Inhibition of interleukin-6 promoter activity by the 24 kDa isoform of fibroblast growth factor-2 in HeLa cells

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The expression of the interleukin (IL-6) gene can be regulated by various activating or inhibitory stimuli. This modulation involves several regulatory binding sites on the IL-6 promoter, and appears to be in general cell-specific. We have previously described that the nuclear 24 kDa isoform of fibroblast growth factor-2 (FGF-2) is able to increase IL-6 gene expression in NIH-3T3 cells. The transduction pathway involved was shown to be distinct from the extracellular mode of action of the smallest 18 kDa FGF-2 isoform. In the present study, we show that 24 kDa FGF-2-encoding vectors transfected into HeLa cells inhibit various co-transfected constructs incorporating the promoter element of the IL-6 gene and either the luciferase or the chloramphenicol acetyltransferase units. This down-regulation occurs dose-dependently with the 24 kDa FGF-2, is IL-6-promoter-specific, and does not involve an autocrine loop of the growth factor, since exogenously added FGF-2 fails to modulate the IL-6 promoter activity. Furthermore, 24 kDa FGF-2 inhibits the activity of both the co-transfected deletion mutants IL-6 (−224) and IL-6 (−158), and the point-mutated IL-6 promoter constructs in which the activating protein-1, nuclear factor (NF)-IL-6 and NF-κB elements are disrupted. We identify a responsive region to 24 kDa FGF-2 between positions −158 and −109 on the IL-6 promoter, which notably contains a retinoblastoma control element.

INTRODUCTION

Interleukin-6 (IL-6) is a multifunctional cytokine described originally as having a crucial role in the immune response. In addition to this, a broad spectrum of biological activities are now linked to the expression of this cytokine and its receptor. In the liver, IL-6 mimics the effects of systemic injury, leading to the induction of acute-phase proteins. IL-6 is also involved in controlling the functions of many cells, including stem cell differentiation, antibody synthesis by B cells, haematopoiesis and in vitro proliferation and survival of cells from a neuronal lineage (for a review, see [1]). In addition, IL-6 potentially plays a major role in oncology relating to its activity as a stimulus of growth for certain types of tumour cells. Whereas it stimulates proliferation of placmacytomas, lymphomas or myelomas, growth-inhibitory effects of IL-6 have also been characterized in breast or colon carcinomas [2]. IL-6 is produced efficiently by many different cells, including most types of cancer cells. It is induced by other cytokines, growth factors, hormones, neuropeptides or microbial products. Its expression can also be controlled by negative regulators, including glucocorticoids [3], retinoic acid [4], IL-4 [5], IL-10 [6], p53 or retinoblastoma [7].

We have previously demonstrated that the nuclear 24 kDa isoform of basic fibroblast growth factor-2 (FGF-2) is able to up-regulate IL-6 gene expression in the fibroblast NIH-3T3 cell line [8]. FGF-2 is a multifunctional cytokine, the activities of which have been extensively reviewed elsewhere [9]. The single-copy human FGF-2 gene encodes five co-expressed isoforms, with apparent molecular masses of 34 [10], 24, 22.5, 22 and 18 kDa, co-translated from a single mRNA transcript [10,11]. Whereas the high-molecular-mass (HMM) FGF-2 isoforms are mainly localized in the cell nucleus [12], the 18 kDa protein is cytoplasmic, and this is secreted via an energy-dependent pathway in which the involvement of the catalytic subunit of Na+K+-ATPase is implicated [13]. Most of the data available on the biological activity exerted by FGF-2 are concerned with the 18 kDa FGF-2. Nevertheless, during the last ten years, several studies have reported specific, distinct effects of HMM FGF-2 on cell phenotypes [8,14–20]. Our aim was therefore to understand the role and mechanism(s) of action of the HMM proteins of FGF-2, by an investigation into the target genes of the nuclear 24 kDa isoform. The regulation of expression of the IL-6 gene appeared to be particularly adapted to the function of FGF-2, since both are implicated in host-defence mechanisms that serve to limit tissue injury, as well as in the proliferation of a broad spectrum of cell lines. A great deal of data has revealed a strong relationship between FGF-2 and IL-6, especially at the level of mRNA stimulation during tumour progression [21,22], but also in relation to common inducers, namely IL-1β and tumour necrosis factor (TNF)-α [23]. Interestingly, in cultured rat hippocampal astrocytes, IL-1β and TNF-α selectively increase the nuclear HMM isoforms of FGF-2 [24]. Moreover, there is substantial evidence to suggest that an increase in IL-6 gene expression in human microvascular endothelial cells is mediated by FGF-2, which is induced endogenously by TNF [25]. In the latter study, the TNF-induced stimulation of expression of IL-6 was only partially reduced by anti-(FGF-2) antibodies, suggesting a possible role for the inducible (HMM) FGF-2 molecules that are not released into the culture medium.

The present work in HeLa cells describes a distinct cell-specific regulation of IL-6 promoter activity by the 24 kDa isoform of FGF-2. We first performed transitory co-transfection experiments on wild-type (WT) HeLa cells using an IL-6 promoter-driven reporter vector and a 24 kDa FGF-2-encoding plasmid. We observed a down-regulation of IL-6 promoter activity that occurs dose-dependently with the 24 kDa FGF-2, is IL-6-promoter-specific, and does not involve an autocrine loop of the growth factor, since exogenously added FGF-2 fails to modulate the IL-6 promoter activity. Furthermore, 24 kDa FGF-2 inhibits the activity of both the co-transfected deletion mutants IL-6 (−224) and IL-6 (−158), and the point-mutated IL-6 promoter constructs in which the activating protein-1, nuclear factor (NF)-IL-6 and NF-κB elements are disrupted. We identify a responsive region to 24 kDa FGF-2 between positions −158 and −109 on the IL-6 promoter, which notably contains a retinoblastoma control element.

Abbreviations used: AP, activating protein; CAT, chloramphenicol acetyltransferase; DME, Dulbecco’s minimal essential medium; FGF, fibroblast growth factor; FCS, fetal-calf serum; HMM, high molecular mass; IL, interleukin; NF, nuclear factor; RCE, retinoblastoma control element; SV40, simian virus 40; TNF, tumour necrosis factor; WT, wild type.

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Cell culture and permanent transfection

HeLa cells were grown in DME containing 5% (v/v) FCS medium supplemented with amphotericin B (2.5 mg/ml), gentamycin (50 mg/ml) and 1-glutamine (2 mM), and incubated at 37 °C in air/CO₂ (19:1). The cells were transfected with Lipo- larmenia Reagent (Gibco BRL), either with the control bi-cistronic pEN vector or the 24kDa FGF-2-encoding vector, pRFGF24 [8]. The cells were cultured in DME containing 5% (v/v) FCS plus 1 mg/ml gentamicin (G418; Gibco-BRL). After 2 weeks, G418-resistant clones were isolated, subcloned in 35-mm dishes, amplified and analysed by an anti-(FGF-2) Western blotting procedure, as described below.

Cell co-transfection and luciferase activity assays

Cells were plated at a density of 150,000 cells/well in 6-well culture plates in DME 5% (v/v) FCS medium. After 48 h of incubation, cells were rinsed with PBS and transfected with the indicated doses of plasmid vectors using 5 μl of Lipofectamine per well. The WT IL-6 promoter (positions −1158 to +11) was fused to the luciferase reporter gene on the pGL3-Basic vector (Promega, Madison, NY, U.S.A.) as described previously [8]. Deletion mutants of the IL-6 promoter (−224 to +11, −158 to +11 and −109 to +11) were obtained as described previously [27]. Two types of 24 kDa FGF-2-encoding vectors were used to co-transfect the cells, into which the cDNA insert allowing the synthesis of only the 24 kDa isoform was cloned from either pEN [8] or pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.) as described previously [27]. After the transfection experiments (48 h), cells were rinsed twice in PBS and suspended in 100 μl of 20 mM Tris/HCl, pH 7.8/2 mM MgCl₂. After two cycles of freezing (−80 °C) and thawing, cells were collected, incubated for 10 min at 65 °C and centrifuged (10,000 g) at 4 °C for 10 min. Aliquots (35 μl) of supernatant were added to 15 μl of a solution containing 100 mM Tris/HCl, pH 8, 100 μM [³⁵]Chloramphenicol and 250 mM n-butyl-N-bicycloexylene. After another 4 h incubation at 37 °C, the reaction was stopped by the addition of 200 μl of a 2:1 mixture of tetramethylpentadecane:xylene and mixed vigorously by vortexing. The samples were then centrifuged for 3 min at room temperature, and 150 μl of the organic phase was counted in 3 ml of Ready Safe reagent (Amersham, Arlington, IL, U.S.A.). Each CAT transfection experiment was carried out in triplicate and repeated at least twice. Unpaired Student’s t tests were performed as for the luciferase assays.

Western blot analysis

Samples of total protein (30 μg) from pRFGF24 or pEN control permanently transfected HeLa cells were analysed by Western blotting, as described previously [8]. The detection of recombinant FGF-2 was performed with a rabbit polyclonal anti-(FGF-2) antibody (SC147; diluted 1:500) and horseradish-peroxidase-labelled anti-rabbit IgG (Amersham; also diluted 1:5000).

RNA isolation and RNase protection

Total RNA was isolated 48 h after transfection experiments by use of RNeasy columns from Qiagen (Courtabœuf, France). RNase protection assays were performed with the RiboQuant System and the hCK-2 human cytokine/chemokine Multi-Probe Template set from Pharmingen (San Diego, CA, U.S.A.) using 15 μg of total RNA for each experiment.

RESULTS

Inhibition of the transfected IL-6 promoter luciferase construct by 24 kDa FGF-2 cDNA in HeLa cells

To investigate the role of intracellular FGF-2 proteins on IL-6 gene regulation in HeLa cells, the ability of the 24 kDa isoform of FGF-2 to act on the IL-6 promoter was examined. In transient experiments, we co-transfected increasing amounts of 24 kDa FGF-2-coding vectors with a luciferase gene construct driven by the DNA sequence from −1158 to +11 of the human IL-6 gene 5'-flanking region. In order to avoid any non-specific plasmid effects on the regulation of the IL-6 promoter, two different types of FGF-2-encoding vectors were used, in which the 24 kDa isoform cDNA was either cloned into pcDNA3 (Invitrogen) or the bi-cistronic pEN plasmids, namely pRFGF24 and pRFGF24. When transfected in COS-7, HeLa or NIH-3T3 cells, these constructs led to overexpression of the 24 kDa protein isoform (results not shown). As shown in Figure 1(A), regardless of the origin of the growth-factor-coding vectors, the luciferase activity was significantly down-regulated, depending on the amount of transfected 24 kDa cDNA used. To test whether the modulation of luciferase activity might be due to an effect of 24 kDa FGF-2 on either the stability of the reporter mRNA or protein activity, the control vector prSV40lac, carrying the luciferase gene driven by the minimal simian virus 40 (SV40)
24 kDa fibroblast growth factor-2 represses interleukin-6 in HeLa cells

Figure 1 Specific repression of the IL-6 promoter activity by 24 kDa FGF-2 encoding vectors in HeLa cells

(A) HeLa cells were transfected with 1 μg of prIL-6, along with increasing doses (0 to 4 μg) of the various 24 kDa FGF-2 expression plasmids, pcFGF24 or pRFGF24. As described in the Experimental section, for each transfection, the total amount of transfected DNA was calibrated to 5 μg with the pcDNA3 or pEN control vectors. (B) HeLa cells were transfected with 1 μg of pSV40Luc and increasing doses of pRFGF24 (0 to 4 μg). Luciferase activity was normalized to Renilla activity driven by the pRLCMV vector (40 ng per transfection) from Promega, and this was represented as a percentage of the relative luciferase activity in the control-vector-only transfected cells. Data shown are means ± S.E.M. for triplicate luciferase measurements repeated four times; asterisks denote a significant variation from the mean (P < 0.05).

Figure 2 Inhibition of IL-6 promoter-deletion mutants by 24 kDa FGF-2 in HeLa cells

(A) Schematic map of the IL-6 promoter and IL-6 promoter-deletion, luciferase-driven constructs. Deletion mutants were obtained as described in the Experimental section, using the restriction enzyme sites underlined. (B) HeLa cells were transfected transiently as before, with 3 μg of pRFGF24 or pEN control vector, and 1 μg of each of the three plasmid constructs containing different lengths of the IL-6 promoter region, as indicated in (A). Asterisks denote a significant difference from the mean (P < 0.01).

Functional studies on IL-6 promoter regulation by 24 kDa FGF-2 in HeLa cells

The effect of overexpression of 24 kDa FGF-2 was then evaluated on deletion mutants of the IL-6 promoter (Figure 2A), under experimental conditions in which we observed a 50% inhibition of the WT promoter activity. Figure 2(B) shows that both the prIL-6(-224) construct, in which a large 5’-region containing the AP-1-binding site had been removed, and the prIL-6(-158) promoter, was tested. Figure 1(B) shows that the relative luciferase activity is not altered by increasing the amount of transfected pRFGF24. Moreover, this dose-dependent inhibition of luciferase expression occurred regardless of the nature of the plasmid (pcDNA3 or pEN) used to calibrate the DNA levels in the co-transfection studies (results not shown).

Taken together, these data strongly suggest that down-regulation of the luciferase activity is a reflection of the inhibitory effect of recombinant 24 kDa FGF-2 on the IL-6 promoter.

Since we have previously shown that extracellular 18 kDa proteins of FGF-2 are able to down-regulate the expression of the IL-6 gene in the fibroblastic cell line NIH-3T3, it was thus important to discriminate against the possibility of such an effect with secreted FGF-2 species in HeLa cells. Although the HMM isoforms of FGF-2 are not usually detected in the media, FGF-2 could reach the extracellular space in the event of either cell injury or lysis. Hence, to address this possibility, 0 to 100 ng of 18 kDa recombinant FGF-2 was added to the cell culture medium 24 h prior to measurement. No significant inhibition of luciferase activity driven by the transfected prIL-6 vector was observed (results not shown), confirming that 24 kDa FGF-2 is acting on the IL-6 promoter via an intracellular pathway.
Figure 3  Analysis of IL-6 promoter site-directed mutants response to 24 kDa FGF-2

HeLa cells were transiently co-transfected with 1 μg of the indicated mutated CAT-driven constructs (as shown by the filled boxes for the mutated motif), and 3 μg of pRFGF24 or pEN control vector. CAT activity was determined as described in the Experimental section 36 h after transfection. The bars show the means for six independent experiments; asterisks denote a significant variation from the mean (P < 0.01).

Figure 4  Endogenous IL-6 mRNA levels in transiently transfected HeLa cells

Ribonuclease protection assays were carried out using the hCK-2 Multi-Probe set (Pharmingen) on total RNA from HeLa cells (lane 1), cultivated with 50 ng/ml of FGF-2 (lane 2), or transiently transfected with the pEN control vector (lane 3, 1 μg; lane 4, 3 μg) or pRFGF24 (lane 5, 1 μg; lane 6, 3 μg). The arrows indicate the protected RNA fragments for IL-6 or the two housekeeping gene transcripts, L32 and glyceraldehyde-3-phosphate dehydrogenase.

reporter plasmid, with a larger deletion that thus removed a cAMP-response element, were also down-regulated by pRFGF24. In contrast, however, significant inhibition was no longer observed with the prIL-6(-109) mutant, suggesting that the (−158/−109) region is involved in the responsiveness of the IL-6 gene promoter to 24 kDa FGF-2. Given that this latter region contains a NF-IL-6-binding site, and that co-operativity between NF-IL-6 and NF-κB components has been previously demonstrated [28], we then examined the effect of 24 kDa FGF-2 on NF-IL-6 5′ and 3′, and NF-κB point-mutated IL-6/CAT constructs (Figure 3). The basal luciferase activity was maximal with the WT IL-6 promoter, and decreased with the mutated constructs. As with the WT IL-6 promoter, all these mutants were also inhibited by pRFGF24. Constructs sharing combinatorial mutations between the different consensus boxes failed to exhibit any detectable luciferase activity (results not shown).

Apart from the indication that none of the NF-IL-6 5′, NF-IL-6 3′ or NF-κB elements are involved in IL-6 repression by 24 kDa FGF-2, the results of these experiments using CAT as a reporter gene also demonstrate further the transcriptional nature of the effect mediated by the growth factor on the IL-6 promoter.

Regulation of endogenous IL-6 mRNA levels in transfected HeLa cells

In further experiments, it was investigated whether transiently transfected 24 kDa FGF-2 could affect in the same way endogenous IL-6 gene expression. This was accomplished by RNase protection studies on total RNA from HeLa cells (Figure 4, lane 1), which was treated with 50 ng of 18 kDa FGF-2/ml of culture medium (Figure 4, lane 2) or transfected with various amounts of control pEN vector (Figure 4, lanes 3 and 4) or pRFGF24 (Figure 4, lanes 5 and 6). The amounts of IL-6 mRNA were normalized to the levels of the glyceraldehyde-3-phosphate dehydrogenase or L32 housekeeping genes. Surprisingly, whereas the extracellular factor caused no modulation of IL-6 mRNA, the transitory transfection of both the control and the 24 kDa-encoding vectors led non-specifically to dramatic increases in endogenous IL-6 mRNA levels. Thus, in HeLa cell systems, it appears that the investigation of the native IL-6 gene regulation in response to transiently transfected vectors is not possible, since the stress associated with transfection induced the accumulation of IL-6 mRNA by itself.

Regulation of IL-6 promoter activity in HeLa cells stably expressing 24 kDa FGF-2

24 kDa-encoding pRFGF24 or control pEN vectors were transfected into HeLa cells, and G418-resistant clones were selected after 2 weeks. About ten clones for each construct were isolated, and permitted to grow further. As shown by Western blot analysis using FGF-2 antibodies (Figure 5A), two clones expressing high levels of 24 kDa FGF-2, namely F24.13 and F24.17, were selected for the following experiments. RNase protection studies were performed to compare the relative amounts of IL-6 mRNA in control and 24 kDa FGF-2-transfected cells. We were not able to observe any down-modulation of IL-6 mRNA levels in F24.13 or F24.17 cells compared with pEN-transfected cell lines. No increase caused by the transfection event was observed (results not shown). The effect of over-
Moreover, the extracellular 18 kDa FGF-2 fails to modulate the IL-6 promoter activity in HeLa cells, which is consistent with the low levels of specific tyrosine-kinase membrane receptors for FGF-2 in these cells. This demonstrates further that the 24 kDa isoform of FGF-2 acts via a specific intracellular transduction pathway.

Distinct cell-specific effects of 24 kDa FGF-2 on IL-6 promoter regulation

We have previously described that the 24 kDa isoform of FGF-2 up-regulates IL-6 transcription in the fibroblastic cell line NIH-3T3 [8]; in the present study we demonstrate that the same protein is able to decrease the IL-6 promoter activity specifically in HeLa cells. Several hypotheses may be proposed to explain such heterogeneous results in these two cellular systems. First, HeLa and NIH-3T3 cells are very different in relation to their respective origin and phenotypes. Whereas the NIH-3T3 cell line corresponds to murine immortalized fibroblasts, HeLa human cells are transformed and highly tumorigenic in nude mice. Their respective genetic programmes and proteinaceous contents are hence very likely to be different, which suggests that distinct cell-specific cofactors for FGF-2 could be responsible for the differential regulation of the IL-6 gene. In addition, opposite effects of the smaller isoform of FGF-2 have been described recently on the tissue-factor gene, depending on whether NIH-3T3 or HUVEC cells were employed as the cellular model [29]. Lastly, our laboratory has recently demonstrated that, in contrast with osteoblastic or HeLa cells, IL-6 expression was not influenced by oestriol treatment in rat vascular smooth-muscle cells [30]. Similarly, the IL-6 gene is activated by a variety of IL-6 inducers in both U-937 and HeLa cells, but alternative inducible enhancer elements can also contribute in a cell-specific manner to IL-6 gene induction [31]. Taken together, these results corroborate those obtained with regard to the differential cell-specific effects of the 24 kDa isoform of FGF-2 on IL-6 gene regulation.

Problems of studying endogenous IL-6 mRNA levels to determine IL-6 gene regulation by transiently transfected 24 kDa FGF-2-coding vectors

By performing RNase protection experiments, we observed that transfection of pRFGF24 vector increased the levels of IL-6 mRNA, whereas in co-transfection experiments it decreased IL-6 promoter-driven reporter vector activity in HeLa cells. We found that this up-regulation of IL-6 mRNA was not dependent on the 24 kDa FGF-2 cDNA, since transfection experiments using either the control vector pEN or other non-coding plasmids (results not shown) led ultimately to the same non-specific IL-6 mRNA accumulation in the treated cells. In addition, RNase protection experiments on stably transfected 24 kDa FGF-2 HeLa cells failed to show significant modification of IL-6 mRNA levels (results not shown). A possible explanation is that other cellular co-factors act to stabilize the IL-6 basal expression, even when 24 kDa FGF-2 is constitutively overexpressed. Another possibility is that the modest inhibition (about 50%) of IL-6 promoter activity is not sufficient to detect any slight differences in amounts of IL-6 mRNA. However, these results strongly suggest that resident IL-6 gene expression is highly sensitive to the cellular stress caused by the transitory transfection event in HeLa cells, and therefore the study of transiently transfected IL-6 promoter-driven reporter vector activity appears to be more appropriate for an investigation into IL-6 gene regulation by the 24 kDa isoform of FGF-2 in HeLa cells.
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