Regulation of interleukin-6 gene expression by pro-inflammatory cytokines in a colon cancer cell line

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The two carcinoma cell lines HeLa and HTM-29 show different behaviour in terms of interleukin-6 (IL-6) production. Analyses of secreted IL-6 by ELISA and of IL-6 mRNA by reverse transcription-PCR revealed that, whereas HeLa cells produced high levels of IL-6 in response to tumour necrosis factor- α (TNF- α) and IL-1 β , the HTM-29 cell line failed to produce both IL-6 protein and mRNA. Nevertheless, the transcription factors nuclear factor- κ B (NF- κ B) and NF-IL6, the main factors involved in IL-6 gene transcriptional activation by cytokines, were activated in both cell lines after treatment with TNF- α or IL-1 β . In order to verify that the lack of IL-6 expression in HTM-29 cells was not due to an endogenous IL-6 gene deficiency or to IL-6 mRNA instability, we carried out transient transfection assays with an IL-6 promoter-reporter construct. Strong activation of the IL-6 promoter by cytokines could be observed in HeLa cells, whereas no induction could be detected in cytokine-treated HTM-29 cells. These cytokines induced a very strong stimulation of NF- κ B-mediated transcription in HeLa cells transfected with a κ B luciferase reporter construct, whereas no induction could be detected in cytokine-stimulated HTM-29 cells. Thus IL-6 promoter repression in HTM-29 cells probably results from a

failure of cytokine-activated NF- κ B to exert its transactivating activities. Western blotting experiments demonstrated that the lack of NF- κ B-mediated transcription was not due to increased expression of I κ B (inhibitor of NF- κ B) proteins in HTM-29 cells. Co-transfection experiments with the κ B Luc reporter construct and the CBP [CREB (cAMP response element binding protein) binding protein] expression vector showed that the impairment in NF- κ B-dependent transcription did not result from a deficiency in the co-activator CBP. Interestingly, both NF- κ B-mediated transcription and IL-6 promoter activation could be restored in HTM-29 cells by transfection with RelA. Furthermore, CBP could have a significant synergistic effect on exogenous RelA-mediated transcription. Since sequencing of the endogenous *relA* gene did not reveal any mutation, it is likely that repression of NF- κ B-mediated transcription results from negative cross-talk between NF- κ B and another nuclear factor specifically expressed or regulated by TNF- α in HTM-29 cells.

Key words: CREB binding protein, NF- κ B, RelA, TNF- α , transcriptional activation.

INTRODUCTION

Interleukin-6 (IL-6) contributes to a multitude of physiological and pathophysiological processes (for reviews, see [1,2]). Among its many functions, IL-6 plays an active role in immunological responses, bone metabolism, reproduction, inflammation, neoplasia and aging. Overexpression of IL-6 has been implicated in the pathology of a number of diseases, including multiple myeloma, rheumatoid arthritis, Castleman's disease, psoriasis, Alzheimer disease and post-menopausal osteoporosis [1,2].

In view of its multiple functions, studies on the regulation of IL-6 gene expression may be of prime importance. This cytokine is produced not only by immune and immune-accessory cells, but also by many non-immune cells, such as osteoblasts, keratinocytes, synoviocytes, epithelial cells, etc. IL-6 expression can be induced by a broad range of cell stimuli (inflammatory cytokines, bacterial products, acute viral infections, etc.), and different mechanisms may act individually or co-ordinately to enhance transcription. Various groups have examined targets of signal transduction within the promoter region of the human IL-6 gene, including an AP-1 (activator protein-1) consensus site [3], a

cAMP response element (CRE) [4], a nuclear factor (NF)-IL6 binding site [5] and an NF- κ B-responsive element [6].

The transcription factor NF- κ B consists of homodimers or heterodimers of proteins that belong to the Rel family (for a review, see [7]). In mammalian cells, this family includes the oncogene c-Rel, p50, p52, p65 (RelA) and RelB. NF- κ B/Rel transcription factors are present in most cells and tissues as inactive cytosolic proteins. The retention of NF- κ B in the cytosol is due to its interaction with inhibitory proteins of the I κ B (inhibitor of NF- κ B) family. Principal members of this family are I κ B α , I κ B β and the two proteins p100 and p105, which are precursors of p52 and p50 respectively. NF- κ B can be induced by a broad spectrum of signals, including mitogens, cytokines, bacterial lipopolysaccharide, viruses, double-stranded RNA and UV light. Despite the differences among signals, one of their targets is the cytoplasmic NF- κ B-I κ B complex. Treatment of cells with inducers of NF- κ B activity leads to phosphorylation, ubiquitination and degradation of the I κ B proteins, thus releasing NF- κ B and allowing it to translocate into the nucleus to activate transcription. In this way, NF- κ B regulates the expression of many genes coding for proteins involved in immune and inflam-

Abbreviations used: AP, activator protein; CRE, cAMP response element; CREB, CRE binding protein; CBP, CREB binding protein; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ERK, extracellular-signal-regulated kinase; I κ B, inhibitor of NF- κ B; IL-6 (etc.), interleukin-6 (etc.); MAPK, mitogen-activated protein kinase; NF, nuclear factor; PKA, protein kinase A; RAI, RelA-associated inhibitor; RBP-J κ , J κ recombination signal sequence binding protein; RT-PCR, reverse transcription-PCR; TNF- α , tumour necrosis factor- α .

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matory responses, such as cytokines, adhesion molecules, acute-phase response proteins, etc.

It has been shown that co-transfection of NF-IL6 with the NF- κ B p65 subunit synergistically activates an IL-6 promoter-reporter construct, indicating that the two factors are sufficient to sustain IL-6 gene activation [8]. These results, however, cannot account for the diversity of tissue-specific expression of the IL-6 gene following treatment with IL-1 β or tumour necrosis factor- α (TNF- α). Indeed, normal liver, for example, does not express IL-6, although hepatocytes require active forms of NF- κ B and NF-IL6 to regulate several genes of the acute-phase response [9–11]. Furthermore, it has been shown that polymorphonuclear cells are not able to produce IL-6 following stimulation with lipopolysaccharide, although they can express the IL-8 gene, which is under the control of the two transcription factors NF- κ B and NF-IL6 [12].

To investigate in more detail the differential transcriptional regulation of the IL-6 gene, we chose two carcinoma cell lines, HeLa and HTM-29, which exhibit opposite behaviour in terms of IL-6 production after treatment with TNF- α and IL-1 β . We demonstrate that in HTM-29 cells, unlike HeLa cells, IL-6 promoter activation in response to cytokines was impaired, and that this deficiency could be correlated with a lack of NF- κ B-mediated transcription. Although TNF- α induced significant NF- κ B activation in both cell lines, we tried to understand why endogenous TNF- α -activated NF- κ B was unable to play its transactivating role in HTM-29 cells. We show that the lack of NF- κ B-mediated transcription in TNF- α -activated HTM-29 cells resulted neither from overexpression of an I κ B protein nor from a deficiency in the CBP [CRE binding protein (CREB) binding protein] co-activator, and that it could be restored by exogenous RelA overexpression. Since the sequencing of the *relA* gene did not reveal any mutation in HTM-29 cells, it is likely that NF- κ B-mediated transcriptional repression results from negative cross-talk between NF- κ B and another nuclear factor specifically expressed or activated by TNF- α in HTM-29 cells.

MATERIALS AND METHODS

Cell lines

The human colon adenocarcinoma cell line HTM-29 was propagated in RPMI 1640 containing ultraglutamine with 10% (v/v) foetal calf serum (BioWhittaker). The human cervix carcinoma cell line HeLa was cultivated in Eagle's minimal essential medium containing glutamine with 10% (v/v) foetal calf serum (BioWhittaker).

Antibodies and reagents

Polyclonal anti-p50, anti-p65 and anti-I κ B α antibodies were purchased from Euromedex, Souffelweyersheim, France. The monoclonal anti-p52 and the polyclonal anti-c-Rel and anti-RelB antibodies were from Santa Cruz Biotechnology. Recombinant human TNF- α and IL-1 β were purchased from Boehringer Mannheim.

Plasmids

The reporter plasmids p1168hu.IL-6P-luc+ and p1168hu.IL-6P(Ig- κ B)-luc+ contained 1168 bp of the human IL-6 promoter, either wild-type or mutated on the κ B site respectively. These plasmids and the p1481.IL-8P-luc+ plasmid, containing an IL-8 promoter fragment of 1481 bp, were gifts from Dr G. Haegeman and Dr W. Vanden Berghe (University of Gent, Belgium) [13]. The reporter construct (κ B)₅Luc was purchased from Strata-

gene. The plasmid RSV- β Gal was a gift from Dr R. Winkler (University of Liege, Belgium). The expression vectors containing the cDNAs for the human p65 subunit (pMT2T-p65) and the full-length mouse CBP protein (pCMXPL2CBP-HA) have been described previously [14,15].

Nuclear and cytoplasmic protein extraction

After stimulation with cytokines, cells were washed with cold PBS, then scraped and harvested by centrifugation (300 g, 5 min). They were resuspended in 1 ml of cold PBS and transferred to 1.5-ml Microfuge tubes. After centrifugation at 15000 g for 15 s, cells were resuspended with 1 ml of cold wash buffer [10 mM Hepes/KOH (pH 7.9), 20 mM KCl, 2 mM MgCl₂ and 0.1 mM EDTA]. Cells were then lysed by incubation for 30 s in 1 ml of cold buffer B [10 mM Hepes/KOH (pH 7.9), 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, protease inhibitors (Boehringer Mannheim) and Nonidet P-40 (0.2%)]. After centrifugation at 15000 g for 30 s, supernatants containing cytoplasmic proteins were removed and stored at -70 °C. The nuclear pellet was washed and nuclear protein extraction was carried out by incubation for 20 min on ice in a cold saline buffer [20 mM Hepes/KOH (pH 7.9), 1.5 mM MgCl₂, 0.2 mM EDTA, 650 mM NaCl, glycerol (25%, v/v), 1 mM DTT, 0.5 mM PMSF and protease inhibitors (Boehringer Mannheim)]. After centrifugation at 15000 g for 30 min at 4 °C, supernatants containing nuclear proteins were transferred to Microfuge tubes and stored at -70 °C. Protein concentrations were measured by the Bradford method using a commercial reagent (Bio-Rad).

Electrophoretic mobility shift assay (EMSA)

The sequences of the oligonucleotide probes used in this work were as follows: wild-type IL-6- κ B probe: sense, 5'-TGTGGG-ATTTTCCCATGACTC-3'; antisense, 3'-CCCTAAAGGGT-ACTGAGAAAC-5'; mutated κ B probe: sense, 5'-GGTTACA-ACTCACTTTCCGCTG-3'; antisense, 3'-TGTTGAGTGAAA-GGCGACGGTT-5'.

For measurement of binding to the κ B probe, nuclear proteins (5 μ g) were incubated for 20 min at room temperature with 0.2 ng of ³²P-labelled oligonucleotide probe in 10 μ l of a reaction mixture containing 2 μ g of BSA, 2.5 μ g of poly(dI-dC)·poly(dI-dC) (Pharmacia Biotech Benelux), 20 mM Hepes/KOH (pH 7.9), 130 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM MgCl₂ and 1 mM DTT. DNA-protein complexes were then resolved on a non-denaturing 6% (w/v) polyacrylamide gel run for 4 h at 180 V in 0.25 × TBE (2.5 mM Tris, 2.5 mM H₃BO₃, 2 mM EDTA, pH 8.5). The gel was dried and autoradiographed on Fuji X-ray film (General Electric). For competition experiments, unlabelled probe was added in excess in the binding buffer. For supershift experiments, protein extracts were preincubated with antibodies for 20 min on ice before addition of the labelled probe. The oligonucleotide probe (Eurogentec) was labelled by in-filling with Klenow DNA polymerase (Boehringer Mannheim) and [α -³²P]deoxynucleotides (3000 Ci/mmol; Du Pont De Nemours International) and then purified on a Sephadex G-25 Quick-spin column (Pharmacia Biotech Benelux). Specific radioactivity was always equal to or greater than 10⁸ c.p.m./ μ g.

Western blot analysis

Cytoplasmic extracts (15 μ g) were added to a loading buffer (10 mM Tris/HCl, pH 6.8, 1% SDS, 25% glycerol, 0.1 mM β -mercaptoethanol and 0.03% Bromophenol Blue), boiled and

electrophoresed on a 10% (w/v) polyacrylamide gel in the presence of SDS. After electrotransfer to Immobilon-P membranes (Millipore), proteins were probed with the appropriate primary and secondary antibodies, and finally analysed using the enhanced chemiluminescence system (ECL[®]) (Amersham) with Fuji X-ray film.

RNA extraction and reverse transcription-PCR (RT-PCR)

Total cellular RNA was extracted with guanidinium thiocyanate (Tripure; Boehringer Mannheim) according to the protocol from the supplier. cDNA was prepared by incubating 1 µg of RNA in 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT and RNase inhibitors (RNAGuard; Pharmacia Biotech) with 250 units of M-MLV (Moloney murine leukaemia virus) reverse transcriptase (Gibco BRL), 1 mM of each dNTP and random primers (0.05 mM; Gibco BRL) for 60 min at 37 °C. PCR was carried out using AmpliTaq DNA Polymerase (Perkin Elmer) according to the recommendations of the supplier. Primer sequences were as follows: for IL-6: sense, 5'-GTCTCCTCAT-TGAATCCAGATTGG-3'; antisense, 5'-AGCTCAGCTATG-AACTCCTTCTC-3'; for β -actin: sense, 5'-GCTTCGGCCG-GAACGTGTAC-3'; antisense, 5'-GACACGACGAGTGGC-TCCGC-3'. PCR was initiated in a thermal cycler programmed at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s (35 cycles). The sizes of the PCR products for IL-6 and β -actin were 338 and 200 bp respectively.

IL-6 determination

IL-6 protein levels in culture supernatants were measured by ELISA (Bender MedSystems, Vienna, Austria).

DNA transfection assays

Cells were transfected by the FUGENE liposome technique according to the protocol of the supplier (Boehringer Mannheim). After 24 h, fresh medium, with or without TNF- α or IL-1 β , was added to the cells for another 24 h. Then cells were washed twice in PBS and lysed. Luciferase activities were measured in cellular extracts (luciferase reporter gene assay; Boehringer Mannheim) and were corrected by normalization to levels of co-expressed β -galactosidase (chemiluminescent β -galactosidase reporter gene assay; Boehringer Mannheim).

RESULTS

Cytokine regulation of IL-6 production in HeLa and HTM-29 cells

The levels of IL-6 secreted by the two human carcinoma cell lines HeLa and HTM-29, untreated or stimulated with IL-1 β or TNF- α , were assessed in cell supernatants by ELISA. HTM-29 cells, both constitutively and after treatment with IL-1 β or TNF- α , failed to produce detectable levels of IL-6 (Figure 1). In contrast, HeLa cells constitutively secreted detectable amounts of IL-6 (Figure 1A). This basal release of IL-6 was significantly increased by stimulation with TNF- α (approx. 4-fold; Figure 1B) and was dramatically induced by stimulation with IL-1 β (approx. 36-fold) (Figure 1C).

Cytokine regulation of IL-6 mRNA accumulation in HeLa and HTM-29 cells

RT-PCR analysis performed on total RNA extracted from uninduced and TNF- α - or IL-1 β -treated HTM-29 cells showed that IL-6 mRNA was undetectable in this cell line (Figures 2B

and 2D). In agreement with the ELISA data, unstimulated HeLa cells expressed very low levels of IL-6 mRNA, which were greatly enhanced after treatment with cytokines (Figure 2). The time course of IL-6 gene induction by IL-1 β (Figure 2A) differed from that obtained on stimulation with TNF- α (Figure 2C). After treatment with IL-1 β , IL-6 mRNA production peaked at 4 h, before decreasing slowly. In the case of TNF- α , the kinetics were more rapid (peak at 2 h) and more transient, since levels began to decrease after 2 h. The IL-6 mRNA level obtained following TNF- α induction was lower than that observed with IL-1 β , but this small difference could not completely explain the much greater IL-6 secretion when HeLa cells were stimulated with IL-1 β (Figure 1).

NF- κ B induction in HeLa and HTM-29 cells treated with cytokines

The transcription factor NF- κ B plays an essential role in IL-6 gene regulation in response to cytokines [8,16]. In order to establish whether the lack of expression of the IL-6 gene in HTM-29 cells might be due to a lack of inducibility of this transcription factor, we examined NF- κ B activation in the two carcinoma cell lines after treatment with TNF- α or IL-1 β .

EMSA were performed using an oligonucleotide corresponding to the NF- κ B binding site from the 5'-regulatory region of the IL-6 gene as a probe. Nuclear extracts were prepared from cells treated for various times with either IL-1 β or TNF- α . Significant binding to the κ B probe was apparent in both cell lines after treatment with either TNF- α or IL-1 β , as illustrated by the two retarded complexes C1 and C2 in Figures 3(A) and 3(B). The lower-molecular-mass complex (C3) was constitutive. Whatever the inducer or the cell line, complexes C1 and C2 reached their maximal level between 0 and 30 min after cytokine addition. Unexpectedly, HTM-29 cells were more inducible, in terms of NF- κ B activation, by TNF- α than by IL-1 β .

Competition experiments with an excess of unlabelled oligonucleotide (either wild-type or mutated) and supershift assays with antibodies directed against the various members of the Rel family allowed us to determine the composition of the C1 and C2 complexes. Figure 3(C) illustrates the results of such experiments performed on nuclear extracts from HTM-29 cells stimulated with TNF- α . Competition experiments showed that the two bands (C1 and C2) resulted from the specific binding of nuclear proteins. Supershift experiments demonstrated that the inducible complexes C1 and C2 were the p65 homodimer and the p50/p65 heterodimer respectively. Identical results were obtained with HTM-29 cells treated with IL-1 β and with HeLa cells stimulated by TNF- α or IL-1 β (results not shown).

The effects of other transcription factors known to modulate IL-6 gene transcription, such as NF-IL6 and AP-1, were also considered. EMSA with oligonucleotides encompassing either the AP-1 or NF-IL6 binding sites of the IL-6 promoter were performed on the same nuclear extracts as those used in the preceding experiments. The two cytokines induced a specific binding activity at the NF-IL6 and AP-1 sites in exactly same manner in both cell lines (results not shown).

An exogenous IL-6 promoter is strongly induced by either TNF- α or IL-1 β in HeLa cells, but not in HTM-29 cells

The preceding experiments demonstrated that the failure of HTM-29 cells to up-regulate IL-6 gene expression does not result from the absence of active transcription factors. In order to verify that this lack of IL-6 expression was not due to a deficiency at the level of the endogenous IL-6 gene promoter, the effects of cytokines on an exogenous IL-6 promoter were studied in the two cell lines.

Figure 1 Time course of IL-6 secretion by HeLa and HTM-29 cells in response to IL-1 β or TNF- α

HeLa (■) or HTM-29 (▲) cells were incubated in serum-containing medium alone (A) or in the presence of TNF- α (100 units/ml) (B) or IL-1 β (100 units/ml) (C). At the indicated times, aliquots of the supernatants were removed and the IL-6 concentration was determined by ELISA. The data shown are means of three experiments.

Figure 2 Time course of IL-6 mRNA synthesis by HeLa and HTM-29 cells in response to IL-1 β or TNF- α

HeLa and HTM-29 cells were incubated in the presence of IL-1 β (100 units/ml) (A, B) or TNF- α (100 units/ml) (C, D). At the indicated times, cells were harvested for total RNA extraction. Detection of IL-6 and β -actin mRNAs was carried out by RT-PCR.

Transient transfection assays were performed with a plasmid containing the entire promoter of the IL-6 gene in front of the luciferase reporter gene (p1168hu.IL6P-luc+). In HTM-29 cells

the exogenous IL-6 promoter was not inducible by either TNF- α or IL-1 β , whereas in HeLa cells it was strongly activated: approx. 3-fold by TNF- α and 9-fold by IL-1 β (Figure 4A). IL-6

Figure 3 NF- κ B activation in HTM-29 and HeLa cells by IL-1 β or TNF- α

Nuclear extracts were prepared from HTM-29 (A) or HeLa (B) cells induced with either IL-1 β (100 units/ml) or TNF- α (100 units/ml) for various times. EMSAs were performed using a 32 P-labelled probe specific for the IL-6- κ B site. Competition experiments with an excess (10–100 $\times$) of unlabelled oligonucleotide [wild-type (wt) or mutated (mut)] and supershift experiments with antibodies (Anti-) against p50, p65, p52 and c-Rel were carried out on nuclear extracts of HTM-29 cells stimulated by TNF- α (C).

promoter stimulation and NF- κ B activation (Figure 3B) were more efficient with IL-1 β than with TNF- α , probably because, on HeLa cells, IL-1 β receptors are more numerous than those for TNF- α .

IL-6 promoter repression in HTM-29 cells does not involve RBP-J κ (J κ recombination signal sequence binding protein)

The IL-6- κ B site specifically binds the constitutive protein RBP-J κ , which acts as a repressor of basal transcription in the absence of detectable amounts of NF- κ B proteins, but which also limits the access of NF- κ B translocated to the nucleus after cytokine induction [17]. Under our EMSA conditions, neither of the bands could be attributed to a DNA–RBP-J κ complex. Nevertheless, in order to test the possible involvement of RBP-J κ in the IL-6 promoter repression observed in HTM-29 cells, the cytokine effect was studied using a mutated IL-6 promoter, in which the IL-6- κ B site was exchanged for the consensus κ B site of the Ig (immunoglobulin) gene promoter, which does not bind RBP-J κ .

Transient transfection assays were performed with a plasmid containing the mutated IL-6 promoter in front of the luciferase reporter gene (p1168hu.IL6P-luc+ mut). Figure 4(B) shows that competition between RBP-J κ and NF- κ B could not explain the lack of IL-6 promoter activity, since the mutated construct did not give rise to an increase in luciferase activity after cytokine stimulation. As expected, the constitutive activity of the mutated IL-6 promoter was increased approx. 2-fold in comparison with the wild-type promoter (results not shown). Unexpectedly, in HeLa cells the IL-6 promoter was less inducible when carrying the consensus Ig- κ B sequence compared with the IL-6- κ B sequence (Figures 4A and 4B). This phenomenon could be due to the binding of different NF- κ B complexes to the consensus Ig- κ B

Figure 4 Induction of exogenous IL-6-promoter-mediated transcription by IL-1 β or TNF- α

HTM-29 (□) and HeLa (■) cells were transiently transfected with reporter plasmids carrying the luciferase gene downstream of either the wild-type IL-6 promoter (p1168hu.IL-6-luc) (A) or a mutated IL-6 promoter [p1168hu.IL-6(Ig- κ B)-luc] (B). After 24 h, transfected cells were incubated in serum-containing medium alone (Co) or in the presence of IL-1 β (100 units/ml) or TNF- α (100 units/ml) for another 24 h. Then cells were harvested and lysed to determine luciferase activity. The values shown are representative of three independent transfections.

site, which would not be able to interact with the other IL-6-specific transcription factors or co-activators. Indeed, in HTM-29 and HeLa cells, the IL-6- κ B site was able to bind the p65 homodimer and the p65/p50 heterodimer (Figure 3C), whereas the consensus Ig- κ B site was recognized by the p50 homodimer and the p65/p50 heterodimer (results not shown).

NF- κ B-mediated transcription after cytokine activation is impaired in HTM-29 cells

In order to verify that IL-6 promoter repression resulted from a failure of NF- κ B to exert its transactivating activities after translocation to the nucleus, transient transfection assays were performed with a synthetic reporter gene construct containing five consensus κ B sites in front of the luciferase gene. A very strong stimulation of NF- κ B-mediated transcription was observed in HeLa cells treated with TNF- α or IL-1 β , whereas no induction could be detected in cytokine-stimulated HTM-29 cells (Figure 5A). Similar observations were made with another NF- κ B-dependent cytokine gene (the IL-8 gene promoter). As shown in Figure 5(B), luciferase activities driven by the IL-8 promoter could be enhanced by both IL-1 β and TNF- α in HeLa cells, whereas no induction could be detected in cytokine-stimulated HTM-29 cells. These observations, together with those reported with the (κ B)₅Luc construct, demonstrate that the lack of inducibility observed in HTM-29 cells is not related to the context of the IL-6 promoter, but only to a failure of the NF- κ B proteins to exert their transactivating functions.

Figure 5 Induction of NF- κ B- and IL-8-promoter-mediated transcription in HeLa and HTM-29 cells by IL-1 β or TNF- α

HTM-29 (□) and HeLa (■) cells were transiently transfected with the reporter plasmid (κ B₅Luc (A) or IL-8-luc (B)). After 24 h, transfected cells were incubated in serum-containing medium alone (Co) or in the presence of IL-1 β (100 units/ml) or TNF- α (100 units/ml) for another 24 h. Then cells were harvested and lysed to determine luciferase activity. The values shown are representative of three independent transfections.

Several hypotheses could explain this lack of NF- κ B-mediated transactivation after cytokine stimulation of HTM-29 cells. On the one hand, the I κ B proteins, depending on whether or not they are strongly expressed, can modulate κ B-dependent transcription. On the other hand, NF- κ B needs to interact with other transcription factors or co-activators to fulfil its transactivating functions, and one of these could be deficient.

The lack of NF- κ B-mediated transcription is not due to increased expression of I κ B proteins in HTM-29 cells

Following degradation of I κ B α and translocation of NF- κ B to the nucleus, I κ B α , the expression of which depends on NF- κ B, is rapidly resynthesized [18]. Newly synthesized I κ B α can migrate to the nucleus and recapture NF- κ B complexes bound to the DNA [19]. This mechanism allows NF- κ B activation to be transient; however, if I κ B α is highly expressed, this process can inhibit NF- κ B-mediated transcription. Moreover, some reports have demonstrated that high expression of the inhibitory p100 protein in various adenocarcinoma cell lines can be responsible for reduced NF- κ B-mediated transcription in response to stimuli such as TNF- α [20]. Indeed, TNF- α does not induce p100 processing. When the majority of the p65 complexes are sequestered by p100, stimulation by TNF- α leads to only weak NF- κ B translocation, which follows the dissociation of the p65 complexes

Figure 6 Expression levels of I κ B proteins in HeLa and HTM-29 cells

HTM-29 and HeLa cells were incubated in serum-containing medium alone (B, C) or in the presence of TNF- α (100 units/ml) for various times (A). Then they were harvested and lysed. Equal amounts of cytoplasmic proteins (15 μ g) were separated on an SDS/12% polyacrylamide gel, transferred to a PVDF membrane and revealed with a monoclonal antibody directed against I κ B α (A), anti-p50/p105 serum (B) or an anti-p52/p100 monoclonal antibody (C).

from I κ B α and is not sufficient to induce transactivation of the target promoter.

In order to determine whether the lack of NF- κ B-dependent transcription in HTM-29 cells could be due to the overexpression of inhibitory proteins, we compared, by immunoblotting, the levels of expression of I κ B α , p100 and p105 in HTM-29 and HeLa cells. As illustrated in Figure 6, the two cell lines constitutively expressed the three inhibitory proteins (I κ B α , p105 and p100) at very similar levels. After stimulation of HTM-29 cells by TNF- α , degradation of I κ B α could be detected in the cytoplasm (Figure 6A). I κ B α proteolysis began after a few minutes and was completed within 10 min. After 20 min of treatment, I κ B α was synthesized *de novo*. The I κ B α degradation kinetics corroborated the time course of NF- κ B translocation (Figures 3 and 6A). The same results were obtained with HTM-29 cells treated with IL-1 β and with HeLa cells stimulated by TNF- α (Figure 6A) or IL-1 β (results not shown). As expected, the inhibitory proteins p100 and p105 were not processed after stimulation of the two cell lines by TNF- α or IL-1 β (results not shown).

Thus it appears that none of the I κ B proteins are involved in the impairment of NF- κ B-mediated transcription in cytokine-stimulated HTM-29 cells.

The lack of NF- κ B-mediated transcription in HTM-29 cells cannot be restored by CBP/p300 alone, but can be rescued in conjunction with exogenous p65 expression or by p65 expression alone

Recent studies have suggested that the activity of many inducible transcription factors is regulated through their interaction with cellular co-activators such as CBP/p300 (for reviews, see [21,22]). In addition to their bridging function between upstream DNA-binding proteins and the basal transcription complex, CBP/p300 co-activators play a role in chromatin remodelling due to their intrinsic histone acetyltransferase activity. The CBP/p300

Figure 7 NF- κ B-mediated transcription and IL-6 promoter activity can be restored by p65, but not by CBP alone

(A) NF- κ B-mediated transcription. HeLa (■) and HTM-29 (□) cells were transfected with the reporter plasmid (κ B)₃Luc alone (Co) or along with the expression plasmids for p65 (0.1 μ g) and/or CBP (1 μ g). (B) IL-6 promoter activity. HeLa (■) and HTM-29 (□) cells were transfected with the reporter plasmid p1168hu.Luc alone (Co) or along with the expression plasmids for p65 (0.1 μ g) and/or CBP (1 μ g). After 24 h, transfected cells were incubated in serum-containing medium alone or in the presence of TNF- α (100 units/ml) for another 24 h. They were then harvested and lysed to determine luciferase activity. The values shown are representative of three independent transfections.

cofactor interacts with a wide range of transcription factors. A recent report has described a very stable, bivalent interaction between phosphorylated p65 and CBP, which is a crucial prerequisite for efficient transcription by NF- κ B [23]. Since bi-allelic inactivating somatic mutations of the CBP gene have been observed in several colon cancer cell lines [24], we wondered whether the lack of NF- κ B-dependent transcription in HTM-29 cells may result from a deficiency in CBP.

To test this hypothesis, we treated HTM-29 cells with TNF- α after co-transfection with a CBP expression plasmid and the κ B-Luc reporter construct. As illustrated in Figure 7(A), exogenous CBP alone could not restore TNF- α -induced NF- κ B-mediated transcription in HTM-29 cells. Various concentrations (1–10 μ g) of the CBP-expressing plasmid were not able to restore NF- κ B-dependent transcriptional activity (results not shown). In HeLa cells transfected with CBP and treated with TNF- α , the stimulation of transcriptional activity was equivalent to that in the absence of CBP, showing that HeLa cells were not limited in terms of their CBP pool (Figure 7A). Interestingly, transfection with exogenous p65 increased luciferase activity very efficiently in HTM-29 cells, to levels even greater than those in HeLa cells (approx. 30- and 13-fold in HTM-29 and HeLa cells respectively) (Figure 7A). When the cells overexpressing p65 were treated with TNF- α , luciferase activities increased further (up to 70-fold) in both cell lines (Figure 7A). This synergistic effect of TNF- α has been reported previously [16]. Indeed, in some cell lines, in addition to inducing NF- κ B nuclear translocation, TNF- α activates p38 and ERK (extracellular-signal-regulated kinase)/MAPK (mitogen-activated protein kinase) pathways, which contribute to transcriptional activation by modulating the trans-activation capacity of the NF- κ B p65 subunit. On the other hand, in line with a study by Plaisance et al. [17], we found a powerful synergistic activation upon co-transfection of CBP with

p65 in the two cell lines (approx. 300-fold; Figure 7A). This stimulation was not increased further by TNF- α treatment (Figure 7A).

Figure 7(B) shows the effects of CBP on IL-6 promoter activity in the two carcinoma cell lines that were not treated, stimulated by TNF- α or co-transfected with p65. The transcriptional co-activator CBP stimulated the basal activity of the IL-6 promoter in both HTM-29 and HeLa cells (approx. 3-fold; Figure 7B), probably by interacting with the transcription factors AP-1 and CREB that are constitutively bound to the IL-6 promoter. This transcriptional activity was further increased in HeLa cells after induction with TNF- α , whereas there was no further increase in HTM-29 cells (Figure 7B). CBP by itself could not rescue IL-6 promoter induction by TNF- α in HTM-29 cells, whereas over-expression of the p65 subunit consistently increased IL-6 promoter activity (Figure 7B). In this case, stimulation of transcription by exogenous p65 could not be increased further by TNF- α treatment in either cell line (Figure 7B). Co-expression of p65 and CBP resulted in a synergistic up-regulation of IL-6 promoter activity (up to 130-fold in HTM-29 cells) (Figure 7B). This synergistic activity could not be further enhanced by treatment with TNF- α (Figure 7B).

From these results, we conclude that the lack of NF- κ B-dependent transcription in HTM-29 cells after TNF- α -induced translocation of NF- κ B to the nucleus is not due to a deficiency in CBP. In addition, this failure does not result from an impairment in other co-activators, since exogenous p65 alone can rescue κ B-dependent transcription in HTM-29 cells to levels very similar to those in HeLa cells.

DISCUSSION

The carcinoma cell lines HeLa and HTM-29 show different behaviour in terms of IL-6 production. Analyses of secreted IL-6 by ELISA and of IL-6 mRNA by RT-PCR revealed that, while HeLa cells produced high levels of IL-6 in response to TNF- α and IL-1 β , the HTM-29 cell line failed to produce both IL-6 protein and mRNA. Nevertheless, the two main transcription factors involved in IL-6 gene regulation by cytokines, i.e. NF- κ B and NF-IL6, were activated by cytokines in both cell lines, as demonstrated by EMSA performed on nuclear extracts using the IL-6- κ B and NF-IL6 sites as probes. The intensity and kinetics of the NF- κ B stimulation and the composition of NF- κ B complexes (p50/p65 and p65/p65) were similar in the two cell lines.

In order to verify that the lack of IL-6 gene expression in response to cytokines in HTM-29 cells did not result from a deficiency in the endogenous IL-6 gene, and because IL-6 expression can be regulated by post-transcriptional mechanisms modulating mRNA stability [25–27], we performed transient transfection assays with an IL-6 promoter-reporter construct. These experiments demonstrated that IL-6-promoter-mediated transcription was not induced by cytokines in HTM-29 cells, whereas the IL-6 promoter was strongly activated in HeLa cells. Thus the lack of IL-6 gene expression in response to cytokines in HTM-29 cells is due, at least in part, to IL-6 promoter repression. Using a IL-6 reporter construct in which the NF- κ B site was exchanged for the consensus Ig- κ B site, which does not bind RBP-J κ , we showed that competition between RBP-J κ and NF- κ B could not explain IL-6 promoter repression in HTM-29 cells.

Transient transfection assays with a κ B reporter construct allowed us to correlate the impairment of IL-6 promoter activation by cytokines with a lack of NF- κ B-mediated transcription. After comparison of I κ B protein expression levels in HeLa and HTM-29 cells, we concluded that none of I κ B α , p100

or p105 was responsible for the deficient NF- κ B transactivating activity in cytokine-treated HTM-29 cells.

Another level of regulation on NF- κ B-mediated transcription is conferred by interactions between NF- κ B subunits and non-DNA-binding co-activator proteins [23] and components of the basal transcription complex [28]. Among these interactions, NF- κ B, similarly to a wide variety of other inducible transcription factors, complexes with the co-activator protein CBP through multiple domains in both proteins [23]. This interaction is a vital prerequisite for efficient transcription by NF- κ B [23]. Since bi-allelic inactivating somatic mutations of the CBP gene have been reported in colon cancers [24], we wondered whether the lack of NF- κ B-dependent transcription in the colon carcinoma cell line HTM-29 results from a deficiency in CBP. Overexpression of CBP alone in HTM-29 cells did not rescue either IL-6 promoter activation or NF- κ B-dependent transcription in response to TNF- α . Very interestingly, when exogenous RelA was overexpressed, both cell lines were able to drive NF- κ B-mediated transcription in a very similar way. RelA overexpression could also induce IL-6 promoter activation in HTM-29 cells, demonstrating that the lack of NF- κ B-transactivating activity in these cells was due to functional impairment of endogenous RelA.

RelA transcriptional activity is subject to control by phosphorylation. For example, RelA phosphorylation on a consensus PKA (protein kinase A) site, which is located in the Rel homology domain, stimulates transcriptional activity by promoting a novel bivalent interaction with CBP [23]. In co-transfection assays with CBP and RelA, CBP had a synergistic effect on exogenous RelA-mediated transcription in HTM-29 cells. The level of stimulation induced by CBP was similar in HeLa and HTM-29 cells. This result excludes the possibility that the lack of NF- κ B-dependent transcription in cytokine-treated HTM-29 cells involves a PKA deficiency, preventing phosphorylation of RelA and its interaction with CBP.

Vanden Berghe and co-workers [16] recently showed that the transduction pathway from the TNF receptor to NF- κ B-mediated transcription occurs in at least two steps: first, NF- κ B becomes activated in the cytoplasm by an I κ B α specific kinase and is translocated to the nucleus; secondly, the 'nuclear' transactivation potential of the DNA-bound complex is increased by (an) additional phosphorylation event(s) via different TNF-activated MAPKs, such as p38 and ERK. Since we observed a significant synergistic effect of TNF- α on exogenous p65-mediated transcription in HTM-29 cells, we cannot attribute the lack of NF- κ B-mediated transcription to a deficiency in TNF signalling leading to p38 or ERK MAPK activation.

Since the lack of NF- κ B-mediated transcription in cytokine-treated HTM-29 cells does not result from a deficiency in RelA co-activators or from an impairment of kinase activity, mutations in the *relA* gene could be at the origin of this transcriptional deficiency. A tumour-associated mutant form of RelA with reduced DNA-binding and transactivating activities has already been identified [29]. In colon carcinoma HTM-29 cells, mutations in the RelA-encoding gene might prevent the p65 homodimer and the p50/p65 heterodimer from exerting their transactivating effect. For example, the RelA-encoding gene could be mutated at the PKA phosphorylation site in the Rel homology domain, or in the transactivation domains, TA1 and TA2, located in the C-terminal region. In order to test this hypothesis, we performed sequencing of the PCR-amplified cDNA coding region of RelA in HTM-29 cells. Neither base substitution nor deletion was identified in the *relA* coding region. We thus conclude that the lack of NF- κ B-dependent gene transcription in HTM-29 cells is not due to mutations in the *relA* coding region.

Cross-talk between transcription factors of distinct families is an important phenomenon in regulating gene transcription, and has become the subject of intensive investigations. A negative cross-talk has recently been described between NF- κ B and p53 [30]. Interestingly, the transcriptional activities of both factors are governed by their relative levels of expression. If NF- κ B is overexpressed comparative to p53, it inhibits p53-dependent transactivation, while still being able to fulfil its own transactivating function. If p53 expression is greater, p53 can also suppress NF- κ B-mediated transcription, although its own transcriptional activity is not affected. This result is in agreement with the divergent cellular outcomes of p53 and NF- κ B induction. Since CBP overexpression makes it possible to overcome this mutual repression between NF- κ B and p53, Webster and Perkins [30] suggested a model in which the two factors compete for a limiting pool of CBP/p300 co-activator protein complexes. A similar negative cross-talk involving CBP has also been suggested between AP-1 and the glucocorticoid receptor [31] and between CREB and NF- κ B [32].

Since Western blot analysis revealed that p53 is stabilized in HTM-29 cells (results not shown), whereas it is degraded through the E6 protein of the human papilloma virus type 18 (HPV-18) in HeLa cells [33], suppression of NF- κ B-mediated transactivation in TNF- α -treated HTM-29 cells might well be due to a negative cross-talk between p53 and NF- κ B. Another possibility to explain the difference in NF- κ B-driven transcriptional activity observed in the two cell lines is the presence of a RelA inhibitor in HTM-29 cells. Such a RelA inhibitor was recently described [34]. This RelA-associated inhibitor (RAI) was shown to abolish the transcriptional activity of the protein in several tissues, such as human heart, placenta and prostate. Since RAI interferes with DNA binding of NF- κ B, it is very unlikely that the presence of RAI or a RAI-related molecule in HTM-29 cells could explain the lack of NF- κ B transcriptional activity in these cells.

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