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IFNα sensitizes ME-180 human cervical cancer cells to TNFα-induced apoptosis by inhibiting cytoprotective NF-κB activation

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Abstract Tumor necrosis factor α (TNFα) induces apoptosis of a variety of tumor cell types. The anti-tumor effect of TNFα is often augmented by interferon (IFN) γ. We hypothesized that IFNα, which shares many biological activities with IFNγ, might also synergize with TNFα for the induction of tumor cell death. We tested our hypothesis using ME-180 human cervical cancer cells exposed to either IFNα or TNFα alone or both. We analyzed the death of ME-180 cells by biochemical and cytological means, and investigated the molecular mechanism underlying cytotoxic synergism between the two cytokines. We found that (i) IFNα/TNFα synergistically induced apoptosis of ME-180 cells, which was accompanied by activation of caspases-3 and -8; (ii) IFNα induced signal transducer and activator of transcription (STAT) 1 phosphorylation, and transfection of phosphorylation-defective STAT1 dominant-negative mutant inhibited IFNα/TNFα-induced apoptosis; (iii) inhibition of nuclear factor κB (NF-κB) by proteasome inhibitor MG-132 sensitized ME-180 cells to TNFα alone; (iv) IFNα treatment attenuated TNFα-induced NF-κB reporter activity, while it did not inhibit DNA binding of NF-κB. Taken collectively, our results indicate that IFNα sensitizes ME-180 cells to TNFα-induced apoptosis by inhibiting TNFα-mediated cytoprotective NF-κB activation, and this sensitizing effect of IFNα is mediated through a STAT1-dependent pathway. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: ME-180 cell; Apoptosis; Caspase; Interferon; Tumor necrosis factor

1. Introduction

Tumor necrosis factor α (TNFα) induces apoptosis of a variety of tumor cell types. The anti-tumor effect of TNFα is often augmented by interferon (IFN) γ [1]. We and others have demonstrated that TNFα and IFNγ synergistically induce apoptosis of tumor cells [2–5]. IFNγ exerts its biological activities by binding to cell surface receptors. Upon ligation of IFNγ receptors, signal transducer and activator of transcription (STAT) 1 is phosphorylated, homodimerized, and translocated into the nucleus to activate transcription of numerous IFNγ-responsive genes [6]. Through the up-regulation of these genes, IFNγ exerts its biological effects such as induction of anti-viral state, inhibition of cellular proliferation, and activation of mononuclear phagocytes and vascular endothelial cells. Many of these biological effects of IFNγ are shared by IFNα. Both IFNγ and IFNα are anti-proliferative, up-regulate class I MHC expression, and inhibit viral replication. Synergism between IFNα and IFNγ has also been reported [7,8]. Signaling cross talk between the two types of IFNs in caveolar membrane domains has been proposed as a molecular basis for their overlapping functions and synergism [9]. However, dissimilar or contrasting effects of the two types of IFNs have also been reported. While IFNγ enhances the expression of class II MHC molecules, IFNα inhibits their expression [6,10]. IFNα produced by natural IFN-producing cells upon viral infection plays an important role in the initiation of adaptive immunity [11,12]. IFNγ has other immunomodulatory activities that are not shared by IFNα, such as promotion of T cell differentiation, B cell class switching, and activation of mononuclear phagocytes, neutrophils, and vascular endothelial cells. Signal transduction by IFNγ and IFNα also involves similar and dissimilar pathways. Unlike IFNγ that activates only STAT1, IFNα activates STAT2 as well as STAT1. After binding to specific receptors, IFNα induces auto- and trans-phosphorylation of Jak1 and Tyk2. Activated Jak1 and Tyk2 catalyze the sequential phosphorylation of STAT2 and STAT1. Heterodimer of activated STAT1 and STAT2 or heterotrimer of STAT1, STAT2, and p48, a member of the interferon regulatory factor (IRF) family of transcription factors, translocates into the nucleus for transcriptional activation of target genes [13]. These slight differences in signaling pathways between IFNγ and IFNα might be the molecular basis for some of the distinct biological activities observed. Compared to IFNγ, the role of IFNα with respect to the synergism with TNFα in tumor cell destruction has not been thoroughly investigated. Several previous reports, however, suggested the possible synergism between IFNα and TNFα in the induction of tumor cell death in vitro as well as in vivo [14–17]. Nevertheless, the underlying mechanisms of IFNα and IFNγ in their synergism with TNFα may be different.

We have recently reported synergism between IFNγ and

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Abbreviations: TNF, tumor necrosis factor; IFN, interferon; STAT, signal transducer and activator of transcription; IRF, interferon regulatory factor; NF-κB, nuclear factor κB

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TNFα in the induction of ME-180 human cervical cancer cell apoptosis and presented evidence that IFNγ inhibits TNFα-induced cytoprotective nuclear factor κB (NF-κB) activation [5]. In the current work, we utilized the same model to investigate whether IFNα synergizes with TNFα to induce tumor cell apoptosis in a manner similar to IFNγ. We also studied the possible regulation of NF-κB activity by IFNα in an attempt to understand the molecular mechanism underlying the cytotoxic synergism between IFNα/TNFα.

2. Materials and methods

2.1. Cell line and reagents

ME-180 human cervical cancer cell line was obtained from ATCC (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, and penicillin–streptomycin (Gibco-BRL, Gaithersburg, MD, USA). Recombinant human IFNγ and TNFα were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human IFNα was generously provided by Dr. Young-Ju Ha at Mogam Institute (Yongin, South Korea). Proteasome inhibitor, MG-132 (carbobenzoxyl-leucinyl-leucinyl-leucinal-H, also called Z-LLL), was from Calbiochem (La Jolla, CA, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless stated otherwise.

2.2. Assessment of cytotoxicity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells (3×10^4/well) were seeded in 96-well plates and treated with various combinations of cytokines for indicated time periods. In some experiments, cells were pretreated with caspase inhibitors or MG-132 for 1 h before cytokine treatment. After cytokine treatment, the medium was removed and MTT (0.5 mg/ml) was added, followed by incubation at 37°C for 2 h in a CO_2 incubator. After a brief centrifugation, supernatants were carefully removed and DMSO was added. After insoluble crystals were completely dissolved, absorbance at 540 nm was measured using a Thermomax microplate reader (Molecular Devices). Results were presented as means ± S.E.M. ($n=3$).

2.3. Morphological and biochemical analysis of apoptosis

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with 2.5 μg/ml of bisbenzimide Hoechst 33342 fluorochrome (Molecular Probes, Eugene, OR, USA), followed by examination on a fluorescence microscope. Oligonucleosomal cleavage of genomic DNA was detected by agarose gel electrophoresis. In brief, genomic DNA isolated as previously described [18] was subjected to 1.5% agarose gel electrophoresis, followed by ethidium bromide staining.

2.4. Assessment of caspase activity

Caspase-3 or -8-like activity was measured using a caspase assay kit (Pharmingen, San Diego, CA, USA) according to the supplier's instruction. In brief, caspase-3 or -8 fluorogenic substrates (Ac-DEVD-AMC or Ac-IETD-AMC) were incubated with cytokine-treated cell lysates for 1 h at 37°C, then AMC liberated from Ac-DEVD-AMC or Ac-IETD-AMC was measured using a fluorometric plate reader with an excitation wavelength of 380 nm and an emission wavelength of 420–460 nm.

2.5. Western blot analysis

Cells were lysed in triple-detergent lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride). Protein concentration in cell lysates was determined using a Bio-Rad protein assay kit. An equal amount of protein for each sample was separated by 10% SDS–PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham, Buckinghamshire, UK). The membranes were blocked with 5% skim milk and sequentially incubated with primary antibodies (rabbit anti-human STAT1 and anti-human phospho-STAT1; New England Biolabs, Beverly, MA, USA) and horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG, Amersham), followed by ECL detection (Amersham).

2.6. Transient transfection

ME-180 cells in 6-well plates were co-transfected with 1 μg of dominant-negative mutant of human STAT1 cDNA (kindly provided by Dr. Hirano, Osaka University, Japan) together with 0.2 μg of *lac*Z gene (pCH110, Pharmacia) using lipofectAMINE reagent (Gibco-BRL). At 48 h after the transfection, cells were treated with cytokines. After another 48 h, the cells were fixed with 0.5% glutaraldehyde for 10 min at room temperature and stained with X-gal (1 mg/ml) in 4 mM potassium ferricyanide/4 mM potassium ferrocyanide/2 mM magnesium chloride at 37°C for detection of blue cells. At least 200 blue cells were counted for each experiment, and transfection efficiency was 10–35%. Results were presented as means ± S.E.M. ($n=3$).

2.7. NF-κB reporter assay

NF-κB reporter activity was measured using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). In brief, ME-180 cells in 12-well plates were co-transfected with 0.5 μg of NF-κB-responsive reporter gene construct carrying two copies of κB sequences linked to luciferase gene (IgGκ NF-κB-luciferase, generously provided by Dr. G.D. Rosen, Stanford University, Stanford, CA, USA) [19] together with 0.1 μg of *Renilla* luciferase gene under HSV thymidine kinase promoter (pRL-TK, Promega) using lipofectAMINE reagent (Gibco-BRL). At 24 h after the transfection, cells were treated with cytokines. After 5 h, activities of firefly luciferase and *Renilla* luciferase in transfected cells were measured sequentially from a single sample using Dual-Luciferase Reporter Assay System (Promega). Results were presented as firefly luciferase activity normalized to *Renilla* luciferase activity. Results were presented as means ± S.E.M. ($n=3$).

2.8. Electrophoretic mobility shift assay

Nuclear extracts were prepared from ME-180 cells treated with cytokines as previously described [20]. Synthetic double-strand oligonucleotides of consensus NF-κB binding sequence, GAT CCC AAC GGC AGG GGA (Promega), were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extract was incubated with the labeled probe in the presence of poly(dI-dC) in a binding buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid at room temperature for 30 min. For supershift assays, a total of 0.2 μg of antibodies against p65 subunit of NF-κB were included in the reaction. DNA–protein complexes were resolved by electrophoresis in a 5% non-denaturing polyacrylamide gel, which was dried and visualized by autoradiography.

3. Results

3.1. IFNα and TNFα synergistically induced the apoptosis of ME-180 cells

Cytotoxicity of IFNα and TNFα toward ME-180 cells was evaluated either alone or in combination. While neither cytokines alone exhibited significant cytotoxicity, the combination of the two cytokines significantly reduced ME-180 cell viability (Fig. 1A). The cytokine cytotoxicity was dependent on the dose of IFNα used (Fig. 1B). However, the use of more than 10 ng/ml of TNFα did not further increase the cytotoxicity (data not shown). The reduction of cell viability was due to apoptosis as demonstrated by Hoechst 33342 staining of nuclei and electrophoresis of genomic DNA. IFNα/TNFα treatment induced nuclear condensation and fragmentation (Fig. 1C) and led to oligonucleosomal cleavage of genomic DNA (Fig. 1A, inset), which are hallmarks of apoptosis. We next investigated whether the activation of caspases is involved in the IFNα/TNFα-induced apoptosis of ME-180 cells. Cytokine-induced apoptosis of ME-180 cells was accompanied by the induction of caspases-3 and -8-like activities as demonstrated by the cleavage of Ac-DEVD-AMC and Ac-IETD-AMC, respectively, in IFNα/TNFα-treated cells (Fig. 2). These results indicate that IFNα/TNFα-induced death of ME-180 cells is a typical apoptosis associated with caspase activation.

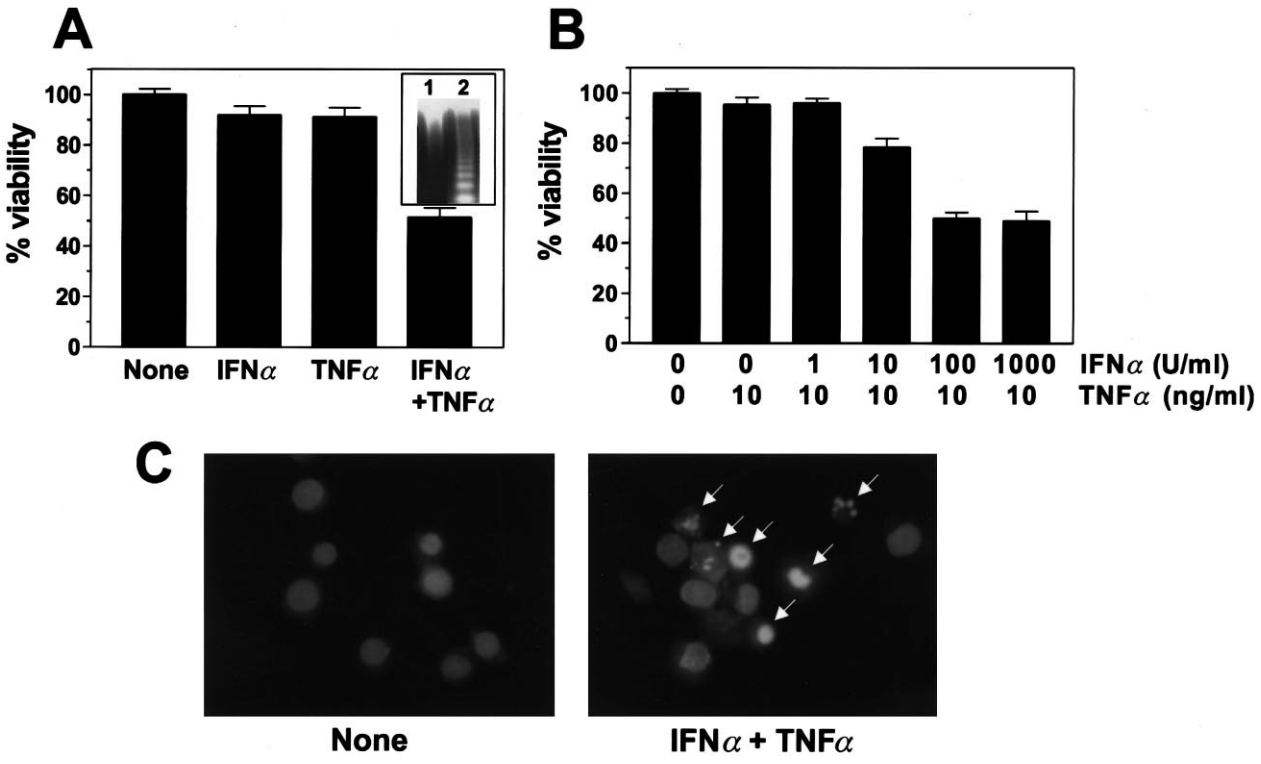

Fig. 1. IFNα/TNFα synergistically induces ME-180 cell apoptosis. A combination of IFNα (100 U/ml) and TNFα (10 ng/ml), but not either cytokines alone, induced ME-180 cell death. Cell viability was assessed by MTT assays after treatment with the cytokines for 48 h (A). Viability of untreated cells was set to 100%. Together with TNFα (10 ng/ml), IFNα dose-dependently decreased the viability of ME-180 cells (B). However, concentrations of IFNα higher than 100 U/ml did not further increase cytotoxicity. Induction of ME-180 cell death was due to apoptosis, because the cytokine treatment for 48 h induced chromatin condensation as detected by Hoechst 33342 staining (C) and oligonucleosomal cleavage of genomic DNA as determined by gel electrophoresis and ethidium bromide staining of genomic DNA (A inset: lane 1, none; lane 2, IFNα (100 U/ml) and TNFα (10 ng/ml)).

3.2. *IFNα-induced STAT1 activation is critically involved in IFNα/TNFα synergism*

Because IFNα induces the expression of a variety of genes via STAT1/STAT2 or STAT1/STAT2/p48, and biological activities of IFNα are mostly mediated through up-regulation of these genes, we hypothesized that IFNα may sensitize ME-180 cells to TNFα-mediated cytotoxicity in a manner dependent on STAT1. We tested this hypothesis by investigating STAT1 induction and phosphorylation in ME-180 cells treated with the cytokines, and evaluated the role of STAT1 in cytotoxic synergism of IFNα/TNFα employing dominant-negative mutant of STAT1. IFNα, but not TNFα, induced STAT1 expression and its phosphorylation (Fig. 3A), and the transfection of phosphorylation-defective dominant-negative mutant of STAT1 significantly inhibited IFNα/TNFα-induced ME-180 cell death, indicating that IFNα-induced STAT1 activation is critical for the induction of TNFα susceptibility (Fig. 3B).

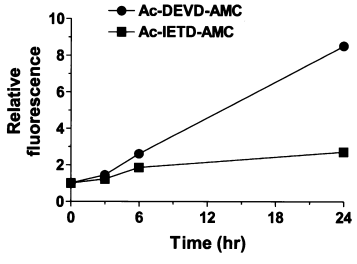

Fig. 2. Activation of caspase-3 and -8-like activities by IFNα/TNFα in ME-180 cells. IFNα/TNFα treatment (IFNα, 100 U/ml; TNFα, 10 ng/ml) induced cleavage of Ac-DEVD-AMC and Ac-IETD-AMC, indicating activation of caspase-3 and -8-like activity, respectively.

3.3. *Inhibition of cytoprotective NF-κB activity by IFNα*

TNFα initiates both death and survival signals, and NF-κB activation is believed to play a pivotal role in TNFα-induced survival signal transduction pathway [21–24]. In order to understand how IFNα induces susceptibility to TNFα-induced cytotoxicity, we examined the role of NF-κB in ME-180 cell death and its possible regulation by IFNα. Treatment of ME-180 cells with a proteasome inhibitor (MG-132), which is known to inhibit NF-κB activation [25], rendered the cells sensitive to TNFα-induced apoptosis (Fig. 4A), suggesting the cytoprotective role of NF-κB. Moreover, NF-κB reporter assays indicated that IFNα pretreatment attenuated TNFα-induced NF-κB activity (Fig. 4B). These results altogether indicate that IFNα synergizes with TNFα for the induction of ME-180 cell apoptosis by inhibiting TNFα-induced cytoprotective NF-κB activity. IFNα pretreatment, however, did not inhibit DNA binding of NF-κB induced by TNFα treatment (Fig. 5).

4. Discussion

Here, we report that IFNα sensitizes ME-180 cells to

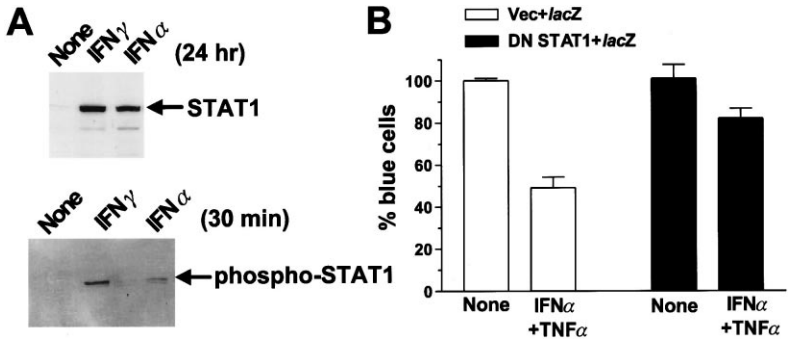

Fig. 3. A key role for STAT1 signaling in IFNα/TNFα synergism in ME-180 cells. (A) Western blot analyses demonstrated that treatment of ME-180 cells with IFNα induced STAT1 expression (24 h treatment) as well as its phosphorylation (30 min treatment). However, TNFα did not induce the expression of STAT1 (data not shown). IFNγ was used for comparison. Concentrations of cytokines used are as follows: IFNγ and IFNα, 100 U/ml; TNFα, 10 ng/ml. (B) Transient transfection of phosphorylation-defective STAT1 dominant-negative mutant (DN STAT1) significantly inhibited IFNα/TNFα cytotoxicity, as demonstrated by counting blue cells co-expressing *lac*Z at 48 h after cytokine treatment (IFNα, 100 U/ml; TNFα, 10 ng/ml). The number of blue cells upon transfection with an empty vector (Vec) without TNFα treatment was set to 100%.

TNFα-induced apoptosis by inhibiting cytoprotective NF-κB activation in a manner dependent on STAT1. This is supported by several lines of evidence. First, the combination of IFNα and TNFα, but not either cytokines alone, induced a significant cytotoxicity toward ME-180 cells. Second, IFNα induced STAT1 phosphorylation, and transfection of phosphorylation-defective STAT1 dominant-negative mutant inhibited IFNα/TNFα-induced apoptosis. Third, the inhibition of NF-κB sensitized ME-180 cells to TNFα alone. Finally, IFNα treatment attenuated TNFα-induced NF-κB reporter activity. These results suggest that TNFα alone does not induce a significant cell death, because it initiates both survival and death signals in ME-180 cells. However, when combined with IFNα, TNFα may induce apoptosis, as IFNα inhibits the survival signal pathway represented by NF-κB. This may be the molecular basis for the cytotoxic synergism between IFNα and TNFα in ME-180 cells. A cytoprotective role of NF-κB in ME-180 cells was demonstrated by MG-132, a proteasome inhibitor. Although treatment of ME-180 cells with MG-132 alone at high concentrations induced cytotoxicity to a certain extent, 0.5 μM of MG-132, which was not significantly toxic by itself, greatly increased TNFα-induced cytotoxicity. A cytoprotective role of NF-κB in ME-180 cells was also demonstrated in our previous work, where inhibition of NF-κB by transfection of dominant-negative mutant of IκB (super-repressor of NF-κB) sensitized ME-180 cells to TNFα-induced cell death [5].

IFNα/TNFα synergism in the induction of ME-180 cell apoptosis was dependent on STAT1. IFNα treatment induced STAT1 expression and its activation. Blockade of STAT1 signaling by dominant-negative mutant of STAT1 suppressed IFNα/TNFα-induced cell death. We have previously demonstrated that IFNγ also synergizes with TNFα for the induction of ME-180 cell apoptosis, and IFNγ also activates the STAT1 signaling pathway in ME-180 cells: IFNγ induces STAT1 expression and its phosphorylation [5]. Thus, in ME-180 cells, both IFNγ and IFNα appear to activate the STAT1 signaling pathway. The cytotoxic priming role of IFNα in IFNα/TNFα synergism presented in the current study does not seem to be restricted to type I IFN. Rather, the STAT1 signaling pathway which can be initiated by either type I or type II IFN appears to be critical for the cytotoxic synergism with TNFα. A central role of STAT1 in IFNα as

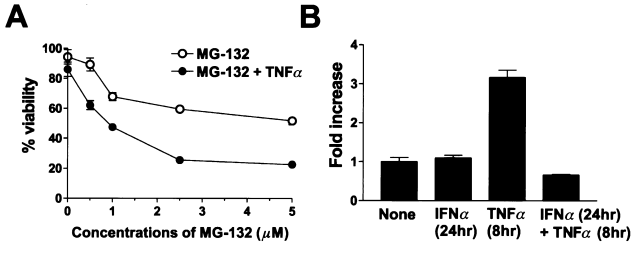

Fig. 4. Inhibition of cytoprotective NF-κB by IFNα. (A) Inhibition of NF-κB by proteasome inhibitor MG-132 sensitized ME-180 cells to TNFα. ME-180 cells were treated with increasing concentrations of MG-132 alone or in combination with TNFα (10 ng/ml) for 48 h, and then cell viability was assessed by MTT assays. (B) NF-κB reporter assays revealed that pretreatment of ME-180 cells with IFNα (24 h, 100 U/ml) inhibited TNFα-induced NF-κB activity. Transiently transfected cells were treated with cytokines for the indicated time period before NF-κB reporter assays.

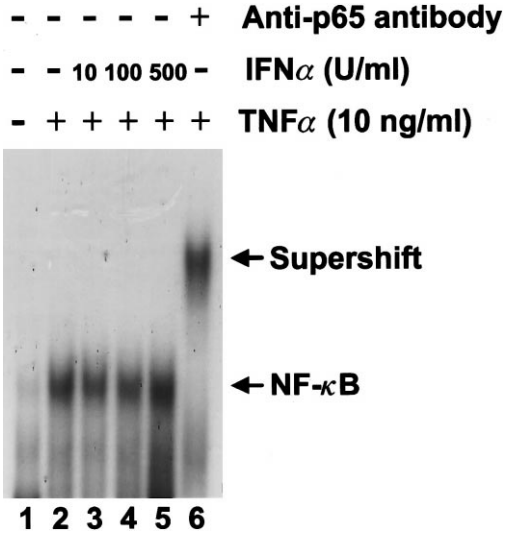

Fig. 5. No significant effects of IFNα on DNA binding of NF-κB protein. IFNα pretreatment (10–500 U/ml, 24 h) did not significantly affect TNFα (45 min)-induced κB sequence binding of NF-κB proteins (lanes 3–5). The identity of DNA-complexed proteins was confirmed by supershift assays using antibodies against p65 subunit of NF-κB (lane 6).

well as IFNγ signaling observed in our work is in agreement with previous gene ablation studies. STAT1-deficient mice showed a complete lack of responsiveness to both IFNα and IFNγ [26,27].

In the current work, we demonstrated that IFNα inhibited NF-κB reporter activity, without suppressing DNA binding of NF-κB. Our previous work demonstrated that IFNγ also sensitized ME-180 cells to TNFα-induced apoptosis by inhibiting NF-κB-mediated activation of anti-apoptosis or survival signals [5]. Like IFNα, IFNγ did not affect TNFα-induced DNA binding of NF-κB or nuclear translocation of NF-κB p65, while inhibiting NF-κB-mediated transactivation. In the case of IFNγ, the STAT1/IRF-1 pathway appeared to be important for the inhibition of NF-κB-mediated anti-apoptotic activity. IFNγ strongly induced both STAT1 and IRF-1. Particularly, IRF-1 transfection alone was able to inhibit NF-κB-mediated transactivation which was abrogated by coactivator p300 overexpression, suggesting a possible interference between transcription factors involving competition for a scanty amount of transcriptional coactivators [5]. However, IFNα did not induce IRF-1 expression over a low constitutive level in ME-180 cells (data not shown). IFNα-activated STAT1 may inhibit NF-κB-mediated transactivation through direct formation of heterodimers with activated STAT2 or heterotrimer involving p48, instead of acting through IRF-1, although the presence of such complexes was not studied in this investigation. Thus, IFNα and IFNγ may use different signaling pathways downstream of STAT1 in their synergism with TNFα, while the apparent outcome seems to be similar.

IFNα is known as leukocyte IFN, since the main cellular source of IFNα has been believed to be mononuclear phagocytes. Recently, however, $CD4^+CD11c^-$ type 2 dendritic cell precursors (pre-DC2s) in human blood have been shown to produce enormous amounts of IFNα in response to viruses [11]. These pre-DC2s were suggested to play master roles in anti-viral immune responses constituting a critical link between innate and adaptive immunity [12]. Pre-DC2s have also been demonstrated to secrete significant amounts of TNFα and interleukin-6 in addition to IFNα and IFNβ after viral infection [12]. However, in inflammatory and anti-tumor immune responses, the main cellular source of TNFα is the activated mononuclear phagocytes. Thus, when produced together by activated mononuclear phagocytes and pre-DC2s, TNFα and IFNα may be able to synergize in the induction of tumor cell death. This type of synergism between IFNα and TNFα may be involved in the in vivo tumor surveillance.

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