Constitutive nuclear factor κB activity is required to elicit interferon-γ-induced expression of chemokine CXC ligand 9 (CXCL9) and CXCL10 in human tumour cell lines

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CXC ligand 10 (CXCL10) and CXCL9 are chemoattractants for activated T cells and possess angiostatic activity. Both CXCL9 and CXCL10 have been considered as important components for the anti-tumour activities of interferon-γ (IFNγ) and interleukin-12 in animal models. In this article we show that the CXCL9 and CXCL10 genes in some types of human tumour cell lines are not inducible by IFNγ and we describe experiments designed to explore the molecular mechanisms involved in this impaired induction. The human oral squamous carcinoma line Ca9-22 and the glioma line A172 failed to express CXCL9 and CXCL10 mRNAs in response to IFNγ, whereas other carcinoma lines including HSC-2 did express these mRNAs. Production of these chemokine proteins was also impaired in Ca9-22 cells. The impaired expression was not due to any deficiency in the IFNγ/signal transducer and activator of transcription 1 (STAT1)-dependent signalling pathway. Instead, analysis of nuclear factor κB (NF-κB) activity revealed that the constitutive low level of NF-κB activity, which is seen in cells that express these chemokines, was absent in Ca9-22 and A172 cells. Activation of NF-κB in Ca9-22 cells restored the expression of IFNγ-stimulated CXCL9 and CXCL10 mRNAs. In contrast, inhibition of the constitutive NF-κB in HSC-2 cells by adenovirus-mediated gene transfer of a dominant-negative IκBα suppressed the IFNγ-induced expression of the CXCL9 and CXCL10 mRNAs. These results indicate that constitutive NF-κB activity, which is often associated with tumour development, is required for the induced expression of CXCL9 and CXCL10 genes in human tumour cell lines in response to IFNγ.

Key words: anti-tumour activity, cancer, chemokine, CXC ligand 9 (CXCL9), interferon-γ, nuclear factor κB (NF-κB), oral squamous carcinoma, signal transducer and activator of transcription 1 (STAT1), transcriptional regulation.

INTRODUCTION

CXC ligand 9 (CXCL9) and CXCL10 were originally identified as interferon (IFN)-inducible genes in cells of the monocyte/macrophage cell lineage [1,2]. The expression of CXCL9/MIG (monokine-induced by IFNγ) is exclusively regulated by IFNγ [2], whereas CXCL10/IFN-inducible protein 10 kDa (IP-10) is inducible by both type I and type II IFNs, as well as by lipopolysaccharide [1,3,4]. CXCL9 and CXCL10 share similar biological activities, including chemoattraction of activated T-cells [5–7] and inhibition of neovascularization [8,9]. Furthermore, these IFNγ-inducible chemokines have been shown to exhibit anti-tumour activity in several rodent model systems [10–15]. Although the expression of CXCL9 and CXCL10 in the tumour environment is a critical determinant for inhibition of tumourigenesis, the regulatory mechanisms involved in the expression of CXCL9 and CXCL10 genes in human tumour cells remain to be elucidated fully.

IFNγ is a pleiotropic cytokine that exerts a number of biological activities involved in host-defence and immunomodulatory functions, including anti-tumour responses [16]. The responsiveness of tumour cells to IFNγ is essential for the effective immune recognition and subsequent tumour-suppressor process elicited by host-defense mechanisms [17,18]. However, many tumour cells have been shown to escape from the cancer immunosurveillance and to exhibit a resistance to treatment by either type I or type II IFN [17–21]. The molecular mechanisms involved in the unresponsiveness to IFN have been attributed to a deficiency of components of the IFN signalling pathway, including expression of the IFNγ receptor, Janus kinases (JAKs), IFN regulatory factor-9 (IRF-9)/p48, STAT1 (signal transducer and activator of transcription) 1 and STAT2 [17,19–21]. Thus, the JAK–STAT pathway is essential for the sensitivity of tumour cells to both type I and type II IFNs and for cancer immunosurveillance.

Because IFNγ is integral to immune surveillance toward cancer and CXCL9 and CXCL10 induced by IFNγ exhibit anti-tumour activity *in vivo*, we wanted to determine whether some human tumour cells fail to express these IFNγ-inducible chemokines, and if so, to explore the molecular mechanisms that underlie their impaired expression. In the present study, we evaluated the IFNγ-induced expression of CXCL9 and CXCL10 genes in several human tumour-cell lines and found that these chemokine genes were not inducible by IFNγ in the human squamous carcinoma cell (SCC) line Ca9-22 and the human glioma cell line A172. However, the impairment for the expression was not due to any defect in IFNγ-activated STAT1. Instead, constitutive nuclear factor κB (NF-κB) activity, which was seen in cells that expressed the IFNγ-induced CXCL9 and CXCL10 genes, was absent in Ca9-22 and A172 cells. Our data indicate that constitutive NF-κB activity, which is often seen in many tumour cells, is a critical determinant for the expression of the IFNγ-induced anti-tumour chemokines CXCL9 and CXCL10, and that downregulation of

Abbreviations used: CXCL, CXC ligand; EMSA, electrophoretic mobility shift assay; GAS, IFNγ activation sequence; IFN, interferon; IKK, IκB kinase; IP-10, IFN inducible protein 10 kDa; IRF, interferon regulatory factor; ISRE, interferon-stimulated responsive element; JAK, Janus kinase; MIG, monokine induced by IFNγ; NF-κB, nuclear factor κB; SCC, squamous carcinoma cells; STAT, signal transducer and activator of transcription; TK, thymidine kinase; TNFα, tumour necrosis factor α.

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constitutive NF-κB activity may inhibit the expression of these IFNγ-inducible chemokines in human tumour cells.

MATERIALS AND METHODS

Reagents

Recombinant human IFNγ and tumour necrosis factor α (TNFα), and ELISA kits for CXCL9 and CXCL10 were purchased from R&D Systems Inc. (Minneapolis, MN, U.S.A.). Antibodies against STAT1 (E23), NF-κB p50 (NLS), RelA (C-20), RNA polymerase II (N-20), the inhibitor of NF-κB (IκBα) (C-21) and β-actin (I-19), and normal rabbit IgG were obtained from Santa Cruz Biotechnology (Hercules, CA, U.S.A.). FuGene transfection reagent was purchased from Roche (Nutley, NJ, U.S.A.). Goat anti-mouse IgG antibody, labelled with Alexa Fluor 488 dye, was obtained from Molecular Probes (Eugene, OR, U.S.A.). Other reagents used in this study were as described previously [22].

Cell culture

The human oral SCC lines HSC-2, HSC-3, HSC-4 and Ca9-22, and the human pharyngeal carcinoma cell line KB were described previously [23,24] and were kindly provided by Dr K. Kusama (Department of Oral Pathology, Meikai University School of Dentistry, Saitama, Japan). HSC-2 was originally isolated from a metastatic oral SCC derived from the floor of the oral cavity, and Ca9-22 was established from SCC of the gingiva [23,24]. These cells were grown in RPMI1640 containing L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 10 % (v/v) foetal bovine serum. The human glioma cell lines T98G and A172 were cultured in Dulbecco's modified Eagle's medium containing the above additives, as described previously [25,26].

Preparation of RNA and Northern hybridization analysis

Confluent monolayers of cells were stimulated as indicated in the legends to Figures 1 and 2. Preparation of total RNA by the guanidine isothiocyanate/cesium chloride method and Northern-hybridization analysis were carried out as described previously [22]. The cDNA probe used to detect human CXCL10 [1] was kindly provided by Dr R. M. Ransohoff (Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, U.S.A.) and was described previously [27]. A cDNA fragment for human CXCL9 (MIG) was prepared by reverse transcriptase–PCR using a set of primers that correspond to the human MIG cDNA sequence obtained from the GeneBank database [2]. The PCR product was subcloned into a pBluescript vector (Stratagene, La Jolla, CA, U.S.A.) and the nucleotide sequence was confirmed. Some Northern blots were quantified by using a Molecular Imager (Bio-Rad, Hercules, CA, USA). The relative magnitude of expression was normalized to values for glyceraldehyde-3-phosphate dehydrogenase expression in the same experiment.

Measurement of CXCL9 and CXCL10 protein contents by ELISA

The levels of CXCL9 and CXCL10 protein in the culture supernatant of HSC-2 and Ca9-22 cells were determined by using an ELISA. Briefly, confluent monolayer of cells cultured in 24-well plates were stimulated as indicated in the legend to Figure 3. After incubation for the indicated times, the culture supernatant was removed and evaluated for CXCL9 and CXCL10 content by using an ELISA kit (R&D Systems) according to the manufacturer's instructions.

Preparation of nuclear extracts

Nuclear extracts were prepared by using a modification of the method of Dignam et al. [28], as described previously [22]. After stimulation, the cells were washed three times with ice-cold PBS, harvested, and resuspended in 300 μl of hypotonic buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT (dithiothreitol), 1 mM PMSF, and 10 μg/ml each of leupeptin, antipain, aprotinin and pepstatin) for 10 min on ice. The cells were then lysed in 0.6 % Nonidet P-40 by vortex mixing for 10 s. Nuclei were separated from cytosol by centrifugation at 12 000 ***g*** for 30 s, and the supernatants were collected as the cytosol fraction. Nuclei were then washed three times with 300 μl of buffer A, and resuspended in buffer C [20 mM Hepes, pH 7.9, 25 % (v/v) glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 10 μg/ml each of leupeptin, antipain, aprotinin and pepstatin] and sonicated briefly on ice. Nuclear extracts were obtained by centrifugation at 12 000 ***g*** for 10 min.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (10 μg of protein) were incubated for 15 min at room temperature in 12.5 μl of 20 mM Hepes, pH 7.9, containing 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 5 % (v/v) glycerol, 200 μg/ml of BSA and 1.25 μg of poly(dI–dC). A ^{32}P-labelled double-stranded oligonucleotide (0.5 ngl 5×10^5 counts/min), 5′-tcgaGCCTGATTTCCCCGAAATGAGGC-3′ from the IRF (interferon regulatory factor)-1 IFNγ activation sequence (GAS) site [29] or 5′-tcgaAGTAGGGTTTTCCCCAGGA-3′ from the CXCL9 κB2 site [30], was then added to the reaction mixture, which was then incubated for 15 min at room temperature. The reaction products were analysed by electrophoresis in a 5 % (w/v) polyacrylamide gel with 0.25× TBE buffer (22.3 mM Tris/HCl, 22.2 mM borate, 0.5 mM EDTA). In some experiments, rabbit anti-NFκB1(p50), anti-RelA(p65) or anti-STAT1 antibodies were added prior to electrophoresis. The dried gels were analysed by autoradiography and by phosphorescence detection.

Western-blot analysis

Nuclear or cytosolic extracts were resolved in SDS/PAGE sample buffer (62.5 mM Tris/HCl, pH 6.8, containing 2 % (w/v) SDS, 20 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol and 0.2 % (w/v) Bromophenol Blue) and separated by SDS/PAGE in a 7.5 % (w/v) polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane by semi-dry blotting. Blots were blocked for 1 h with 5 % (w/v) non-fat milk in TBS-T [50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl and 0.1 % (v/v) Tween-20] and then incubated with primary antibodies overnight at 4 °C. After washing with TBS-T, the blots were incubated for 1 h at room temperature with secondary antibodies conjugated to horseradish peroxidase and were washed again with TBS-T. The blots were developed by using a SuperSignal West Pico chemiluminescence substrate kit (Pierce, Rockford, IL, U.S.A.).

Immunocytochemistry

Cells grown on Lab-tek chamber slides (Nunc, Rochester, NY, U.S.A.) were stimulated with IFNγ or TNFα for 30 min. They were then fixed at room temperature in 95 % (v/v) ethanol containing 5 % (v/v) acetic acid and 0.5 % (v/v) Triton X-100, and subsequently, were reacted with anti-STAT1 or anti-RelA (p65) antibodies at room temperature for 1 h, after which the unbound

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antibody was removed by washing with PBS. Bound antibody was detected with goat anti-rabbit IgG conjugated with Alexa Fluor 488 dye. Immunofluorescence was detected by confocal laser scanning microscopy (LSM 510; Carl Zeiss, Göttingen, Germany).

Reporter assay

The luciferase reporter construct pTK GASluc, in which four copies of the GAS motif from IRF-1 [29] were placed in front of the herpes simplex virus thymidine kinase (TK) promoter, was described previously [31]. pTK κB2luc, which contains five copies of the κB motif from the CXCL10/IP-10 gene, was described earlier [32]. The pCMVluc control luciferase plasmid was kindly provided by Dr Ganes Sen (Department of Molecular Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, U.S.A.). Cells were transiently transfected with luciferase reporter plasmids and pRL-TK reference *Renilla* luciferase plasmid (Promega, Madison, WI, U.S.A.) by using FuGene transfection reagents (Roche, Nutley, NJ, U.S.A.), according to the manufacturer's instructions. After 24 h, the cells were treated with IFNγ for 8 h. Firefly and *Renilla* luciferase activities were assayed by using reagents provided by Promega, according to the manufacturer's instructions. For standardization of the transfection efficiencies, the luciferase activity from pTK GASluc or pTK κB2luc was normalized to the *Renilla* luciferase activity, and the relative luciferase activities in HSC-2 and Ca9-22 cells were normalized to the activity of pCMVluc.

Adenovirus transfection

The recombinant replication-defective adenovirus encoding the cDNA for the mutant form of IκBα (Ad5IκB) and control recombinant adenovirus Ad5LacZ, which contains the β-galactosidase gene, were kindly provided by Dr R. W. Ganster (Department of Surgery, University of Pittsburgh, Pittsburgh, PA, U.S.A.) and were described previously [33]. Ad5mIκB contains an IκBα sequence with S32A and S36A mutations [34]. These recombinant adenovirus were scaled up and purified using two CsCl density gradient centrifugations. Virus titres were determined by a standard serial dilution assay using the 293 cell line, and the recombinant viruses were stored in storage buffer [10 mM Tris/HCl, pH 7.5, 135 mM NaCl, 1 mM $MgCl_2$ and 10 % (v/v) glycerol] at – 80 °C.

For the adenovirus transfection, confluent monolayers of HSC-2 cells were infected with Ad5mIκB or Ad5LacZ at a multiplicity of infection of 50. After the infection, the cells were washed with PBS, replenished with fresh medium and cultured for another 48 h. The infected cells were then stimulated with IFNγ and/or TFNα as indicated in the legends to Figures 9 and 10. Transfection efficiency for adenovirus Ad5LacZ was approx. 90 %, as measured by β-galactosidase staining. Levels of mutant IκBα protein in Ad5mIκB transfected cells were determined by Western-blot analysis with an anti-IκBα antibody.

RESULTS

Impaired expression of IFNγ-induced chemokines CXCL9 and CXCL10 in human oral carcinoma cells

IFNγ-dependent expression of CXCL9 and CXCL10 was earlier shown to exert an anti-tumour effect *in vivo*, and the sensitivity to IFNγ was found to be a critical determinant for cancer immunosurveillance [11–15]. To examine the responsiveness of human tumour cell lines to IFNγ for the expression of CXCL9 and CXCL10, we assessed the expression of the mRNAs of these chemokines in the five oral SCC lines indicated in Figure 1. These cells were treated with IFNγ for 4 h and total RNA was prepared and analysed by Northern hybridization. Although IFNγ markedly induced the expression of CXCL9 and CXCL10 mRNAs in HSC-2, HSC-3 and HSC-4 cells, the expression of both was severely impaired in Ca9-22 and KB cells (Figure 1). A time-course experiment (Figure 2A) showed that, although the

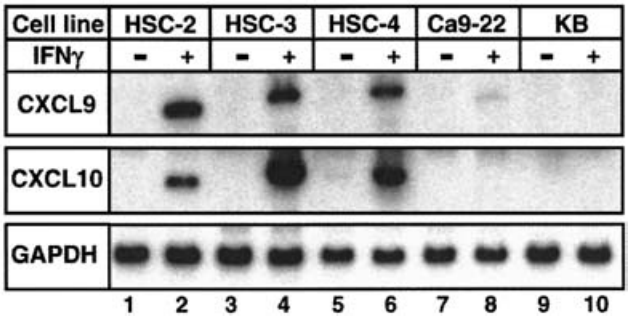

Figure 1 Impaired expression of IFNγ-induced CXCL9 and CXCL10 mRNAs in human oral carcinoma cell lines

Human oral SCC lines were either left untreated or treated with IFNγ (10 ng/ml) for 4 h before preparation of total RNA and analysis for specific mRNA levels by Northern hybridization. Total RNA (5 μg) was analysed in each lane. Blots were hybridized with the indicated radiolabelled cDNA probes. Similar results were obtained in three individual experiments.

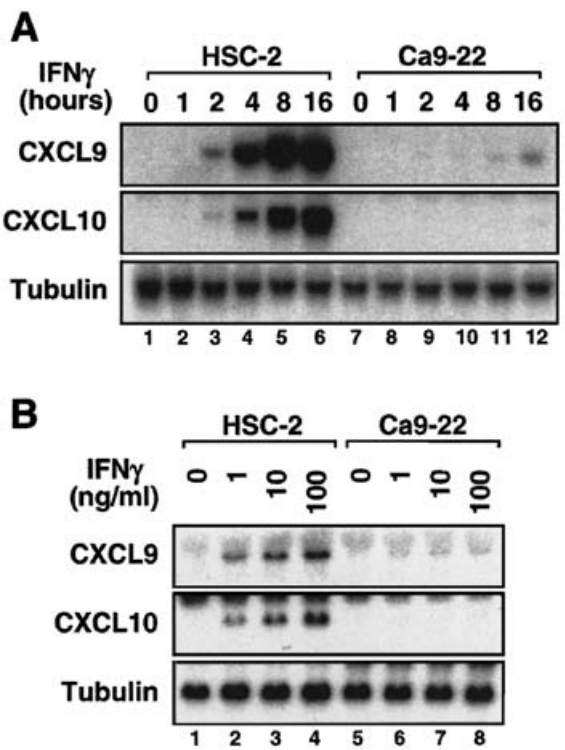

Figure 2 Time- and dose-dependent expression of CXCL9 and CXCL10 mRNAs in HSC-2 cells in response to IFNγ

(**A**) HSC-2 and Ca9-22 cells were either left untreated (0 h) or treated with IFNγ (10 ng/ml) for various periods time as indicated before preparation of total RNA and analysis of specific mRNA levels by Northern hybridization. (**B**) HSC-2 and Ca9-22 cells were either left untreated (0 ng/ml) or treated with increasing concentrations of IFNγ for 4 h as indicated before preparation of total RNA and analysis of specific mRNA levels by Northern hybridization. Similar results were obtained in three individual experiments.

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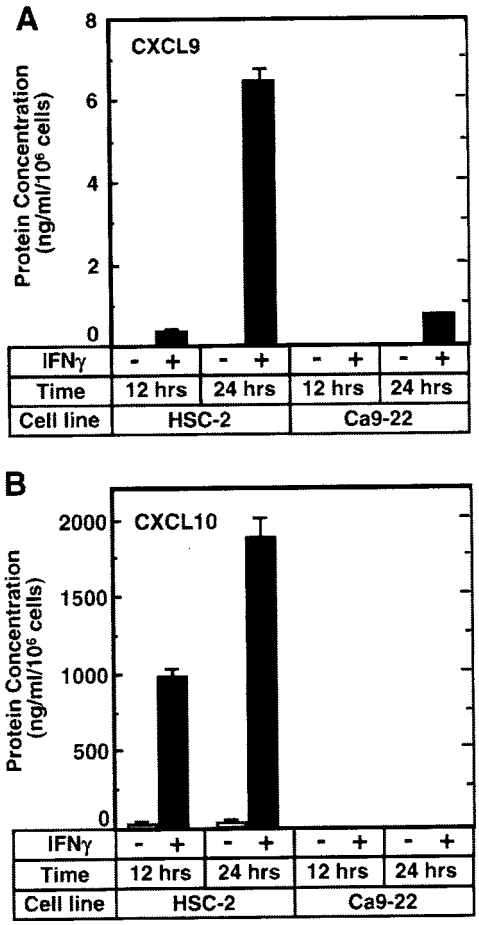

Figure 3 Protein contents of CXCL9 and CXCL10 in culture supernatants from IFNγ-stimulated HSC-2 and Ca9-22 cells

Confluent monolayers of cells cultured in 24-well plates were stimulated with IFNγ (10 ng/ml) for the times indicated or were left untreated. After incubation, the culture supernatant was removed and measured for CXCL9 (**A**) and CXCL10 (**B**) protein content by ELISA. The protein contents are shown as ng $\cdot$ ml^{-1} $\cdot$ 10^6 cells^{-1}. Each column and bar represent the mean $\pm$ S.E.M. of three independent experiments.

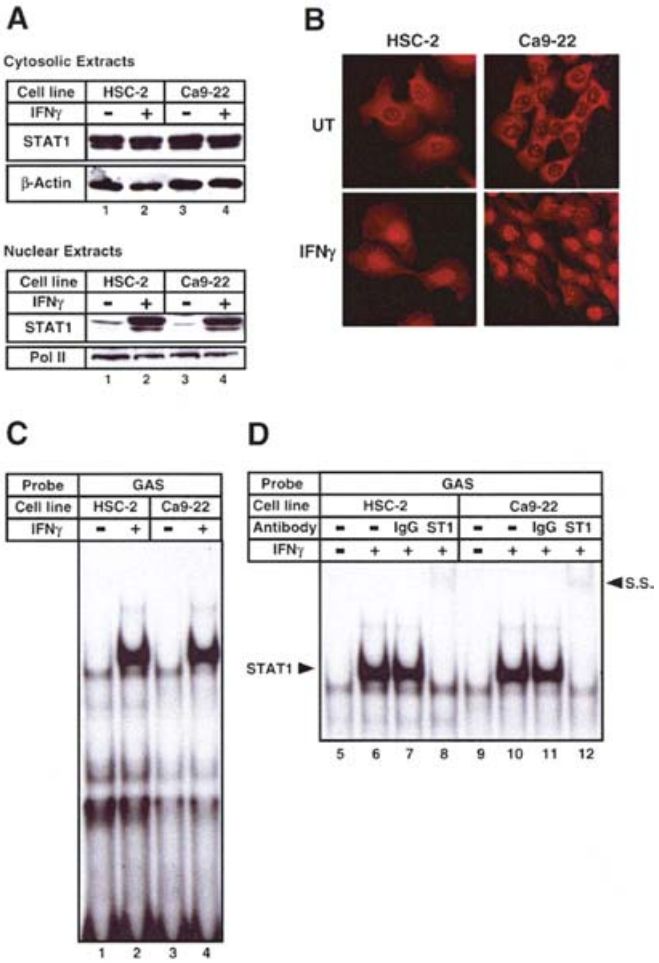

Figure 4 IFNγ activates STAT1 in HSC-2 and Ca9-22 cells

(**A**) Levels of cytoplasmic and nuclear STAT1 protein in IFNγ-treated oral carcinoma cell lines. HSC-2 and Ca9-22 cells were either left untreated or treated with IFNγ (10 ng/ml) for 30 min before preparation of cytoplasmic and nuclear extracts. Equal amounts of cytoplasmic or nuclear protein (20 μg/lane) were loaded on the gel and analysed by Western blotting using anti-STAT1, anti-β actin or RNA polymerase II (Pol II) antibodies. The anti-β actin or anti-Pol II antibodies were used to evaluate the loading of equal amounts of cytosolic and nuclear proteins respectively. (**B**) Immunofluorescence microscopy of HSC-2 and Ca9-22 cells stained with an anti-STAT1 antibody. HSC-2 and Ca9-22 cells were either left untreated or treated with IFNγ (10 ng/ml) for 30 min. Immunofluorescence was detected by confocal laser scanning microscopy. (**C**) DNA-binding activity of STAT1 in nuclear extracts from IFNγ-stimulated cells. HSC-2 and Ca9-22 cells were treated with IFNγ as described above, prior to preparation of nuclear extracts. Each nuclear extract (10 μg) was analysed for GAS-binding activity by EMSA using radiolabelled oligonucleotides as described in the Materials and Methods section. (**D**) An antibody-supershift assay for STAT1. Nuclear extracts were incubated with the indicated antibodies (1 μg) before analysis of the binding activities. Super-shifted complexes (s.s.) are shown. ST1; anti-STAT1 antibody. Similar results were obtained from three independent experiments.

levels of CXCL9 and CXCL10 mRNAs in HSC-2 cells increased in a time-dependent manner in response to IFNγ (10 ng/ml), only low levels of these chemokine mRNAs were observed in the Ca9-22 cells. Furthermore, a higher concentration of IFNγ (100 ng/ml) failed to induce an increase in the expression of these chemokine mRNAs in the Ca9-22 cells (Figure 2B, lane 8). We did not observe any induction of these chemokine mRNAs in KB cells (results not shown). To determine whether the impaired expression of these chemokine mRNAs correlates with the differences in protein production, we measured the amounts of these chemokine proteins in culture supernatants by using an ELISA. As shown in Figure 3, although marked increases in the protein contents of CXCL9 and CXCL10 were detected in the culture supernatant of HSC-2 cells stimulated with IFNγ for 24 h, only small amounts of these chemokines were induced in Ca9-22 cells (0.73 ng/ml for CXCL9 and 3.87 ng/ml for CXCL10 after 24 h of stimulation with IFNγ). Thus, these results indicate that the IFNγ-induced expression of CXCL9 and CXCL10 mRNAs and their protein was impaired in the Ca9-22 cells.

IFNγ-induced STAT1-dependent pathway is intact in Ca9-22 cells

Previous studies demonstrated that certain tumour cells are unresponsive to IFNγ and that this unresponsiveness results from deficiencies, including a defective STAT1 protein in the JAK–STAT-signalling pathway [17,19]. Because the IFNγ-induced expression of the CXCL9 and CXCL10 genes depends upon the activation of STAT1 [22,35,36], we investigated whether the impaired expression of the IFNγ-induced chemokines in Ca9-22 cells might be attributed to a defective STAT1-dependent signalling pathway. Initially, we assessed levels of STAT1 protein in cytosolic and nuclear extracts from HSC-2 and Ca9-22 cells by Western-blot analysis. As shown in Figure 4(A), the

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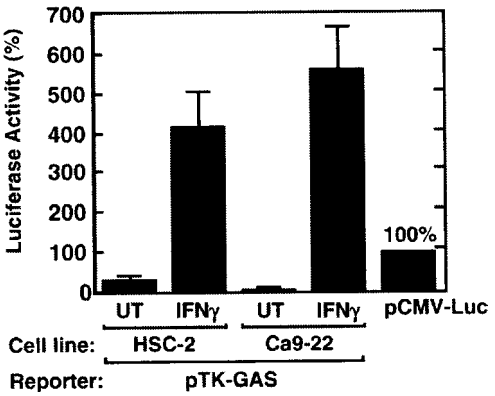

Figure 5 STAT1-dependent transcriptional activities in IFNγ-stimulated HSC-2 and Ca9-22 cells

HSC-2 and Ca9-22 cells were transiently transfected with the indicated pTK-GAS or pCMVluc luciferase reporter construct (3 μg). After transfection (24 h), the cells were either left untreated or treated with IFNγ (10 ng/ml) for 8 h prior to measurement of luciferase activity. Relative luciferase activities are shown as percentages of the activities from pCMV-Luc-tranfected cells. Each column and bar represents the mean ± S.E.M. of three independent experiments.

levels of STAT1 protein in the cytosolic extracts from Ca9-22 cells were comparable with those from HSC-2 cells. When either cell type was stimulated with IFNγ, a similar amount of STAT1 was translocated to the nucleus (nuclear extracts; Figure 4A, lanes 2 and 4). β-Actin and RNA polymerase II were used as loading controls for cytoplasmic and nuclear protein respectively. Consistent with this finding, immunostaining for STAT1 demonstrated that the treatment with IFNγ led to nuclear translocation of STAT1 in both HSC-2 and Ca9-22 cells (Figure 4B). In addition, we assessed nuclear extracts from IFNγ-treated cells for their ability to bind the STAT1-binding motif in DNA (Figure 4C). IFNγ induced prominent DNA binding to IRF-1 GAS, a high-affinity binding motif for STAT1 [29], in nuclear extracts from both cell types (Figure 4C, lanes 2 and 4); and the DNA-binding complex contained STAT1, as demonstrated by the results of the antibody-supershift assay (Figure 4D, lanes 8 and 12). A similar IFNγ-induced STAT1-binding activity was observed in KB cells (results not shown). To determine whether the IFNγ-activated STAT1 in Ca9-22 cells might have a functional deficiency in trans-activating capacity, we performed a luciferase reporter assay using a heterologous promoter construct containing four copies of the GAS motif (pTK-GAS), which mediates STAT1-depdendent transactivation [29,31]. As shown in Figure 5, strong IFNγ-dependent luciferase activities were observed in both HSC-2 and Ca9-22 cells. Taken together, these results indicate that the IFNγ-induced STAT1-dependent transcriptional activity was intact in Ca9-22 cells.

Constitutive NF-κB activation in HSC-2 cells, but not in Ca9-22 cells

Although previous studies demonstrated that IFNγ-induced STAT1 is indispensable for the transcriptional activation of the CXCL9 and CXCL10 genes, NF-κB is also required to induce optimal transcription of these genes in concert with STAT1 [22,30,36]. Furthermore, several earlier studies showed that NF-κB is constitutively activated in some human tumour cells [37–43]. From these lines of evidence, we postulated that the impaired expression of the CXCL9 and CXCL10 genes in Ca9-22 cells might have resulted from the loss of constitutive NF-κB activation. To determine the involvement of constitutive NF-κB, we

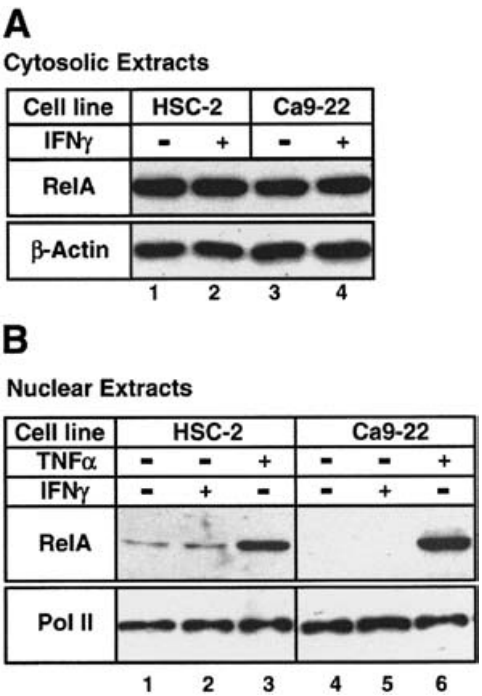

Figure 6 Constitutive NF-κB activity in the nucleus of HSC-2 cells

Levels of cytosolic (**A**) and nuclear (**B**) RelA (p65) protein in HSC-2 and Ca9-22 cells. HSC-2 and Ca9-22 cells were either left untreated or treated with IFNγ (10 ng/ml) or TNFα (10 ng/ml) for 30 min before preparation of cytosolic and nuclear extracts. Equal amounts of cytoplasmic or nuclear protein (20 μg/lane) were loaded on the gel and then analysed by Western blotting using anti-RelA, anti-β actin or anti-RNA polymerase II (Pol II) antibodies. Pol II was used to monitor the addition of equal amounts nuclear protein. Similar results were obtained from three separate experiments.

assessed the levels of NF-κB protein in HSC-2 and Ca9-22 cells by Western-blot analysis (Figure 6). In the cytosolic extracts, similar levels of RelA protein were observed in the extracts from both HSC-2 and Ca9-22 cells (Figure 6A). Interestingly, a low level of the RelA protein was detected in the nuclear extracts from unstimulated HSC-2 cells (Figure 6B, lane 1). However, such constitutive levels RelA protein were not seen in the extracts from Ca9-22 cells (Figure 6B, lane 4). Treatment with IFNγ had no effect on the levels of RelA protein in both cell types (Figure 6B, lanes 2 and 5), although TNFα increased the nuclear translocation of the RelA protein (Figure 6B, lanes 3 and 6). The increased level of the constitutive nuclear RelA protein in HSC-2 was not due to a difference in the amount of the nuclear extracts loaded, as the levels of RNA polymerase II in the nuclear extracts from both cells were comparable.

To characterize further the constitutive NF-κB, we performed an EMSA using an oligonucleotide that contained the κB2 site from the CXCL9 gene [30]. As shown in Figure 7(A), a prominent constitutive NF-κB-binding activity was observed in the nuclear extracts from untreated HSC-2 cells (Figure 7A, lane 1; C1 and C2 complexes). Although faints bands were detected in Ca9-22 cells (Figure 7A, lane 4), such prominent constitutive NF-κB-binding activity (C1 and C2 complexes) was absent in the extracts from these cells. Consistent with the results from Western blots (Figure 6), treatment with IFNγ failed to induce DNA-binding activity by NF-κB in either cell type. In contrast, TNFα markedly induced this activity in nuclear extracts from Ca9-22 cells (Figure 7A, lane 6), indicating that cellular components necessary for the NF-κB activation were intact in these cells. An antibody-supershift assay demonstrated that the constitutive

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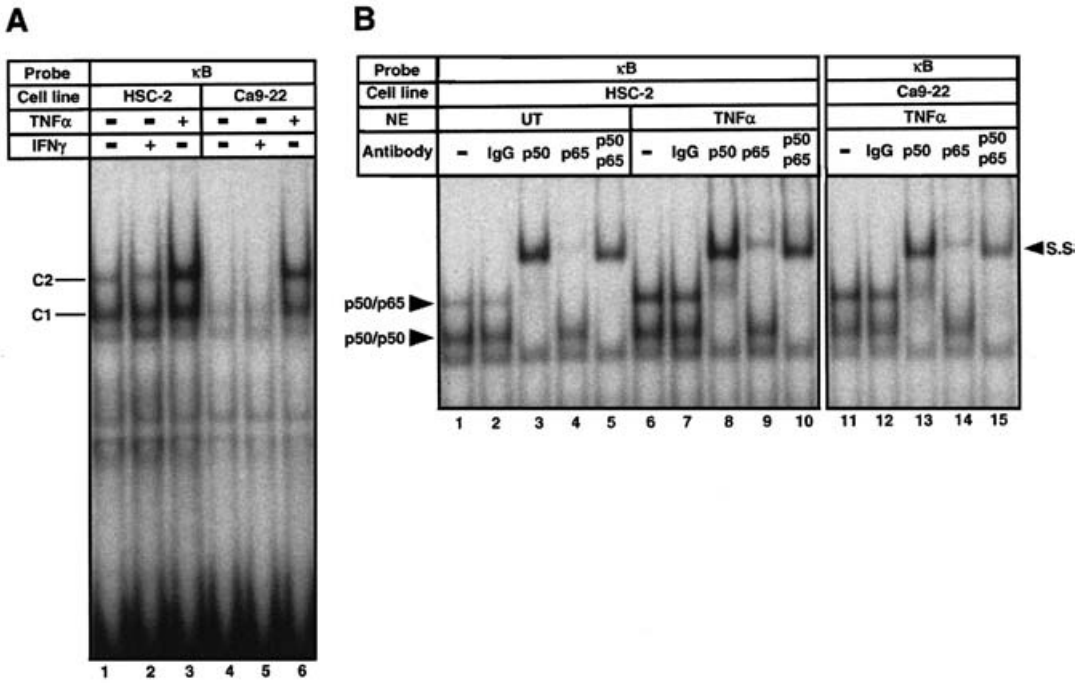

Figure 7 NF-κB DNA-binding activity in HSC-2 and Ca9-22 cells

(**A**) HSC-2 and Ca9-22 cells were either left untreated or treated with IFNγ (10 ng/ml) or TNFα (10 ng/ml) for 30 min before the preparation of nuclear extracts. Each nuclear extract (10 μg) was analysed for NF-κB-binding activity by EMSA using radiolabelled oligonucleotides as described in the Materials and Methods section. Similar results were obtained from three separate experiments. (**B**) An antibody supershift-assay for NF-κB. Nuclear extracts were incubated with the indicated antibodies (1 μg) before analysis of the binding activities. Super shifted complexes (s.s.) are shown. NE, nuclear extracts; UT, untreated.

NF-κB binding complexes in the nuclear extracts from HSC-2 cells were composed of RelA (p65)/NF-κB1 (p50) heterodimers and NF-κB1 (p50) homodimers (Figure 7B, lanes 1–5). The composition of NF-κB subunits was similar to that seen in nuclear extracts from TNFα-induced cells (Figure 7B, lanes 6–15). Thus, these results demonstrate that prominent constitutive NF-κB activity was detected in HSC-2 but not Ca9-22 cells, and suggest that the impaired expression of CXCL9 and CXCL10 in Ca9-22 cells can be accounted for by the lack of the constitutive NF-κB.

Activation of NF-κB needed for IFNγ-mediated induction of CXCL9 and CXCL10 mRNA expression in Ca9-22 cells

If the low level of constitutive NF-κB is required to induce the expression of CXCL9 and CXCL10 genes, then exogenous activation of NF-κB should restore the expression of these chemokines in Ca9-22 cells in response to IFNγ. Because TNFα was able to induce NF-κB activation in Ca9-22 cells (Figure 7), we next examined the effect of TNFα on the expression of the CXCL9 and CXCL10 mRNAs in these cells. As shown in Figure 8, although TNFα alone had no stimulatory effect on the expression of CXCL9 and CXCL10 mRNAs in Ca9-22 cells (Figure 8, lane 3), a marked induction of these mRNAs was observed in the cells treated with IFNγ and TNFα (Figure 8, lane 4). In HSC-2 cells, simultaneous treatment with IFNγ and TNFα also potentiated the IFNγ-induced expression of CXCL9 and CXCL10 mRNAs (Figure 8, lane 8), which is consistent with the results from the EMSA that showed that the treatment with TNFα enhanced further the DNA-binding activity of NF-κB (Figure 7A, lane 3). Thus, these results suggest that the impairment of the expression of the IFNγ-stimulated CXCL9 and CXCL10 mRNAs is due to the lack of the constitutive NF-κB activity in Ca9-22 cells.

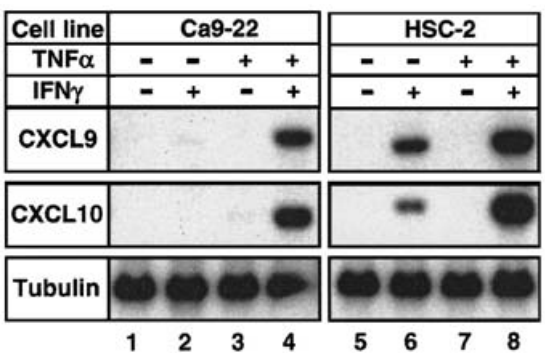

Figure 8 Exogenous activation of NF-κB by TNFα treatment restores the IFNγ-induced expression of CXCL9 and CXCL10 mRNAs in Ca9-22 cells

Ca9-22 and HSC-2 cells were either left untreated or treated with IFNγ (10 ng/ml) and/or TNFα (10 ng/ml) for 4 h before preparation of total RNA and analysis of specific mRNA levels by Northern hybridization. Total RNA (5 μg) was analysed in each lane. Similar results were obtained in three independent experiments.

Constitutive active NF-κB is required for the IFNγ-induced CXCL9 and CXCL10 mRNA expression in HSC-2 cells

We analysed the functional significance of the constitutive NF-κB activity in the expression of the CXCL9 and CXCL10 mRNAs in HSC-2 cells. If constitutive NF-κB is required to induce mRNA expression of these chemokines in HSC-2 cells, inhibition of constitutive NF-κB activity should decrease the expression of these IFNγ-induced chemokines. To test this possibility, we transfected HSC-2 cells with an adenoviral expression vector that encodes a dominant-negative form of IκBα, which is non-degradable IκBα and functions as a super-repressor of NF-κB

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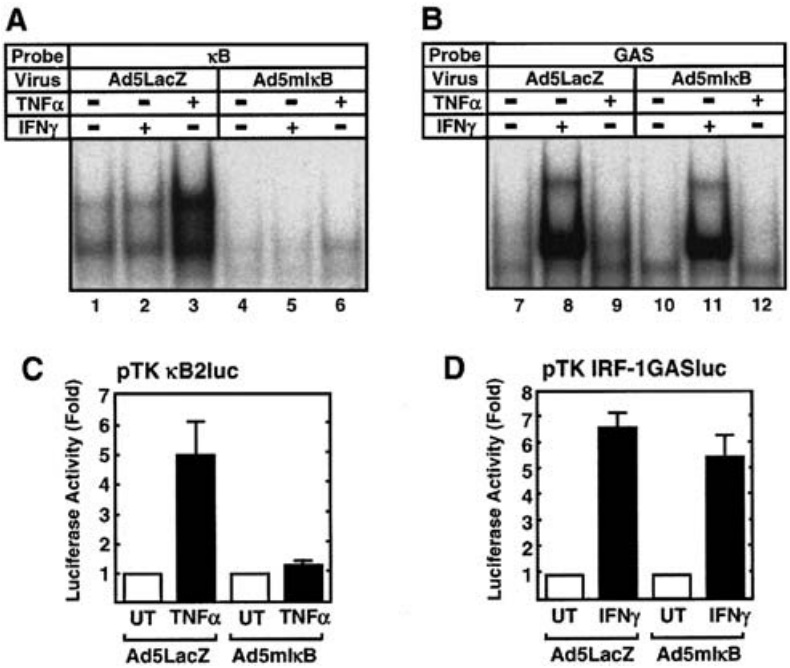

Figure 9 Dominant-negative IκBα inhibits constitutive NF-κB activity in HSC-2 cells

HSC-2 cells were infected with recombinant replication-defective adenovirus encoding the cDNA for the mutant form of IκBα (Ad5mIκB) or with control recombinant adenovirus Ad5LacZ. After the infection, the cells were washed with PBS, replenished with fresh medium and cultured for a further 48 h. The infected cells were then stimulated with IFNγ (10 ng/ml) or TFNα (10 ng/ml) for 30 min before preparation of nuclear extracts. Each nuclear extract (10 μg) was analysed for NF-κB (**A**) or GAS DNA-binding activity (**B**) by EMSA using radiolabelled oligonucleotides as described in the Materials and Methods section. Similar results were obtained from three separate experiments. (**C**) and (**D**) Reporter-gene analysis for NF-κB- and STAT1-dependent transactivation in the adenovirus-infected HSC-2 cells. The cells were transfected with the indicated luciferase reporter constructs (3 μg). After transfection (24 h), the cells were infected with Ad5mIκB or Ad5LacZ as described above and stimulated with IFNγ (10 ng/ml) (**D**) or TFNα (10 ng/ml) (**C**) for 8 h, before being analysed for luciferase activity. The relative luciferase activity is shown as a fold induction compared with the activity of unstimulated samples. Each column and bar represent the mean ± S.E.M. of three independent experiments.

[33,34]. As shown in Figure 9(A), transfection with the dominant-negative IκBα (Ad5mIκB), but not with the control virus (Ad5LacZ), significantly decreased the constitutive as well as the TNFα-induced NF-κB binding activity in HSC-2 cells (Figure 9A, lanes 4–6) without affecting the IFNγ-induced STAT1-binding activity (Figure 9B, lane 11). The transfection efficiency for the control virus (Ad5LacZ) was approx. 90%, as measured by β-galactosidase staining. When the effect of dominant-negative IκBα on κB-dependent transcriptional activity was analysed, the Ad5mIκB virus markedly inhibited the NF-κB-dependent transcriptional activity, as assessed by the luciferase reporter assay (Figure 9C). In contrast, the dominant-negative IκBα had no inhibitory effect on the IFNγ-induced GAS-dependent transcriptional activity (Figure 9D), indicating that the dominant negative IκBα specifically inhibited the NF-κB-dependent transcriptional activity.

To determine the functional significance of the constitutive NF-κB activity for the IFNγ-induced chemokine expression in HSC-2 cells, we transfected HSC-2 cells with either Ad5mIκB or Ad5LacZ, and stimulated the cells with IFNγ for 4 h before preparation of total RNA for Northern-blot analysis (Figure 10A). Although the treatment with IFNγ induced expression of CXCL9 and CXCL10 mRNAs in the control virus-transfected cells (Figure 10A, lane 2), a significant decrease in the levels of these IFNγ-induced chemokine mRNAs was observed in cells transfected with Ad5mIκB (Figure 10A, lane 6). Quantitative analysis of these blots showed that the transfection with Ad5mIκB decreased the levels of CXCL9 and CXCL10 mRNAs by 70% and 95% respectively (Figure 10B). Taken together, these results indicate that the constitutive NF-κB activity in HSC-2 is required to induce the IFNγ-induced expression of CXCL9 and CXCL10 mRNA.

Impaired expression of IFNγ-induced CXCL9 and CXCL10 mRNA in a human glioma cell line

To determine whether constitutive NF-κB activity is required for the IFNγ-induced expression of CXCL9 and CXCL10 in other tumour cells, we sought additional tumour cell lines that might be unresponsive to IFNγ in terms of the expression of these chemokines. Of the cell lines that we examined, the human glioma cell line A172 also exhibited barely detectable levels of these chemokine in response to IFNγ (Figure 11A, lane 6). In contrast, IFNγ markedly induced the expression of CXCL9 and CXCL10 mRNAs in another human glioma cell line T98G (Figure 11A, lane 2). The levels of STAT1 protein were comparable between these glioma cells (results not shown), and comparable levels of IFNγ-induced STAT1 DNA-binding activity were observed in both cells, as demonstrated by EMSA (Figure 11B, lanes 2 and 6). However, as we expected, constitutive NF-κB was not observed in the nuclear extracts from A172 cells (Figure 11C, lane 4). In contrast, T98G cells exhibited a detectable level of constitutive NF-κB activity (Figure 11C, lane 1), and treatment with IFNγ slightly increased DNA-binding activity of NF-κB (Figure 11C, lane 2). The antibody-supershift assay demonstrated that faster- and slower-migrating complexes contained the NF-κB1(p50) homodimer and NF-κB1(p50)/RelA(p65) heterodimer respectively (results not shown). As in the case of the oral SCC line Ca9-22, activation of NF-κB by TNFα restored the expression

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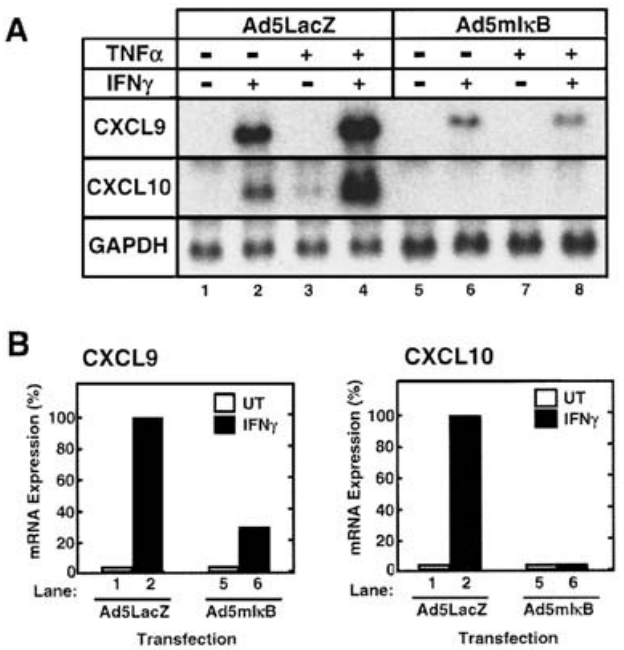

Figure 10 Inhibition of IFNγ-induced expression of CXCL9 and CXCL10 mRNAs by dominant-negative IκBα

(**A**) HSC-2 cells were either infected with dominant-negative IκBα (Ad5mIκB) or control adenovirus Ad5LacZ, as described in the legend to Figure 9. After the infection, the cells were stimulated with IFNγ (10 ng/ml) and/or TFNα (10 ng/ml) for 4 h, before preparation of total RNA and analysis of specific mRNA levels by Northern hybridization. Similar results were obtained in three separate experiments. (**B**) Northern blots were quantified by PhosphorImager analysis, and relative mRNA levels are presented as a percentage of the IFNγ-stimulated expression in cells infected with control virus (Ad5LacZ).

of the CXCL9 and CXCL10 mRNAs in A172 cells, and a marked increase in the chemokine mRNA levels was observed (Figure 11A, lane 8). Thus, taken together, these results indicate that a low level of constitutive NF-κB activity is required to induce the expression of IFNγ-stimulated CXCL9 and CXCL10 genes in human tumour cells.

DISCUSSION

Certain types of tumour cells are unresponsive to IFNγ and escape from immunosurveillance by the host-defence system. These deficiencies are often associated with defective components of the JAK–STAT signalling pathway [17,19]. In the present study, we found that the CXCL9 and CXCL10 genes were not expressed in human oral SCC line Ca9-22 and the glioma cell line A172 in response to IFNγ stimulation. However, the impaired induction of these IFNγ-inducible chemokines was not due to any defect in the IFNγ-activated, STAT1-dependent pathway. Instead, we found that low-level constitutive NF-κB activity was a critical requirement for the IFNγ-induced expression of these chemokine genes in human tumour cell lines. These conclusions are based on the following observations. (i) IFNγ failed to induce the expression of the CXCL9 and CXCL10 genes in Ca9-22 and A172 cells, whereas the induction of these chemokines in HSC-2 and T98G cells was sensitive to IFNγ. (ii) IFNγ induced activation of STAT1 and stimulated STAT1-dependent transcription in both Ca9-22 and HSC-2 cells. (iii) Constitutive NF-κB was observed in nuclear extracts from HSC-2 and T98G cells, but not from Ca9-22 and A172 cells. (iv) Inhibition of the constitutive NF-κB by dominant-negative IκBα suppressed the IFNγ-induced expression of these chemokine genes in HSC-2 cells, without

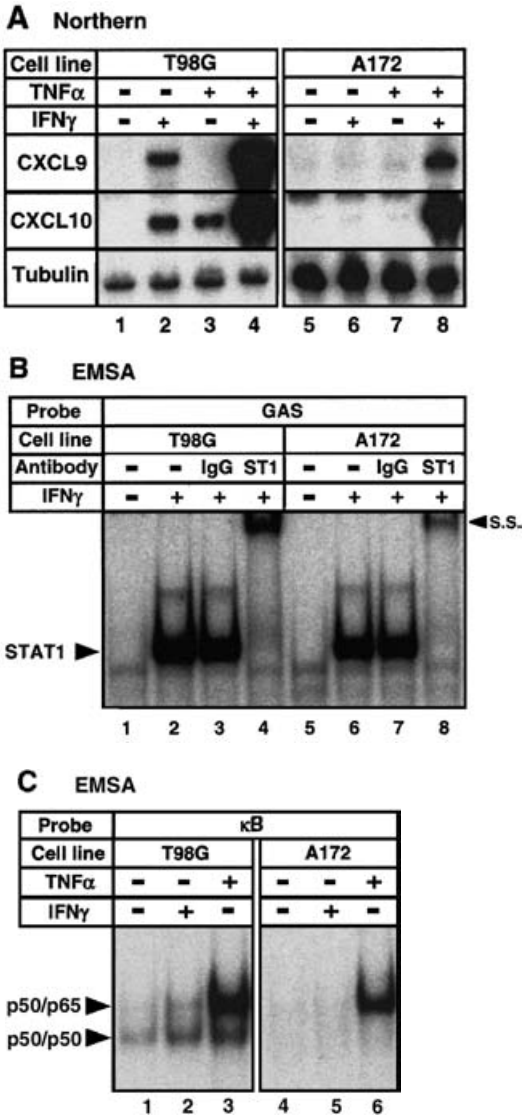

Figure 11 Impaired expression of IFNγ-induced CXCL9 and CXCL10 mRNAs in a human glioma cell line

(**A**) Human glioma cell lines T98G and A172 were either left untreated or treated with IFNγ (10 ng/ml) and/or TNFα (10 ng/ml) for 4 h before preparation of total RNA and analysis of specific mRNA levels by Northern hybridization. Each total RNA (5 μg) was analysed in each lane. Blots were hybridized with the indicated radiolabelled cDNA probes. Similar results were obtained in three separate experiments. (**B**) STAT1 DNA-binding activity in nuclear extracts from IFNγ-stimulated glioma cells. T98G and A172 cells were treated with IFNγ as described above, before to preparation of nuclear extracts. Each nuclear extract (10 μg) was analysed for GAS DNA-binding activity by EMSA using radiolabelled oligonucleotides, as described in Materials and Methods. Nuclear extracts were incubated with the indicated antibodies (1 μg) before analysis of the binding activities (lanes 3, 4, 7 and 8). Super-shifted complexes (s.s.) are shown. Similar results were obtained from three separate experiments. ST1, anti-STAT1 antibody. (**C**) Constitutive NF-κB DNA-binding activity in T98G glioma cells. Each nuclear extract (10 μg) from T98G and A172 cells was analysed for κB DNA-binding activity by EMSA. Similar results were obtained in three separate experiments.

affecting STAT1-dependent transcriptional activity. (v) Exogenous activation of NF-κB by TNFα restored the impaired expression of these IFNγ-inducible chemokines in Ca9-22 and A172 cells.

Although both the CXCL10/IP-10 and CXCL9/MIG genes were originally identified as IFNγ-inducible genes in cells of the monocyte/macrophage lineage [1,2], other extracellular stimuli are also known to modulate the expression of these chemokine genes [3,4]. We reported previously that the interferon-stimulated

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responsive element (ISRE) and a NF-κB-binding site in the promoter region of the CXCL10 gene were required to induce optimal expression of IFNγ-induced promoter activity in mouse macrophages [36], and that NF-κB and STAT1 or IRF-9 (p48) co-operatively induced transcriptional activation of the CXCL10/IP-10 gene [27,36]. IFNγ-induced expression of the CXCL9/MIG gene depends on a tandem STAT1-binding element [35], and several potential NF-κB-binding sties have been identified in the promoter region of the gene [22]. Recently, we identified a NF-κB2 site in the promoter region of the CXCL9 gene and found that it was critical to the transcriptional synergy between IFNγ-induced STAT1 and TNFα-induced NF-κB [30]. Thus, although activation of NF-κB alone had only a minimal effect on the transcription of these chemokine genes, co-operation between IFNγ-induced STAT1 and the constitutive or inducible NF-κB appeared to be essential for the transcriptional activation of these IFNγ-inducible chemokine genes. In this regard, we demonstrated previously that the co-operation between STAT1 and NF-κB recruits co-activator CBP (CREB-binding protein; CREB is cAMP-response-element-binding protein) at the promoter region of the CXCL9 gene and provides a stable scaffold for RNA polymerase II [30]. Since the promoter region of many IFNγ-inducible genes, including IRF-1 and intercellular adhesion molecule 1, contain one or more NF-κB-binding sites, our present results suggest the possibility that the IFNγ-induced STAT1-dependent genes are also positively regulated by a low level of constitutive NF-κB activity.

Members of the NF-κB/Rel family of transcription factors have been implicated in the regulation of a wide variety of genes, including those of the cytokines and adhesion molecules, and those involved in cell survival. Recent studies have shown that constitutive NF-κB activity is present in many tumour cells and that this activity is associated with the induction of genes involved in cell survival and in tumour progression and metastasis [37–43]. In this study, we also observed a constitutive NF-κB activation in human oral SCC line HSC-2 and the glioblastoma line T98G, and showed that the constitutive NF-κB activity was required for the IFNγ-induced expression of the CXCL9 and CXCL10 genes. Since many tumour cells exhibit constitutive NF-κB activity, the activation of NF-κB may lead to alteration of the chromatin structure of the target genes. It is likely that the constitutive NF-κB maintains an open chromatin structure, which could facilitate induction of transcriptional activation of the IFNγ-stimulated chemokine genes. Whether the status of the chromatin conformation is different between in the IFNγ-responsive and non-responsive cells remains to be determined.

Although the mechanisms that underlie the constitutive activation of NF-κB in these cells are unknown at present, it is conceivable that a constitutively active IκB kinase (IKK) complex regulates the NF-κB activation. Indeed, our results showing that a dominant-negative form of IκBα, which is resistant to phosphorylation by IKKs [34], inhibited the IFNγ-induced chemokine expression suggest that constitutive IKK activity leads to the activation of NF-κB in these cells. Furthermore, our preliminary data showed that sulindac, an IKK inhibitor [44], suppressed the IFNγ-induced expression of the CXCL9 and CXCL10 genes (results not shown). Although IKK activity has been shown to be regulated by multiple upstream signalling pathways, the autocrine production of tumour-derived cytokine appears to be involved in the NF-κB activation pathway in certain types of tumour cell [45]. In this regard, Yang and Richmond [46] demonstrated that the autocrine production of CXCL1 in human melanoma directly activated NF-κB via induction of IKK activity and that the activated NF-κB resulted in further induction of CXCL1 expression. Whether HSC-2 or T98G cells produce a factor(s) that activates NF-κB remains to be determined.

TNFα appears to have disparate effects on CXCL9 and CXCL10 expression in HSC-2 and T98G cells (Figures 8 and 11). TNFα alone induced CXCL10, but not CXCL9, mRNA expression in T98G cells (Figure 11), whereas such inducibility was not seen in HSC-2 cells (Figure 8). Although the mechanism involved in the differential induction of CXCL10 is currently unknown, several possible interpretations may be considered. First, it is conceivable that TNFα may induce type I IFNs in T98G cells and the IFNs could mediate ISRE-dependent transcription of the CXCL10 gene, which would co-operate with TNFα-induced NF-κB [36]. In contrast, the promoter region of the CXCL9 gene does not contain an ISRE. Therefore, type I IFNs would not induce the transcriptional activation of this gene. In fact, type I IFNs have been shown to exhibit no stimulatory effect on the CXCL9 expression in many cell types [2]. Alternatively, the composition or activity of TNFα-induced NF-κB in T98G cells might be different from that in HSC-2 cells. Indeed, there appeared to be an increase in the content of the p50/p65 heterodimer in TNFα-stimulated T98G cells, compared with its content in HSC-2 cells (Figure 11C, lane 3 and Figure 7A, lane 3). Furthermore, previous studies have demonstrated that the requirement for NF-κB in transcriptional activation differs between these two chemokine genes; NF-κB-inducing stimuli such as lipopolysaccharide or TNFα alone has been shown to induce CXCL10 [36,47], although these stimuli only have a minimal effect on the CXCL9 expression [2]. Thus, it is possible that the CXCL9 and CXCL10 genes are differentially regulated by multiple mechanisms in different cell types.

Constitutive NF-κB activity is required for tumour survival and cytokine production by many types of tumour cells [37–43]. However, more recent studies have also indicated that inhibition of NF-κB leads to cell proliferation and development of squamous carcinoma [48]. Furthermore, some types of tumour cells such as colon cancer cells showed a decreased NF-κB activity [49]. These lines of study, together with the results presented here, suggest that downregulation of NF-κB activity might lead to tumourigenesis in certain types of cancer cells via increased cell proliferative activity, as well as possibly, immune evasion, in which recruitment of activated T cells by CXCL9 and CXCL10 and angiostatic activity of these chemokines might be impaired. Hence, although downregulation of NF-κB activity has been considered as a therapeutic target for cancer chemotherapy, the efficacy of such treatment may depend upon the type of cancer cell. Further work will be necessary to elucidate the roles of constitutive NF-κB activity in expression of these chemokines and their anti-tumour activities *in vivo*.

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