Transcriptional Silencing of Perlecan Gene Expression by Interferon-γ

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Perlecan, a heparan sulfate proteoglycan of basement membranes and cell surfaces, has been implicated in the control of tumor cell growth and metastasis because of its ability to bind and store growth factors and its activity as an inducer of angiogenesis. Because interferon-γ (IFN-γ), a cytokine with known antiproliferative and antitumoral activity, binds with high affinity to the heparan sulfate side chains of perlecan, we investigated the activity of IFN-γ on perlecan gene expression and cell growth in colon carcinoma cells. We found that IFN-γ rapidly and efficiently blocked perlecan gene expression with concurrent growth suppression, a phenomenon that was independent of a functional p21WAF1/CIP1. These effects were transcriptionally mediated, did not require new protein synthesis, and were fully reversible. Moreover, we found these IFN-γ-induced effects to be generalizable because they could be reproduced in a variety of cells with various histogenetic backgrounds. The transcriptional repression of the perlecan gene required intact Stat1 protein, and these effects were likely mediated by Stat1-binding sites in the distal promoter region. Thus, the IFN-γ-mediated transcriptional repression of perlecan may represent a novel antitumoral effect of this cytokine through which it eliminates a powerful angiogenic stimulus.

Proteoglycans play key roles during inflammation, tissue remodeling, and cancer growth primarily through their ability to act as modifiers of growth factors and by actively participating in the fine tuning of receptor/ligand interactions (1). An important member of the proteoglycan gene family is perlecan, a ubiquitous heparan sulfate proteoglycan that has been involved in the control of cell proliferation, tumor invasion, and angiogenesis (2). The complex structure of perlecan protein core (3) and its widespread distribution (4, 5) suggest that this gene product is involved in several fundamental biological processes. For instance, increased perlecan levels are found in metastatic melanomas (6) and correlate with a more aggressive behavior (7). In tumor xenografts induced by subcutaneous injection of human prostate carcinoma cells in nude mice, human-derived perlecan was deposited along the basement membrane of newly formed murine vessels (2). Thus, perlecan may act as a scaffold upon which proliferating capillaries grow and eventually form functional blood vessels (2). Indeed, perlecan binds basic fibroblast growth factor (FGF-2) (8, 9) and acts as a low affinity receptor for FGF-2 thereby promoting angiogenesis (10). FGF-2 binds directly to the heparan sulfate side chains located in the N-terminal Domain I, and its release by proteolytic processing (11) may represent a biological mechanism by which dynamic molecules can be functionally disengaged at the active site of tissue remodeling and tumor invasion (12). In support of this view, reduction of endogenous perlecan levels by stable antisense transfection causes a marked suppression of autocrine and paracrine function of FGF-2 in melanoma cells (13). In contrast, antisense expression of perlecan cDNA induces tumorigenesis in fibrosarcoma cells and a faster appearance of tumors in nude mice (14). Thus, the cellular context is important in mediating the functions of perlecan.

A systematic study of murine embryogenesis has revealed that perlecan expression appears early in tissues of vasculogenesis such as the heart primordium and major blood vessels (15). Subsequently, perlecan mRNA levels increase, and this correlates with the onset of tissue differentiation of various organs including kidneys, lungs, spleen, liver, and gastrointestinal tract. In adult human tissues, perlecan epitopes are distributed along all vascularized organs, including the sinusoidal spaces of the liver, spleen, and lymphoreticular organs (4). The latter suggests that perlecan may play a role in the normal development and homeostasis of lymphoid organs.

In addition to binding a variety of extracellular matrix proteins, perlecan binds to transforming growth factor-β and is transcriptionally induced by this cytokine (16) and also binds to interferon-γ (IFN-γ) (17) with high affinity (KD = 10−9 M) (18). Interferon-γ is a glycoprotein synthesized primarily by T lymphocytes and is involved in the regulation of the immune response (19). It possesses antiviral, antiproliferative, and antiangiogenic activity in a number of cells and triggers a signaling cascade that leads to the expression of early response genes through the activation of the signal transducers and activators of transcription (STAT) proteins (19, 20). This activation occurs through both Tyr phosphorylation, which is critical for the translocation and binding to the DNA, and Ser/Thr phosphorylation, which is crucial for maximal transcriptional activation (19). Upon activation, STAT proteins dimerize and translocate into the nucleus where they bind to the IFN-γ activated site (GAS) (19).

Because one of the main goals of our laboratory is to understand how specific cytokines regulate perlecan gene expression, we embarked on a study to determine whether IFN-γ affects...
perlecan gene expression and, if so, to characterize the mechanism of action. We discovered that IFN-γ concurrently suppressed tumor cell growth and perlecan gene expression. These effects were transcriptionally mediated, did not require new protein synthesis, were fully reversible, and were general since they could be reproduced in a variety of cells with various histogenetic backgrounds. The results also showed that the transcriptional repression of the perlecan gene required a functional Stat1 protein and that these effects were likely mediated by Stat1-binding sites located in the distal promoter region. Thus, the IFN-γ-mediated transcriptional repression of perlecan may represent a novel antitumoral effect of this cytokine through which it eliminates a powerful angiogenic stimulus from the tumor microenvironment.

EXPERIMENTAL PROCEDURES

Materials and Cell Cultures—[α-32P]dCTP and [14C]chloramphenicol were obtained from Amersham Corp. Human recombinant IFN-γ was purchased from Boehringer Mannheim. HeLa cervical carcinoma, WiDr/HT29 colon carcinoma, Saso-2 osteosarcoma, HT-1080 fibrosarcoma, and Hep G2 hepatoma cells were obtained from the American Type Culture Collection. HCT116 p21 (wt) (27) and its isogenic mutant p21−/−, WiDr/HT29 colon carcinoma cells (21) and U3A (Stat1−/−) and 2FTGH (Stat1+/+) cells (22) were described previously. Cells were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mM glutamine, and 100 units/ml penicillin. Perlecan promoter-CAT constructs were used as described previously (16). The CDNA HS15 was described before (3).

Transient Cell Transfection and CAT Assays—Transient cell transfection was performed by the calcium phosphate procedure as described before (16). Briefly, subconfluent cells were co-transfected in suspension with 20 μg of perlecan promoter-CAT and 10 μg of pSV-β-galactosidase plasmid to provide an internal standard for normalization of products. The CAT assay and the nuclear run-on transcription assay were performed with the TRI-Reagent TM (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer’s recommendations. The RT was performed using 5 μg of total RNA (24). Briefly, the reaction was performed at 42 °C in RT buffer containing 5 mM dNTPs, 1 μM perlecan antisense primer from Domain IV (bp 9259–9237), and 1 μM GAPDH antisense primer. For PCR, 5 μl of the RT reaction was added to a mixture containing 2 μM dNTPs, PCR buffer, two perlecan primers from bp 7950–7966 (sense primer) and bp 9067–9051 (nested antisense primer) of Domain IV, and the GAPDH-specific sense primer. The PCR reaction comprised 25 cycles with 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C. A 25-μl aliquot of each PCR reaction was separated on a 1% agarose gel and transferred to nitrocellulose in 10× SSC. The membrane was hybridized with either a probe spanning Domain IV of perlecan (HS166) or GAPDH, washed in 0.5× SSC/0.1% SDS at 65 °C, air-dried, and exposed to x-ray films for 10–30 min.

Nuclear Run-on Transcription Assay—Nuclear run-on was performed as described before (25) with minor modifications. Each reaction consisted of 210 μl of nuclei and 60 μl of 5× run-on buffer (25 mM Tris-HCl, pH 8.0, 12.5 mM MgCl₂, 750 mM KCl, and 1.25 mM triphosphates of A, G, and C). [α-32P]UTP (30 μl, 3000 Ci/mm) was added, and the nuclear suspension was incubated at 30 °C for 30 min, after which DNase I (5 μg/ml) was added for 5 min. The reaction was then made 1× SDS (20 μl, 5% SDS, 0.5 μM EDTA, pH 7.4), and 200 μg/ml proteinase K was added for 45 min at 37 °C. Following phenol/chloroform extraction and isopropyl alcohol precipitation, the pelleted nuclei were purified further through a G-50 spin column. The eluate was made 0.2 M in NaOH and after 10 min on ice, HEPES was added to a concentration of 0.24 M. Following ethanol precipitation for 18 h at −20 °C, the purified pellets were resuspended in hybridization buffer (10 mM Tris, pH 7.4, 0.2% SDS, 10 mM EDTA, 0.3 M NaCl, 1× Denhardt’s solution, and 250 μg/ml salmon sperm DNA). Nitrocellulose filters containing the plasmid DNAs were hybridized for 36 h at 65 °C, washed twice for 15 min in 0.1% SDS, 2× SSC at room temperature, washed once at 60°C (0.1% SDS, 0.1× SSC) for 30 min, and processed for autoradiography as above.

RESULTS AND DISCUSSION

The Effects of IFN-γ on the Growth of Human Colon Carcinoma Cells: A Block in Cell Cycle Progression That Does Not Require a Functional p21—In the first sets of experiments we investigated the effects of IFN-γ on the growth of the human colon carcinoma cells WiDr/HT29 (26). These cells represent a useful model system to study growth and proteoglycan biology because they synthesize high levels of perlecan (27) and respond to a variety of factors including transforming growth factor-β (28), nerve growth factor (6), and phorbol ester (24). The results showed a complete growth arrest, without any evidence of cell death, when the tumor cells were exposed to ~9 nM IFN-γ (160 ng/ml) for various periods of time (Fig. 1A). This growth suppression was fully reversible (Fig. 1B), and dose-response studies performed by exposing the cells to increasing amounts of IFN-γ for 2 (Fig. 1C) or 3 (not shown) days revealed nearly complete inhibition at 80 ng/ml, with an IC50 of ~50 ng/ml (3 nM). To determine whether the inhibitory effects of IFN-γ were due to a block in the onset of DNA synthesis, the growth state of the cells was tested by FACS analysis. Interferon-γ increased the proportion of cells in G1 from 50% to 73% and decreased the proportion of cells in S and G2 phases (Fig. 1D). Because it was previously shown that activation of Stat1 by IFN-γ led to growth suppression via induction of p21 (29), we tested HCT116 colon carcinoma cells that harbor a targeted disruption of both p21 alleles (21). The results clearly showed that p21 is not required for growth arrest in these colon carcinoma cells since comparable growth suppression was observed in HCT116 cells with either a p21+/+ (Fig. 1E) or p21−/− (Fig. 1G) genotype. Similarly, FACS analysis revealed a proportional increase in the percentage of cells in G1 phase of the cell cycle (Fig. 1, F and H).

Collectively, these findings indicate that WiDr/HT29 colon carcinoma cells are growth-suppressed by IFN-γ and that these effects do not require endogenous p21, a powerful inhibitor of cyclin-dependent kinase activity (21). This is in contrast with a recent report (29) that has shown significant cell death under identical conditions and dosage of IFN-γ. In our study, it was found that after 6 days of treatment with IFN-γ, only ~30% of the WiDr/HT29 cells were viable as measured by trypan blue exclusion (29). We note that our cell proliferation assay provides an accurate and reproducible method for calculating live, fully metabolizing cells (23), and we did not detect any appreciable cell death.

IFN-γ Markedly Suppresses Perlecan Gene Transcription and Its Effects Are Fully Reversible—Because perlecan has been linked to tumor progression by virtue of its ability to modulate angiogenic stimuli such as FGF-2 and FGF-7 (9, 10), we tested whether the expression of this key proteoglycan was modulated by IFN-γ. We used RT-PCR and a primer set that...
would amplify a perlecan-specific 1.1-kb fragment encoding part of Domain IV (24). The analysis was performed in the linear range thus allowing quantitative assessment of the perlecan levels vis à vis those of the housekeeping gene GAPDH which was concurrently amplified in the same test tube. The levels of perlecan mRNA were markedly suppressed (Fig. 2A, lane 2), and these effects were maintained for up to 48 h (Fig. 2A, lane 3). Moreover, the suppression of perlecan mRNA transcript was fully reversible (Fig. 2A, lanes 4 and 5). The down-regulation of perlecan mRNA steady state levels was corroborated by a time course analysis of perlecan protein core synthesis by Western immunoblotting using a monoclonal antibody directed against perlecan Domain III (Fig. 2B). The amount of secreted perlecan following IFN-γ treatment was markedly reduced by ~80% after 2 h and by ~90% after 4 h, with a calculated T1/2 of ~1 h. Notably, this inhibition remained relatively constant for up to 48 h, in agreement with the mRNA data. The slight increase at later time points (Fig. 2B) is likely due to the decline of activity of the IFN-γ because we used one single bolus of the cytokine. Because the half-life of perlecan in WiDr/HT29 colon carcinoma cells is relatively long, ~75 min (30), the inhibitory effects of IFN-γ on perlecan gene transcription would have been even higher than calculated.

Next we determined whether the IFN-γ-mediated down-regulation of perlecan synthesis was a general phenomenon. Several cell lines with various histogenetic backgrounds, including tumor cells derived from bone (Saos-2), liver (Hep G2), uterus (HeLa), colon (HCT116), and fibrous tissue (HT-1080), all showed a marked suppression of perlecan protein core biosynthesis (Fig. 2C). Because some of these tumor cell lines are defective in p53, retinoblastoma protein, or p21, these results suggest that none of these growth-regulating factors are directly involved in IFN-γ-mediated suppression of perlecan gene expression. Our results are in agreement with recent studies that have shown an inhibition of [³⁵S]sulfate incorporation into

**FIG. 1. Growth suppression of human colon carcinoma cells upon exposure to IFN-γ.** A, WiDr/HT29 human colon carcinoma cells were seeded in 48-well plates with (○) or without (●) 160 ng/ml (~9 × 10⁵ IFN-γ and counted every day with an hemocytometer. Values are the mean ± S.D. of quadruplicate determinations. B, experimental procedures were similar to A except that, after 3 days, IFN-γ was removed (▼) and cells were counted for 4 additional days. Values are the mean ± S.D. of quadruplicate determinations. C, dose-response curve of IFN-γ-treated WiDr/HT29 colon carcinoma cells following a 2-day exposure. Growth curves were performed by a nonradioactive cell proliferation assay where the absorbance units are directly proportional to the number of viable cells. Values are the mean ± S.D. of quadruplicate determinations (notice that the S.D. is smaller than the symbols). D, FACS analysis of the DNA content of untreated or IFN-γ-treated cells. About 10⁶ cells were analyzed by FACS. The ordinate indicates the relative fraction of cells in each phase of the cell cycle as a percentage of total DNA. E and G, number of viable HCT116 p21+/+ and p21−/− cells in the absence (●) or presence (○) of IFN-γ (160 ng/ml). Values are the mean ± S.D. of quadruplicate determinations. F and H, FACS analysis of cells with the designated genotype. Notice the relative increase in the proportion of cells in G1, phase of the cell cycle following IFN-γ (160 ng/ml) for either 24 h (empty bars) or 48 h (stippled bars) as compared with control untreated cultures (filled bars). Values are the mean ± S.D. of quadruplicate determinations.

**FIG. 2. IFN-γ drastically and reversibly inhibits perlecan gene expression.** A, Southern blotting of cDNAs generated by RT-PCR of total RNA extracted from WiDr/HT29 cells in the absence (lane 1) or presence of IFN-γ for either 24 (lane 2) or 48 (lane 3) h. Lanes 4 and 5 represent samples that were incubated with IFN-γ for 24 or 48 h and subsequently incubated in the absence of the cytokine for either 24 or 48 h, respectively. Notice the full recovery of perlecan-specific mRNA levels vis à vis those of the housekeeping gene GAPDH. The primer set used for the PCR corresponds to a 1.1-kb fragment between exons 58 and 65 encoding a portion of Domain IV. Equal aliquots of the PCR reactions (less than 10 ng cDNA) were separated on a 1% agarose gel, transferred to a nitrocellulose membrane, and hybridized under stringent conditions with a cDNA probe spanning almost the entire Domain IV. The bottom panel shows the fragment obtained by RT-PCR using a primer set specific for human GAPDH. Blots were exposed to x-ray film for ~30 min. B, Western immunoblotting analysis of serum-free media conditioned by WiDr/HT29 cells exposed to 160 ng/ml IFN-γ for varying times as indicated and performed as described under “Experimental Procedures.” The values are the mean of three separate experiments with S.D. < 10% of the mean. C, immunoblotting analysis of several cell lines exposed to 160 ng/ml IFN-γ. The values are the mean of triplicate determinations ± S.D. .
heparan sulfate chains in human skin fibroblasts (31) and provide a plausible explanation for this effect; that is a reduction in the synthesis of perlecan protein core, a major gene product of fibroblasts (2).

Transcriptional Suppression by IFN-γ—The dramatic decrease in perlecan mRNA and protein core levels described above upon treatment with recombinant IFN-γ could be accounted for by a decrease in transcriptional rates, a decline in mRNA stability, or by alterations in nuclear processing of the nascent RNA molecules (or by a combination of these processes). To measure transcription of the perlecan gene directly, we employed nuclear run-on assay, a direct measure of RNA polymerase density, and hence transcriptional activity (32). Nascent RNA labeled with [³²P]UTP from nuclei isolated from control or IFN-γ-treated cells was hybridized to membranes preslotted with various pBluescript plasmids containing perlecan, GAPDH, or insert-less vector. The results showed a marked suppression of perlecan transcriptional activity within 2 h of exposure to IFN-γ, and these effects were maintained for 4 h (Fig. 3A). Under the same conditions the transcriptional rates of GAPDH were slightly increased, also confirming the lack of cytotoxic effects of our assay.

To establish whether this transcriptional repression required new protein synthesis, we performed additional experiments where the levels of perlecan protein core were determined following various incubations with cycloheximide, an established inhibitor of protein synthesis that blocks RNA on polysomes (32). In these experiments we used a concentration of cycloheximide in which over 95% of protein synthesis is blocked within 1 h (30). The results showed that the IFN-γ-induced transcriptional repression was totally independent of new protein synthesis. Specifically, we observed ~80% reduction in perlecan protein core synthesis by Western slot-blot analysis following a 2-h exposure to cycloheximide, an overall inhibition comparable with that obtained in the absence of the drug (Fig. 3B). These data indicate that preexisting proteins were modified in a ligand-dependent manner to affect silencing of the perlecan gene and suggest that the differences in perlecan gene expression were not due to post-transcriptional events. Moreover, these results suggest that perlecan is an early response gene, in agreement with our previous data in human melanoma cells in which perlecan mRNA levels were rapidly induced (within 10 min) by nerve growth factor (6). Thus, perlecan levels are finely tuned by opposing negative and positive cytokines that modulate the growth state and angiogenic potential of tumorigenic cells.

The transcriptional repression was further corroborated by transient cell transfection assays utilizing perlecan promoter-CAT reporter gene constructs. The full-length perlecan promoter, a 2.5-kb segment of DNA shown before to be the functional promoter of this gene (16), and two deletion constructs comprising 1.8 and 0.46 kb were transfected into WiDr/HT29 cells. After a 48-h incubation to allow expression of the transgene, the cells were incubated in the absence or presence of IFN-γ for an additional 18 h. In several independent experiments, only the full-length promoter exhibited marked transcriptional repression by ~70% (Fig. 3C). In contrast, the other two deletion constructs were totally unresponsive. These data confirm the transcriptional silencing of the perlecan gene by utilizing a functional assay in live cells and further indicate that the distal promoter region (~660 bp) contains IFN-γ-responsive elements. Interestingly, the consensus sequence for STAT binding elements is the GAS sequence TTTNAA, which is found in IFN-γ-inducible genes (19). Notably, there are eight GAS elements within the perlecan promoter, located at −851, −931, −1539, −1553, −1789, −2265, −2261, and −2549 bp, respectively (16). Thus, at least three GAS elements in the distal promoter region, between −2.5 and −1.8 kb, could potentially mediate the IFN-γ action on the perlecan gene.

The Transcriptional Repression of Perlecan by IFN-γ Requires a Functional Stat1 Protein—Next, we wanted to dissect the potential mechanism through which IFN-γ may regulate perlecan gene transcription. To this end, we determined whether the effects of IFN-γ required a functional Stat1, a transcription factor known to be regulated by IFN-γ (19). We utilized Stat1-deficient U3A cells and the parental Stat1+/+ 2FTG7 cells. The mutant U3A cells are derived from HT-1080 fibrosarcoma cells and are defective in their response to IFN-γ because of a lack of Stat1 (22). As predicted, the U3A cells were insensitive to IFN-γ in terms of growth suppression whereas the parental cell line responded quite efficiently (Fig. 4A). As in

![Fig. 3.](http://www.jbc.org/)
FIG. 4. The IFN-γ-mediated transcriptional repression of perlecan gene expression requires a functional Stat1 protein. A, cell growth analysis of 2FTGH (Stat1+/+) and U3A (Stat1−/−) cells in the absence or presence of IFN-γ as indicated. The values are the mean of 5 determinations with S.D. < 5% of the mean (the error bars are smaller than the symbols). B, Western immunoblotting of serum-free media conditioned for either 4 or 24 h by cells treated with or without IFN-γ as indicated. The values are the mean ± S.D. of triplicate determinations. C, perlecan promoter-CAT activity in both cell types as indicated. The transient cell transfection studies were done as in the legend to Fig. 3 using the full-length promoter construct. The values are the mean ± S.D. of triplicate determinations.

...lates with growth suppression and indicate that the effects of IFN-γ on perlecan gene expression are transcriptionally controlled and require a functional Stat1 protein. Our data further suggest that a region encompassing three GAS elements located in the distal promoter of perlecan may mediate its transcriptional repression. Because perlecan is located at strategic locations, i.e., the cell surface and basement membranes, where specific interactions between lymphocytes and the growing tumor cells take place, the IFN-γ-mediated transcriptional repression of perlecan may represent an additional anti-tumoral effect of this cytokine. Thus, IFN-γ could affect tumor growth either directly or indirectly by acting on the tumor extracellular matrix via two important mechanisms: blocking collagen production (32) and repressing perlecan, a potential angiogenic stimulus. Future studies need to address how specific these IFN-γ-mediated effects are for perlecan or for other heparan sulfate proteoglycans that are also known to be angiogenic. Eventually, novel therapeutic interventions focused on targeting key modulators of tumor progression could be designed.

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