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Loss of IL-6 Receptor Expression in Cervical Carcinoma Cells Inhibits Autocrine IL-6 Stimulation: Abrogation of Constitutive Monocyte Chemoattractant Protein-1 Production

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IL-6 is synthesized in human papilloma virus (HPV)-transformed cervical carcinoma cell lines and is supposed to stimulate these cells in an autocrine manner. We studied IL-6 production and responsiveness in nonmalignant HPV-transformed keratinocytes and cervical carcinoma cells in detail. IL-6 was detected in cervical carcinomas in situ. Correspondingly, HPV-positive carcinoma cell lines expressed high IL-6 levels. However, these carcinoma cell lines showed low responsiveness to IL-6 as revealed by low constitutive STAT3 binding activity, which was not further enhanced by exogenous IL-6. In contrast, in vitro-transformed nonmalignant keratinocytes without endogenous IL-6 production strongly responded to exogenous IL-6 with activation of STAT3. STAT3 protein expression levels were comparable in both responsive and nonresponsive cell lines. Also, gp130, the upstream signal-transducing receptor subunit conveying IL-6 signals into the cell, was expressed in all tested cell lines. However, the IL-6 binding subunit gp80 was lost in the malignant cells. Addition of soluble gp80 was sufficient to restore IL-6 responsiveness in carcinoma cells as shown by enhanced activation of STAT3 binding activity. As a consequence of the restored IL-6 responsiveness, carcinoma cells strongly produced the chemokine monocyte chemoattractant protein-1 (MCP-1). Our data demonstrate that cervical carcinoma cells producing high amounts of IL-6 only weakly respond to IL-6 in an autocrine manner due to limited gp80 expression. While production of IL-6 might contribute to a local immunosuppressive effect, silencing an autocrine IL-6 response prevents constitutive production of the mononuclear cell-attracting chemokine MCP-1. Both mechanisms might help the tumor to escape the immune system. The Journal of Immunology, 2000, 165: 1939–1948.

Malignant progression of tumors is characterized by unbalanced growth, gain of invasive potential and metastasis, and the acquisition of mechanisms to escape the immune system. These phenotypic changes involve dysregulation on different levels, e.g., by directly affecting the cell cycle, signal-transduction mechanisms, production of soluble mediators, or altered functions of cell surface receptors.

Premalignant lesions of the cervix uteri and cervical carcinomas, in particular, harbor human papillomaviruses (HPV) in up to 95% of all investigated cases. Either as a consequence of the HPV infection or independently thereof, a variety of cellular changes have been described in cervical carcinomas. Thus, malignant progression is accompanied by the up-regulation of the anti-apoptotic factor bcl-2 (1). Moreover, cellular receptors and cytokines are up-regulated, including the receptor CD40 (2), the epidermal growth factor receptor (3), different growth factors, like endothelin-1 (4) or insulin-like growth factor-1 (5), and the cytokine IL-6 (6, 7).

IL-6 has been characterized as a multifunctional member of the cytokine family. It is synthesized by a variety of cells upon stimulation and acts on a wide range of different target cells to regulate cell growth, differentiation, and gene expression (for review see Refs. 8–10). This includes up-regulation of the monocyte chemoattractant protein-1 (MCP-1) (11–13). IL-6 is inducible by appropriate stimuli such as IL-1, TNF, IFN-γ, IL-4 (14–17), and CD40 (18–20) or by viral and bacterial infections (21). In multiple myeloma (22) and a number of epithelial tumor cell lines, including renal cell carcinoma, bladder cell carcinoma, and cervical carcinoma cells, IL-6 was found to be constitutively expressed (6, 7, 23, 24). Dependent on the target cell, IL-6 may act as a positive or negative regulator of cell growth. While it inhibits the growth of breast carcinoma cell lines, melanocytes, and certain B cell lymphomas (25, 26), it is an important regulator of growth and survival of multiple myeloma cells (22, 27). Moreover, it is supposed to promote the growth of normal and EBV-transformed B cells, normal keratinocytes, mesangial cells (14, 28–30) and also of renal, bladder, and cervical carcinoma cells (7, 23, 24, 31). In contrast, IL-6 may also act as a potent antiinflammatory and immunosuppressive cytokine (reviewed in Tilg et al.; Ref. 32). Thus, IL-6 blocks TNF and IL-1 production (33, 34) and induces IL-1 receptor antagonist in macrophages (32) and inhibits the degradation of the extracellular matrix (35).
Signaling of IL-6 involves binding of the cytokine to the IL-6R (gp80). Both molecules form a binary complex that then associates with gp130 and induces its dimerization (36, 37). Of note, a functional IL-6R complex is formed even when gp80 is lacking its transmembrane and intracellular domains, as it is found in the naturally occurring soluble form of gp80 (sgp80). Thus, cell types with low or lacking surface expression of gp80 may signal through gp130 if they are stimulated with IL-6 in the presence of sgp80 (12, 38). In a sgp80 transgenic model, it was shown that in fact sgp80 strongly sensitized the mice for IL-6 effects and significantly prolonged the plasma half-life of IL-6 in vivo (39). Gp130 is shared by a number of cytokines as a signal-transducing receptor component. It does not bind IL-6 by itself. Dimerization of gp130 is followed by the rapid activation of tyrosine kinases of the Janus kinase (Jak) family and subsequent activation of transcription factors of the STAT family, STAT3 and STAT1 (40–44). STAT factors participate in transcriptional regulation of genes comprising STAT-specific binding sites, e.g., tissue inhibitor of metalloproteinases-1 (TIMP-1) (45).

In this study, we investigated the production of IL-6 by HPV-transformed cell lines with different malignant potential and analyzed in detail their responsiveness to this cytokine. We demonstrate that cervical carcinoma cells producing IL-6 at high levels only weakly respond to IL-6 in an autocrine manner due to limited gp80 expression. Production of IL-6 but silencing an autocrine IL-6 response prevents the tumor cells from constitutively producing the mononuclear cell-attracting chemokine MCP-1, possibly helping the tumor to escape the immune system.

Materials and Methods

Cells and cell culture

Malignant and nonmalignant, in vitro HPV-transformed keratinocyte cell lines were used in this study. The HPV16- or HPV18-positive cervical carcinoma cell lines SiHa (HTB-35, HPV16; American Type Culture Collection, Manassas, VA), CaSkI (CRL-1550, HPV16; American Type Culture Collection) HeLa (CC-2, HPV18; American Type Culture Collection), SW756 (HPV18; kindly provided by Dr. M. von Knebel-Doeberitz, Heidelberg, Germany), the keratinocyte cell line Skv (HPV16) derived from a vulvar bowenoid papule (6), and the HPV-negative cervical carcinoma cell line C33A (HTB-31) were cultured in DMEM medium. The nonmalignant HPV16-transformed foreskin keratinocyte cell line HPKIA (46) was kindly provided by Dr. M. von Knebel-Doeberitz and cultured in DMEM. The nonmalignant in vitro HPV18 E6/7-transformed keratinocyte strains K51 and I56 (kindly provided by Dr. L. A. Laimins, Chicago, IL) were maintained in RPMI containing 25% FCS, 0.4 μg/ml gentamicin, 0.04 μg/ml hydrocortisone, 10−9 M cholera toxin, 5 μg/ml transferrin, 2 × 10−11 M triiodothyronin, 1.8 × 10−7 M adenine, 5 μg/ml insulin (all from Sigma, Deisenhofen, Germany), and 10 ng/ml epidermal growth factor (Life Technologies, Eggenstein, Germany). U266 cells (TIB-196; American Type Culture Collection) were maintained in RPMI 1640 medium. All media were supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, and 2 mM L-alanyl-L-glutamin (all from Life Technologies).

Immunohistochemistry

Six specimens with squamous cell carcinoma (SCC) of the cervix uteri, high-grade CIN (CIN III), and nontumorous cervical epithelium derived from the files of the Institute of Pathology (University of Cologne, Cologne, Germany) were fixed in 5% formalin and were embedded in paraffin. After deparaffinization, tissue sections were stained applying the the Immunomax technique (47) as described previously (2) with the following modifications: as primary Abs affinity-purified rabbit anti-IL-6 (kindly provided by Dr. D. Novick, Weizmann Institute of Science, Rehovot, Israel) or rabbit control Abs (Dianova, Hamburg, Germany) were diluted to 0.1 μg/ml in 10% normal goat serum and incubated for 1 h at 37°C. Biotinylated goat anti-rabbit Ab (Dianova) was added for 30 min at room temperature. Then the sections were incubated with biotinylated tyramine solution (20 mg N-hydroxy-succinimido sulfo-LC-biotin (Pierce, Rockford, IL) dissolved in 0.5 ml DMSO allowed to react with 6.4 mg tyramine (Sigma) in 10 ml 0.1 M borate buffer, pH 8.0) for 10 min at room temperature. StreptAB-alkaline-phosphatase complex (K391; Dako, Hamburg, Germany) was applied for 30 min at room temperature followed by a peroxidase-coupled streptavidin-biotin-complex (K355; Dako) for 30 min at room temperature. Finally, the reaction products were visualized using naphthol-AS-biphosphate and new fuchsin as chromogens. Nuclei were counterstained with hematoxylin.

FACS analysis

Adherent cells were detached with PBS containing 0.005% trypsin-EDTA (1:10 diluted solution from Life Technologies). Following blocking with 7% BSA (Sigma) in PBS, cells were incubated with anti-gp130 mAb (HB-2022, clone 2H4, IgG1, hybridoma supernatant; American Type Culture Collection), anti-gp80 mAb (IgG1; Diacalone, Besançon, France), or MOPC-21 (Sigma) as an isotype-matched control. Cells were then stained with FITC-conjugated goat anti-mouse F(ab′)2 (Dianova), and expression was determined by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA).

Stimulation experiments

Cells were seeded in 24-well plates at a density of 1.5 × 103 cells/well. After 20 h, they were stimulated with DMEM or indicated stimulants in a 300-μl volume. Twenty-four hours later, cellular supernatants (SN) were collected, centrifuged, and stored at −20°C.

Cytotoxicity assay

Cells were seeded in flat-bottom microtiter plates (Renner, Damstadt, Germany) at a density of 2.5 × 104 cells/well. Twenty-four hours later, they were stimulated with medium or 100 ng/ml IL-6 in the presence or absence of 500 ng/ml sgp80. After 8 h, the cells were challenged with serial dilutions of anti-Fas mAb in the presence of 50 μg/ml cycloheximide. Cell viability was assessed 16 h later by the neutral red uptake method according to Finter (48) as described previously (49).

Detection of cytokines by ELISA

Cytokine ELISAs were essentially performed as described (2, 19). Maxisorp plates (Nunc, Wiesbaden, Germany) were coated with 1 μg/ml anti-IL-6 or anti-MCP-1 mAb (PharMingen, Hamburg, Germany) overnight. After blocking of the plates for 1 h with PBS containing 0.5% BSA, 0.05% Tween 20 (Serva, Heidelberg, Germany), and 0.02% NaN3, SN or serial dilutions of the respective recombinant human cytokines (Tebu, Frankfurt, Germany) as standards were added for 6 h. Plates were then incubated with anti-IL-6 or anti-MCP-1 polyclonal Ab (pAb) (Tebu) at 0.5 μg/ml overnight. After 2 h incubation with peroxidase-labeled goat anti-rabbit F(ab′)2, the substrate was applied and the extinction was measured with an SLT ELISA reader at 405 nm.

Western blot analysis

A total of 3.5 × 106 cells were grown in 10-cm dishes, washed twice with PBS, and scraped off with a cell scraper. Cells were pelleted and resuspended in 100 μl buffer containing 20% glycerol, 50 mM Tris (pH 7.9), 1 mM DTT, and 0.1% Nonidet P-40. A total of 46 μg of each sample were separated on a 10% SDS-PAGE. After transfer onto nitrocellulose membranes (Hybond ECL; Amersham, Braunischweig, Germany), the membranes were blocked with 5% skim milk and 0.1% sodium azide in PBS. Detection was performed with anti-STAT3 pAb (Santa Cruz Biotechnology, Santa Cruz, CA) at 2 μg/ml, peroxidase-labeled goat anti-rabbit Ab (Dianova, Hamburg, Germany), and the enhanced chemiluminescence detection system (Amersham) according to the manufacturer’s instructions.

EMSA

Cervical carcinoma cell lines were plated in 10-cm culture dishes reaching confluence the next day. They were then stimulated with medium, 100 ng/ml IL-6, or 1000 U/ml IFN-γ. Stimulation took place in the absence or presence of 500 ng/ml soluble IL-6 receptor (sgp80, R&D Systems, Wiesbaden, Germany) or 2 μg/ml anti-IL-6 pAb as indicated. After 15 min incubation, 10 μl of each sample were separated on a 10% SDS-PAGE. After transfer onto nitrocellulose membranes (Hybond ECL; Amersham, Braunischweig, Germany), the membranes were blocked with 5% skim milk and 0.1% sodium azide in PBS. Detection was performed with anti-STAT3 pAb (Santa Cruz Biotechnology), anti-Fas mAb, or MOPC-21 (Sigma) as an isotype-matched control. Cell extracts were harvested and nuclear extracts were prepared according to Andrews et al. (50). Five micrograms of the respective extracts were analyzed for STAT3 binding activity in a buffer containing 10 mM K-HEPES, 1 mM EDTA, 5 mM MgCl2, 10% glycerol, 50 μg/ml poly(dI:dC), 1 mg/ml BSA, 5 mM DTT, and 2 mM PMSF using the sis-inducible element (SIE) of the c-fos promoter (double-stranded oligonucleotide, 5′-CATGGGAGGGATTTACGGGAAATGCTA-3′) as a probe for supershift analyses, 200 ng anti-STAT3 or anti-STAT1 (Santa Cruz Biotechnology) pAbs were added to the samples for 40 min at 4°C. DNA-protein complexes were then separated for 1 h in a 4.5% polyacrylamide gel...
gel in 0.25× TBE buffer. After fixation of the gels in 10% acetic acid and 10% methanol, autoradiograms were taken.

**RT-PCR**

Cells were seeded at a density of 3.5 × 10⁴ cells/10-cm dish. Total RNA was extracted using the RNAzol B (Wak-Chemie Medical, Bad Homburg, Germany) according to the manufacturer’s instructions. One microgram of RNA was reverse transcribed with 200 U MMLV-RT in 20 μl reaction volume using the Bioline RT primer mix (Bioline, Luckenwalde, Germany). The cDNA samples were stored at −20°C in the dark until analyzed.

PCR amplification of STAT3, Gp80, and B-actin were performed in 50 μl reaction volume containing 125 ng cDNA, 0.5 μmol of each primer, 0.25 mmol/dl dNTPs, 1 U Taq polymerase (Bioline), and 2.5% agarose gels, respectively, and photographs were taken. The amplification conditions were as follows: 35 cycles with denaturation at 94°C for 45 s, annealing at 45°C for 45 s, and extension at 72°C for 2 min. Gp80-specific RT-PCR was performed using the 5′ primer AGTCTGTATTGCTGATGTC and the 3′ primer CATTGCCATTGTTCTGAGGTTC and the 3′ primer AGTAGTCTGTATTGGATGTGTC. The amplification conditions were the same as for the β-actin RT-PCR. PCR products were separated on 1% or 2.5% agarose gels, respectively, and photographs were taken.

**Results**

**IL-6 expression in cervical carcinoma in situ and in vitro**

Applying immunohistochemistry, IL-6 was detected in all cervical SCC investigated. Carcinoma cells showed a diffuse cytoplasmic Ag distribution (Fig. 1A, a). Specific immunostaining of IL-6 was also observed in some dysplastic lesions, especially in the epithelial cells of high-grade CIN (Fig. 1A, b). Of note, basal and suprabasal cell layers of nontumorous cervical epithelium were devoid of IL-6 staining (Fig. 1A, c). These cell layers are known to represent the proliferating compartment of an epithelium in which transformation processes take place and from which carcinomas may arise. Upper, more differentiated cell layers of the normal epithelium showed staining with anti IL-6 Ab. However, this was also seen with nonspecific control Ab (Fig. 1A, f), suggesting that binding to these cell layers was nonspecific. In the inflammatory infiltrate of the tumors, preferentially mononuclear cells were stained for the IL-6 protein. Here, staining was less than in the tumor cells themselves, further underlining the autocrine IL-6 production of the cancer cells.

To examine IL-6 expression levels of the epithelial cells, in vitro analyses were performed. The HPV16- or HPV18-positive cervical carcinoma cell lines SiHa, CaSki, SW756, and HeLa and the HPV16-positive keratinocyte cell line Skv derived from a vulvar Bowenoid papule were compared with the in vitro-transformed, nonmalignant keratinocyte lines HPKIA, I56, and K51 for constitutive IL-6 production. While IL-6 was not detectable in SN of the in vitro-transformed cell lines by ELISA (detection limit at 30 pg/ml), carcinomas secreted IL-6 into the culture supernatants at high levels (Fig. 1B). SiHa and SW756 even reached ng/ml concentrations. Of note, the HPV-negative cervical carcinoma cell line C33A did not produce IL-6.

**Exogenous IL-6 does not activate STAT3 in cervical carcinoma cells**

Because IL-6 had been proposed as a cytokine acting in an autocrine manner on cervical carcinoma cells (7) we were interested to study the signaling events of IL-6 in these cells. To this end, we analyzed constitutive and IL-6-inducible STAT binding activity as an overall measure of gp130 activation. Using the double-stranded radiolabeled SIE-oligonucleotide from the c-fos promoter in EMSAs, we obtained two weakly shifted complexes (I and II) in the carcinomas. While complex I was strongly inducible in the in vitro-transformed keratinocytes after IL-6 stimulation (Fig. 2A, lanes 2, 4, and 6), to our surprise hardly any changes were observed in the carcinomas (Fig. 2A, lanes 10, 12, 14, and 16). A slight increase was seen in the vulvar Bowenoid carcinoma cell line Skv (Fig. 2A, lane 8). Supershift analysis revealed that complex I consisted of STAT3 protein in all tested cell lines as it was shifted by anti-STAT3 (Fig. 2B, upper panel) but not anti-STAT1 Abs (Fig. 2B, lower panel) or control pAb. As a positive control, IFN-γ-induced STAT1 complexes where efficiently shifted by STAT1 Abs (Fig. 2B, lower panel, lane 18). Complex II was not inducible and was neither affected by the addition of STAT3-specific, STAT1-specific, nor control Abs. Therefore, the nature of the non-inducible protein complex II remains elusive. These data indicated that cervical carcinoma cells show weak constitutive activation of STAT3. However, STAT3 was not activated by exogenous IL-6 as observed in the in vitro-transformed keratinocytes.

**STAT3 protein expression is not altered in cervical carcinoma cells**

Reduced IL-6-mediated STAT3 activation in cervical carcinoma cells might either be due to a generally suppressed response to IL-6 activation or to a specific defect in the STAT3 signaling pathway. Based on the fact that IL-6 had been described as an autocrine growth factor for these cells, we first assumed that the unresponsiveness to exogenous IL-6 might be restricted to the STAT3 pathway. One explanation for the lack of STAT3 activation could have been a reduced expression of the STAT3 protein itself. To test for this hypothesis, we prepared whole-cell extracts of the cell lines and examined STAT3 expression by Western blot analysis. In EMSAs, only phosphorylated STAT3 protein, which has translocated into the nucleus and specifically binds to DNA, is investigated. In contrast, Western blot analysis of whole-cell extracts detects activated as well as nonphosphorylated STAT protein located either in the nucleus or in the cytoplasm of the cell. As shown in Fig. 3, the cervical carcinoma cells expressed STAT3 at least at the same levels as the in vitro-transformed keratinocytes. Therefore, STAT3 protein expression levels could not account for the altered responsiveness of the carcinomas.

**Lack of gp80 surface expression in cervical carcinoma cell lines**

To investigate a disruption of IL-6 signal transduction already at the level of IL-6 binding or the initiation of IL-6 signaling, we measured the expression of the respective receptor chains by FACS analysis. The signal transducing chain gp130 was detectable in all cell lines and appeared to be expressed even slightly higher in the malignant cells (Fig. 4A). In contrast, using an anti-gp80 mAb (Dialclone), gp80 was only weakly detectable in the in vitro-transformed keratinocytes and undetectable on the surface of the carcinoma cell lines (Fig. 4A). The same results were obtained with a different anti-gp80 mAb provided by Dr. D. Novick (Weizmann Institute of Science, Rehovot, Israel; data not shown). These data suggested that lack of gp80 surface expression could account for the observed differences in IL-6 signaling.

Not only surface expressed but also shedded sgp80 is able to bind IL-6. Unlike other soluble receptors that bind and thereby neutralize cytokines in the extracellular space, this complex transduces intracellular signals via gp130 dimerization. However, shedded gp80 is not detectable by FACS analysis. Hence, a negative result in FACS analysis might point to a defective gp80 expression on the cellular surface but does not exclude the presence of a functional IL-6R complex. To study whether gp80 expression is affected at transcriptional levels, semiquantitative RT-PCR was performed. As shown in Fig. 4B, serial dilutions (1:2) of cellular cDNAs that gave similar results in cDNA-specific PCR for the constitutively expressed β-actin transcript were compared with a gp80-specific RT-PCR. Corresponding to the protein data obtained by FACS analysis, the nonmalignant keratinocytes expressed gp80 mRNA, while in the carcinomas a gp80-specific transcript was only weakly (CaSki, SiHa) or not detectable (HeLa, SW756).
FIGURE 1. A, IL-6 expression in malignant, dysplastic, and normal cervical tissues in situ. Sections were immunostained with affinity-purified anti-IL-6 pAb (a, b, and c) or nonspecific rabbit control pAb (d, e, and f) in cervical carcinoma (a and d), high-grade CIN (b and e) and nontumorous cervical tissue (c and f) using the ImmunoMax technique as described in Materials and Methods. B, IL-6 production in cultured nonmalignant and malignant keratinocyte cell lines. Cells were seeded at equal densities (1.5 × 10⁵ cells/well) and cultured for 20 h. Twenty-four hours after the addition of fresh medium, supernatants were harvested and IL-6 was determined by ELISA. Shown are the mean values of duplicate determinations in one of three experiments.
In additional experiments, we examined whether the unresponsiveness of the carcinoma cell lines to exogenous IL-6 might be due to constitutive endogenous IL-6 production. In fact, long-term stimulation of the IL-6 responsive cell line HPKIA for 10 days with IL-6 resulted in the down-regulation of gp80 (Fig. 5A, upper panel), but not β-actin (Fig. 5A, lower panel), mRNA expression as shown by semiquantitative RT-PCR analysis. Therefore, we neutralized IL-6 in the supernatants of the carcinoma cells with anti IL-6 pAb. As shown in Fig. 5B, application of IL-6-specific pAb diminished the constitutive STAT3 binding activity at least in Skv, CaSki, HeLa, and SiHa cells (lanes 17 and 18) were supershifted with anti-STAT1 Abs (lane 18). Neutralization and immediately stimulated with exogenous IL-6 at a high dose. In none of the cells tested, neutralization of endogenously produced IL-6 restored the cellular responsiveness to the exogenously applied cytokine (Fig. 5B, lane 4). Thus, down-regulation of IL-6 signaling in cervical carcinomas could not be reverted by abolishing the continuous IL-6 stimulus.

Addition of sgp80 restores IL-6 responsiveness in cervical carcinomas

If gp80 is the limiting factor restricting IL-6 signaling in cervical carcinomas, sgp80 should be able to complete a functional IL-6R-signaling complex. Therefore, we investigated the effect of sgp80 addition to the carcinoma cell lines. In fact, addition of sgp80 was able to restore their IL-6 responsiveness. Applying sgp80, the cell lines did not only strongly react to exogenous IL-6 with STAT3 activation (Fig. 6, lane 4) but also to endogenously produced IL-6 (Fig. 6, lane 3). These experiments indicated that the impaired responsiveness of cervical carcinomas was not due to a defect in intracellular signaling but related to gp80 expression.

Soluble gp80 does not influence Fas-mediated apoptosis but induces the chemokine MCP-1 in cervical carcinoma cells

In view of the fact that carcinoma cells do produce high amounts of IL-6 but do obviously hardly respond to it, we were interested...
STAT3 signaling had been implicated in the regulation of apoptosis. With few exceptions, it had been described to promote the survival of tumor cells, e.g., conferring resistance to Fas-mediated apoptosis (51). Therefore, we investigated whether STAT3 signaling might influence the sensitivity to apoptosis in cervical carcinoma cells. Only CaSki cells were strongly sensitive, all other cells were not or only weakly sensitive to Fas-mediated apoptosis. However, neither in CaSki (Fig. 7A) nor in the other cell lines (not shown) did preactivation of the IL-6 signaling pathway by application of IL-6 in combination with sgp80 change the sensitivity to Fas-mediated cell death.

Therefore, we hypothesized that strong IL-6 signaling might be disadvantageous for the tumor in a way different from the modulation of apoptosis. One factor described to be important for the immunological control of cervical carcinomas is the chemokine MCP-1 attracting mononuclear cells (52, 53). This CC-chemokine was previously shown to be inducible in cervical carcinoma cells by CD40 ligand in synergism with IFN-γ (2), a cytokine that induces STAT1 activation. As MCP-1 may be induced by IL-6 in other cell types (11–13), we concentrated on the IL-6-mediated induction of MCP-1 in cervical carcinoma cells. Constitutive production of MCP-1 into the culture supernatants was low as shown previously. As expected, stimulation of the carcinomas with exogenous IL-6 could not enhance MCP-1 production. However, in the cell lines with high constitutive IL-6 secretion, addition of sgp80 was sufficient to induce an 8-fold induction of MCP-1 in SiHa and a 6-fold induction in SW756, respectively (Fig. 7B). These results underlined the observation that IL-6-producing cervical carcinomas efficiently responded to IL-6 only after addition of the receptor subunit sgp80. However, full autocrine responsiveness to IL-6 would lead to chemokine production, which would be disadvantageous for the tumor.

**Discussion**

IL-6 up-regulation has been observed in a variety of malignant cell types. Depending on the nature of the target cell, this cytokine displays a broad spectrum of activities ranging from modulation of cell growth, apoptosis, and metastasis to local and systemic effects on the immune system. Here, we show that cervical carcinoma cells producing IL-6 at high levels only weakly respond to IL-6 in an autocrine manner as a consequence of limited IL-6R expression. Thereby, the cells are prevented to constitutively produce the mononuclear cell attracting chemokine MCP-1, a mechanism that may help the tumor to escape the immune system.

IL-6 had been described as an autocrine factor for cervical carcinoma cells (7), as it had been done for other cell types, especially plasmocytomas (22, 27). Therefore, we were interested to study the signals that mediate this effect. Our data show that IL-6 is expressed in cervical carcinomas in vivo. The fact that IL-6 was not found in cervical tumor cells in a different study (54) might be related to the different immunostaining techniques applied. In fact, similar to Tartour et al., we were unable to detect IL-6 using a conventional peroxidase-based staining protocol (data not shown). Applying the ImmunoMax technique, which reached a higher sensitivity than the immunoperoxidase method, IL-6 was detected in the cytoplasm of cervical carcinoma cells and high-grade dysplasias. Staining was even stronger than in the infiltrate of the tumor.
stroma, underlining that IL-6 was produced by the tumor cells themselves. Keratinocytes in the basal and suprabasal cell layers of normal epithelium were devoid of IL-6. These layers have proliferative capacity and represent the compartment where dysplastic transformation may be initiated. IL-6 staining of carcinoma cells was obtained not only with a commercial anti-IL-6 pAb (rabbit anti-human polyclonal IL-6 500-P26; Tebu; data not shown) but also with affinity-purified anti-IL-6 pAb as shown here. In contrast, nonspecific control pAb did neither stain the cervical carcinoma cells nor dysplastic lesions. Moreover, in vitro, HPV-positive cervical carcinoma cells constitutively produced IL-6, some of them in nanogram amounts. This is in concordance with a previous report of Malejczyk et al. demonstrating that the HPV16-harboring cell line Skv expresses and releases this cytokine (6). IL-6 was neither detected in the SN of nonmalignant keratinocytes transformed with HPV16 or HPV18 in vitro nor of the HPV-negative carcinoma cell line C33A. Transcription of the IL-6 gene is positively regulated by control elements comprising the binding sites for the transcription factors NF-κB, NF-IL-6 (c/EBPβ), and AP-1. In contrast, the tumor suppressor proteins p53 and pRb suppress IL-6 transactivation (55–60). Whether or not dysregulation of the HPV oncogenes E6 and E7 plays a role in the up-regulation of the cytokine in the carcinoma cells, e.g., by E7, which transactivates AP-1 (61) and neutralizes pRb (62), or E6, which promotes p53 degradation (63), remains to be determined.

When IL-6 signaling was analyzed in the HPV-positive cervical carcinomas, we were surprised that we did not observe IL-6-inducible activation of the transcription factor STAT3, a hallmark of IL-6 signal transduction (40), nor of STAT1. Only weak constitutive STAT3 binding activity was detectable. Altered expression of the STAT3 protein could not account for the reduced binding activity as the protein was expressed in the carcinomas at similar levels as in in vitro-transformed keratinocytes, which responded well to IL-6. In fact, all cell lines that did not constitutively secrete IL-6 could be activated by the cytokine. This corresponds to the observations of Igelsias et al. showing IL-6 responses in cervical cell lines producing only low IL-6 levels (31). At least in some cell lines (Skv, HeLa, CaSki, SiHa), the weak constitutive STAT3 binding activity was due to weak autocrine IL-6 signaling as it was abolished after neutralization of IL-6 in the cellular SNs. FACS analysis demonstrated a lack of sgp80 surface expression, the ligand binding chain of the IL-6 receptor complex, on the carcinoma cells. However, loss of gp80 on the cell surface could also be a consequence of receptor release from the surface. The gp80 protein is efficiently shedded in different cell types resulting in a truncated but nevertheless functionally active receptor chain (64). Semiquantitative RT-PCR analysis revealed that the loss of gp80 expression was regulated on transcriptional level (Ref. 65 and this paper). Moreover, in experiments where sgp80 was added to the cervical carcinomas, we could fully restore the responsiveness to endogenously produced and exogenously applied IL-6. This indicated that indeed gp80 was the limiting factor in cervical carcinomas. IL-6 neutralization in the cellular SN did not restore the responsiveness to exogenously applied IL-6. This argued against a sole functional down-regulation of gp80, which can be observed on

FIGURE 5. A, Long-term IL-6 stimulation down-regulates gp80 expression in in vitro-transformed keratinocytes. HPKIA cells were stimulated with medium or 100 ng/ml IL-6 for 10 days. Total RNA was prepared and reverse transcribed to cDNA. To analyze gp80 expression at semiquantitive levels, serial dilutions of cDNA (1:2) were subjected to gp80-specific (upper panel) or β-actin-specific RT-PCR (lower panel) as a control. B, Neutralization of IL-6 in SN of cervical carcinomas does not alter their IL-6 responsiveness. Cells were incubated for 15 min with the indicated reagents. IL-6 was used at 100 ng/ml, anti-IL-6 pAb was used at 2 µg/ml. Nuclear extracts were analyzed for STAT binding activity by EMSA. Extracts used in lane 4 were prepared from cells that had been stimulated with anti-IL-6 pAb for 24 h, washed twice, and then incubated with IL-6 for 15 min.

FIGURE 6. Soluble gp80 restores IL-6 responsiveness. Cells were incubated for 15 min with the indicated reagents. IL-6 was used at 100 ng/ml and sgp80 at 500 ng/ml. Nuclear extracts were analyzed for STAT binding activity by EMSA. Lane 1 and 2 show the same controls (medium and IL-6, respectively) as in Fig. 3. For extracts used in lane 4, cells had been simultaneously stimulated with IL-6 and sgp80.
RNA level after long-term stimulation of IL-6-responsive keratinocytes (as shown here) or on protein level after stable transfection of hepatoma cells with a CDNA-encoding IL-6 (66).

From these observations two questions arose as to 1) why the IL-6-producing carcinomas shut off their IL-6 responsiveness and 2) what could be the in vivo role of IL-6 produced by cervical carcinoma cells. One can only speculate about the answers to these questions.

IL-6 might only be beneficial for the carcinomas when intracellular signaling is low. This is in contrast to myeloma cells, which profit from strong IL-6 signaling sustained by autocrine sgp80 production (67). Low STAT3 signaling in the cervical carcinoma cells did not lead to a higher sensitivity for apoptosis signals as one could have expected from the results of previous studies in other cell types (51, 68, 69). In fact, although SiHa, CaSki, SW756, and HeLa expressed Fas on their surface (data not shown), only CaSki cells were strongly susceptible to Fas apoptosis. Activation of the STAT3 in these cells did not confer protection. This indicated that STAT3 signaling does not represent an advantage in cervical carcinoma cells with respect to cell death regulation. A clue for the cellular restriction of the IL-6 signal came from a different observation. When the high-level IL-6-producer cell lines SW756 or SiHa were stimulated with sgp80, enabling the cell to transduce a strong autocrine IL-6 signal, the chemokine MCP-1 was induced. MCP-1 is hardly detectable in nonactivated cervical carcinoma cells (2, 52). However, when HeLa cells transfected with the MCP-1 gene had been inoculated into nude mice, significant growth retardation and macrophage infiltration was observed (53). Thus, blockade of autocrine signaling via IL-6 may serve the malignant cells to prevent constitutive production of MCP-1 and the attraction of mononuclear cells to the tumor, which in turn could provide the tumor cells with shedded sgp80.

In vivo, IL-6 may also act in a paracrine fashion. There is increasing evidence that IL-6, which had been regarded as a proinflammatory cytokine, may in some respect act as an antiinflammatory mediator (32). Among various mechanisms, IL-6 is able to inhibit IL-1 and TNF production in human blood mononuclear cells (33, 34), enhances the expression of IL-1 receptor antagonist (70), and inhibits the degradation of extracellular matrix (35). Thus, while production of IL-6 might contribute to a local immunosuppressive effect, silencing an autocrine IL-6 response prevents constitutive production of the mononuclear cell-attracting chemokine MCP-1. Both mechanisms might help the tumor to escape the immune system.

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References


