Interferon-inducible Gene Expression in HL-60 Cells: Effects of the State of Differentiation

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Abstract

The promyelocytic leukemia line HL-60 can be terminally differentiated in vitro to either monocyte/macrophages or granulocytes. We used this cell line to test whether the state of differentiation of a cell changes its response to interferon (IFN). The characteristics of expression of several IFN-α- and IFN-γ-inducible genes in undifferentiated and differentiated HL-60 cells were examined. p56, an IFN-γ-inducible protein, was induced similarly in three cell types, whereas another IFN-γ-inducible protein, p56, was induced strongly only in undifferentiated cells. In contrast, two isozymes of 2,5(A)-synthetase were induced better in differentiated cells in response to either IFN. Several IFN-α-inducible mRNAs, e.g., 561, 6-16, 1-8, and 2A, were induced much more strongly in granulocytes than in macrophages or in undifferentiated cells. Electrophoretic mobility shift assays using the IFN-stimulated response element of gene 561 and nuclear extracts of IFN-α-treated cells revealed the appearance of one complex and the disappearance of another one, concomitant with differentiation of the cells to granulocytes. These observations suggest that expression of IFN-inducible genes in HL-60 cells is regulated by trans-acting factors whose activity changes with the state of differentiation of the cells. Our data may have implications in the optimal clinical use of IFNs. Inducing cellular differentiation may augment the efficacy of IFNs as antitumor agents.

Introduction

IFNs are potent biological response modifiers with a wide range of effects on cells of different lineages. In addition to their antiviral and antiproliferative activities, IFNs profoundly influence the functioning of many cells of the immune system (1, 2). The diverse biological actions of IFNs are carried out by the products of IFN-inducible genes (3, 4). Some of these genes, such as 561 and 6-16, are induced primarily by IFN-α/β (5, 6); some, such as p56 and p67, are induced primarily by IFN-γ (7, 8); whereas others, such as 2,5(A)-synthetases and 1-8, are induced by both types of IFNs (6).

ISRE, a cis-acting sequence present in many of these genes, mediates the stimulatory response to IFN-α/β (9-18). Three trans-acting factors which bind to ISRE have been identified so far. ISGF1 is present constitutively, whereas ISGF2 is induced by both IFN-α and IFN-γ. A third factor, ISGF3, is induced by IFN-α but not by IFN-γ, and it appears to be the crucial positive regulatory factor which is responsible for transcriptional activation of the IFN-α-responsive genes (13, 19, 20). In addition to these three known trans-acting factors, there may be other nuclear factors that can bind to ISRE and thereby influence, either positively or negatively, the expression of the corresponding genes.

The promyelocytic leukemia cell line HL-60 has been extensively used as a model system for studying cellular differentiation-related changes in the monocyte and granulocytic lineages (21). Treatment of this cell line with DMSO or retinoic acid leads to terminal differentiation to mature granulocytes (22). On the other hand, treatment of this continuously proliferating line with chemicals such as PMA or 1,25-dihydroxy-vitamin D3 leads to differentiation to cells of the monocytic-macrophage phenotype (23). A concomitant effect of HL-60 differentiation is cessation of growth. The terminally differentiated cells do not multiply, and they have a finite life span. Expression of several genes is affected by the differentiation process. c-myc is down-regulated upon differentiation to either lineage (24-26). c-fms, c-los, and c-jun are induced by PMA (27, 28). Levels of three protein kinase C isozymes are elevated during differentiation to granulocytes (29).

There are several reports in the literature on the effects of IFNs in undifferentiated HL-60 cells. Both IFN-α and IFN-γ have antigrowth effects on undifferentiated HL-60 cells (30). They may also cause cellular differentiation. 2-5(A)-Synthetase activity increases in undifferentiated HL-60 cells after IFN-α treatment (31). The basal level of this enzyme also increases upon differentiation to granulocytes (32). IFN-γ induces HLA class II antigens in undifferentiated HL-60 cells (33).

In the study reported here, we observed that the state of differentiation of HL-60 cells profoundly affects the degree of expression of different IFN-inducible genes. Several IFN-α-inducible mRNAs were expressed at a much higher level in the granulocytes than in undifferentiated cells. There was a corresponding difference in the pattern of ISRE-binding complexes formed in the nuclear extracts of the two cell types.
Results
Expression of IFN-\(\gamma\)-inducible Proteins. In a series of experiments, we investigated whether expression of IFN-\(\gamma\)-inducible proteins is affected by the stage of differentiation of HL-60 cells. Expression of two proteins, p67 and p56, which are inducible primarily by IFN-\(\gamma\) in fibroblasts and other cells, was examined. p67 is a member of the IFN-inducible guanylate-binding protein (4), and p56 is identical to tryptophanyl tRNA synthetase (34). Antibodies to both proteins have been raised which were used to quantitate their cellular levels in undifferentiated and differentiated cells before and after IFN treatment.

As shown in Fig. 1, untreated and IFN-\(\alpha\)-treated cells did not contain detectable levels of p67 protein. IFN-\(\gamma\) induced the protein strongly in all three cell types. The level of induction was higher in the macrophages, and the band appeared to be more diffuse. In contrast, the pattern of expression of p56 was quite different (Fig. 2). The undifferentiated cells had a low level of this protein, which was elevated upon IFN-\(\gamma\) treatment. Both macrophages and granulocytes derived from these cells, however, had high basal levels of this protein which were only slightly boosted by IFN-\(\gamma\) treatment. In addition, the protein from granulocytes appeared as a closely migrating doublet.

These results demonstrated that the state of differentiation influences the degree of induction of genes by IFN-\(\gamma\). They also showed that the effects of differentiation were different for the two proteins examined. The most marked difference was with respect to the basal levels of these proteins in untreated cells. Whereas the differentiated cells contained a much higher level of p56 as compared to the undifferentiated cells, this was not true for p67.

Induction of 2,5(A)-Synthetase Isozymes. 2,5(A)-Synthetases are a family of proteins with similar enzymatic activities (4). They are inducible by both IFN-\(\alpha\) and IFN-\(\gamma\) in most cell types. There are at least three families of 2,5(A)-synthetases: small, medium, and large. The large family is represented by a 100 kD protein, the medium isozyme is a 67 kD protein, and the small size class has several members, including two proteins of 46 kD and 42 kD. A specific antipeptide antibody immunoreacted with several of these proteins (35). We used this antibody to investigate which isoforms are induced by IFN in HL-60 cells before and after differentiation (Fig. 3). HeLaM cells were used as a control. HeLaM cells have a high basal level of the 42 kD protein which was not induced further by IFN-\(\alpha\); the 46 kD protein was slightly induced, and the 100 kD protein was highly induced in these cells.

In undifferentiated HL-60 cells, none of these proteins was detectable even after IFN treatment. In contrast, in macrophages and granulocytes, both the 100 kD and the 46 kD species were IFN inducible. Surprisingly, the 100 kD form was primarily induced by IFN-\(\alpha\), and the 46 kD form was primarily induced by IFN-\(\gamma\). These results demonstrated that the induction of 2,5(A)-synthetase proteins by both IFN-\(\alpha\) and IFN-\(\gamma\) is enhanced by the terminal differentiation of these cells. Moreover, the specific isoforms induced by the two types of IFNs were different.

Expression of Other IFN-\(\alpha\)-inducible Genes. The observation that the state of differentiation profoundly affects 2,5(A)-synthetase induction by IFN-\(\alpha\) (Fig. 3) prompted us to examine the induction of other IFN-\(\alpha\)-inducible genes. Their levels of expression were assessed at the mRNA level in order to establish that the regulation is at a pretranslational level. Northern analysis of two such mRNAs, 561 and 6-16, are shown in Fig. 4. Both mRNAs had identical induction patterns. As expected, their levels were undetectable in untreated and IFN-\(\gamma\)-treated cells. Surprisingly, they were induced by IFN-\(\alpha\) very strongly in the granulocytes but only slightly in the undifferentiated cells and even less in the macrophages.

The same pattern also was true for mRNAs 1-8 and 2A, although the absolute degrees of induction were differ-
ent (data not shown). These results demonstrated a profound difference in IFN-α responsiveness of HL-60 cells before and after differentiation. Moreover, the specific route of terminal differentiation was critical. Differentiation to macrophages reduced the responsiveness, whereas differentiation to granulocytes increased it dramatically.

Trans-acting Factors for IFN-α-inducible Genes. In the next series of experiments, we explored the molecular mechanism responsible for the observed differential responsiveness to IFN-α of the granulocytes and the undifferentiated HL-60 cells. All IFN-α-responsive genes analyzed to date contain the cis-acting ISRE sequence, which receives the signal generated by IFN-α by binding specific trans-acting factors (13–16). We inquired whether the statuses of these factors were different in undifferentiated HL-60 cells and differentiated granulocytes. Since the magnitudes of induction of 561 mRNA in the two cell types were very different, the ISRE sequence of this gene was used as the probe for electrophoretic mobility shift assays (Fig. 5A). As reported earlier (36), in nuclear extracts of IFN-α-treated HeLaM cells, this probe formed three distinct complexes (Fig. 5B). The slowest complex, ISGF3, was not formed in extracts of untreated and IFN-γ-treated cells. Various lines of evidence indicate that formation of this complex is critical for enhanced transcription of IFN-α-inducible genes (19, 20, 36). As expected, ISGF2 was induced by both IFN-α and IFN-γ, whereas ISGF1 was present in both untreated and IFN-treated cells.

When the same assay was performed with nuclear extracts of HL-60 cells, a much more complex picture emerged (Fig. 5C). At least, six distinct complexes (Bands A–F) were formed in extracts of IFN-α-treated undifferentiated cells. All of them were specific for the probe, since they were competed out by excess unlabeled probe (data not shown). Of these complexes, only Complex F was induced by IFN-α treatment. The same complex, F, was also induced by IFN-α in the differentiated granulocytes. On the other hand, Complex A, the slowest moving complex, was not present in extracts of either IFN-α-treated or untreated granulocytes, although it was present in extracts of undifferentiated cells. Complexes B–E were present in all extracts. These results demonstrated the existence of a differentiation-dependent factor (Complex A) and an IFN-α-induced factor (Complex F) which, in addition to the four common factors (B–E), bound to the ISRE sequence.

We examined carefully whether the ISGF3 complex was formed in these extracts, since formation of this complex correlates very well with transcriptional induction of gene 561 in other cells. Although the ISGF3 complex was not readily visible (Fig. 5C), a longer exposure of the same autoradiogram revealed its presence (Fig. 5D). The identity of this complex as ISGF3 is based upon its comigration with the ISGF3 complex formed in HeLaM extract and its presence in the cytoplasm (data not shown). As shown in Fig. 5D, this complex was induced well by IFN-α in the granulocytes but only very weakly in the undifferentiated cells. This pattern parallels the pattern of transcriptional induction of 561 mRNA in these cells (Fig. 4).

Discussion

In this study, we have systematically examined how the state of differentiation affects IFN-inducible gene expression. We selected the HL-60 line for experimental convenience as well as to enable us to take advantage of the wealth of information available about its differentiation characteristics (21–23, 29, 37). Functioning of the IFN system in relation to cell differentiation has been examined in a few other cell lines. The level of 2,5A-synthetase is higher in peripheral mature T-lymphocytes than in thymocytes (38). The level of this enzyme also increases upon in vitro differentiation of Friend erythroleukemia cells and the histiocytic lymphoma line U937 (39, 40). However, these increases could be a consequence of endogenous IFN production. Embryonal carcinoma cells are only partially responsive to IFN, as judged by their antiviral state, before retinoic acid-induced differ-
Table 1. IFN-inducible gene expression in differentiated and undifferentiated HL-60 cells

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<tr>
<th>Gene product</th>
<th>Undifferentiated mRNA</th>
<th>Macrophages mRNA</th>
<th>Granulocytes mRNA</th>
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<td>1133</td>
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<td>Synthetase, 100 kD</td>
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<td>6-16</td>
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*Results shown in Figs. 1-4 are summarized here in a semiquantitative form. C, untreated control; α, IFN-α treated; γ, IFN-γ treated. 0, 5, relative abundances in an arbitrary scale.*

receptors. If such was the case, one would expect coordinate changes in the levels of expression and inducibility of different IFN-inducible genes. Although in this study we have focused mechanistically on transcriptional regulation, there were indications that differentiation may also affect regulations at other levels. For example, in the granulocytes, p56 protein appeared as a doublet, whereas in undifferentiated cells and in macrophages, we observed a single band. One can speculate that the observed doublet in the granulocytes was a consequence of a cell-specific posttranslational modification of the protein.

The information obtained from the gene induction studies is summarized in Table 1. The results clearly show that expression of both IFN-α- and IFN-γ-inducible genes is affected by the stage of differentiation. There were also gene-specific differences. For example, the basal level and the inducibility of p67 were hardly affected by the stage of differentiation. But expression of p56, 2,5(A)-synthetases, and several IFN-α-inducible mRNAs was considerably affected by differentiation. The observed dual effects on different genes indicate that the crucial differentiation-related change was not due to endogenous IFN production or up- or down-regulation of IFN

Fig. 5. Mobility shift assay using nuclear extracts of HeLaM and HL-60 cells. A, the sequence of two strands of the synthetic double-stranded oligonucleotide corresponding to the human genome sequence -125 to -94 of the IFN-α gene containing the ISRE region. Lower case letters, extraneous linker sequence; R, nuclear extracts of HeLaM cells were prepared from untreated cells (C) or cells treated with either IFN-α (α) or IFN-γ (γ) and used for gel shift assay using the above-mentioned probe as previously described (34). Arrow, position of ISGF3 bands. C, the procedure for granulocyte differentiation was the same as described for Fig. 1. Nuclear extracts were prepared either from untreated undifferentiated cells and granulocytes (C) or after treatment with IFN-α (α). A, J, band positions. Bracket, the position used for a longer exposure, which is shown in D.
contained many factors which bound to this probe (Fig. 5C). At least six complexes were easily distinguishable by their electrophoretic mobility. Among these, four (Complexes B–E) were present in comparable quantities in extracts of IFN-α-treated and untreated undifferentiated HL-60 cells and granulocytes, suggesting that their presence is not instrumental for expression of this gene. Similarly, Band F was present in both cell types, although only after IFN-α treatment. Only Band A showed a cell-type difference. Its presence in the undifferentiated cells but not in granulocytes suggests a possible negative role of this complex in preventing transcriptional induction of ISGF3 mRNA. Absence of formation of this complex may be necessary for this process, but it definitely is not sufficient, since untreated granulocytes do not express ISGF3 mRNA.

Among positive trans-acting factors, there were two candidates: band F, whose mobility did not match with any known ISGF, and ISGF3, which was formed to a lesser extent as compared to HeLa extracts. It was interesting, however, that the ISGF3 complex was more abundant in the extract of granulocytes. This, by itself, or in conjunction with the absence of Band A in granulocytes, may determine the degree of transcription of IFN-α-inducible genes. The observed low intensity of the ISGF3 band in HL-60 cells could be an indication of the presence of a lower concentration of this trans-acting factor in these cells. However, it is also possible that, due to the presence of so many other ISRE-binding proteins in these cells, the particular assay used here underestimated the level of ISGF3. Nonetheless, the difference in ISGF3 formation between IFN-α-treated granulocytes and undifferentiated cells was clear. This difference indicates a possible difference in IFN-α-elicited signal transduction between the two cell types. Further investigation is needed to determine whether both subunits of ISGF3 are deficient in undifferentiated cells and whether the ISGF3α subunit becomes activated in their cytoplasm upon IFN-α treatment.

In principle, it is possible that the observed cell-specific difference in gene expression is mediated by another cis-acting element present in IFN-α-inducible genes. This putative element may act either positively or negatively. If it is a positive element, binding of the cognate factor present in granulocytes will promote transcription, whereas the absence of such a factor in undifferentiated cells will prevent it. If it works negatively, undifferentiated cells contain the cognate trans-factor which blocks expression of the genes. For testing these various models of regulation, future transfection experiments using reporter genes containing different portions of the regulatory regions of 561 gene will be required.

Generalization of the observations reported here may have important implications in the clinical use of IFNs as anticancer agents. Since all biological actions of IFNs are mediated by the IFN-inducible proteins, their high level expression ensures potent effects of the IFNs. It follows, therefore, that inducing cellular differentiation will augment the antitumor effects of IFNs. Hence, a combined therapy with differentiating agents and IFNs may provide the optimal modality. Our studies also indicate, however, that the chosen lineage of differentiation can also profoundly influence the putative beneficial effects of IFNs.

Materials and Methods
Materials. Cell culture materials were purchased from GIBCO. PMA and DMSO were from Sigma Chemical Co. Sources of pure IFNs were described in our earlier publication (36). Protein electrophoresis reagents and agarose were obtained from Bio-Rad. Guanidine thiocyanate was from Fluka.

Cell Culture and Differentiation. The human promyelocytic HL-60 leukemia cells from American Type Culture Collection (Rockville, MD) were maintained in suspension culture with RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum supplemented with 2 mM glutamine, penicillin (100 μg/ml) and streptomycin (100 μg/ml) in humidified 5% CO₂-95% air at 37°C (21). Differentiation to macrophage lineage was induced by treatment of cells with 100 ng/ml of PMA in 150-mm tissue culture dishes for 2 days. Stock PMA was dissolved in DMSO, and the maximum concentration of DMSO in the culture medium was 0.01%, which by itself has no effect on differentiation. Although commitment to differentiation to the monocytic lineage was complete by 2 days, for ensuring the elimination of undifferentiated cells, only the attached cells (macrophage characteristic) were used for IFN treatment after washing three times with phosphate-buffered saline. Cells were treated with 500 units/ml of either IFN-α or IFN-γ for 16 h or left untreated. For granulocyte differentiation (22), cells were treated with 1.25% of DMSO for 2 days. Cells were washed with PBS, and suspensions in medium were made for untreated control or for treatment with 500 unit/ml of either IFN-α or IFN-γ for 16 h.

Electrophoresis and Immunoblotting. Cell extracts containing equal amounts of proteins were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (42) and then transferred to nitrocellulose paper. Nonspecific binding was blocked by 5% Carnation non-fat dry milk in PBS, and the nitrocellulose papers were incubated with specific immune serum at 1:300 dilution for 2 h at 37°C. Blots were incubated with 125I-Protein A (~10⁶ cpm/ml) at 37°C for 1 h and then washed, dried, and autoradiographed (35, 43). Antibody to 25A-synthetase (antipeptide B) was obtained from J. Cheetham (35). The antibodies against the p56 and p67 proteins were described in our previous publications (7, 8).

RNA Isolation and Blot Analysis. Cells were washed three times with PBS, and RNA was prepared by guanidine solution according to the method described earlier (44). RNA samples were electrophoresed in 1% agarose-2.2 M formamide gels and then transferred to nylon membrane (Nytran from Schleicher and Schuell). Membranes were prehybridized and then hybridized for 16 h at 42°C in the presence of 2 × 10⁶ cpm/ml of 32P-labeled specific DNA probe. Probes were prepared by random priming using a Boehringer-Mannheim kit to an efficiency of >0.5 × 10⁶ cpm/μg of DNA. Filters were washed at a final stringency of 0.2X standard saline citrate and 1% sodium dodecyl sulfate at 65°C and autoradiographed (45).

Cell Extract Preparation and Gel Mobility Shift Assay. Cellular extracts and mobility shift assays were performed according to the method described in detail in our earlier publication (36) using salmon sperm DNA instead of polydeoxyinosinic-deoxyctydilic acid. Oligonucleotides
corresponding to the human genomic sequence \(-125\) to \(-93\) of IFI-56K gene containing the ISRE region were synthesized in an applied Biosystem DNA Synthesizer Model 3808. Oligonucleotides were gel purified, annealed, and end labeled by polynucleotide kinase using [\(\gamma^{32}\)P]ATP from Amersham. After electrophoresis, gels were dried and different exposures were taken for autoradiograms.

Acknowledgments

IFN-\(\alpha\) was a kind gift from Hoffman LaRoche, and IFN-\(\gamma\) was a kind gift from Genetech Co. We would especially like to thank J. Chebath and M. Revel for the 25S RNA-synthesize antibody; we also thank Margaret Leet for typing the manuscript.

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