Cheng M, Yasukawa K, Suzuki H, Saito T, Osugi Y, Tokunaga T, Kishimoto T: Human soluble IL-6 receptor: its detection and enhanced release by HIV infection. J Immunol 1992, 148:2175–2180
19. Lowy DR, Kirnbauer R, Schiller JT: Genital human papillomavirus infection. Proc Natl Acad Sci USA 1994, 91:2436–2440
20. Koutsky LA, Holmes KK, Critchlow CW, Stevens CE, Paavonen J, Beckmann AM, DeRoven TA, Galloway DA, Vernon D, Kiviat NB: A cohort study of the risk of cervical intraepithelial neoplasia grade 2 or 3 in relation to papillomavirus infection. N Engl J Med 1992, 327:1272–1278
21. Schmauz R, Okona P, de Villiers E-M, Dennin R, Brade L, Lwanga SK, Owor R: Multiple interactions in cases of cervical cancer from a high-incidence area in tropical Africa. Int J Cancer 1989, 43:805–809
22. Woodworth CD, Bowden PE, Doniger J, Pirisi L, Barnes W, Lancaster WD, DiPaolo JA: Characterization of normal human exocervical epithelial cells immortalized *in vitro* by papillomavirus types 16 and 18 DNA. Cancer Res 1988, 48:4620–4628
23. Pirisi L, Yasumoto S, Feller M, Doniger J, DiPaolo JA:

Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. J Virol 1987, 61:1061–1066

24. Cook PW, Mattox PA, Keeble WW, Pittelkow MR, Plowman GD, Shoyab M, Adelman JP, Shipley GD: A heparin sulfate-regulated human keratinocyte autocrine factor is similar or identical to amphiregulin. Mol Cell Biol 1991, 11:2547–2557
25. Plowman GD, Green JM, McDonald VL, Neubauer MG, Disteche CM, Todaro GJ, Shoyab M: The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity. Mol Cell Biol 1990, 10:1969–1981
26. Rosenthal A, Lindquist PB, Bringman TS, Goeddel DV, Derynck R: Expression in rat fibroblasts of a human transforming growth factor-α cDNA results in transformation. Cell 1986, 46:301–309
27. Piechaczyk M, Blanchard JM, Marty L, Dani CH, Panabrieres F, El Sabouty S, Fort P, Jeanteur P: Post-transcriptional regulation of glyceraldehyde-3-phosphate-dehydrogenase gene expression in rat tissues. Nucleic Acids Res 1984, 13:6951–6963
28. Sato JD, Kawamoto T, Le AD, Mendelsohn J, Polikoff J, Sato GH: Biological effects *in vitro* of monoclonal antibodies to human epidermal growth factor receptors. Mol Biol Med 1983, 1:511–529
29. Lahm H, Petral-Malec D, Yilmaz-Ceyhan A, Fischer JR, Lorenzoni M, Givel JC, Odartchenko N: Growth stimulation of a human colorectal carcinoma cell line by interleukin-1 and -6 and antagonistic effects of transforming growth factor β1. Eur J Cancer 1992, 28A:1894–1899
30. Miki S, Iwano M, Miki Y, Yamamoto M, Tang B, Yokokawa K, Sonoda T, Hirano T, Kishimoto T: Interleukin-6 (IL-6) functions as an *in vitro* autocrine growth factor for renal cell carcinomas. FEBS Lett 1989, 250:607–610
31. Klein B, Zhang X-G, Jourdan M, Content J, Houssiau F, Aarden L, Piechaczyk M, Bataille R: Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. Blood 1989, 73:517–526
32. Eustace D, Han X, Gooding R, Rowbottom A, Riches P, Heyderman E: Interleukin-6 (IL-6) functions as an autocrine growth factor in cervical carcinomas *in vitro*. Gynecol Oncol 1993, 50:15–19
33. Saito T, Yasukawa K, Suzuki H, Futatsugi K, Fukunaga T, Yokomizo C, Koishihara Y, Fukui H, Ohsugi Y, Yawata H, Kobayashi I, Hirano T, Taga T, Kishimoto T: Preparation of a soluble murine IL-6 receptor and anti-murine IL-6 receptor antibodies. J Immunol 1991, 147: 168–173
34. Coffey RJ Jr, Derynck R, Wilcox JN, Bringman TS, Groustin AS, Moses HL, Pittelkow MR: Production and autoinduction of transforming growth factor-α in human keratinocytes. Nature 1987, 328:817–820
35. Barrandon Y, Green H: Cell migration is essential for sustained growth of keratinocytes colonies: the roles of transforming growth factor-α and epidermal growth factor. Cell 1987, 50:1131–1137
36. Derynck R: Transforming growth factor α. Cell 1988, 54:593–595
37. Ciardiello F, Kim N, Saeki T, Dono R, Persico MG, Plowman GD, Garrigues J, Radke S, Todaro J, Salomon DS: Differential expression of epidermal growth factor-related proteins in human colorectal tumors. Proc Natl Acad Sci USA 1991, 88:7792–7796
38. LeJeune S, Leek R, Horak E, Plowman GD, Greenall M, Harris AL: Amphiregulin, epidermal growth factor receptor, and estrogen receptor expression in human primary breast cancer. Cancer Res 1993, 53:3597–3602
39. Normanno N, Selvam MP, Qi C-F, Saeki T, Johnson G, Kim N, Ciardello F, Shoyab M, Plowman G, Brandt R, Todaro G, Salomon DS: Amphiregulin as an autocrine growth factor for c-Ha-*ras*- and c-*erb*B-2-transformed human mammary epithelial cells. Proc Natl Acad Sci USA 1994, 91:2790–2794
40. Piepkorn M, Lo C, Plowman GD: Amphiregulin-dependent proliferation of cultured human keratinocytes: autocrine growth, the effects of exogenous recombinant cytokine, and apparent requirement for heparin-like glycosaminoglycans. J Cell Physiol 1994, 159:114–120
41. Grossman MM, Krueger J, Yourish D, Granelli-Piperno A, Murphy DP, May LT, Kupper TS, Sehgal PB, Gottlieb AB: Interleukin 6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. Proc Natl Acad Sci USA 1989, 86:6367–6371
42. Krueger JG, Krane JF, Carler DM, Gottlieb AB: Role of growth factors, cytokines, and their receptors in the pathogenesis of psoriasis. J Invest Dermatol 1990, 94-(suppl):135S–140S
43. Hirano T, Kishimoto T: Interleukin-6 (IL-6). Handbook Exp Pharmacol 1991, 95:633–665