Soluble interleukin-6 receptor activates the human papillomavirus type 18 long control region in SW756 cervical carcinoma cells in a STAT3-dependent manner

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Cervical carcinoma cells producing high levels of interleukin-6 (IL-6) were shown to be unresponsive to the cytokine IL-6 due to the loss of their IL-6 receptor. Addition of IL-6 receptor in a soluble form restores IL-6 signalling in SW756 carcinoma cells. This leads to a rapid and strong activation of the transcription factor signal transducer and activator of transcription 3 (STAT3). Nuclear factor IL-6 (NF-IL6, C/EBPβ) was induced only as a late event. While C/EBPβ significantly repressed the human papillomavirus type 18 long control region (HPV18-LCR), IL-6 signalling unexpectedly activated the HPV18-LCR in these cells. This IL-6 receptor-mediated induction could be completely reverted by transfection of a dominant-negative STAT3 but not STAT1 expression construct, indicating that STAT3 might play an important role in HPV18 oncogene promoter activation.

Interleukin-6 (IL-6) is expressed in cervical carcinomas in vitro and in vivo (Eustace et al., 1993; Hess et al., 2000; Malejczyk et al., 1991) and may be further induced by activation of the receptor CD40 in these cells (Altenburg et al., 1999). IL-6 is a multifunctional member of the IL-6 family of cytokines, which also includes oncostatin M, leukaemia inhibitory factor, IL-11, ciliary neurotrophic factor and cardiophrin-I. It regulates cell growth, differentiation and gene expression in a wide range of different cell types (for review see Heinrich et al., 1998; Hirano et al., 2000; Taga & Kishimoto, 1997). While IL-6 is known to be an important autocrine growth stimulator of multiple myeloma cells (Kawano et al., 1988; Klein et al., 1995), it may also inhibit the growth of breast carcinoma cell lines, melanocytes and certain B cell lymphomas (Chen et al., 1988; Morinaga et al., 1989). IL-6 binds to the IL-6 receptor chain, gp80, which is functional either in its membrane or its soluble form, sgp80 (Romano et al., 1997; Tamura et al., 1993), leading to dimerization of the signalling subunit gp130 (Murakami et al., 1993; Taga et al., 1989). Dimerized gp130 in turn activates constitutively associated tyrosine kinases of the Janus (Jak) family, which phosphorylate transcription factors of the STAT (signal transducers and activators of transcription) family, STAT3 and STAT1 (Hibi et al., 1990; Lutticken et al., 1994; Rakemann et al., 1999; Stahl et al., 1994; Zhong et al., 1994). IL-6 may also lead to the activation of the mitogen-activated protein (MAP) kinase pathway and induces the expression of the transcription factor C/EBPβ (Akira et al., 1990; Baumann et al., 1992; Boulton et al., 1994; Daipour et al., 1993). Recently, it has been suggested that STAT3 activation is essential for IL-6-mediated C/EBPβ induction. In that study, STAT3 activated the C/EBPβ promoter despite the lack of a sequence-specific STAT DNA-binding site (Niehof et al., 2000).

Similar to multiple myeloma, IL-6 was postulated to be an autocrine growth factor for cervical carcinoma (Eustace et al., 1993). However, as shown previously, cervical carcinoma cells producing IL-6 in large quantities do not respond to it due to the loss of gp80 expression (Bauknecht et al., 1999; Hess et al., 2000). Detailed analysis revealed that other components of the signalling cascade are intact, as responsiveness to IL-6 could be completely restored by the addition of gp80 in a soluble form (Hess et al., 2000). Cervical carcinoma cell lines like SW756, which produces nanogram amounts of IL-6, even responded to their own IL-6 in the presence of sgp80, leading to strong activation of STAT3 and production of the chemokine MCP-1 (monocyte chemoattractant protein-1). As MCP-1 may enhance an anti-tumour response by attracting mononuclear cells into the tumour tissue, loss of gp80 expression might be a helpful immune escape mechanism for the carcinoma cells (Hess et al., 2000).

The effects of IL-6 signalling on human papillomavirus (HPV) regulation in cervical carcinoma cells are less well understood. Whereas IL-6 activated the HPV18 promoter in the hepatoma cell line HepG2, it was postulated that IL-6 might repress the HPV18 long control region (HPV18-LCR) in cervical carcinoma cells through the activation of C/EBPβ.
However, this hypothesis could not be tested in the latter cells due to the fact that they have lost gp80 expression.

To further analyse this question we restored responsiveness to IL-6 by adding sgp80 to the IL-6-producing cervical carcinoma cell line SW756. Cells were kept in DMEM 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-glutamine (all from Gibco BRL). They were seeded at a density of 1·5 × 10^5 cells per well in 6-well plates, grown overnight and co-transfected with 1 µg of the vector p4321-luc and 1 µg of CMV-β-gal reporter vectors. After 24 h they were stimulated with medium (lane 1), 100 ng/ml IL-6 (lane 2), 500 ng/ml sgp80 (lane 3) or 100 ng/ml IL-6 in the presence of 500 ng/ml sgp80 (lane 4). At 48 h post-transfection cells were harvested and analysed. Shown are the mean values ± SD of three experiments with duplicate determinations. All values are normalized on CMV–β-gal values. Medium control was set to the value 1.

In order to identify intracellular signals mediating this effect, we analysed the induction of the transcription factor C/EBPβ by IL-6 in the presence of sgp80. SW756 were seeded in 6 cm dishes and were stimulated with 100 ng/ml IL-6 in the presence of 500 ng/ml sgp80 for different time intervals. Nuclear extracts were prepared according to Schreiber et al. (1989). Each extract (5 µg) was assayed for C/EBPβ-binding activity in an electrophoretic mobility shift assay (EMSA) using the ^32P-labelled double-stranded oligonucleotide 5' GGACGTCACATTGCCAATCTTAATAA 3' (Akira et al., 1990). As shown in Fig. 2 (A, left panel), SW756 cells displayed constitutive C/EBPβ-binding activity. IL-6/sgp80 was able to induce C/EBPβ-binding activity; however, this was a late event, as it was not observed within the first 4 h. The identity of the shifted band was proven to be C/EBPβ in supershift analysis, where the extracts were pre-incubated with a polyclonal rabbit anti-C/EBPβ antibody (Santa Cruz Biotechnology) (Fig. 2A, right panel).

In order to test C/EBPβ for its activity on the HPV18-LCR, the C/EBPβ cDNA was PCR-amplified and cloned into BamHI and EcoRI sites of the pcDNA3.1+ vector (Invitrogen), resulting in pcD-C/EBPβ. SW756 cells were seeded at equal densities (9·2 × 10^5 cells per well) in 6 cm dishes and co-transfected with various doses of pcD-C/EBPβ together with 3 µg of p4321-luc and 3 µg of CMV–β-gal reporter vectors, 2 µg pBluescript SK+ II (Stratagene) and the indicated amounts of pcD-C/EBPβ using FuGene. The amount of pcDNA vector was adjusted with empty pcDNA3.1+ vector in all transfections. In accordance with the findings of Baurknecht et al. (1999), pcD-C/EBPβ strongly suppressed HPV18-LCR activity even at very low doses (Fig. 2B). Thus, C/EBPβ could not be responsible for the observed stimulatory effect of IL-6 in the presence of sgp80.

Earlier studies had revealed rapid and strong activation of STAT3 by sgp80 in SW756 cells (Hess et al., 2000). Computer analysis of the HPV18-LCR using the MatInspector program did not reveal a consensus STAT-binding site, which does not exclude the existence of an atypical yet functional STAT-binding site. Seven potential sites showing at least similarity with a STAT-binding site were investigated by EMSA, and may regulate transcription without direct DNA binding (Niehof et al., 2000). We were interested to investigate whether STAT factors are functionally involved in HPV-LCR activation. In order to block either STAT1 or STAT3 activation specifically, dominant-negative forms specific for the respective transcription factors were used. These STAT mutants (STAT1F and STAT3F) cloned into the vector pCAGGS-Neo (pCAGGS-Neo-HA-STAT1F and pCAGGS-Neo-HA-STAT3F) were kindly provided by Dr T. Hirano, Osaka, Japan (Hirano et al., 1997; Nakajima et al., 1996). In transient transfection analysis, dominant-negative STAT1 and STAT3 were tested for their...
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Fig. 2. IL-6 signalling in SW756 induces C/EBPβ-binding activity in SW756 as a late event (a). Cells were cultured in 6 cm dishes overnight. Then they were stimulated with 100 ng/ml IL-6 in the presence of 500 ng/ml sgp80 for the indicated time intervals (left panel). Nuclear extracts were analysed for C/EBPβ-binding activity by EMSA. Nuclear extracts of cells stimulated for 24 h were investigated by supershift analysis using an anti-C/EBPβ polyclonal antibody (right panel). C/EBPβ transfection in SW756 cell represses the HPV18-LCR (b). Cells were seeded at equal densities (9-2 x 10⁵ cells per well) in 6 cm culture plates and cultured for 20 h. Then they were co-transfected with 3 µg of p4321-luc and 3 µg of CMV-β-gal reporter vectors, 2 µg pBluescript and the indicated amounts of pcD-C/EBPβ. At 48 h post-transfection cells were harvested and analysed. Shown are the mean values ± SD of three experiments with duplicate to triplicate determinations each. All values are normalized on CMV-β-gal values. Transfection containing no pcD-C/EBPβ plasmid was set to the value 1.

Thus, IL-6 cannot be generally regarded as a HPV-suppressive cytokine but may activate the HPV18-LCR even in cervical carcinoma cells. The induction involves the transcription factor STAT3. In SW756 cervical carcinoma cells, IL-6 is produced in large amounts, but the cells do not respond due to a loss of the IL-6R ligand-binding chain (Bauknecht et al., 1999; Hess et al., 2000). It was speculated that autocrine IL-6 signalling may be inhibited because C/EBPβ, a potential downstream effector of IL-6 signalling, suppresses transcription by the HPV18-LCR (Bauknecht et al., 1996). For the HPV16-LCR the effects of C/EBPβ have been controversially discussed, as this factor has been reported to activate (Struyk et
Fig. 3. Dominant negative STAT3 blocks IL-6/sgp80-mediated activation of the HPV18-LCR. Cells were seeded at equal densities (1.5 × 10⁵ cells per well) in 6-well culture plates and cultured for 20 h. Then they were co-transfected with 1 μg of p4321-luc vector, 1 μg of CMV-β-gal reporter vector and 1 μg of the indicated expression vectors: empty vector pCAGGS-Neo (lane 1), pCAGGS-Neo-HA-STAT1F (lane 2) or pCAGGS-Neo-HA-STAT3F (lane 3). After 24 h all cells were stimulated with 100 ng/ml IL-6 in the presence of 500 ng/ml sgp80. At 48 h post-transfection cells were harvested and analysed. Shown are the mean values ± SD of three experiments with duplicate determinations. All values are normalized on CMV-β-gal values. The transfection of empty vector pCAGGS-Neo was set to the value 1.

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al., 2000) or to repress (Kyo et al., 1993) transcription. In fact, C/EBPβ, which was induced upon reconstitution of IL-6 signalling in SW756, also strongly repressed the HPV18-LCR in our experiments when overexpressed by transfection in the same cells. How can these on first glance contradictory effects of IL-6 and C/EBPβ be explained? We had previously shown that IL-6 signalling does not only result in C/EBPβ induction but also activates other transcription factors of the STAT family, STAT3 and STAT1. Recent data suggests that both signalling events are interrelated as IL-6 appears to induce STAT3-dependent IL-6 signal. However, from luciferase assays these possibilities are difficult to dissect, as the half-life of the luciferase reporter enzyme and of the corresponding mRNA might not permit the detection of an up- and down-response. It is still unclear how STAT3, which probably does not directly bind to the HPV18-LCR, might nevertheless alter its activity. It will be interesting to investigate whether there are atypical STAT3-binding sites or whether STAT3 is tethered to a complex bound to the HPV18-LCR as has recently been suggested for the C/EBPβ promoter. Finally, STAT3 involvement might be indirect via induction of a different transcription factor that binds to the LCR.

We are well aware of the fact that reporter construct analyses might not completely reflect the response of the integrated genes in carcinomas, as their regulation is undoubtedly much more complex and might also additionally depend on cellular factors acting at the viral integration sites. However, our study provides evidence that IL-6, produced by SW756 carcinoma cells, can clearly provide positive regulatory signals which induce the HPV18-LCR.

In conclusion, in cervical carcinoma cells a major role of shutting off the autocrine IL-6 stimulation might not be the repression of the viral oncogene promoter, but possibly an immunological role, e.g. to prevent chemokine production, which might help the malignant cells to escape the immune surveillance.

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References


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