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Stimulation of Primary Human Endothelial Cell Proliferation by IFN

Dolores Gomez and Nancy C. Reich

The IFN family of cytokines has pleiotropic roles in immunity and development. In this study, we provide evidence that IFN can stimulate the proliferation of primary human endothelial cells. This is in contrast to the growth-suppressive effects of IFN observed on transformed human cells, thereby underscoring the distinctive responses of primary human cells. The growth-stimulatory effect of IFN was determined by an increase in DNA synthesis assessed with [1H]thymidine incorporation, an increase in G1 and M cell cycle phases assessed with flow cytometric analysis, and an increase in cell number. Distinct cell types, including primary human fibroblast and smooth muscle cells, were also growth stimulated by IFN. Neutralizing Abs to IFN were used to demonstrate the growth response was mediated specifically by the IFN cytokine. The signaling pathway of type I IFNs activates STAT1 and STAT2. In primary endothelial cells, we demonstrate that STAT3 and STAT5 are also activated, and these STATs may contribute to cellular proliferation. To evaluate possible effectors of positive growth, DNA microarray analyses were performed to assess gene induction in response to IFN. These results reveal changes in the RNA levels of genes in endothelial cells that encode proteins involved in cellular proliferation. The Journal of Immunology, 2003, 170: 5373–5381.

Clinical use of IFN as a cancer therapeutic in the treatment of vascular neoplasias led us to investigate the effects of IFN on normal primary endothelial cells (1, 2). The endothelium is a continuous monolayer of cells forming the innermost layer of blood vessel walls, and impacting numerous physiological and pathological processes, including angiogenesis, inflammation, blood coagulation, tissue repair, reproduction, hypertension, atherosclerosis, and tumor growth (3, 4). Endothelial cells are an essential barrier between the vascular circulation and underlying tissue, and they respond to various homeostatic stimuli as well as to damage signals with actions that vary from new blood vessel formation to lymphocyte recruitment at sites of tissue injury (5). Many recent studies have investigated the promotion of angiogenesis by tumor cells through the release of factors that promote the proliferation and migration of endothelial cells (6). The stimulated endothelial cells form vessels that are required to support the growth of solid tumors beyond a critical mass (7). In addition to their role in tumor angiogenesis, growth-disregulated endothelial cells can form vascular neoplasias such as hemangio- mas and Kaposi sarcomas (8, 9). Although IFN has been found to inhibit the growth of tumorigenic cells, in this study we demonstrate that its normal physiological role is to promote proliferation of primary human adherent cells.

Type I IFNs (IFNα/β/ω) induce biological changes in target cells by stimulating the activation of latent cytoplasmic transcription factors designated STATs (10–15). In response to IFN-α, STAT1 and STAT2 are tyrosine phosphorylated by Janus kinases (JAK1 and TYK2) associated with the type I IFN cell surface receptor. Following phosphorylation, the STATs dimerize and in association with the IFN regulatory factor-9 translocate as a complex to the nucleus and bind DNA target sites containing an IFN-stimulated response element (16). In response to IFN-α, the STAT1 molecule can also homodimerize and translocate to the nucleus to bind to a distinct response element, the IFN-γ activated site, in the promoters of target genes (17). The binding of STAT factors to these DNA elements regulates gene expression and elicits subsequent biological changes. A biological change that has been correlated with STAT1 activation is the inhibition of cellular proliferation in transformed human cells (18, 19). In addition to signaling tyrosine phosphorylation of STAT1, there is evidence that IFN can activate the STAT3 and STAT5 factors in a subset of cell types (20–22). In contrast to a role of STAT1 in growth inhibition, studies indicate that the STAT3 and STAT5 transcription factors are involved in the promotion of cellular growth. Persistent STAT3 and STAT5 phosphorylation has been shown to play a critical role in proliferation stimulated by growth factors and in cellular transformation by oncogenes (23–28). Also, introduction of constitutively active forms of STAT3 or STAT5 has been shown to induce unregulated proliferation and/or tumorigenesis (29, 30). In this study, we demonstrate the concomitant stimulation of primary human endothelial cell growth by IFN-α with the phosphorylation of STAT1, STAT3, and STAT5. Our findings clearly demonstrate a role for IFN-α as a stimulator of primary human cell proliferation. DNA microarray analyses of gene expression in response to IFN-α support our observation of proliferative effects, and indicate induced expression of genes associated with cellular growth and survival. These findings provide provocative evidence for a diverse role of IFN in normal human physiology vs aberrant transformed conditions.

Materials and Methods

Cell culture and reagents

HUVEC were collected from umbilical cords by collagenase perfusion of the umbilical vein (31). HUVEC were maintained in medium 199 (M199) containing 20% FBS (HyClone Laboratories, Logan, UT). HUVEC from several cords were pooled to account for individual variability and were...
used in experiments at passage 2. Human aortic endothelial cells (Clonetics, Walkersville, MD) were maintained in M199 with 20% FBS. Normal human dermal fibroblasts (Clonetics) were cultured in M199 with 10% FBS, HT1080 cells (American Type Culture Collection, Manassas, VA) and U3A cells (STAT1 deficient) (gift of G. R. Stark, Cleveland Clinic Foundation Research Institute, Cleveland, OH) were cultured in DMEM with 8% FBS. Murine embryo fibroblasts (gift of P. J. Hertzog, Monash University, Clayton, Victoria, Australia) prepared from wild-type BALB/c mice were maintained in DMEM with 10% FBS. Anti-STAT3 (H-190) Ab and anti-STAT5a Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). STAT1 mAb was generated in our laboratory (16). Abs specific for the tyrosine-phosphorylated forms of STAT1, STAT3, and STAT5 were obtained from Cell Signaling Technology (Beverly, MA).

Results

IFN-α promotes primary HUVEC proliferation

The growth-inhibitory effect of IFN-α on tumor cells has led to its clinical use in the treatment of several malignancies. To investigate whether IFN-α regulates cell growth in healthy tissue, we focused our studies on human primary endothelial cells as a model system. Primary endothelial cells were isolated from three to four human umbilical cords and pooled in each analysis to account for individual variability. HUVEC were treated with 0, 50, 100, 500, 1000, or 2000 U/ml IFN-αA for 72 h, then assayed for DNA synthesis by [3H]thymidine pulse incorporation during the last 4 h of treatment. A dose-dependent increase in [3H]thymidine incorporation was observed in response to IFN-αA treatment, which indicated a physiological progression of cells into S phase of the cell cycle (Fig. 1A). The minimum dose of IFN-αA administered, 50 U/ml, induced [3H]thymidine incorporation by 1.5-fold, and the highest dose of 2000 U/ml stimulated a 4.5-fold increase in DNA synthesis in this representative experiment. The HUVEC cultures used in these studies were a pure population of endothelial cells.PECAM-1 (or CD31), a protein expressed at endothelial cell junctions, was detected by Western blot analysis (32). A 130-kDa protein was reactive with specific PECAM-1 Ab in HUVEC lysate, but not in lysate of negative control HT1080 fibrosarcoma cells (Fig. 1B). The homogeneity of the HUVEC population was evident by immunofluorescence assays performed with PECAM-1 specific Ab. PECAM-1 specific expression was observed throughout the

![FIGURE 1. IFN-αA promotes primary endothelial cell proliferation. A, HUVEC were either mock treated or treated with 50, 100, 500, 1000, or 2000 U/ml IFN-αA for 72 h. The cells were radiolabeled with [3H]thymidine for the final 4 h, and the data are presented as fold increase in [3H]thymidine incorporation. The data shown are means (n = 3) ± SD and are representative of similar experiments. B, Cell protein lysates were prepared from HUVEC and HT1080 fibrosarcoma cells, and Western blot analysis was performed with human anti-PECAM-1 Ab C, Immunofluorescence studies were performed on HUVEC with mineral oil induced plasmacytoma Ab (control) or PECAM-1 Ab.](http://www.jimmunol.org/)
culture at the endothelial cell junctions, indicating the population was of endothelial origin (Fig. 1C).

To examine the kinetics of endothelial cell proliferation promoted by IFN-α, HUVEC were treated with IFN-α for 12, 24, 48, 72, or 96 h with 1000 U/ml human IFN-αA. The cells were radiolabeled with [3H]thymidine for the last 4 h of treatment and assayed for metabolic incorporation into DNA. A significant increase in [3H]thymidine incorporation was seen following a 24-h incubation with IFN-α, and by 72 h of IFN-α treatment there was a 4-fold increase in [3H]thymidine incorporation (Fig. 2A). The confluency of the culture at 96 h of IFN-α treatment was reflected in the plateau of the increase in DNA synthesis. Different IFN-α gene subtypes have been characterized, and some reports indicate that IFN-α subtypes can elicit unique biological responses (33). To determine whether the proliferative response was specific to the IFN-αA subtype, HUVEC were treated with a hybrid IFN-α molecule generated from recombinant IFN-αA and IFN-αD, designated the universal type I IFN or IFN-αA/D. Treatment with either IFN-αA or IFN-αA/D was found to stimulate a 3- to 4-fold increase in HUVEC DNA synthesis relative to the mock treated control (Fig. 2B). Similar results were observed in cells treated with distinct members of the IFN family of cytokines, including IFN-β and IFN-γ (data not shown). These results provide evidence that the proliferative response of HUVEC to IFN-α is not specific to the IFN-αA subtype.

Endothelial cells respond to numerous stimuli, including various cytokines, growth factors, and bacterial products. To test whether the proliferative response of the HUVEC was specifically stimulated by IFN-α and not by a contaminant in the rIFN preparation, HUVEC were treated with IFN-αA or IFN-αA/D in the presence or absence of IFN-α neutralizing Ab. Treatment of cells with the IFN-α neutralizing Ab was found to ablate the proliferative response of IFN-αA and IFN-αA/D treated cells, while control Ab had no effect (Fig. 2B), clearly demonstrating that the increase in DNA synthesis observed in IFN-treated cells was stimulated specifically by the IFN. These thymidine incorporation analyses indicated that IFN-αA promoted cellular progression into S phase of the cell cycle. To confirm that IFN-α stimulated complete cell cycle progression of endothelial cells, flow cytometric analyses of the cell cycle distribution of IFN-αA treated HUVEC were performed. In these experiments, HUVEC were either maintained in 20% serum and treated with 1000 U/ml IFN-αA for 24 h, or maintained in 1% serum for 24 h to induce quiescence, then mock treated or treated with 1000 U/ml IFN-αA, or 1× endothelial cell growth factor (ECGF) for 48 h. ECGF is a cocktail of factors developed to support the growth of endothelial cells in culture. Following treatment of the cultures, cells were harvested and DNA content was evaluated by propidium iodide staining and flow cytometric analysis to determine cell cycle distribution. At 24 h, treatment of HUVEC with IFN resulted in a 7.2-fold increase in the number of cells entering S phase of the cell division cycle, and a 1.4-fold increase in the number of cells in G2/M (Table I). In the presence of 1% serum at 48 h, treatment of cells with IFN-αA resulted in a 2-fold increase in the percentage of HUVEC entering S phase compared with the mock treated sample (Table I). In addition, both IFN-αA and ECGF increased G2/M phase entry of HUVEC by 5-fold above the mock treated control. To confirm that IFN promoted complete progression through the cell cycle, direct cell counts of subconfluent mock treated or IFN treated HUVEC were compared. After 1 day in culture, the cell numbers were relatively equivalent with 20,000 (±8,165) cells in the mock treated samples and 17,500 (±5,000) cells in the IFN treated sample. However, by 5 days in culture, a 2-fold increase in cell number was observed in the presence of IFN with 52,500 (±5,000) cells in the IFN-treated samples, but only 27,500 (±5,000) in the mock treated control. Taken together, these results demonstrate that IFN-αA stimulate S phase entry and complete cell cycle progression of HUVEC.

IFN-α specifically stimulates the proliferation of primary human cells

HUVEC are large vessel endothelial cells that are exposed to deoxygenated blood. Such cells have unique properties that may influence responsiveness to extracellular stimuli, including cytokines. To evaluate whether the proliferative response of HUVEC to IFN-α was shared with other endothelial cells, we examined cells obtained from distinct vascular regions. Human aortic endothelial cells that line large vessels that carry oxygenated blood were found to proliferate in response to 500 U/ml IFN-αA by 3-fold in the [3H]thymidine incorporation assay above the mock treated control (Fig. 3A). This effect was ablated in the presence of IFN-αA neutralizing Ab. In addition, microvascular human endothelial cells lining the microvessels that carry deoxygenated blood were also growth stimulated by IFN-αA (data not shown). These results suggest that IFN-αA induction of human endothelial cell proliferation is not specific to HUVEC. To determine whether the

<table>
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<tr>
<th>Cell Treatment</th>
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<tr>
<td>24 h 20% serum</td>
<td>1.5</td>
<td>7.6</td>
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<td>10.8</td>
<td>10.7</td>
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<td>48 h 1% serum</td>
<td>4.9</td>
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<td>1% serum + IFN</td>
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<tr>
<td>1% serum + ECGF</td>
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a Flow cytometric analyses of HUVEC based on ~10,000 individual events.
observed proliferation of primary human cells to IFN-α was specific to the endothelial cell type, the response of primary human dermal fibroblasts was examined following treatment with 500 U/ml IFN-α-A in the presence or absence of IFN-α-neutralizing Ab. IFN-α-A induced a 2.5-fold increase in the 4-h [3H]thymidine incorporation assay that was inhibited by the inclusion of the neutralizing IFN Ab (Fig. 3A). Similar results were observed with human coronary artery smooth muscle cells (data not shown), providing evidence that IFN-α induced growth was not specific to endothelial cells.

Previous studies have reported the growth inhibitory effect of human IFN-α on tumor cells and transformed cell lines. Our observations confirm these results. When HT1080 fibrosarcoma cells maintained in medium with 8% FBS were treated with 500 U/ml IFN-α-A, proliferation was inhibited in comparison to mock treated controls, as indicated by a 3-fold decrease in [3H]thymidine incorporation (Fig. 3B), supporting observations of the growth-inhibitory properties of IFN (34). This antiproliferative effect was reversed in the presence of IFN-α-neutralizing Ab. Although IFN-α is known to inhibit HT1080 cell proliferation, it did not stimulate apoptosis in these cells (data not shown). To determine whether inhibition of cellular proliferation by IFN-α was dependent on the activation of the STAT1 transcription factor, cells deficient in STAT1 were evaluated. The STAT1-deficient U3A cell line derived from an HT1080 parental cell line was treated with 500 U/ml IFN-α and assayed for [3H]thymidine incorporation (35). Consistent with previous reports, the growth of U3A cells was unaffected by IFN-α, indicating that STAT1 was required for the growth inhibitory effect observed in the HT1080 cells (Fig. 3B) (19). Similar results were obtained in cells deficient in STAT2, which suggested that STAT2 expression was also critical for the growth-inhibitory effects of IFN-α (data not shown).

Our results indicate that IFN-α stimulates the proliferation of human primary cells, but inhibits the proliferation of human transformed cells. These dichotomous effects of IFN appear to be specific to cells of human origin. The growth of primary murine embryo fibroblasts was evaluated in response to the universal IFN-α/A, and results clearly indicated a suppression of proliferation (Fig. 3C). The observed growth inhibition of primary murine fibroblasts is consistent with previous observations that IFN-α hinders primary mouse cell growth (36). These data demonstrate a specific proliferative effect of IFN-α in primary human cells.

IFN-α-induced endothelial cell proliferation is independent of p38 mitogen-activated protein kinase (MAPK), MAP kinase kinase (MEK), phosphatidylinositol-3 (PI-3) kinase, and NF-κB activity

IFN-α has been reported to stimulate various signaling pathways in target cells in addition to the STAT1 and STAT2 transcription factors. Findings indicate IFN-α can activate p38 MAP kinase, MEK1, PI-3 kinase, and NF-κB (37–42). To determine the potential involvement of these signaling proteins in IFN-α-induced cellular proliferation, HUVEC were treated with IFN-α/A in the presence or absence of inhibitory molecules specific for p38 MAP kinase (SB202409190), MEK (PD98059), PI-3 kinase (LY294002), or NF-κB (BAY 11-7085), and assayed for DNA synthesis by the 4-h [3H]thymidine incorporation assays. Inhibiting the function of p38 MAP kinase, MEK, PI-3 kinase, or NF-κB activity did not alter the ability of IFN-α to stimulate proliferation of HUVEC (Fig. 4, A–D). These results indicate that IFN-α induced cellular proliferation is independent of these signaling molecules.

IFN-α stimulates tyrosine phosphorylation of STAT1, STAT3, and STAT5 in endothelial cells

The STAT1 transcription factor is a critical signaling protein in the response to IFN, and lack of STAT1 expression results in an inability to inhibit tumor cell growth by IFNs (Fig. 3B) (18, 19). To determine the activation status of the STAT1 protein in IFN-α treated HUVEC, the cells were treated with IFN-α-A for 30 min and assayed for STAT1 phosphorylation by Western blot analysis with anti-phosphotyrosine STAT1 Ab. IFN-α treatment of HUVEC resulted in STAT1 tyrosine phosphorylation, and the level of phosphorylation in HUVEC appeared comparable to that observed in the IFN-α growth inhibited HT1080 cells (Fig. 5A). We also assayed for the DNA-binding activity of STAT1 on the IFN-stimulated response element or IFN-γ activated site DNA targets and detected characteristic transcriptional complexes (data not shown). Reports indicate that additional STAT family members are activated by IFN-α in a subset of target cells (20–22). The critical role of STAT3 in cellular proliferation by growth factors and transformation by oncogenes led us to investigate the activation of this molecule in IFN-α growth stimulated HUVEC. STAT3 activation was assayed by Western blot analysis with Abs specific for the tyrosine-phosphorylated form of this protein. Tyrosine phosphorylation of STAT3 was detected in IFN-α treated HUVEC. However, STAT3 tyrosine phosphorylation was also detected in transformed HT1080 cells that are growth inhibited by IFN-α, making it unlikely that STAT3 activation was sufficient for HUVEC proliferation (Fig. 5B). To pursue the possible role of STAT3 phosphorylation in HUVEC proliferation, cells were
treated with IL-6, a known STAT3-activating cytokine, and assayed for growth. IL-6 did not stimulate HUVEC growth, despite detectable levels of STAT3 phosphorylation (data not shown). These results indicate that STAT3 phosphorylation is not sufficient for HUVEC proliferation.

The STAT5 protein is an additional STAT family member involved in numerous cellular growth and transformation processes. To determine whether STAT5 was activated in response to IFN treatment of HUVEC, STAT5 phosphorylation was assayed by Western blot analysis with Abs specific for the tyrosine-phosphorylated form of this protein. Tyrosine phosphorylation of STAT5a was detected in IFN-α treated HUVEC (Fig. 5 C). Conversely, transformed HT1080 cells that are growth inhibited by IFN-α did not respond with STAT5 tyrosine phosphorylation (Fig. 5 C). There are two STAT5 genes that encode STAT5a and STAT5b proteins, and using discriminating Abs it was apparent that HT1080 cells had very low basal levels of STAT5a protein. To test the effect of overexpression of STAT5a in HT1080 cells, transient transfections were performed. Although STAT5a was overexpressed, the protein was not tyrosine phosphorylated in response to IFN-α treatment in HT1080 cells (data not shown).

If STAT5a were mediating the growth effect of IFN in HUVEC, treatment of cells with other activators of STAT5 would be expected to stimulate proliferation. For this reason, we tested the effects of IL-2, hepatocyte growth factor, growth hormone, and IL-15, but these agents did not stimulate STAT5 tyrosine phosphorylation or proliferation in HUVEC (data not shown). The inability of these factors to stimulate STAT5 phosphorylation and proliferation may result from a lack of specific receptor expression in HUVEC. In contrast, the ability of IFN-α to activate endogenous STAT5 correlates with endothelial cell proliferation.

Global changes in HUVEC gene expression in response to IFN-α

IFN-α induces biological effects in target cells by changing the expression of a subset of genes. To gain insight into the nature of the changes that occur in endothelial cells as they are promoted to proliferate in response to IFN-α, we evaluated mRNA expression by Affymetrix DNA microarrays. RNA was isolated from HUVEC that were treated with 1000 U/ml IFN-α for 0, 5, or 12 h and evaluated for alterations in expression by hybridization to the Affymetrix HG-U95A GeneChip microarray. A partial list of resultant mRNAs that change expression by at least 3-fold following IFN-α treatment is provided in Table II. Most of the changes in gene expression corresponded to increases in mRNA levels; however, there were some specific mRNAs that decreased expression, which may reflect either transcriptional repression or mRNA turnover. Treatment with IFN-α for 5 h produced the greatest number of changes, and these changes were either maintained for 12 h or returned to basal levels (Fig. 6). Expression of a subset of mRNAs increased only following 12 h of IFN-α, and these may therefore
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<th>Gene Description</th>
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have been the consequence of a secondary response to induced effectors. Evaluation of the genes encoded by the mRNAs revealed the induction of known IFN-α stimulated genes, including the MHC, 2′-5′ oligoadenylate synthetase, Mx, IFN-α stimulated gene 54, etc., demonstrating an effective response to IFN-α (43, 44). Induced gene expression also included RNAs that correlate with proliferation, such as RNAs encoding 18S and 28S ribosomal RNAs and histone H2B RNAs, supporting the observed growth response of HUVEC to IFN-α. In addition, IFN-α increased the levels of mRNAs encoding proteins known to be able to stimulate proliferation of primary cells. These include extracellular ligands and cell surface receptors such as the IL-3R, IL-15, IL-15R, hepatocyte growth factor receptor, and oncostatin-M receptor. The regulated expression of a subset of genes, including SnoA, hepatocyte growth factor receptor, and guanylate-binding protein-2, was confirmed by real-time PCR (data not shown).

Discussion
Knowledge of the mechanisms by which hormones modulate cellular function is fundamental to understanding human development and immune defense. In addition to the antiviral effects of IFN hormones, studies with human tumor cells and murine model systems indicate that IFNs can be inhibitors of cellular growth. In this study, we evaluated the growth effects of IFN-α on primary human tissue, and clearly demonstrated that IFN stimulated the proliferation of normal human adherent cells ex vivo. Our studies focused on the direct effect of IFN-α on adherent cells, specifically...
primary human endothelial cells. These cells serve critical functions as the primordial tissue of vascular remodeling or angiogenesis, as physical barriers between the vascular circulation and underlying tissue, and as mediators of inflammatory responses. Endothelial cells are long-lived, and in the absence of any pathology or injury, the endothelial monolayer is estimated to have a turnover measured in years (45). It is essential to maintain the integrity of the blood vessel wall, and so any local damage must be sensed and restored to maintain homeostasis. IFNs function as modulators of native immunity and are produced in response to viral and bacterial infections, as well as to immune cell activation. An infected endothelial cell may succumb to the lytic action of a virus or to a defensive apoptotic response, but the infected cell will first produce and secrete type I IFNs. IFNs can act locally to confer resistance to viral infections, and as we have demonstrated in this study, they can also stimulate endothelial cell proliferation to restore damaged tissue. The proliferative effects of IFN also have been demonstrated in vivo with human blood vessels in an animal model (46).

Our findings bring to light a significant difference in the way that human primary cells respond to IFN in comparison with mutant transformed cells (34, 47). However, it should be noted that there are a limited number of previous studies with primary adherent cells reporting a negative growth effect of IFN (48–50). Most of these reports are primarily based on the influence of IFNs on the effects of other growth factors, with or without serum depletion. Because IFNs stimulate the production of negative modulators such as suppressors of cytokine signaling that can negatively regulate both cytokine signaling and growth factor signaling, these results may have been influenced by such a negative feedback (51). Another more relevant observation is the ability of IFN to inhibit human hematopoietic colony formation (52, 53). In these assays, progenitor cells are enumerated by their ability to grow ex vivo in the presence of CSFs. The ability of IFN to induce members of the suppressors of cytokine signaling family of negative regulators that can inhibit erythropoietin or CSF signaling may contribute to an inhibition of proliferation by these factors. In our studies, we used experimental conditions to evaluate the direct effects of IFN on cells that were maintained in 20% serum, but had no additional growth factor added to avoid aberrant consequences of serum depletion or growth factor inhibition that can stimulate responses of apoptosis or anoikis.

The mechanisms by which IFN stimulates cellular proliferation remain to be determined and may result from a primary signal at the IFN receptor complex, and/or a downstream secondary pathway. Although STAT activation is the best-characterized primary signaling pathway of IFN, we also considered the possible involvement of other growth-signaling molecules such as p38 kinase, MEK1 kinase, PI-3 kinase, and the NF-κB transcription factor. However, inclusion of cell-permeable inhibitors of these signaling molecules did not block the proliferative effects of IFN. For this reason, we turned our attention to STAT activation and new gene expression induced in response to IFN-α. STAT1 and STAT2 are activated by tyrosine phosphorylation in cells that are stimulated by type I IFNs, but reports have also indicated the activation of STAT3 and STAT5α in some cell types (20–22). The activation of these STAT molecules may confer distinct cellular growth responses to IFN because STAT1 has been associated with growth-inhibitory effects of IFN on tumor cells, whereas STAT3 and STAT5α have been associated with stimulation of cellular proliferation and transformation (18, 19, 23–26). We therefore evaluated the possibility that IFN-α could stimulate the activation of STAT3 or STAT5α in primary human endothelial cells, and found unexpectedly that both STAT3 and STAT5α were activated. Activation of STAT3 by other cytokines did not stimulate cellular proliferation in HUVEC. These results suggest that activation of STAT5α in endothelial cells may be a determining factor in their growth response to IFN-α. Examination of human fibrosarcoma HT1080 cells that are growth inhibited in response to IFN-α revealed that they do not have detectable activation or expression of STAT5α. Even HT1080 cells that exogenously expressed STAT5α were not able to tyrosine phosphorylate STAT5 in response to IFN (data not shown). The reason for the selective activation of STATs in response to IFN remains to be determined. Future studies to specifically inhibit STAT5α activation in endothelial cells are challenging because primary cells have a limited life span in culture and are refractory to transfection techniques, but such analyses are needed to determine whether STAT5α plays an essential or cooperative role in IFN-induced proliferation. The growth response of cells to IFN may reflect a balanced influence of STAT1 and STAT5α pathways that have opposing effects on cell cycle progression.

To evaluate the molecular changes that occur in response to IFN-α treatment of primary endothelial cells, we examined mRNA expression after 5 or 12 h of IFN stimulation by Affymetrix DNA microarray analysis. Modulation of new gene expression by IFN has been reported previously for the HT1080 transformed cell line, and although a distinct GeneChip was used and a precise comparison is not possible, there are both shared and distinct induced genes (43, 44). Some of the induced genes in endothelial cells encode proteins that are involved in cellular proliferation. These range from secreted growth factors such as IL-15 and nerve growth factor, to cell surface receptors that signal proliferation of primary cells such as the hepatocyte growth factor receptor, IL-3R, IL-15R, and oncostatin M receptor. Induction of signaling molecules such as SnoA that can block TGFβ inhibition of growth may also be involved in a proliferative response to IFN (54). In addition, overexpression of the guanylate-binding protein-2 gene has previously been shown to stimulate cellular proliferation (55). Conversely, some mediators of apoptosis are also induced by IFN-α, including the TNF-related apoptosis inducing ligand, Bak, and caspase 1. The proliferative response to IFN in primary endothelial cells appears to reflect the prevailing effect of positive regulators. It is not known whether specific STAT factors are responsible for regulation of these individual genes, and in fact, very few direct target genes of specific STATs have been identified. We evaluated our microarray data for the induction of reported STAT5 target genes oncostatin M, Bcl-xL, Mcl-1, cyclins D1/D2, c-myc, hnf-6, perforin, and Cis. Of these genes, hnf-6 (hepatocyte nuclear factor-6) mRNA expression is increased in response to IFN-α treatment (Table II). It should be noted, however, that these genes were identified as STAT5 targets in transformed cell lines, and it is not clear whether they are STAT5 responsive in primary human cells.

The mechanism of action of IFN-α in clinical settings for the treatment of leukemias, hemangiomas, and melanomas may involve direct inhibition of tumor cell proliferation, and/or enhanced immune surveillance with increased expression of MHC molecules and activation of T cells, macrophages, and NK cells (56). Some studies have indicated that IFN can block the production of fibroblast growth factor, which stimulates endothelial cell growth; however, our microarray analysis did not detect any change in fibroblast growth factor expression (57). Solid tumors depend on a developed blood supply for continued growth, and our studies indicate that the antitumor effects of IFN are not a result of inhibiting the proliferation of normal endothelial cells. In fact, our results demonstrate that IFN stimulates the proliferation of primary adherent cells, and this effect may serve a protective role during infections, wound healing, and normal developmental processes.
Acknowledgments
We thank all the members of the laboratory for their helpful discussions, and in particular Sarah Van Scy for her support with real-time PCR experiments and Dr. Janet Andersen for critical suggestions. We are grateful to Dr. Martha Furie (Stony Brook University) for her expertise and guidance in harvesting HUVEC, and John Schwedes (Stony Brook University) for his assistance in compiling the microarray data.

References