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Response to IL-6 of HPV-18 Cervical Carcinoma Cell Lines

Tobias Bauknecht,*[,1] Bettina Randelzhofer,† Beate Schmitt,† Zoltan Ban,†
Juan-Jose Hernando,† and Thomas Bauknecht†[,1,2]

*Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum Heidelberg, 69120 Heidelberg; and †Universitäts-Frauenklinik Freiburg, 79106 Freiburg, Germany

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The human papillomavirus type 18 (HPV-18) upstream regulatory region (URR) controls cell type-specific expression of the viral oncoproteins E6 and E7. The HPV-18 URR is active in the cervical carcinoma cell line HeLa but inactive in the hepatoma cell line HepG2. C/EBPß (NF-IL-6) was shown to participate as an important regulator in HPV transcription dependent on the cell type. The finding that C/EPBß is critical for HPV-18 URR activity and that C/EPBß is induced by IL-6 offers the opportunity of manipulating HPV activity by specific cytokine treatment. In this report, we show that treatment with IL-6 results in activation of HPV-18 URR activity in HepG2 cells. In contrast, the HPV-18 URR is not inducible by IL-6 in three cervical carcinoma cell lines. In all three cell lines we found decreased expression of the IL-6 receptor compared to the IL-6-responsive HepG2 cells, whereas the level of expression of the signal transduction component gp130 is present in all cells. These results suggest that cervical carcinoma cells may circumvent the IL-6-induced cellular defense mechanism through downregulation of the IL-6-receptor.

INTRODUCTION

Interleukin-6 (IL-6) is a pluripotent growth-regulatory cytokine which exerts diverse biological effects depending on the target cell type (Akira *et al.,* 1993; Kishimoto, 1989; Seghal, 1990). The levels of IL-6 and its receptor within a given microenvironment, as well as the physiology of the IL-6 pathway, are critical factors required for both responsiveness and regulating normal cell function (Rose-John and Heinrich, 1994).

The IL-6 signal cascade is initiated after interaction of IL-6 with a ligand binding extracellular receptor subunit, IL-6R. In addition, the IL-6 receptor subunit is able to release a soluble IL-6 receptor by proteolytic cleavage (Akira *et al.,* 1993; Rose-John and Heinrich, 1994). The corresponding transmembranic subunit, gp130, which seems to be almost unique to various cell types, is required for signal transduction. Binding of IL-6 to IL-6R initiates dimerization of IL-6R and gp130, following signal transduction by tyrosin phophorylation of gp130 (Mackiewicz *et al.,* 1993; Rose-John and Heinrich, 1994). A soluble form of human IL-6 receptor has been shown to bind to IL-6 in solution and to induce biological response to $IL\text{-}6R^{-}/gp130^{+}$ cells that are normally unresponsive to IL-6 (Mackiewicz *et al.,* 1993; Narazaki *et al.,* 1993).

In malignant cells, IL-6 may act as a potent growth activator, particularly for plasmocytomas and myelomas (Kawano *et al.,* 1988; Klein *et al.,* 1989) and other nonhematopoetic tumors (Miki *et al.,* 1989; Miles *et al.,* 1990; Watson *et al.,* 1993). In contrast, in other malignancies such as leukemia, melanoma, and breast and lung cancer, it was shown that IL-6 exerts an inhibitory effect to *in vitro* and animal tumor systems (Chen *et al.,* 1988; Lu *et al.,* 1992; Revel, 1992; Takizawa *et al.,* 1993; Sun *et al.,* 1995).

Some cervical carcinoma cell lines have shown to synthesize IL-6, and autocrine as well as paracrine mechanisms have been suggested to promote proliferation (Eustace *et al.,* 1993; Tartour *et al.,* 1994; Iglesias *et al.,* 1995; Castrilli *et al.,* 1997). The high-risk papillomavirus types, especially human papillomavirus HPV-16 and HPV-18, are believed to be the causal agents of cervical cancer (zur Hausen, 1989, 1991a,b, 1996; Schiffman *et al.,* 1993). E6 and E7 oncogenes are known to be relevant for progression to malignant growth. Their loss of function causes proliferation arrest and cell death (von Knebel-Doeberitz *et al.,* 1988, 1992). A number of transcription factors have been reported to be important in the regulation of HPV transcription (Kyo *et al.,* 1993; Hoppe-Seyler and Butz, 1994; McCaffery and Jackson, 1994; Bauknecht *et al.,* 1996; Wang *et al.,* 1996). In recent studies it was shown that HPV-11, -16, and -18 as well as bovine papillomavirus type 4 are strongly repressed by C/EBPß (Kyo *et al.,* 1993; McCaffery and Jackson, 1994; Bauknecht *et al.,* 1996; Wang *et al.,* 1996). C/EBPß (NF-IL-6) was first characterized as a protein whose mRNA synthesis is

[1] Present address: Medical Molecular Diagnostic GmbH, Sigmund-Freud-Str. 25, 53105 Bonn-Venusberg, Germany.
[2] To whom correspondence and reprint requests should be addressed at Universitäts-Frauenklinik, Sigmund-Freud-Str. 25, 53105 Bonn-Venusberg, Germany. Fax: +49-228-287-6091.

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regulated by IL-6 and other cytokines (Akira *et al.,* 1990). Members of the C/EBP family consist of highly related isoforms, which bind to CCAAT box of several promoters and to a distinct core homology of viral enhancers (Akira *et al.,* 1993; Wedel and Ziegler-Heitbrock, 1995). The C/EBP family of proteins have common basic region leucine zipper (bZIP) domains. Through the leucine zipper region, C/EBP proteins form homo- and heterodimers with other family members, with leucine zipper proteins outside the family as well as non-leucine-zipper-containing proteins (Landschulz *et al.,* 1989; Wedel and Ziegler-Heitbrock, 1995). Members of the C/EBP family have a highly conserved tissue distribution (Birkenmeier *et al.,* 1989; Akira *et al.,* 1993; Wedel and Ziegler-Heitbrock, 1995) and targets for C/EPB proteins are liver- and differentiation-specific genes, cytokines genes, and in addition enhancer regions of diverse viruses (Birkenmeier *et al.,* 1989; Maire *et al.,* 1989; Majello *et al.,* 1990; Akira *et al.,* 1993; Wedel and Ziegler-Heitbrock, 1995).

In the present study, we examined the possibility of regulating HPV-18 activity in response to the cytokine IL-6 by comparing three established HPV-18-positive cervical carcinoma cell lines and an established hepatoma cell line, HepG2. Transfection studies of HPV-18 upstream regulatory region (URR) reporter gene constructs revealed a strong induction of URR activity by IL-6 in HepG2 cells. This IL-6-induced activation was dose dependent. In contrast, HPV-18 URR activity was not induced by IL-6 in the three HPV-18-positive cervical carcinoma cell lines HeLa, C4-1, and SW756. To understand the mechanisms by which IL-6 has no effect on the HPV-18 URR activity in these cervical carcinoma cells, we showed by Northern blot and RT–PCR analysis that expression of IL-6 receptor is strongly reduced in the tested cervical carcinoma cell lines but not in HepG2 cells. Interestingly, different passages of HPV-16-transformed human keratinocytes revealed a reduced IL-6 receptor expression in Northern blot analysis, depending on the increasing passage number. In addition, flow cytometry studies demonstrate a reduced IL-6 receptor expression in the cervical carcinoma cell lines as well as in the high-passage HPV-16-transformed keratinocyte cell line HPK IAP359. However, we found that the signal transduction component gp130 is present in all cells.

In summary, by using the HPV-18 URR as a test system we have provided evidence that IL-6 has no effect on URR activity, probably through a low-level expression of the IL-6 receptor in different cervical carcinoma cells compared to the IL-6-responsive cell line HepG2. The fact that C/EBPß is strongly induced by IL-6 and that HPV-18 and other HPV types can be repressed by overexpression of C/EBPß points to a possible mechanism by which cervical carcinoma cells could avoid a cellular defense mechanism against transcription of viral oncoproteins E6/E7.

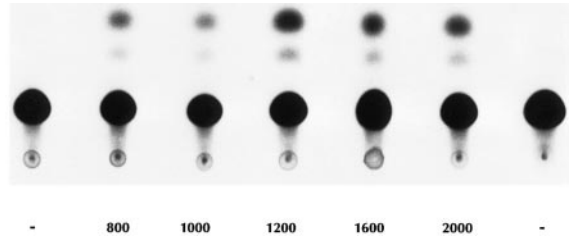


FIG. 1. Induction of HPV-18 URR in HepG2 cells by IL-6 treatment. Treatment by IL-6 after 6 h of transfection significantly induced HPV-18 URR-driven CAT expression. CAT assays were carried out with extracts prepared from HepG2 cells transfected with 18 μg p18URR together with 0.5 μg of RSV/L as an internal control. CAT activities were quantified relative to the activity obtained with p18URR without IL-6 treatment (lanes 1 and 7), which was set at 0.1. The results of a representative CAT assay are shown. Lanes 1 and 7, without IL-6 treatment; lanes 2–6, increasing amounts of IL-6 from 800 to 2000 u IL/6/ml as shown. Relative CAT activities were as follows: p18URR without IL-6, 0.1; with 800 u IL-6/ml, 0.6; with 1000 u IL6/ml, 0.7; with 1200 u IL6/ml, 1.2; with 1600 u IL6/ml, 1.0; with 2000 u IL6/ml, 0.95.

RESULTS

IL-6 activates HPV-18 expression in HepG2 cells

Active expression of HPV-18 E6 and E7 oncoproteins is regulated by the URR. In recent reports it was shown that the HPV-18 URR exhibits stringent cell type specificity and functions only in certain human epithelial cells (Bauknecht *et al.,* 1992; Thierry *et al.,* 1987; Bernard *et al.,* 1989; Mack and Laimins, 1991). Mutational analyses of the HPV-18 URR have shown that a sequence termed the switch region contributes in a cell type-specific manner to the high-level activity of the HPV-18 URR in HeLa cells (Bauknecht *et al.,* 1995). It was shown that in HeLa cells C/EBPß forms an activating complex with YY1 which binds to the switch region OL22 (Bauknecht *et al.,* 1996). In contrast, the HPV-18 URR is virtually inactive in HepG2 cells and this is believed to be due to the lack of C/EBPß–YY1 interaction, which ensures that YY1 functions as an activator of the HPV-18 URR, as is the case in HeLa cells. Overexpression of C/EBPß in HepG2 cells restores the C/EBPß–YY1 switch region interaction and therefore activates the HPV-18 URR (Bauknecht *et al.,* 1996). The finding that C/EBPß is critical for HPV-18 activity and that C/EBPß is regulated by the cytokine IL-6 offers the opportunity of manipulating HPV-18 promoter activity by IL-6 treatment. Therefore, we were interested in analyzing the effect of IL-6 treatment on URR activity in different cervical carcinoma cell lines.

To determine if URR activity can be influenced by IL-6 we first analyzed the HPV-18 URR in HepG2 cells (Fig. 1). HPV-18 URR CAT plasmid p18URR was transfected into HepG2 cells, and after transfection cells were maintained in medium treated with various concentrations of human recombinant IL-6. After a 24-h period of IL-6 treatment extracts of transfected cells were prepared and assayed for CAT activity. As shown in Fig. 1, HPV-18

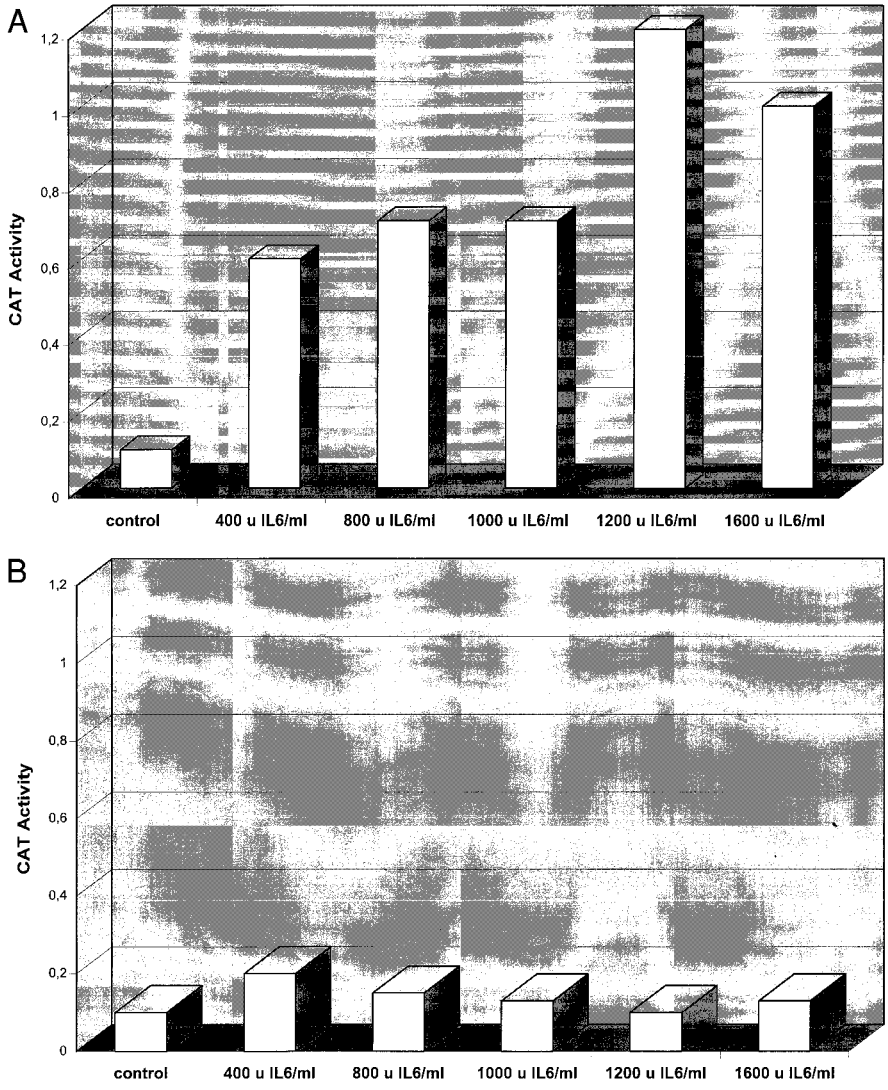
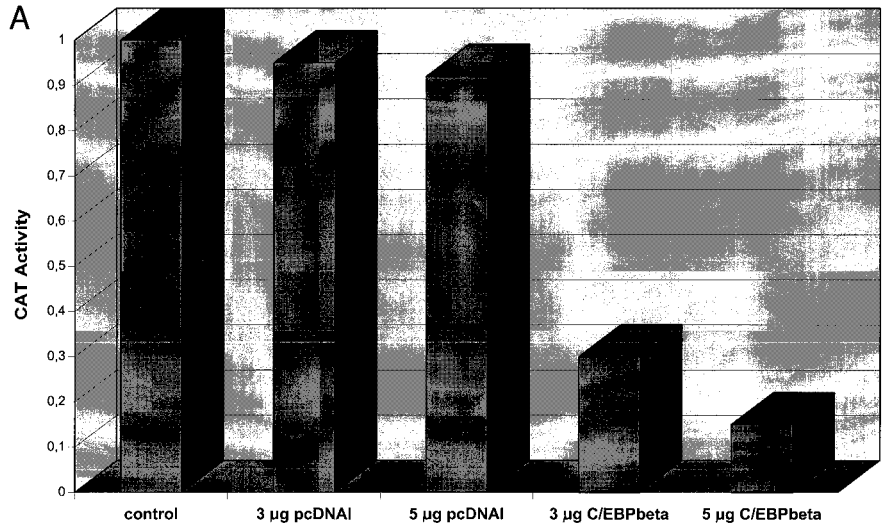


FIG. 2. In contrast to wild-type HPV-18 URR, mutation of the C/EBPβ–YY1 switch region OL22 prevents induction of HPV-18 URR by IL-6 in HepG2 cells. (A) HepG2 cells were transfected with 18 μg of the wild-type construct p18URR together with 0.5 μg RSV/L as an internal control. Column 1, control without IL-6 treatment; columns 2–5, increasing amounts of IL-6 up to 1600 u/ml. (B) HepG2 cells were transfected with 18 μg of p18URR-22M1, the HPV-18 URR CAT plasmid containing the mutated C/EBPβ/YY1 site OL22M1, together with 0.5 μg RSV/L as an internal control. In A and B, CAT activities were quantified relative to the activity of the control transfection (without IL-6 treatment), which was set at 0.1. (A) p18URR without IL-6, 0.1; with 400 u IL-6/ml, 0.58; with 800 u IL-6/ml, 0.66; with 1000 u IL-6/ml, 0.68; with 1200 u IL-6/ml, 1.18; with 1600 u IL-6/ml, 0.98. (B) p18URR-22M1 without IL-6, 0.1; with 400 u IL-6/ml, 0.2; with 800 u IL-6/ml, 0.15; with 1000 u IL-6/ml, 0.13; with 1200 u IL-6/ml, 0.1; with 1600 u IL-6/ml, 0.13.

URR (p18URR) has a very low-level activity in HepG2 cells. Addition of IL-6 to the medium activated p18URR in HepG2 cells and URR activity reached a maximum level with about 1200 units IL-6/ml medium (Fig. 1). Activation of the HPV-18 URR in HepG2 cells through overexpression of C/EBPß is accompanied by formation of the C/EBPß–YY1 switch region complex (Bauknecht *et al.,* 1996). Mutations in the switch region that abolished the complex formation also abrogated C/EBPß-induced transcriptional activation in HepG2 cells (Bauknecht *et al.,* 1996). To determine if IL-6-induced activation of the HPV-18 URR is dependent on a functional switch region, HPV-18 CAT plasmids containing mutations in the switch region (p18URR-22M1) were transfected into HepG2 cells and treated with the same amount of IL-6 as in Fig. 1 and extracts of transfected cells were analyzed for CAT activity (Fig. 2). In parallel, HepG2 cells were transfected with wild-type p18URR to control for the quality of the applied IL-6. In contrast to IL-6-induced activation of wild-type HPV-18 URR (p18URR) (Fig. 2A) mutations of the C/EBPß–YY1 binding site in the context of the HPV-18 URR (plasmid p18URR-22M1, containing OL22M1, the mutated switch region) prevented activation of the HPV-18 URR by IL-6 in HepG2 cells (Fig. 2B). These results demonstrate that activation of the HPV-18 URR by IL-6 in HepG2 cells is restricted to a functional switch



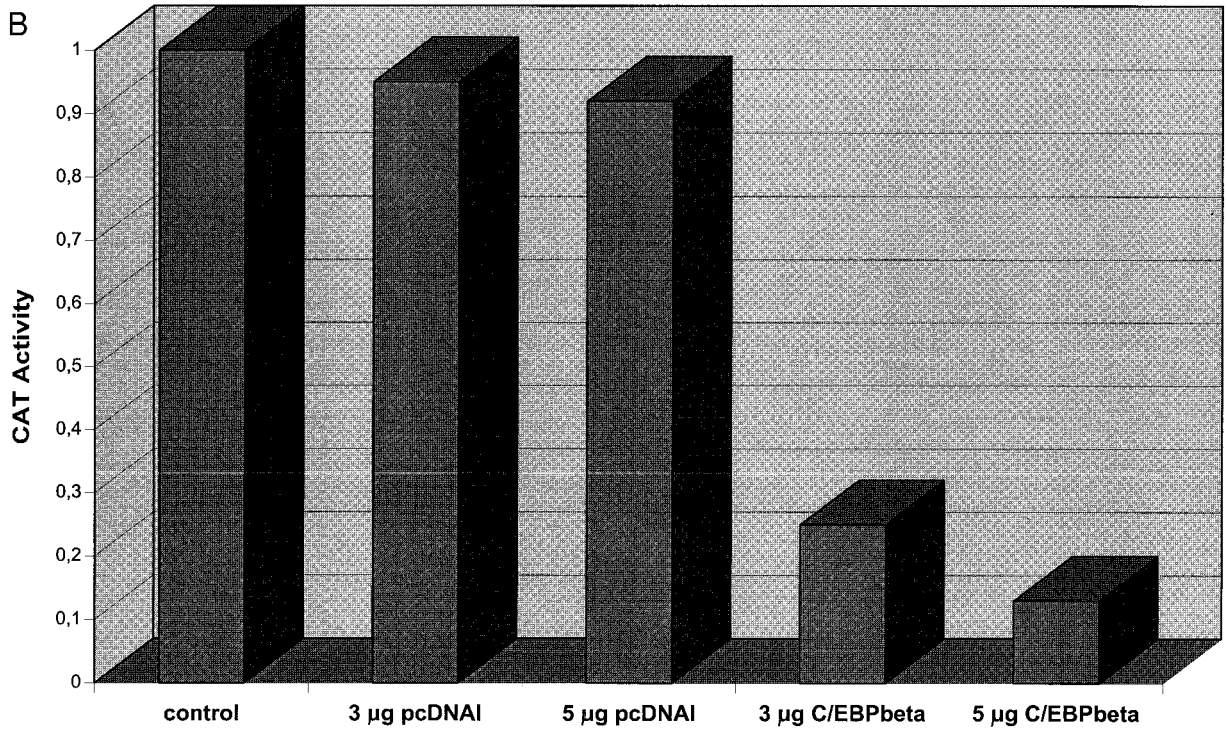


FIG. 3. (A and B) Overexpression of C/EBPβ in the cervical carcinoma cells C4-1 and SW756 results in downregulation of HPV-18 URR activity. Each cell line was transfected with 18 μg of p18URR together with increasing amounts of CMV-C/EBPβ (lanes 2–5) and 1.0 μg RSV/L as an internal control. Cell lines: (A) C4-1 and (B) SW 756. Column 1, control; columns 2–5, increasing amounts of cotransfected CMV-C/EBPβ plasmid. CAT activities were quantified relative to the activity of the control transfection, with only 18 μg p18URR and 1.0 μg RSV/L, which was set at 1.0. (A) Relative CAT activities were as follows: control, 1.0; 3 μg pcDNAI, 0.95; 5 μg pcDNAI, 0.92; 3 μg C/EBPβ, 0.3; 5 μg C/EBPβ, 0.16. (B) Control, 1.0; 3 μg pcDNAI, 0.95; 5 μg pcDNAI, 0.93; 3 μg C/EBPβ, 0.25; 5 μg C/EBPβ, 012.

region, which is in accordance with what was shown for C/EBPß-induced activation of the HPV-18 URR in HepG2 cells (Bauknecht *et al.,* 1996).

In contrast to HepG2 cells where overexpression of C/EBPß was shown to activate the HPV-18 URR, HPV-18 URR activity is repressed by C/EBPß in HeLa cells (Bauknecht and Shi, 1998). To determine the effect of C/EBPß in other cervical carcinoma cells, we performed cotransfection studies of the HPV-18 URR CAT construct and the C/EBPß expression plasmid in C4-1 and SW756 cells (Fig. 3). Overexpression of C/EBPß resulted in a downregulation of the HPV-18 URR in both cell lines, similar to that shown in HeLa cells.

Next we analyzed the effect of IL-6 on HPV-18 URR activity in HeLa cells in which the URR activity is strongly suppressed by overexpression of C/EBPß. As shown in Fig. 4, addition of IL-6 (up to 2000 units IL-6/ml medium) had no effect on HPV-18 promoter activity. Similar results

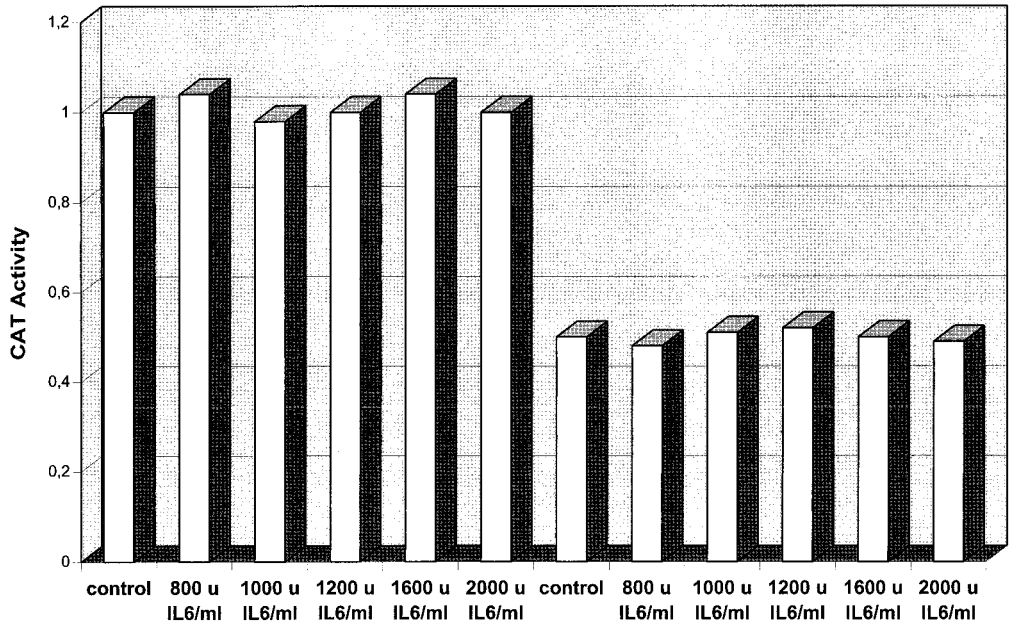


FIG. 4. In HeLa cells, HPV-18 URR activity is not affected by the C/EBPβ inducer IL-6. HeLa cells were transfected with 2 μg (lanes 1 to 6) or 1 μg (lanes 7 to 12) of p18URR together with 0.5 μg RSV/L as an internal control. Treatment with IL-6 was carried out 6–24 h after transfection for up to 48 h. CAT activities were quantified relative to the activity obtained with p18URR without IL-6 treatment (2 μg of transfected DNA) (lane 1), which was set at 1.0 [for 1 μg of transfected DNAs (lanes 7 to 12), the activities are quantified relative to p18URR set at 1, lane 1]. Lanes 1 (2 μg of p18URR) and 7 (1 μg of p18URR), without IL-6 treatment; lanes 2 to 6 (2 μg of p18URR) and lanes 7 to 12 (1 μg of p18URR), increasing amounts of IL-6 from 800 to 2000 u IL-6/ml. Relative CAT activities were as follows: lane 1 (control), 1.0; lane 2 (800 u IL6/ml), 1.04; lane 3 (1000 u IL6/ml), 0.98; lane 4 (1200 u IL6/ml), 1.0; lane 5 (1600 u IL6/ml), 1.04; lane 6 (2000 u IL6/ml), 1.0; lane 7 (control), 0.5; lane 8 (800 u IL6/ml), 0.48; lane 9 (1000 u IL6/ml), 0.51; lane 10 (1200 u IL6/ml), 0.52; lane 11 (1600 u IL6/ml), 0.5; lane 12 (2000 u IL6/ml), 0.49.

were observed with the HPV-18-positive cervical carcinoma cell lines SW756 and C4-1, as the transfected HPV-18 URR CAT construct p18URR was not affected in either cell line by IL-6 treatment (data not shown). These results suggest that in HeLa, SW756, and C4-1 cells one or several components of the IL-6/IL-6 receptor complex or a mechanism in signal transduction might be affected and prevent induction of C/EBPß in these cells.

IL-6 receptor complex: Expression of the 80-kDa IL-6 binding receptor protein (IL-6R)

As described above, reduced expression of one of the IL-6 receptor complex components could be responsible for the nonresponsiveness of the HPV-18 URR to IL-6 treatment in the cervical carcinoma cell lines HeLa, SW756, and C4-1. To define this component, we first analyzed the expression of the 80-kDa IL-6R component in each cervical carcinoma cell line and in HepG2 cells by RT–PCR and Northern blot. When we analyzed the cells by RT–PCR for expression of IL-6R (Fig. 5A) we observed a strong signal in HepG2 cells (lane 9) compared to slight signals in HeLa (lane 8), C4-1 (lane 7), and SW756 cells (lane 6). Lanes 1 to 4 demonstrate the β-actin control with each sample of the different cell lines. Lanes 5 and 10 demonstrate the controls with water. The same difference was observed when we analyzed IL-6 receptor mRNA expression by Northern blot (Fig. 5B). As shown in Fig. 5A, in contrast to HepG2 cells, in each of the three cervical carcinoma cell lines we detected only a very weak expression of endogenous IL-6 receptor mRNA by Northern blot analyses (Fig. 5B).

Flow cytometry analyses for quantification of the IL-6R-positive cells were performed. In addition to several cervical carcinoma cell lines we add to our analyses of the IL-6R expression two different passages of HPV-16-transformed human keratinocytes, low-passage-number HPK IAP49 cells, and high-passage HPK IAP359 cells. Recently, high-passage-number HPK cells were shown to be tumorigenic (Dürst *et al.,* 1995). Cell surface expression of IL-6 receptor was examined in comparison of the binding reactivities of a specific anti-IL-6 receptor MAb with that of an isotype-matched control MAb. Their fluorescence profiles are shown in Fig. 6. Significant staining was observed in HepG2, with a positivity of over 66% for IL-6R. When we analyzed the cervical carcinoma cell lines HeLa, C4-1, and SW756 we observed with HeLa cells an over 50% reduced expression of IL-6R compared to HepG2 cells (Fig. 6). Moreover, our analysis of C4-1 as well as SW756 cells revealed a complete downregulation of the IL-6 receptor in these cells. Interestingly, when we analyzed HPK IAP49 and HPK IAP359 cells, we observed that the low-passage-number HPK IAP49 cells of HPV-16-transformed human keratinocytes give rise to a more intensive IL-6R expression signal than the high-passage-

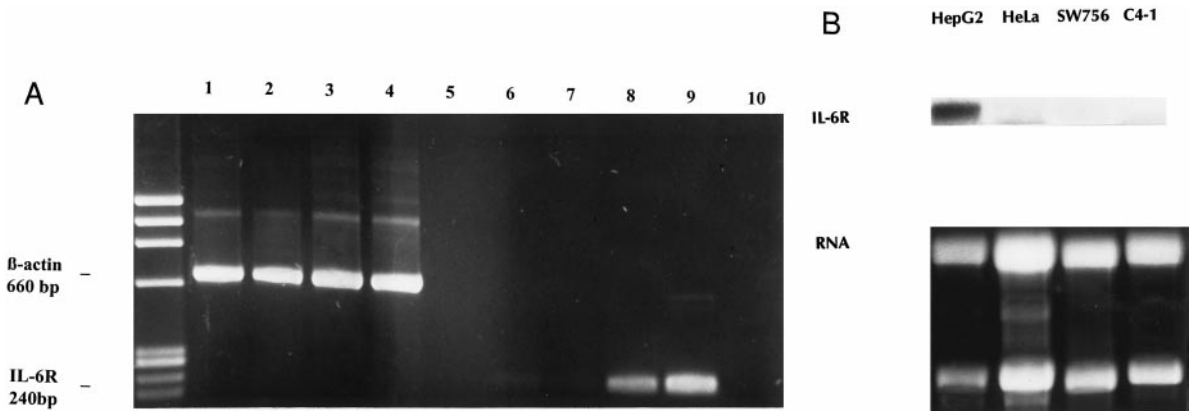


FIG. 5. RT–PCR and Northern blot analysis of IL-6 receptor (IL-6R) expression in cervical carcinoma and HepG2 cells. (A) Analysis of IL6-R expression by RT–PCR. Only weak signal intensities were observed in SW756 and C4I cells. The marker X174 was digested with *Hae*III. As controls in lanes 1–4, β-actin was amplified to normalize PCR products obtained from different cell lines. Additional controls with H_2O are shown in lanes 5 and 10. Lane 1, SW756; lane 2, C4-1; lane 3, HeLa; lane 4, HepG2; lane 5, H_2O; lane 6: SW756; lane 7, C4-I; lane 8, HeLa; lane 9, HepG2; lane 10, H_2O. (B) Analysis of IL-6R expression by Northern blot. On the bottom, RNA loading of the gel is shown. HepG2 cells (lane 1) were used as a positive control for IL-6 receptor expression. Lanes 2–4 show weak expression of IL-6 receptor in different cervical carcinoma cell lines.

number HPK IAP359 cells and the cervical carcinoma cell line HeLa (Fig. 6).

IL-6 receptor complex: Expression of the 130-kDa signal transducing protein (gp130)

After binding of IL-6 to its receptor, the ligand–receptor complex is associated with gp130, the signal-transducing component of the IL-6 receptor (Rose-John and Heinrich, 1994). The exact mechanism of signal transduction by the IL-6 receptor complex is still not known, although it has been reported that dimerization of gp130 initiates signal transduction via the JAK/STAT pathway (Akira *et al.,* 1994; Lutticken *et al.,* 1994; Stahl *et al.,* 1994; Wegenka *et al.,* 1994; Zhong *et al.,* 1994). IL-6 stimulation has also been shown to activate RAS and mitogen-activated protein kinases (MAPK) that are essential for C/EBPß (NF-IL-6) activation (Nakajima *et al.,* 1993).

Determination of expression of gp130 at the mRNA level by RT–PCR indicates that gp130 is expressed in all cell lines (Fig. 7). The expression of gp130 mRNA in the IL-6-responsive cell line HepG2 was used as a control. These results are consistent with previous reports showing that gp130 is expressed in nearly all examined human and mouse cell lines (Taga and Kishimoto, 1992; Ganapathi *et al.,* 1996). This is not surprising since gp130 has been shown to be signal transducer for several other cytokines with overlapping biological functions, including IL-11, leukemia inhibiting factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) (Gearing *et al.,* 1992; Ip *et al.,* 1992; Liu *et al.,* 1992; Yin *et al.,* 1993; Pennica *et al.,* 1995).

DISCUSSION

The HPV URR controls transcription of the E6 and E7 oncogenes which are involved in the carcinogenesis of HPV-induced cervical cancer. The transcription factor C/EBPß plays an important role in the transcriptional regulation of HPV-18. In HeLa cells, C/EBPß forms a complex with YY1 and contributes to about 50% of the URR activity. In several reports it was shown that the HPV-18 URR has a very high activity in HeLa cells but is inactive in HepG2 cells (Bauknecht *et al.,* 1995, 1996). Overexpression of C/EBPß in HepG2 cells was shown to activate the HPV-18 URR by forming a C/EBPß–YY1 complex which binds to the switch region OL22 in the HPV-18 URR (Bauknecht *et al.,* 1996). In contrast, a further increase of C/EBPß in HeLa cells by its overexpression results in strong repression of HPV-18 URR activity (Bauknecht and Shi, 1998). It was shown that overexpression of C/EBPß in HeLa cells specifically interferes with TBP binding to the TATA box of the HPV-18 URR, but not in HepG2 cells, where it activates the URR (Bauknecht and Shi, 1998).

C/EBPß (NF-IL-6) is regulated by IL-6 at the level of both transcription and posttranslational modifications (Akira *et al.,* 1990; Wedel and Ziegler-Heitbrock, 1995). The strong repression effect of C/EBPß on HPV-18 URR activity by its overexpression in HeLa cells lets us suggest that IL-6 treatment should result in similar repression of HPV-18 URR activity.

In this report we have shown that HPV-18 URR is activated in HepG2 cells by IL-6 treatment (Fig. 1). This activation is similar to that seen when C/EBPß is overexpressed in HepG2 cells (Bauknecht *et al.,* 1996) and demonstrates an intact C/EBPß induction pathway. Recently, the OL22M1 mutation of the switch region sequence was shown to be defective in competition for the formation of the C/EBPß–YY1 complex in gel retardation assays. This provided a correlation between binding of C/EBPß to the switch region and the ability of C/EBPß to

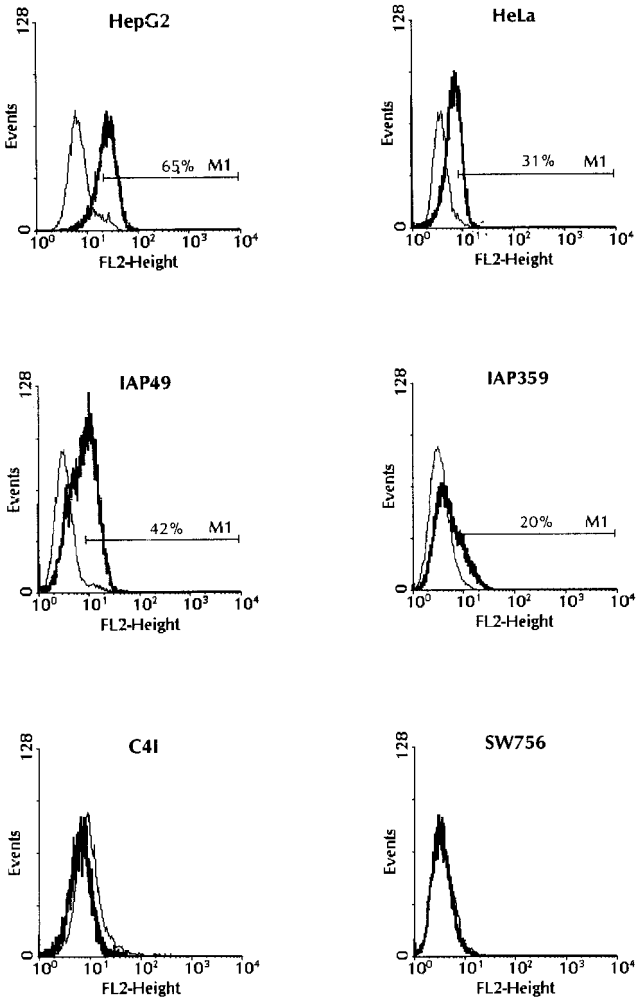


FIG. 6. Flow cytometric analysis of the cell surface expression of IL-6R by various tumor cell lines stained with an anti-IL-6R MAb (heavy line) and an isotype-matched control MAb (thin line). Bound antibodies were detected with biotinylated goat F/ab$_2$, anti-mouse IgG1, and R-ttPE-SA. *X*-axis, log$_{10}$ of FL-2 intensity; *Y*-axis, relative cell number. (%) Percentage of cells being positive for IL-6R expression. Details are described under Materials and Methods.

activate the HPV-18 URR in HepG2 cells (Bauknecht *et al.,* 1996). As we could demonstrate that p18URR-22M1 is not activated by IL-6 in contrast to wild-type HPV-18 URR (p18URR) (Fig. 2), we conclude that activation of URR by IL-6 is restricted to the switch region of the HPV-18 URR. However, it was not possible to induce any IL-6 response in the HPV-18-positive cervical carcinoma cell lines HeLa (Fig. 4). No effect on URR activity was observed when C4-1 and SW756 cells were treated with IL-6 (data not shown). In this regard it will also be interesting to analyze the effect of IL-6 on the HPV-18 URR after introduction into primary keratinocytes.

It can be postulated that induction of C/EBPß would have a strong repression effect on HPV-18 URR activity, similar to that seen when C/EBPß is overexpressed in HeLa cells. In addition it was shown that overexpression of C/EBPß also causes significant downregulation of the HPV-18 E6/E7 mRNA level in HeLa cells (Bauknecht and Shi, 1998). It was reported that reduced E6/E7 expression negatively affects the growth rate of cervical carcinoma cells (von Knebel-Doeberitz *et al.,* 1988; Kyo *et al.,* 1993). Therefore, it is not unexpected that cervical carcinoma cells seem to lack an intact IL-6/C/EPBß pathway. This is in accordance with our analysis of two other cervical carcinoma cells demonstrating that overexpression of C/EBPß strongly represses HPV-18 URR activity (Figs. 3A and 3B), as in the case in HeLa cells. In this context it seems interesting to analyze if introduction of an E6/E7 heterologous expression system results in downregulation of HPV-18 URR responsiveness to IL-6 in HepG2 cells.

Iglesias *et al.* (1995) reported that in normal cervical epithelium, the presence of HPV-16 or -18 DNA significantly decreased the expression of cytokines, including IL-1ß, IL-8, and IL-6, compared to normal epithelium. Both IL-6 receptor and its signal transducer gp130 could be detected in all cell lines by RT–PCR (Figs. 5 and 7). However, Northern blot analyses revealed in all cervical carcinoma cells lines strongly reduced expression levels of IL-6 receptor compared to the IL-6-responsive HepG2 cells (Fig. 5B). The percent quantification of flow cytometry data with the different cell lines supports this observation (Fig. 5A). As shown in Fig. 6, flow cytometry analysis detected a significantly higher amount of IL-6 receptor in the HepG2 and HPK IAP49 cell lines than in the three cervical carcinoma cell lines HeLa, C4-1, and SW756 and the high-passage-number cell line HPK IAP359, which was shown to be tumorigenic (Dürst *et al.,* 1995). The reported results point to an involvement of the IL-6 expression level in the process of HPV-induced carcinogenesis. Our current analyses of cervical tumors support this suggestion (Bauknecht and Bauknecht, unpublished results). We are planning to analyze a larger number of tumors to clarify if reduced levels of IL-6R are HPV specific. In this context it will be interesting to analyze if there is a difference in IL-6 responsiveness of

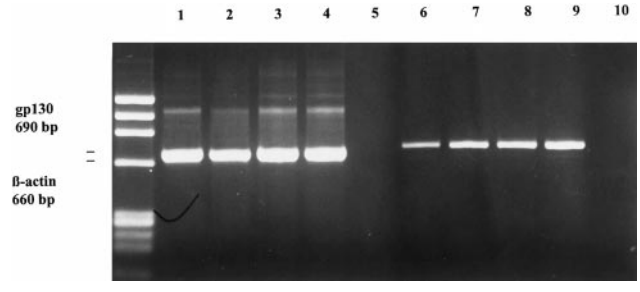


FIG. 7. Expression of gp130 mRNA in different cell lines analyzed by RT–PCR. RT–PCR was performed with 25 cycles. Expected signals were detected with a 690-bp fragment (lanes 6–9). Lanes 1–4, β-actin was amplified to normalize PCR products from different cell lines (660-bp fragment). Lanes 5 and 10 show controls with H_2O. Lane 1, SW756; lane 2, C4-1; lane 3, HeLa; lane 4, HepG2; lane 5, H_2O; lane 6, SW756; lane 7, C4-1; lane 8, HeLa; lane 9, HepG2; lane 10, H_2O.

the HPV-16 URR in early versus late passage HPV-16 immortalized keratinocytes by analyzing endogenous HPV-16 mRNA expression in the two cell lines HPK IAP49 and HPK IAP359.

Reduced expression of IL-6 receptor may not be the sole cause of the nonresponsiveness of the cervical carcinoma cell lines against IL-6 treatment. The mechanism for IL-6 resistance in cervical carcinoma cells may involve defects not only on the level of IL-6 receptor expression but also steps further downstream of the signal cascade. As shown in our flow cytometry studies, HeLa cells express significantly more IL-6 receptor molecules than C4-1 or SW756 cells (Fig. 6). We are currently analyzing if other components of the signal transduction cascade are affected in HeLa cells (Bauknecht and Bauknecht, unpublished results).

Tumor development and progression are often accompanied by the alteration of response to cytokines and growth factors and the disruption of the balance between negative and positive proliferation signals may provide growth advantages. IL-6 has been reported to act as a growth factor of normal squamous epithelial cells (Yoshizaki *et al.,* 1990). In view of the lack of the IL-6 receptor it seems to be controversial that IL-6 was shown to act as a growth factor in cervical carcinoma cells. Iglesias *et al.* (1995) demonstrated in HPV-16- and HPV-18-transformed cervical cell lines an EGF-receptor-dependent pathway involving autocrine stimulation of growth factors by IL-6. In our study we could not detect changes in growth behavior after IL-6 treatment of HeLa, C4-1, SW756, HPK IA49, and HPK IA359; this was analyzed by cell counting, BrdU proliferation assay, and cell cycle analysis (data not shown). However, we have not used IL-6 antibodies. Their application to the medium of cultured cervical carcinoma cell lines has been shown to induce growth repression (Eustace *et al.,* 1993; Tartour *et al.,* 1994). Takano *et al.* (1996) reported a high diversity of IL-6 production and its receptor expression in several cervical dysplasia and carcinoma cell lines. Due to this observation he concluded that there was a loss of IL-6 signal transduction capability in the course of progression of dysplasia to cervical carcinoma (Takano *et al.,* 1996). In conclusion, this study supports the theory that there is a heterogenic mechanism by which tumor cells acquire changes and resistance to specific signal transduction pathways, achieving more growth advantage. Further investigations have to focus on expression patterns of IL-6 receptor in the course of different steps of dysplasias to carcinoma tissues.

MATERIALS AND METHODS

Cell culture and reagents

HepG2 (human hepatocellular carcinoma) as well as the HPV-18-positive cell lines C4-1, HeLa, and SW756 (cervix carcinoma) were obtained from ATCC (Rockville, MD). HPK IAP49 and HPK IAP359, representing increasing passages of HPV-16-transformed human keratinocyte cell lines, were kindly provided by Dr. M. Dürst (gynäkologische Molekularbiologie, Universität Jena, Germany). Tumor cells were maintained as monolayers in antibiotic-free D-MEM medium supplemented with *N*-acetyl-L-alanyl-L-glutamine and 10% heat-inactivated FCS (PAA, Linz, Austria) in a humidified atmosphere of 5% CO_2 at 37°C.

Human recombinant IL-6 was purchased from Boehringer (Mannheim). Phycoerythrin-conjugated streptavidin (R-PE-SA) was purchased from Coulter-Immunotech (Hamburg, Germany). Anti-IL-6R MAb (B-N12, anti-gp80 IgG1) was obtained from BioSource (Ratingen, Germany), and biotin-conjugated goat $F(ab')_2$ anti-mouse IgG1 from Coulter-Immunotech. Isotype-specific mouse IgG1 was purchased from Southern Biotechnology Associates (Birmingham, AL).

Transfection and CAT assay

HeLa, SW756, and C4-1 were transfected by the calcium phosphate coprecipitation method (Chen and Okayama, 1987). Transfections were performed as described (Chen and Okayama, 1987) and were done with 1 to 18 μg HPV-18 URR CAT containing reporter constructs, 3 and 5 μg of the cytomegalie (CMV)-driven C/EBPβ expression plasmid (CMV-C/EBPβ), and 0.5 to 1 μg RSV/L as an internal control (de Wet *et al.,* 1987). Plasmid p18URR contains the complete HPV-18 URR (nucleotides 6930 to 103 of the HPV-18 genome) fused to the CAT gene of pBLCAT3 as previously described (Bauknecht *et al.,* 1992). p18URR-22M1 contains HPV-18 URR with a mutation in the switch region (Bauknecht *et al.,* 1996). Cytokines were applied after transfections. CAT assays were performed by using extracts with equal luciferase counts. To circumvent a possible effect of the effector plasmid expressing C/EBPβ on the cotransfected RSV–luciferase construct RSV/L, CAT assays were also performed in parallel, using the same protein concentrations. To circumvent a possible IL-6 induction of the cotransfected RSV–luciferase construct, transfected cells were, after an overnight exposure to the precipitates, split in a 1:2 ratio on tissue plates and medium was added to each transfection with and without IL-6. After treatment, CAT assays were performed by using equal amounts of luciferase counts of the nontreated cells with the same protein amount of the appropriate IL-6-treated cells. All transfections were repeated at least five times. Quantification of CAT assays was performed only when the percentage of acetylated CAM was below 80%, for the assay to be within the linear range. CAT activities were quantified by cutting spots from thin-layer chromatography plates and determining the radioactivity by liquid scintillation. Results from individual transfection varied less than 20%.

RNA extraction and RT–PCR

Total cellular RNA was prepared using the guanidium thiocyanate method (Chomczynski and Sacchi, 1987). Reverse transcription was performed on 1 μg of total RNA followed by PCR amplification. β-actin was amplified as an external control for equalizing the obtained cDNA from the different cell lines.

For interleukin-6 receptor (IL-6R) analysis, a primer pair corresponding to nucleotide positions 544 to 564 and 763 to 784 of psBSF2R.236 was used (Yamasaki *et al.,* 1988). Amplification of gp130 was initiated by primers corresponding to nucleotides 1767–1791 of the coding strand, respectively, 2480 to 2456 to the noncoding strand (Hibi *et al.,* 1990). A single amplified product of the predicted size was obtained for IL-6R (240 bp), gp130 (690 bp), and β-actin (661 bp). Thirty cycles for IL-6R and 25 cycles for gp130 were performed and the PCR products were electrophoresed on an 2% agarose gel.

Northern blot

Total cellular RNA (15 μg) was denatured and electrophoresed in 1% agarose gels and transferred to nitrocellulose filters (Hybond N,$^+$ Amersham, Buckinghamshire, UK). Filters were prehybridized for 1–3 h at 42°C in 50% deionized formamide, 5× SSPE, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 20 μg/ml denatured salmon DNA. The 1.7-kb *Xho*I–*Xho*I fragment of the human IL-6 receptor cDNA (pBSF2R.236) was used as a probe and labeled with ^{32}P by the random oligonucleotide method. Hybridization was carried out at 42°C in the same buffer, with the addition of 1×10^6 cpm/ml of the labeled probe. After hybridization, the filters were washed three times in 2× SSC plus 0.1 sodium dodecyl sulfate for 5 min at room temperature, followed by three washes in 0.1× SSC plus 0.1% sodium dodecyl sulfate for 15 min at 50°C. Filters were exposed to Kodak X-OMAT film for 2–5 days. Filters were subsequently rehybridized to a human β-actin probe to ascertain the differences in RNA loading among the different samples.

Flow cytometric analysis

Cell surface expression of IL-6R by tumor cells was assessed on a FACScan cytometer with laser excitation at 488 nm (Becton–Dickinson, Mountain View, CA) using standard instrument parameters. Flow data was collected on forward- and side-scattered gated populations. Ten thousand events per sample were acquired as list mode data and the files analyzed using Lysis II software. Data were evaluated by determining the difference of the mean channel fluorescence intensity (MFI) of cell populations stained with the specific and control MAbs. Cells (0.5×10^6/10 μl) were harvested with PBS without Ca^{2+}, Mg^{2+} containing trypsin/EDTA (0.05%/0.02%, w/v). In outline, the method depends on a high-sensitivity three-layer staining, all steps being performed at 4°C with ice-cold labeling puffer (PBS without Ca^{2+}, Mg^{2+} supplemented with 3% heat-inactivated FCS and 0.05% NaN_3) and using the following incubation sequence: (1) unconjugated anti-IL-6R MAb (25 μl, 12 μg/ml/10^6 cells), 120 min in the dark, (2) biotin-conjugated goat $F(ab')_2$ anti-mouse IgG1 (5 μl, 5 μg/ml), 45 min in the dark, and (3) R-PE-SA (25 μl, 2.5 μg/ml), 45 min in the dark. After centrifugation, cells were resuspended in 200 μl propidium iodide (5 μg/ml labeling puffer) to stain and exclude dead cells. Control stainings included an irrelevant, isotype-matched mouse IgG1 MAb.

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