Overexpression of the FGF-2 24-kDa isoform up-regulates IL-6 transcription in NIH-3T3 cells

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Abstract We have isolated NIH-3T3 cell lines overexpressing the nuclear 24-kDa isoform of fibroblast growth factor (FGF)-2 and characterized its regulatory effect on the expression of interleukin-6 (IL-6) in these cells. The clone pRF5 expressing the highest level was able to grow in 1% serum medium to a high saturation density and acquired a radioresistance advantage. In pRF5 and another clone pRF1, IL-6 RNA levels were markedly increased. Studies with IL-6 promoter constructs revealed that IL-6 gene up-regulation occurred at the transcriptional level and did not involve the AP-1 binding site. Exogenously added 18-kDa isoform of FGF-2 (100 ng/ml) produced down-regulation of IL-6 involving an AP-1 binding site, thus suggesting a receptor-independent pathway for the intracellular 24-kDa isoform.

Key words: Fibroblast growth factor-2; Interleukin-6; NIH-3T3 cell; Interleukin-6 promoter; Gene regulation

1. Introduction

Basic fibroblast growth factor (FGF-2) is a multifunctional cytokine involved in the proliferation and differentiation of a broad spectrum of mesodermal and neuro-ectodermal cell types [1]. It has been also implicated in angiogenesis [2] and injury-repair responses.

Different molecular forms of FGF-2, representing alternative translation products from a single mRNA, have been characterized [3]. Initiation of translation at CUG codons located 5′ to the AUG codon accounts for the 24-, 22.5- and 22-kDa isoforms, whereas the AUG codon is used to generate an 18-kDa protein. The small isoform is mainly cytoplasmic and when secreted, subsequently activates the FGF receptor pathway [4] eliciting the cascade of second messenger production. The high molecular weight isoforms of FGF-2 (HMW FGF-2) are NH_2-terminal extensions of the 18-kDa form and contain nuclear localization sequence-like signals responsible for their preferential nuclear targeting [5]. Accordingly, the biological specificity of the different isoforms of FGF-2 might depend upon the subcellular localization of these molecules and their ability to be secreted and to activate a membrane receptor.

Although there is an increasing amount of published data relating to a specific intracellular mode of action of HMW FGF-2, the mechanisms by which these FGF-2 molecules produce their specific activities is not clear. They may act as transcription factors [6,7] or indirectly by forming complexes with transcription factors or other intracellular partners [8]. Indeed, the secreted 18-kDa isoform which acts in a receptor-dependent manner, can modulate gene transcription in a cell-free system [7], emphasizing that the FGF-2 core can act in vivo in the nucleus at the promoter level.

In addition to its subcellular location [5] and affinity for DNA [6], there is also evidence to suggest that some of the biological activities of HMW FGF-2 are specifically receptor-independent. For instance, it has been shown that the expression of different forms of FGF-2 results in different NIH-3T3 transformed cell phenotypes [9]. These observations have been confirmed in FGF-2 transfected ABAE cells where the nuclear HMW FGF-2 isoforms induce immortalization and the cytoplasmic 18-kDa form is transformant [10]. Moreover, in agreement with the oncogenic theory, the coexpression of these forms allowed a typical malignant transformation [10].

Therefore, to understand how cellular responses to HMW FGF-2 are produced, our efforts have been focused on the study of stably transfected cell models and the subsequent expression of genes such as interleukin-6 (IL-6) gene whose products are possibly related to the growth factor activity [11–14].

We report here the existence of two distinct pathways for FGF-2-induced IL-6 gene regulation in NIH-3T3 cells. The AP-1 binding motif on the IL-6 promoter is not required for induction by the intracellular 24-kDa isoform, but is necessary for the down-regulation of IL-6 expression by the extracellular 18-kDa isoform.

2. Materials and methods

2.1. Materials

Rabbit polyclonal anti-bFGF antibody (Ab-2) was purchased from Oncogene Science (Paris, France). Recombinant FGF-2 was produced in our laboratory. Molecular biology reagents, newborn calf serum (CS) and Dulbecco's medium without phenol red were from Gibco-BRL, Gaithersburg, USA. Amphotericin B, gentamicin and L-glutamine were from Seromed, Biochrom, Berlin, Germany. Cells were irradiated with a clinical cobalt-60 machine (Theratronics 1000; AECL, Ottawa, Canada).

2.2. Cell culture and transfection

NIH-3T3 cells were grown in DME containing either 10% or 1% calf serum medium supplemented with amphotericin B (2.5 μg/ml), gentamicin (50 μg/ml) and L-glutamine (2 mM) and incubated at 37°C, 5% CO_2. The cells were transfected with Lipofectin Reagent (Gibco-BRL), either with the eukaryotic bicistronic expression control pEN vector [15], or with pRFGF24 vector which is similar to pEN but contains a cDNA insert where the CUG305 of FGF-2 was mutated in AUG, allowing the synthesis of the 24-kDa FGF-2 isoform only. The cells were cultured in DME containing 10% CS plus 1 mg/ml geneticin (G418: Gibco-BRL). After two weeks, G418-resistant clones were isolated and subcloned in 35-mm dishes. These G418-resistant cells were amplified and then analyzed for their recombinant FGF-2 in Western blotting as described below.

2.3. Western blot analysis

Frozen cell pellets were treated and 30 μg of total protein was

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analyzed as previously described [15] for FGF-2 detection with a rabbit polyclonal anti-FGF-2 antibody (Ab-2; diluted 1:200) and horseradish peroxidase-labeled anti-rabbit IgG (Amersham) diluted 1:10 000. For β-actin detection, the same nitrocellulose membranes were rinsed overnight in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20), reblocked for 1 h in TBST supplemented with 3% non-fat milk, and treated with a monoclonal mouse anti-β-actin antibody (1:120 000; Sigma-Aldrich) and an anti-mouse HRP antibody (Sigma-Aldrich) diluted 1:5000.

2.4. Cell proliferation assays

Cells were seeded at 7000 cells/well in 12-well culture dishes in 10% CS DME medium. After overnight incubation at 37°C, and then every three days, cells were rinsed twice with PBS and refed with DME without phenol red containing 1% CS. The cells were counted with a Coulter counter (Coulter Electronics, Hialeah, FL, USA) every three days. All experiments were performed twice in triplicate.

2.5. Radiation survival curves

Cells were plated at a density of 2000 cells/25 cm^2 in fresh medium. After 48 h incubation, irradiation was carried out with a clinical cobalt-60 machine at a dose rate of 65 cGy/min. Single doses ranging from 1 to 10 Gy were delivered and the cells were returned to the incubator for one week. Survival after irradiation was calculated as previously described [16] as the ability of the cells to maintain their clonogenicity.

2.6. RNA isolation and RNase protection

Total RNA was isolated from appropriately treated cells by use of Rneasy columns from Qiagen. RNase protection assays were performed with the RiboQuant System and the mck-1 mouse cytokine/chemokine set from Pharmingen (San Diego, CA, USA), using 15 μg of total RNA for each experiment. Assays were quantitated with a Molecular Dynamics PhosphorImager and IL-6 mRNA signal intensity was normalized to the GAPDH housekeeping gene signal.

2.7. Cell cotransfections and luciferase activity assays

Cells were plated at a density of 50 000 cells/well in 6-well culture plates. Twenty-four hours later, medium was changed to 1% CS DME. After another 24-h incubation, cells were transfected with the indicated doses of plasmid vectors with the Fugene 6 transfection reagent (Boehringer Mannheim) following the manufacturer's instructions. The reporter plasmid prIL-6 was obtained by insertion of the human IL-6 DNA fragment −1158 to +11 released by *Xba*I and *Xho*I digestion of the plasmid pBRgHIL-61 (kindly supplied by Walter Fiers, Laboratory of Molecular Biology, University of Gent, Belgium) into pGL3-Basic vector (Promega, Madison, WI, USA) which was digested by *Nhe*I and *Xho*I. The pRL-CMV *Renilla* reporter vector from Promega was used as an internal control of transfections. The Bluescript II KS vector (Stratagene, La Jolla, CA, USA) was added to adjust the transfected DNA amounts to 5 μg. After 24 h, the cells were replaced in 1% CS medium containing or not recombinant FGF-2 to various doses. Luciferase activities in the lysate were determined 48 h later using the Dual-Luciferase Reporter Assay System from Promega. The activity of the firefly luciferase experimental reporter (PrIL-6 or PrIL-6ΔAP1) was normalized to the activity of this internal control. The luciferase activity measurements were performed with a plate-reading luminometer (Labsystems Luminoskan). All values are expressed as mean ± S.D. Unpaired Student's tests were employed to determine the significance of changes. A significant difference was taken for P values < 0.05.

3. Results

3.1. Construction and characterization of NIH-3T3 cell lines stably expressing the 24-kDa isoform of FGF-2

The bicistronic vector pRFGF24 coding for the high molecular weight isoform of FGF-2 [10], and the pEN control vector were transfected into NIH-3T3 cells. From 15 G418-

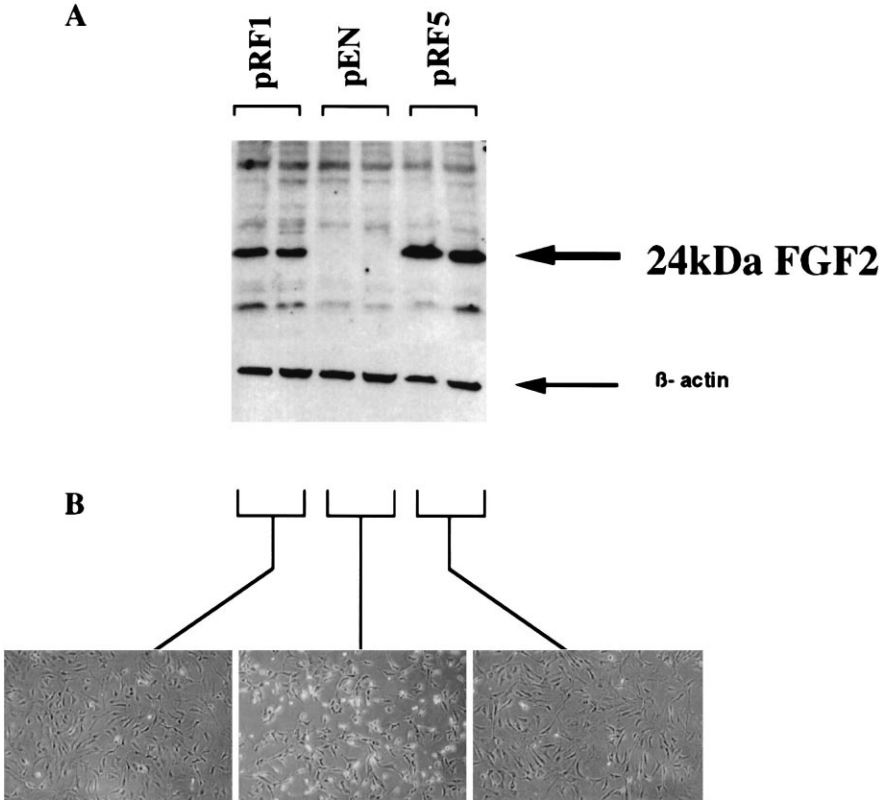

Fig. 1. 24-kDa FGF-2 stably transfected cell lines: pRF1 and pRF5. A: FGF-2 protein expression from vector alone and pFGF24 transfected NIH-3T3 cells. Thirty micrograms of extracted proteins from each cell line were loaded on 12.5% SDS polyacrylamide gels, and Western blotting was performed as described in Section 2. Arrows indicate the positions for the 24-kDa FGF-2 and β-actin proteins. Under the same conditions, no recombinant 24-kDa species of FGF-2 can be detected in wild-type (not shown) or in vector-transfected 3T3 cells, pEN. B: Morphological changes induced by transfection with the 24-kDa FGF-2 cDNA depending on the recombinant protein content.

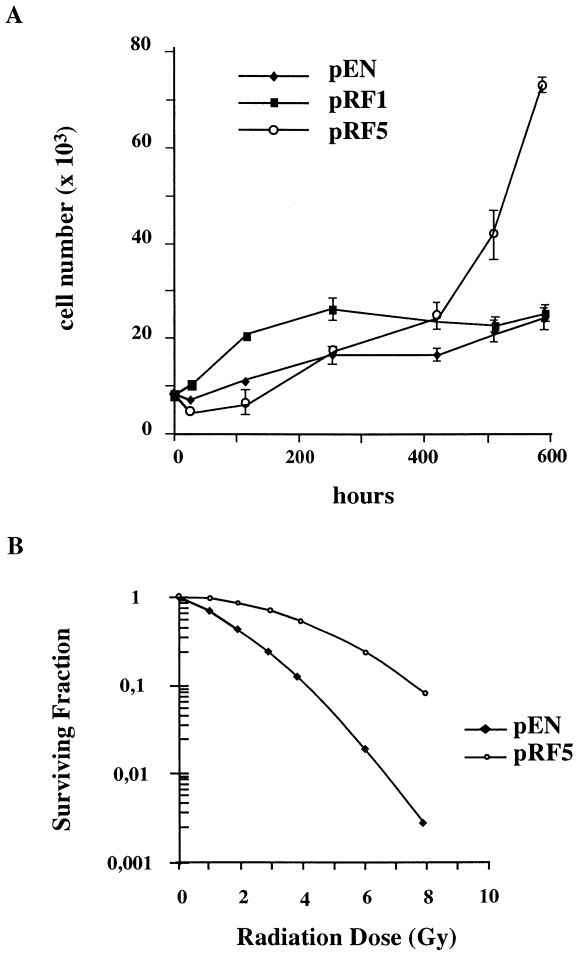

Fig. 2. The biological activities of 24-kDa FGF-2 transfected NIH-3T3 cells. A: Proliferation of cells expressing 24-kDa FGF-2 with two different levels: pRF1 and pRF5, compared to mocked transfected cells: pEN. The experiment was carried out as described in Section 2. The data represent mean ± S.D. of triplicate samples and are representative of two independent experiments. B: Radiation survival curves of pRF5 and pEN cells exposed to various ionizing radiation doses. The radiosensitivity of the different cell lines was calculated using the clonogenic survival assay. Data represent a typical curve from three different experiments for each clone.

resistant clones isolated, we selected two representative pRFGF24-transfected clones expressing FGF-2 at a different level.

As shown by Western blot using anti-FGF-2 antibody (Fig. 1A), pRF5 and pRF1 express the recombinant 24-kDa isoform at high level in a ratio of 2.5:1, respectively. The amount of endogenously overexpressed FGF-2 is about 1 ng/mg protein for pRF5. We carried out subcellular compartmentalization studies by immunocytochemical analysis and confirmed the preferential nuclear and perinuclear localization of FGF-2 in pRF5 cells (data not shown).

FGF-2 transfected cells are morphologically different from vector alone transfected cells (Fig. 1B). In low serum medium, the control cells are polygonal, fusiform and refractile. With increasing levels of recombinant FGF-2, the cells become larger and better organized.

To further characterize these cells, we performed proliferation tests with clones expressing the 24-kDa FGF-2 protein and vector alone-transfected cells in culture medium containing 1% calf serum. Fig. 2A shows typical proliferation curves with two representatives clones pRF1 and pRF5. At early time points, we observed a growth disadvantage for pRF5 which might result from a low plating efficiency and the need of cell to cell contacts for NIH-3T3 growth. As previously described, using adenoviral FGF-2 coding vector [17], we found that the overexpressed 24-kDa isoform of FGF-2 confers a high saturation density growth of pRF5 cells as well as the ability to grow in low serum medium compared to the pEN mocked transfected cells. Similarly, the pRF1 cells, expressing lower levels of the recombinant growth factor, displayed a proliferative advantage compared to pEN, but reached a density to saturation identical to the one observed for the control cells.

It has recently been described that the endogenously expressed 24-kDa isoform of FGF-2 confers a selective advantage to γ-radiation of transfected HeLa cells [16]. Fig. 2B shows representative clonogenic survival curves of pRF5 and pEN cells exposed to graded doses of radiation. The pRF5 cells displayed a significant increase in clonogenic survival compared with the pEN cells ($P < 0.01$). The mean inactivation dose (MID) was 182 ± 18 cGy for pEN and 410 ± 36 cGy for pRF5. We also verified that the endogenous murine isoforms levels of FGF-2 were not modified under these transfection conditions (data not shown).

3.2. Constitutive overexpression of recombinant 24-kDA FGF-2 up-regulates the amount of interleukin-6 mRNA

As a strong relationship exists between FGF-2 and IL-6

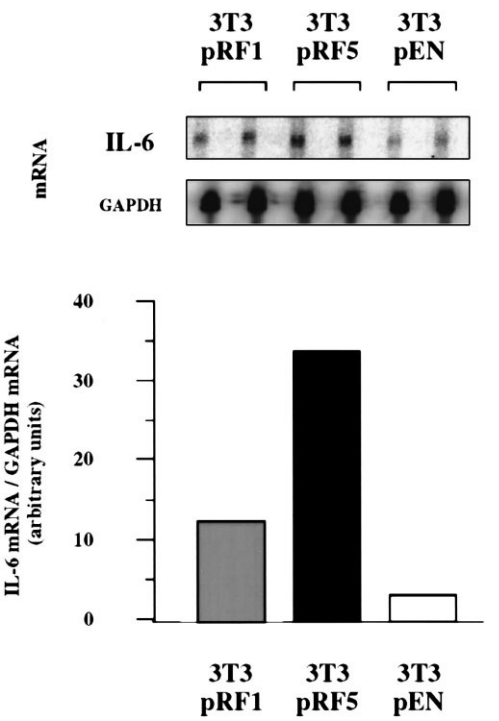

Fig. 3. Activation of IL-6 mRNA expression by endogenous 24-kDa FGF-2. Fifteen μg of total RNA from pEN, pRF1 and pRF5 cells were used for each hybridization experiment. IL-6 and GAPDH mRNA expression were quantitated simultaneously by RNase protection as described in Section 2. The autoradiogram shows mRNA signals for IL-6 and GAPDH in the different cell lines and a quantitative analysis of the RPA results normalized to the GAPDH mRNA level was performed with a phosphorimager. Data are presented in arbitrary units and are means of two experiments sharing similar results.

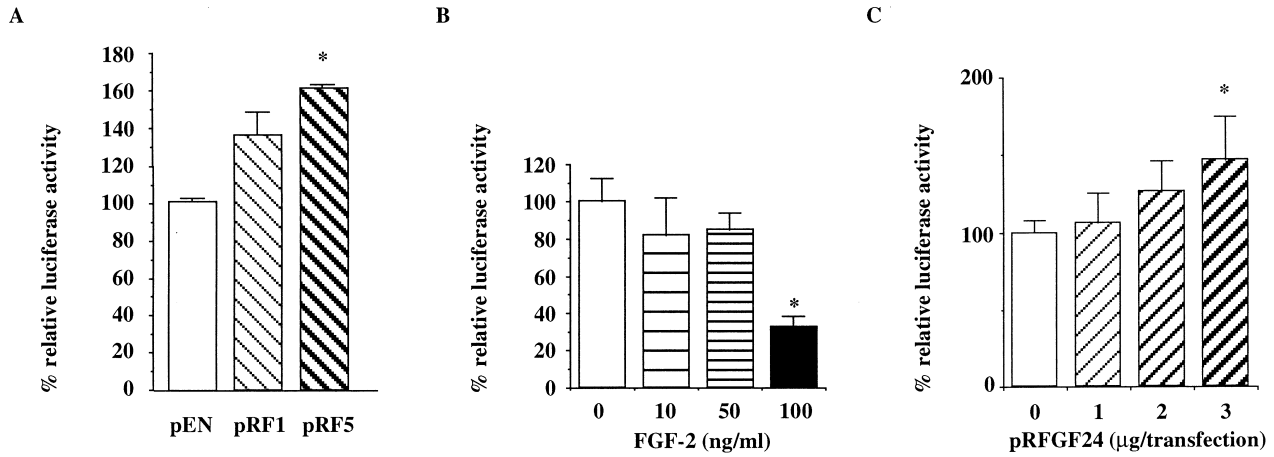

Fig. 4. Effects of FGF-2 on the activity of IL-6 promoter. A: pEN, pRF1 and pRF5 NIH-3T3 cells were transiently transfected with 1 μg prIL-6 and 40 ng pRL-CMV vectors per well in 6-well culture plates, as explained in Section 2. The relative firefly luciferase activity is the actual IL-6 promoter activity when calibrated against the *Renilla* luciferase signal which is representative of the transfection efficiency for each experiment. Results are presented in percentage of the relative firefly luciferase activity in the control pEN cells. *Statistically significant variations with $P < 0.01$. B: NIH-3T3 cells were transfected as below, with prIL-6 and pRL-CMV vectors. Twenty-four hours after transfection, various doses of FGF-2 were added to the cell low serum medium during an additional 24 h. Data shown are means and standard deviations for triplicate measurements from one representative transfection. *Statistically significant ($P < 0.01$). C: NIH-3T3 cells were transiently cotransfected with 1 μg pIL-6 as the reporter vector, 0–3 μg pRFGF24 as the inducer vector and 3–0 μg pKSII to adjust the amount of DNA to 5 μg in each transfection experiment. Forty ng pRL-CMV (Promega) was included in each case as an internal control. Data shown are means and standard deviations for triplicate luciferase measurements from one representative transfection. *Significant variation, $P < 0.01$.

[11–13,18–20], we further investigated the molecular mechanisms involved in the proliferative and radioprotective activities of HMW FGF-2 by RNase mapping analysis with an interleukin-6 probe.

Fig. 3 represents the expression pattern for the IL-6 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNAs in control or 24-kDa isoform-transfected NIH-3T3 cells. We have calibrated the level of the interleukin-6 mRNA amount using the GAPDH mRNA signal as a control. The relative expression histograms show that IL-6 mRNA levels increased in transfected cells. In pRF1 cells, IL-6 expression was enhanced 4-fold when compared to the pEN control cells, while a 10-fold increase of IL-6 mRNA levels occurred in the clone pRF5. Consequently, the constitutive expression of this growth factor up-regulates the intracellular IL-6 mRNA content in an apparent dose-dependent fashion. This was confirmed by RT-PCR performed with synthetized specific IL-6 primers (data not shown).

3.3. Stably transfected 24-kDa FGF-2 but not exogenous FGF-2 up-regulates the interleukin-6 promoter activity

To evaluate whether it was a transcriptional regulation, we transfected IL-6 reporter constructs in our cells. In the PrIL-6 plasmid, the 5′ flanking region (−1158 to +11) of the IL-6 gene corresponding to the entire IL-6 promoter [20] was fused to the firefly luciferase reporter gene, and activation of the IL-6 promoter was determined by measuring luciferase activity in transient transfection assays.

As shown in Fig. 4A, the IL-6 promoter activity was up-regulated and again it was dependent on the amount of recombinant FGF-2 expressed in the transfected clones. The induction was weak for pRF1 and highly significant ($P < 0.01$; $t = 5.4$) for pRF5. These results suggest that the differences in interleukin-6 mRNA levels observed in our previous RNase protection studies very likely reflect the transcriptional regulation of the IL-6 gene.

Exposure to exogenous 18-kDa FGF-2 enhanced the IL-6 gene expression in several cellular systems, such as human microvascular endothelial cells [13] and osteoblasts [21]. We investigated therefore whether the up-regulation observed with pRF1 and pRF5 cells was due to extracellular release of the growth factor. Accordingly, we tested different amounts of the 18-kDa isoform on the IL-6 promoter regulation. Interestingly, as shown in Fig. 4B, large amounts of the growth factor (up to 100 ng/ml) led to a significant down-regulation of the IL-6 promoter ($P < 0.01$; $t = 8.4$). Thus, the IL-6 promoter up-regulation described for the pRF1 and pRF5 cells is not due to an extracellular release of the growth factor.

To further demonstrate that the opposing effects of the 24-kDa isoform was not as a result of the integration site of the transfected plasmid, we performed transitory cotransfection assays with increasing amounts of the pRFGF24 24-kDa FGF-2 coding vector and PrIL-6 vector. The total transfected DNA level was standardized with a neutral plasmid pKSII to avoid any effect of the DNA levels in our studies. IL-6 promoter activity was up-regulated by transiently transfected 24-kDa FGF-2, reaching significance when the 3 μg pRFGFG24/ml transfection medium was used ($P < 0.05$; Fig. 4C), thus confirming the results obtained with pRF cells.

3.4. Distinct pathways involved for exogenous and endogenous FGF-2

As our results suggested distinct signal transduction pathways for exogenous and endogenous FGF-2, we analyzed whether the same regulatory elements were involved in this differential activity.

We constructed a 5′ deletion mutant (positions −224 to +11) of the IL-6 promoter which results in removal of the AP-1 (c-Fos and c-Jun) binding motif. Our transfection experiments showed that deletion of the AP-1 site resulted in a major decrease of the basal level of the IL-6 promoter activity (approx. 6-fold, data not shown), confirming that the AP-1

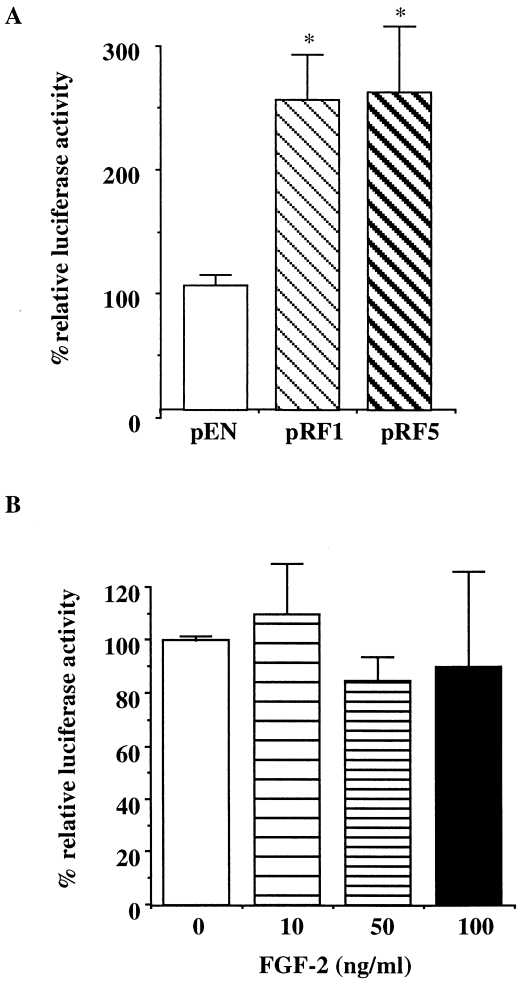

Fig. 5. Effect of the AP-1 site deletion on the regulation of the IL-6 promoter activity by FGF-2. Cells were transfected as before except the prIL-6ΔAP-1 vector replaced the prIL-6 vector. A: Regulation of the AP-1 motif deleted IL-6 promoter by constitutive overexpression of 24-kDa FGF-2 in pRF1 and pRF5 cells. *Significant variation ($P < 0.01$). B: Regulation of the AP-1 site deleted construct by increasing doses of exogenous FGF-2. Data represent means and S.D. for three separate transfection experiments.

sequence is essential for the constitutive expression of the IL-6 gene.

Fig. 5A shows the results obtained when pRF1 and pRF5 cells were transiently transfected with the PrIL-6ΔAP1 reporter vector. The deletion of AP-1 site did not modify the up-regulation of luciferase activity ($P < 0.01$). The more effective and dose-independent stimulation of the promoter might be related to the drastic decrease of basal activity for the AP-1 deleted constructs.

Interestingly, the inhibition of promoter activity containing the AP-1 deletion by the 18-kDa isoform was totally abolished (see Fig. 5B). These results suggest that the AP-1 binding site regulatory element is necessary for the down-regulation of the promoter activity by the extracellular growth factor.

4. Discussion

FGF-2 is synthesized as different molecular weight isoforms with multiple biological functions depending on their localization in specific cell compartments. As an approach to the study of the functional properties of intracellular HMW FGF-2, we have established permanent cell lines that express the recombinant 24-kDa isoform which is not secreted and mainly localized in the cell nucleus [6,22–25].

Depending on the growth factor content in the cells, we typically observed either an increase of the proliferation rate for the pRF1 clone or both a proliferative advantage and a high density growth at saturation (pRF5 clone) in low serum medium. Interestingly, transfection of the same isoform with an adenoviral vector which leads to a very high expression of the protein, promoted growth to a high saturation density and enhanced proliferation in low serum [17]. These data suggest that a high level of the 24-kDa isoform expression is required to alter the parameters of cell growth.

Besides change in cell growth, overexpression of the 24-kDa isoform factor was also able to confer a decreased sensitivity to radiation-induced death. A similar effect was recently demonstrated in HeLa cells [16].

FGF-2 and IL-6 have both been implicated in proliferation of a broad spectrum of cell lines as well as in ionizing radioresistance [26–31]. Further support for the link between these cytokines is the molecular relationship, especially on mRNA stimulation during tumoral progression [11,12], but also relative to common inducers, namely interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα) [18–20]. Interestingly, in human microvascular endothelial HOME cells, the TNFα-induced stimulation of IL-6 expression is preceded by an increase in FGF-2 isoforms, raising the possibility that this effect is mediated by these TNFα-inducible FGF-2 molecules [13]. Therefore the induction of IL-6 gene in the cells transfected by the 24-kDa isoform provides a molecular basis for the change of cell parameters.

In the latter study [13], addition of an FGF-2 antiserum only partially decreased this response. Such a result argues in favor of an intracrinal role for the HMW FGF-2 molecules. It was thus of interest to show that addition of the extracellular 18-kDa isoform led to a different regulation of the IL-6 gene in NIH-3T3 cells. Indeed, such opposite effects between differentially localized FGF-2 isoforms, have already been described. Estival et al. [32] provided evidence that the expression of either the cytosolic 18-kDa or the nuclear-targeted 24-kDa FGF-2 isoform produced an increase in the cell surface high affinity receptors, and by contrast, exogenously added FGF-2 exerted opposite effects resulting in FGF receptor down-regulation.

As such, we investigated both extra- and intracellular FGF-2 effects on the regulation of AP-1 site deleted IL-6 promoter. Indeed, FGF-2 is known to induce early response genes such as *c-jun* and *c-fos* in various cell types [33], and IL-6 expression can be regulated through at least four transcription factors [34], notably the activator protein-1 binding element (AP-1). It was thus interesting to show that deletion of the AP-1 element did not modify the response induced by the 24-kDa isoform whereas it abrogated the down-regulation induced by the 18-kDa isoform. Consequently, our data strongly suggest that the 24-kDa FGF-2-induced up-regulation of IL-6 gene expression does not occur through the activation of the cell surface receptor but rather through an intracrine mechanism. In addition, the opposite effects as well as the differential regulation of IL-6 expression outline the activation of distinct intracellular pathways by 18-kDa and 24-kDa isoforms of FGF-2.

Taken together, our data provide evidence for an intracrine effect of the overexpressed 24-kDa FGF-2 isoform on IL-6 gene up-regulation. Furthermore, whilst regulating the same gene, both isoforms display opposite effects and distinct mechanisms of regulation. Finally, it is of interest to investigate further the link between the 24-kDa FGF-2 isoform and IL-6 in the change in proliferation rate and radiation survival.

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