Downregulation of interleukin 8 gene expression in human fibroblasts: Unique mechanism of transcriptional inhibition by interferon

(cytokine network/tumor necrosis factor/nuclear run-on assay/neutrophil chemotaxis/inflammation)

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ABSTRACT The chemotactic cytokine interleukin 8 (IL-8) is produced upon stimulation by various agents in many cell types, including connective-tissue fibroblasts. Tumor necrosis factor (TNF) and IL-1 are potent inducers of IL-8 expression. Earlier we showed that TNF-induced stimulation of IL-8 mRNA accumulation in human FS-4 fibroblasts was inhibited by interferon-β (IFN-β) or IFN-γ. Here we show that this inhibition is not specific for TNF, since IFN-β also reduced IL-8 mRNA accumulation induced by IL-1 or the double-stranded RNA poly (I:C). Treatment with IFN-β also decreased TNF-induced IL-8 protein accumulation. Interestingly, the inhibitory effect was much less pronounced when IFN-β was added 1 hr before TNF. The inhibitory action of IFN-β on IL-8 RNA accumulation was undiminished in the presence of inhibitors of protein synthesis. Nuclear run-on assays demonstrated that IFN-β caused a marked inhibition of TNF-induced IL-8 gene transcription; the transcriptional activation of several other TNF-induced genes was not inhibited by IFN-β. The results suggest that the specific inhibition of the transcriptional activation of IL-8 by IFN is due either to a transient inactivation of a factor required for IL-8 transcription or to the activation of a selective inhibitory factor.

Cytokines exert most of their biological actions by altering the level of gene expression in target cells (1). For example, exposure of cells to interferon α/β (IFN-α/β) leads to rapid transcriptional activation of a characteristic set of genes (2, 3). Other cytokines known to produce the activation of a common set of cellular genes are tumor necrosis factor (TNF) and interleukin 1 (IL-1) (4, 5). The target genes activated by TNF or IL-1 partly overlap the genes activated by IFN-α/β or IFN-γ (6–8). Much less is known about the inhibitory actions of cytokines on gene expression. Expression of c-myc (9, 10), c-fos (11), immunoglobulin heavy-chain μ mRNA (12), lamin A and C genes (13), and the mitochondrial genes encoding cytochrome b, subunit I of cytochrome c oxidase, and NADH dehydrogenase subunit 5 (14) was inhibited by treatment with IFN-α/β or IFN-γ in some cells. An earlier study described a set of genes activated by TNF in human fibroblasts (8). Some of the genes activated by TNF were also inducible by IFN-β or IFN-γ. In contrast, IFN-β or IFN-γ inhibited the TNF-induced expression of one of these genes, termed TSG-1. Sequencing of the TSG-1 cDNA revealed that this gene was identical to the gene encoding an important inflammatory cytokine known as IL-8 or NAP-1 (8). IL-8 is a member of the family of 8- to 10-kDa chemotactic cytokines that includes platelet factor 4, IP-10, Gro, macrophage inflammatory peptides 1 and 2, macrophage chemotactic and activating factor, and several others (reviewed in refs. 15 and 16). In the present study we investigated the inhibitory actions of IFN-β on TNF-induced IL-8 gene expression in human fibroblasts. Our data indicate that IFN treatment leads to a selective depletion of IL-8 mRNA; this inhibitory effect correlates with a decrease in the rate of IL-8 mRNA transcription. The mechanism responsible for this action appears to be unique in that the inhibitory effect of IFN is established very rapidly and it is not diminished in the presence of inhibitors of protein synthesis, consistent with the idea that this effect is due to a transient modification of a preexisting factor.

MATERIALS AND METHODS

Materials. Recombinant human TNF-α (specific activity, 3 × 10⁷ units/mg) and IFN-γ (5 × 10⁹ units/mg) were from M. Tsujimoto, Suntory Institute for Biomedical Research, Osaka, Escherichia coli-derived recombinant human IFN-β (Betaseron, 2 × 10⁹ units/mg) was from Triton Biosciences, Alameda, CA, and recombinant human IFN-β derived from CHO cells (Betaseron, 3 × 10⁹ units/mg) was provided by Wieland Wolf, Bioferon, Laupheim, Germany. The E. coli-derived IFN-β preparation was used unless specified. Recombinant human IL-1α (3 × 10⁹ units/mg) was a gift from Peter Lomedico and Alvin Stern, Hoffmann-La-Roche, Nutley, NJ.

Cell Culture. Human diploid FS-4 fibroblasts were maintained in Eagle's minimum essential medium (GIBCO/BRL) supplemented with 5% fetal bovine serum, 6 mM Heps, 3 mM Tricine, and 50 μg of gentamicin per ml. Cultures were seeded in 175-cm² flasks (2 × 10⁶ cells per flask), 75-cm² flasks (8.5 × 10⁵ cells per flask), or 175-cm² dishes (2 × 10⁶ cells per dish) and grown to confluence (9–11 days). The cultures were then switched to medium with 0.25% fetal bovine serum and incubated for an additional 4–6 days. All agents were used were added directly to the medium, without prior medium change.

Nuclear Run-On Analysis. Nuclear run-on assays were performed as described (3). Briefly, FS-4 cells were rinsed and scraped off the dish in ice-cold phosphate-buffered saline. The nuclei were collected by centrifugation after disruption of the cells with a Dounce homogenizer (B pestle) and washed once in reaction buffer (20 mM Tris, pH 7.5/10 mM MgCl2/140 mM KCl/20% glycerol/0.5 mM dithiothreitol). The nuclei were then resuspended in the same buffer containing 0.5 mM ATP, CTP, and GTP, 5 μM UTP, and 0.2–0.3 mCi of [α-3²P]UTP (3000 Ci/mM; ICN; 1 Ci = 37 GBq), and the elongation reaction was allowed to proceed for 9049
15 min at 30°C. The templates were digested with 200 units of RNase-free DNase I (Boehringer Mannheim), and the labeled RNA was extracted with 65°C phenol and phenol/chloroform and precipitated with trichloroacetic acid. Plasmids containing the sequences of interest were alkali-denatured and immobilized on a nylon membrane (Schleicher & Schuell). After prehybridization for 2–3 hr in TESS2D (10 mM Tes, pH 7.4/2× Denhardt’s solution/400 mM NaCl/4 mM EDTA/0.1% SDS with 200 µg of E. coli tRNA per ml), the filters were hybridized overnight with the labeled nuclear RNA probe.

**Northern Blot Analysis.** Total cytoplasmic RNA was isolated (8), and samples (10 µg) were subjected to electrophoresis in a formaldehyde/Mops/1% agarose gel and transferred to a nylon membrane (Schleicher & Schuell). Blotting, prehybridization, hybridization, and washing conditions were as described (17). The TSG-1/IL-8 cDNA probe (8) and the pHe7 cDNA probe (from P. B. Sehgal, Rockefeller University, NY) were 32P-labeled with a nick-translation kit (Boehringer Mannheim). The filters were exposed to a Kodak XAR-5 film with two intensifying screens at −70°C. Further quantification was performed with the aid of a laser densitometer. The results are expressed as relative mRNA accumulation, with pHe7 mRNA as the internal standard.

**RESULTS**

**Inhibitory Effect of IFN-β and IFN-γ on IL-8 mRNA Accumulation Induced by TNF or Other Agents.** Earlier we reported that the induction of IL-8 mRNA by TNF in human FS-4 fibroblasts was inhibited when cells were treated simultaneously with TNF and IFN-β or IFN-γ (8). This inhibition was highly specific for IL-8, since IFN-β or IFN-γ showed no inhibitory action on the ability of TNF to induce seven other types of mRNA in the same cells. We examined the effects of different doses of IFN-β or IFN-γ on IL-8 mRNA levels in FS-4 cells exposed to TNF and IFN-β or IFN-γ for 2 hr (Fig. 1). IFN-β or IFN-γ produced a dose-dependent decrease in the steady-state levels of TNF-induced IL-8 mRNA, with IFN-β showing a more potent inhibitor effect at lower doses than IFN-γ. An inhibitory effect of ≥90% was produced with IFN-β at ≥100 units/ml or IFN-γ at ≥500 units/ml. However, the maximal inhibition of IL-8 mRNA levels produced by IFN treatment varied somewhat from experiment to experiment. Only IFN-β was used in subsequent experiments.

To determine whether the inhibitory action of IFN-β on IL-8 mRNA accumulation was specific for induction by TNF, we examined the action of IFN-β on IL-8 mRNA levels induced by IL-1α or poly(I:C) (8). FS-4 cells were exposed for 2 hr to TNF, poly(I:C), or IL-1α in the presence or absence of IFN-β (Fig. 2). The inhibitory effect of IFN-β on IL-8 mRNA accumulation was pronounced with all inducing agents used and, hence, is not specific for TNF. It is noteworthy that poly(I:C) is best known as a potent inducer of IFN-β (18, 19), but since IFN-β inhibits rather than stimulates IL-8 induction, the ability of poly(I:C) to induce IL-8 mRNA must be separate from its IFN-β-inducing capacity.

**Inhibitory Effect of IFN-β on TNF-induced IL-8 Protein Accumulation.** Cultures were treated with TNF alone or simultaneously with TNF and IFN-β. Culture fluids were collected at intervals for the determination of IL-8 protein by a specific immunoassay (Fig. 3). IL-8 was first demonstrated in the culture supernatants 3–6 hr after stimulation with TNF and continued to increase throughout the duration of the experiment (96 hr). The addition of IFN-β (100 units/ml) reduced the levels of IL-8 protein in the culture medium at the various time points examined by 60–75%.

**Transient Nature of the Inhibitory Effect of IFN-β on IL-8 mRNA Accumulation.** Most actions of IFN are due to a stimulation of expression of selected genes in the target cells (20–22). If the inhibitory action of IFN-β on IL-8 mRNA accumulation also were mediated by IFN-induced protein(s), one would expect that preincubation of cells with IFN-β, followed by the addition of TNF, would lead to a greater inhibition of IL-8 mRNA accumulation than simultaneous treatment of cells with IFN-β and TNF for 2 hr. To elucidate this point, cells were first treated with IFN-β for 1 or 4 hr. Thereafter, TNF was added and the cultures were incubated in the presence of both IFN-β and TNF for 2 hr, at which point steady-state IL-8 mRNA levels were determined (Fig. 4). In parallel, one group of cells was treated simultaneously with TNF and IFN-β for only 2 hr. Surprisingly, the inhibitory effect of IFN-β decreased with the length of preincubation, and little inhibition was seen when IFN-β was added 4 hr before TNF.

One possible explanation of these results was that IFN-β somehow inhibited the generation of a signal required for IL-8 induction by TNF, but only when IFN-β and TNF were added at about the same time. To test this idea we first exposed FS-4 cells to TNF for 2 or 4 hr and then added IFN-β. IL-8 mRNA levels were determined by Northern blot analysis 2 or 4 hr after the addition of IFN-β. Compared with cells exposed to TNF alone, there was a marked decrease in IL-8 mRNA in the IFN-β-treated cultures (data not shown), indicating that the inhibitory action of IFN-β was not directed selectively at an early event in the stimulation of IL-8 gene expression.

**Reduction in IL-8 mRNA by IFN-β Is Undiminished in the Presence of Inhibitors of Protein Synthesis.** Earlier we showed that the induction of IL-8 mRNA by TNF was direct, because cycloheximide not only failed to reduce but in fact "superinduced" IL-8 mRNA levels (8). A similar superinduction was seen in the experiment shown in Fig. 5. Interestingly, the inhibitory effect of IFN-β on TNF-induced IL-8 mRNA also was not reduced in the presence of cycloheximide, suggesting that this action too did not require protein synthesis. In addition, anisomycin, a protein synthesis inhibitor acting by a mechanism different from that of cycloheximide, also caused a superinduction of IL-8 mRNA by TNF and failed to diminish the inhibition of IL-8 mRNA induction by IFN-β (data not shown).

**IFN-β Inhibits the Rate of IL-8 mRNA Transcription.** Nuclear run-on assays were carried out in FS-4 fibroblasts
treated for 2 hr with TNF alone, TNF in the presence of IFN-β, or IFN-β alone (Fig. 6A). No IL-8 transcription was detected in untreated or IFN-β-treated cells. Treatment with TNF resulted in a transcriptional induction of IL-8 message. Cells treated simultaneously with TNF and IFN-β showed a reduced transcriptional activation of IL-8 (about 25% of that seen in cultures treated with TNF alone, according to densitometric determination). To prove that simultaneous treatment with TNF and IFN-β did not cause a general inhibition of transcription, the same nuclear RNA probes were also hybridized with β-actin DNA (provided by E. B. Ziff, New York University Medical Center) and with cDNAs specific for metallothionein II/TSG-37 and TSG-6 (8) and for class I histocompatibility antigen HLA-B7 (23). IFN-β did not inhibit the transcription of TNF-induced TSG-6 mRNA, and transcription of the metallothionein II and HLA-B7 genes, known to be inducible by IFN-β (21, 22), was in fact enhanced by IFN-β. Thus simultaneous addition of TNF and IFN-β was found to result in a synergistic induction of IL-8.

FIG. 3. Effect of IFN-β on TNF-induced IL-8 protein accumulation. FS-4 cells were treated with TNF-α (20 ng/ml) alone (○) or in the presence of IFN-β (100 units/ml) (□). At the indicated intervals, culture fluids were collected and the IL-8 protein concentration was determined by immunoassay (Quantikine ELISA kit, R&D Systems, Minneapolis).

FIG. 4. Inhibitory effect of IFN-β on TNF-induced IL-8 mRNA accumulation: effect of IFN-β added before the onset of treatment with TNF. FS-4 cells were left untreated or treated with TNF (20 ng/ml), IFN-β (100 units/ml), or both TNF and IFN-β for the times indicated. Arrow indicates that IFN-β was added 1 or 4 hr before TNF; in the latter groups TNF was added to the IFN-β-containing medium, and further incubation for 2 hr was in the presence of both IFN-β and TNF. RNA was extracted and analyzed by Northern blotting and laser densitometry.
IFN-β did not produce a general transcriptional downregulation.

The inhibitory effect of IFN-β on IL-8 transcription was maximal when IFN-β and TNF were added simultaneously (Fig. 6B). Addition of IFN-β 2 hr before TNF diminished the inhibitory effect, and its addition 6 hr earlier abolished the effect. These results are in general agreement with the data shown in Fig. 4, further illustrating that when IFN-β is added to cells prior to TNF, the inhibitory effect is relatively short-lasting.

**DISCUSSION**

One characteristic feature of many cytokines is that their actions involve intricate interacting networks or cascades. In many instances the interactions are stimulatory; i.e., cytokines stimulate the production of other cytokines in the target cells (8, 24). Less frequently, a cytokine network involves an inhibitory action on cytokine production (1). One such interaction, the inhibition of IL-8 production by IFN (8), has been analyzed in the present study. The decrease in steady-state mRNA levels seen after IFN-β treatment was matched by a decrease in the IL-8 mRNA signal generated in the run-on assay, indicating an inhibition at the level of transcription.

Most IFN actions are mediated by intracellular proteins induced by IFN in the target cells (20–22). It seemed plausible that the inhibitory action on IL-8 transcription also could be mediated by IFN-induced protein(s). To begin to address the question whether an IFN-induced protein(s) is involved, we examined the action of IFN-β added at different times before TNF. The inhibition was the greatest when IFN-β and TNF were added at the same time, and the effect on steady-state IL-8 mRNA levels (Fig. 4) or on IL-8 transcription (Fig. 6B) decreased with the increase in the time between IFN-β addition and TNF addition. The fact that the inhibition was marked when IFN-β and TNF were both present for only 2 hr, together with the rapidly diminishing effectiveness of IFN-β when it was added to cells before TNF, speaks against the involvement of IFN-β-induced protein(s). In addition, the inhibitory action of IFN-β on TNF-induced IL-8 mRNA levels was not reduced in the presence of inhibitors of protein synthesis, cycloheximide (Fig. 5) and anisomycin (data not shown). If the inhibitory action on IL-8 transcription is not mediated by de novo synthesized IFN-induced protein(s), it could be due to the modification of preexisting factor(s). The modification brought about by IFN might lead to the generation of factor(s) that act as specific transcriptional inhibitors of the IL-8 gene. Mukaida _et al_. (25), who studied cis-acting elements responsible for transcriptional activation of IL-8 by TNF, IL-1, or phorbol 12-myristate 13-acetate in a human fibrosarcoma cell line, concluded that two elements are necessary and sufficient for IL-8 induction: an NF-κB-like and an NF-IL-6-like element. Whether IFN might affect the protein factors recognized by these elements remains to be examined.

Although the data in Fig. 6 indicate that IFN-β inhibits TNF-induced transcription of IL-8 mRNA, these results do not rule out the possibility that IFN-β affects IL-8 synthesis also at some other point(s). Despite the transient nature of the inhibitory action of IFN-β, accumulation of IL-8 protein remained significantly reduced in the presence of IFN-β over
a period of at least 4 days (Fig. 3). One possible explanation is that in addition to inhibiting IL-8 mRNA transcription, IFN-β also blocks IL-8 synthesis at a posttranscriptional, translational, or posttranslational level. It is not unusual for a single agent to inhibit the synthesis of a protein at more than one level (26). That IFN-β also might destabilize IL-8 mRNA, in addition to inhibiting its transcription, was suggested by a rapid decrease in the IL-8 mRNA level seen when IFN-β was added to FS-4 cells 4 hr after TNF, despite the fact that in actinomycin D-treated cells IL-8 mRNA was stable for several hours (data not shown).

Although IFN-α/β and IFN-γ are best known as activators of gene expression (20–22), the IL-8 gene is not the only gene whose expression is inhibited by IFN. One gene on which IFN was shown to exert an inhibitory effect is c-myc in human lymphoblastoid (Daudi) cells (9, 10). Whether this effect was due to an inhibition of transcription (10) or to a decrease in the stability of c-myc mRNA (9, 27) has not been fully resolved. It does not appear that the inhibition of c-myc expression and the inhibition of IL-8 gene expression are mediated by the same IFN-activated mechanism. The inhibitory action of IFN-α or IFN-β on c-myc was seen in cells that were exposed to IFN for 20–48 hr (9, 10). In contrast, the inhibitory effect on IL-8 mRNA diminished rapidly with the length of incubation of cells with IFN-β (Figs. 4 and 6B). Other genes whose expression was shown to be inhibited by IFN include the c-fgr (28) and c-fos (11) protooncogenes, immunoglobulin heavy-chain μ mRNA (12), lamin A and C genes (13), and a set of mitochondrial genes (14). Most of these systems differ from the one described here. Thus, the inhibitory effect on c-fos mRNA seen with IFN-γ in murine macrophages, was found to be due to a decrease in mRNA stability and not to an effect on transcription (11). The inhibitory action on heavy-chain μ mRNA, seen with IFN-α in the Daudi lymphoblastoid cell line, was thought to occur at the level of termination and/or posttranscriptional processing of mRNA (12). The inhibitory action of IFN-α/β on the expression of several mitochondrial genes is different from our system in the unique nature of the target genes and in that the former inhibitory effect is completely blocked in the presence of cycloheximide (14). The mechanisms of the inhibitory action of IFN on the expression of some other genes (13, 28) have not been analyzed, making a comparison with our data difficult.

We believe that the inhibitory effect on IL-8 synthesis represents a heretofore unknown mechanism of IFN action because (i) the inhibitory effect is not on the induced, constitutive, expression of a gene; (ii) the inhibition is pronounced at the level of transcription; (iii) establishment of the inhibitory action is unusually rapid and is not diminished in the presence of inhibitors of protein synthesis; and (iv) the modification brought about by IFN is short-lasting. In addition to the FS-4 line of human diploid foreskin fibroblasts, we demonstrated an inhibition of steady-state accumulation of TNF-induced IL-8 mRNA by IFN-β in the WI-38 line of human diploid lung embryo fibroblasts (data not shown). An inhibitory effect of IFN-γ on IL-8 production in human thymic epithelial cells was also reported (29). However, several virus-transformed or tumor-derived cell lines, in which IL-8 mRNA levels were induced by TNF, proved refractory to the inhibitory action of IFN-β on IL-8 mRNA (data not shown). Hence, it appears that the mechanism responsible for the inhibition of IL-8 mRNA synthesis is not operative in most transformed cells. The most important function of IL-8 is thought to be its ability to recruit neutrophils and T lymphocytes to inflammatory sites (15, 16). Both IFN-α/β and IFN-γ can be produced at inflammatory sites (20, 30). Moreover, under some conditions IFNs have anti-inflammatory activity in animal models of delayed-type hypersensitivity (20). These findings suggest that the inhibitory action of IFN-α/β and IFN-γ on IL-8 synthesis may have pathophysiological significance in inflammation.

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