Human Malignant Mesothelioma Cell Growth: Activation of Janus Kinase 2 and Signal Transducer and Activator of Transcription 1α for Inhibition by Interferon-γ

Annie Buard, Claire Vivo, Isabelle Monnet, Christian Boutin, Yannick Pilatte, and Marie-Claude Jaurand

Institut National de la Santé et de la Recherche Médicale U139, Faculté de Médecine [A. B., C. V., M.-C. J., Y. P.], Service de Pneumologie, Centre Hospitalier Intercommunal de Créteil [I. M.], 94010 Créteil Cedex, and Hôpital de la Conception, Marseille [C. B.], France

ABSTRACT

Intraperitoneal injections of recombinant human IFN-γ have shown some efficacy in reducing tumor growth in early stages of diffuse malignant mesothelioma (DMM). Here, we have addressed the potential therapeutic effect of IFN-γ in DMM by investigating the activation of the JAK/STAT signaling pathway in seven human malignant mesothelioma cell lines (HMCLs) that were differentially responsive to the antiproliferative activity of IFN-γ. We showed that Janus kinase 2 (JAK2) and signal transducer and activator of transcription 1 (STAT1) were phosphorylated on tyrosine residues within 15 min in all the HMCLs in which IFN-γ (500 units/ml) inhibited proliferation. In addition, STAT1 binding activity to the gamma-activated sites DNA sequence was detected within 15 min in all the HMCLs in which IFN-γ (500 units/ml) inhibited DNA synthesis after 72 h of treatment. Conversely, in several HMCLs, absent or limited growth suppressive effect (less than 22% inhibition of DNA synthesis) was associated with alterations in expression or activation of JAK2 or STAT1 or, downstream, with low induction of IFN regulatory factor-1 (IRF-1) expression following IFN-γ treatment. These data suggest that at least part of the IFN-γ effect on proliferation of HMCLs is mediated directly through activation of the JAK/STAT1 signaling pathway, and it could account for the antitumoral activity reported in DMM patients treated with IFN-γ.

INTRODUCTION

DMM is a severe primary tumor of the pleura that is frequently associated with asbestos exposure, and it has poor prognosis (1, 2). Current therapies, including surgery, chemotherapy, and radiotherapy, have been unsuccessful in improving patient survival. Nevertheless, immunotherapy has emerged as an alternative therapy since inoculation of recombinant human IFN-γ in the pleural cavity of mesothelioma patients was shown to be effective in reducing progression of early stages of malignant mesothelioma (3, 4). Clinical trials with other cytokines, such as IFN-α2a and IL-2, were also reported to result in partial responses (5, 6). In addition to immunomodulatory activity, IFN-γ is thought to act directly on cell growth, as demonstrated by the antiproliferative effect that was observed in various cell types, including epithelial and hematopoietic cells (7-11). These results suggest that cytokine therapy might prove successful in some particularly drug-resistant diseases, such as DMM. New therapeutic methods are currently being developed to improve the effects of cytokines in tumors treatment. Gene therapy using vectors that encode for cytokines genes has drawn particular interest (12). However, as it was recently emphasized (13), it is very important to document the mechanisms of action of these therapeutic agents at the cellular level to understand the outcomes in clinical studies.

Although modulation of the immune response likely accounts for part of the antitumoral effects of IFN-γ in DMM (4, 14), we have demonstrated previously that IFN-γ elicited a direct antiproliferative effect in vitro in 50% of well-characterized HMCLs isolated from DMM patients (15). Further examination of the mechanisms by which IFN-γ exerts its antitumoral action in HMCLs indicated that the differential growth-suppressive effect of IFN-γ in HMCLs was independent of the activation of nitric oxide synthase and indoleamine 2,3-dioxygenase (14-16). Because the IFN-γR had been found to be expressed in all these cell lines at both the RNA and protein levels (14, 16), here, we examined the capacity of IFN-γ to activate its signaling pathway and whether molecular alterations were present downstream of the receptor in unresponsive cell lines.

Elements of the signal transduction pathway that are specific to IFN-γ have been identified previously using mutagenized cells (17-19). Briefly, binding of IFN-γ to its heterodimeric receptor induces oligomerization of the receptor subunits and causes phosphorylation of the IFN-γR α chain by two associated tyrosine kinases, JAK1 and JAK2. The activity of JAK1 and JAK2 results in recruitment and phosphorylation of the latent cytoplasmic transcription factor p91/STAT1α, which is then dimerized and translocated to the nucleus, where it binds to GAS consensus sequences that present in the promoter of IFN-γ-activated genes.

Biological models containing mutations in the IFN-γ-signaling pathway have brought into light the roles of JAK1, JAK2, and STAT1 proteins in IFN-γ-mediated effects, such as immune and antiviral responses (7, 8). For example, STAT1-deficient mice exhibited a lack of IFN-γ-induced responses, such as gene activation, induction of immunomodulatory molecules, and antimicrobial and antiviral activities (20, 21). Similarly, mutant cells defective in either JAK1 or JAK2 were found to be unresponsive to IFN-γ (18, 22). More recently, expression of a kinase-negative mutant of JAK2 resulted in a blockade of IFN-γ-inducible gene expression (23). Less is known regarding genes involved in IFN-γ-mediated regulation of cell growth. Nonetheless, recent data have supported a direct link between activation of the IFN-γ-signaling pathway and control of proliferation through activation of STAT1 (24). The mutagenized human cell line U3A, which does not express STAT1, is not growth inhibited by IFN-γ, but growth response was restored by transfection of STAT1 (24). However, no such alterations have been found naturally so far in human cells isolated from tumors.

No prior data have been previously reported with regard to the mechanisms of IFN-γ-mediated growth inhibition in HMCLs. Our in vitro data indicated that several cell lines were resistant or poorly responsive to the cytokastic effect of IFN-γ (15, 16). Therefore, we investigated whether JAK2 and STAT1 were activated in response to...
IFN-γ treatment in a series of HMCLs and whether activation of the signaling pathway correlated with differential sensitivity in response to IFN-γ-antiproliferative effect. We show that expression and/or activation of these major components of the transduction pathway were altered in HMCLs that were not growth inhibited by IFN-γ. Our findings are significant in the context of the developing clinical use of IFN-γ as immunotherapy for human malignant mesothelioma and might be relevant to other human pathologies.

MATERIALS AND METHODS

Materials. Purified recombinant human IFN-γ (specific activity, 3 × 10^7 units/mg) was a generous gift from Boehringer Ingelheim (Reims, France). Anti-STATT1 and anti-JAK2 antibodies were purchased from Santa Cruz Biotechnology (TEBU s.a., Paris, France). Antiphosphotyrosine antibody (4G10) was provided by Upstate Biotechnology, Inc. (Euromedex, Souffelweyersheim, France). Anti-IFN-γR antibody was obtained from Genzyme (Paris, France).

Human Mesothelioma Cell Lines. Specimens were obtained from seven patients with confirmed malignant mesothelioma of the pleura and were provided by the Departments of Pulmonary Medicine of Hôpital de la Conception (Marseille, France) and of Centre Hospitalier Intercommunal (Créteil, France). Mesothelioma cell lines were then established from pleural effusions or tumors, as described previously (15). The clinical data and cell line characteristics are presented in Table 1. Cell lines were routinely maintained in RPMI 1640 (Life Technologies, Inc., Cergy-Pontoise, France), supplemented with 20% fetal bovine serum (Life Technologies, Inc.), 1 μg/ml of gentamycin, 50 μg/ml streptomycin, and 50 units/ml penicillin (ATGC Biotechnologie, Noisy-Le-Grand, France) and buffered with 10 mM HEPES (ATGC). The HMCLs used here have been studied previously with regard to their immunocytochemical characteristics (14, 15, 25). Here, HMCLs (passages 6–20) were treated with 500 units/ml for 15 min, or 5 min.

Immunoprecipitations and Western Blots. Cells were plated at 2 × 10^4 cells/cm² in 75-cm² flasks, and medium was changed the following day. Two days later (50% confluence), cells were either left untreated or treated with 500 units/ml IFN-γ for 15 min or as indicated at 37°C. Cultures were washed twice with ice-cold PBS and scraped in extraction buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 50 mM sodium fluoride, 1 mM EDTA, 0.5 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate]. Concentration of NaCl in the lysates was brought to 400 mM by addition of 5 mM NaCl. After 1 h of incubation on ice, the mixture was centrifuged at 18,000 × g for 15 min at 4°C. Supernatants were collected and kept at −80°C until use. The oligonucleotide probe (5'-CTAGTATCCCTCTGAAAAGGANACATGATGATTG-3') for the GAS present in the promoter of the high-affinity Fcγ receptor gene was a generous gift from Dr. van Weyenberg (Institut National de la Santé et de la Recherche Médicale U365, Institut Curie, Paris, France). The fragment was end-labeled with [α-32P]dCTP using the Ready-To-Go DNA-labeling kit (Pharmacia Biotech) and purified on G5 Sephadex columns. Protein extracts (10–15 μg) were incubated for 30 min on ice with the radiolabeled probe (60,000 cpm) in a final volume of 16 μl of 1× binding buffer [50 mM HEPES (pH 7.9), 25 mM Tris-Cl, 2.5 mM EDTA, 1 mM NaCl, 50% glycerol, and 500 μg/ml BSA], containing 1 μg of sonicated salmon sperm DNA. Competition assays were performed by adding a 100-fold molar excess of the unlabeled oligonucleotide. Supershift experiments were performed by incubating protein extracts with 1 μg of anti-STATT1 antibody for 1 h at 4°C, prior to the addition of the radiolabeled oligonucleotide. Additional control consisted in preincubating the reaction with 1 μg of antibody unrelated to STATT1 (anti-p21^WAF1 antibody was used; Transduction Laboratory, Medgene Science, Pantin, France). The DNA-protein complexes were separated from the free probe on 5% nondenaturing polyacrylamide gels in 1× TGE buffer (50 mM Tris-Cl, 0.2 mM glycine, and 2 mM EDTA). The dried gels were exposed at −80°C.

Northern Blot Analysis. Total RNA was prepared using the standard guanidium-isothiocyanate method. Total RNA extracts prepared from HeLa cells, treated with or without IFN-γ, were kindly provided by Dr. Alcaide (Institut National de la Santé et de la Recherche Médicale U276, Institut Pasteur, Paris, France) and were used as positive controls. Twenty or 10 μg of HeLa cells extracts were resolved on a denaturing 1.2% agarose gel containing 0.4 M formaldehyde, transferred to a nylon membrane, and hybridized overnight at 65°C to [α-32P]dCTP-labeled probe (2 × 10^6 cpm/ml). cDNA probe was a 1-kb fragment of the IRF-1 gene (provided by Dr. H. Uedo, Hyogo, Japan). Probe was labeled by random priming using the Ready-To-Go labeling kit (Pharmacia Biotech). Following hybridization, filters were washed twice in 1× SSC-0.1% SDS for 10 min at room temperature and once in 0.1× SSC-0.1% SDS for 10 min at 65°C.

Thymidine Incorporation Assay. The antiproliferative effect of IFN-γ was assessed on cell lines cultured on 12-well plates. For this assay, cells were plated at 2 × 10^4 cells/cm² and grown for 24 h in complete medium. Cells were either treated with 500 units/ml IFN-γ or left untreated. After 72 h of treatment, cells were pulsed with [3H]thymidine ([3H]tdU) (3 μCi/ml; specific activity, 40–60 μCi/ml; ICN Pharmaceuticals, Inc., Orsay, France) for 60 min. Cells were washed three times with Dulbecco’s PBS (Seromed, Berlin, Germany) and incubated with 1 ml of ice-cold 10% TCA for 10 min. The TCA-precipitable fraction was solubilized in 200 μl of 0.2 M NaOH–1% SDS, and [3H]thymidine incorporation was determined with a LS6000SC scintillation counter (Beckman, Gagny, France). [3H]Thymidine incorporation in triplicates of IFN-γ-treated cultures was expressed as a percentage of incorporation in untreated control cultures.

RESULTS

Activation of JAK2 in Response to IFN-γ Treatment in HMCLs. We have reported previously that HMCLs were differentially responsive to IFN-γ antiproliferative activity (15). Because the

<table>
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<th>Tumors</th>
<th>Cell lines</th>
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<td>7</td>
<td>M</td>
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</table>

*See Ref. 4 for patient staging information.

E, epithelial; M, mixed.

Pe, pleural biopsy; Pe, pleural effusion.

—, not available.

Table 1 Patient and cell line data
lack of response to IFN-γ in HMCLs was not related to the absence of receptor (14, 16), we investigated whether differential sensitivity to the antiproliferative activity of IFN-γ was related to molecular differences in the capacity to activate the major downstream components of the IFN-γ signaling pathway. The central role played by JAK2 in the IFN-γ-signaling pathway led us to first analyze its activation in various HMCLs treated with IFN-γ.

With the exception of one cell line, FER, phosphorylation of JAK2 on tyrosine residues was detected in all of the HMCLs tested following a 15-min treatment with IFN-γ (Fig. 1, top, and data not shown). In the cell line FER, no phosphorylation was observed in response to IFN-γ treatment (Fig. 1, left), and reblotting of the same membrane with an anti-JAK2 antibody indicated that the protein could not be detected in these cells (Fig. 1, bottom). The presence of a defect in the IFN-γ signaling pathway in the cell line FER was confirmed by examining phosphorylation of IFN-γR in response to IFN-γ treatment (Fig. 2). Lysates from the cell lines MORC and FER, treated with or without 500 units/ml IFN-γ, were subjected to immunoprecipitation with an anti-IFN-γR antibody, followed by immunoblotting with an antiphosphotyrosine (Fig. 2, top). Tyrosine phosphorylation of the receptor was detected within 2 min in the cell line MORC, with a peak at 5 min (data not shown), and it was still detected after 15 min. In contrast, no modifications were observed in the cell line FER treated with IFN-γ, and it was still detected after 15 min. In other words, addition of an antibody unrelated to STAT1 had no effect on STAT1-DNA binding activity to assess whether STAT1 was functional in HMCLs. Cellular extracts were subjected to immunoprecipitation with an anti-STAT1 antibody, followed by SDS-PAGE and blotting with antiphosphotyrosine. STAT1 was activated by tyrosine phosphorylation within 15 min following addition of IFN-γ in most of the cell lines, i.e., HIB, HUO, RAV, COS, and MORC (Fig. 3 top). In contrast, tyrosine phosphorylation of STAT1 was very low in CORO and absent in FER (Fig. 3). Blots were reprobed with anti-STAT1 antibody to ascertain that comparable amounts of STAT1 were immunoprecipitated from treated and untreated cultures (Fig. 3, bottom).

Therefore, we next analyzed phosphorylation of STAT1 and IFN-γ-induced STAT1-DNA binding activity to assess whether STAT1 was functional in HMCLs. Cellular extracts were subjected to immunoprecipitation with an anti-STAT1 antibody, followed by SDS-PAGE and blotting with antiphosphotyrosine. STAT1 was activated by tyrosine phosphorylation within 15 min following addition of IFN-γ in most of the cell lines, i.e., HIB, HUO, RAV, COS, and MORC (Fig. 3, top). In contrast, tyrosine phosphorylation of STAT1 was very low in CORO and absent in FER (Fig. 3). Blots were reprobed with anti-STAT1 antibody to ascertain that comparable amounts of STAT1 were immunoprecipitated from treated and untreated cultures (Fig. 3, bottom).

To determine whether the phosphorylated STAT1 was functionally active, we examined the capacity of STAT1 to bind to the GAS consensus sequence. Detection of STAT1-DNA-binding activity in EMSA analysis after 15 min of IFN-γ treatment confirmed that STAT1 was activated in the cell lines MORC (Fig. 4A, Lanes 1–7), COS (Fig. 4B, Lanes 1–7), HIB (Fig. 4C, Lanes 1–4), HUO (Fig. 4C, Lanes 5–8), and RAV (Fig. 4C, Lanes 9–15). Although this was not readily detectable, some of the STAT1 protein was able to bind to the GAS DNA sequence in the cell line CORO treated with IFN-γ (Fig. 4B, Lane 9). In contrast, STAT1-DNA binding activity was absent in FER (Fig. 4A, Lanes 8–13). Excess unlabeled GAS oligonucleotide competed with formation of labeled GAS-STAT1 complexes, as assessed by reduced intensity of the band detected in MORC (Fig. 4A, compare Lanes 2 and 4), HIB (Fig. 4C, compare Lanes 2 and 3), HUO (Fig. 4C, compare Lanes 6 and 7), and RAV (Fig. 4C, compare Lanes 10 and 12). Specificity of the GAS DNA binding was verified by preincubating lysates with an anti-STAT1 antibody, which altered migration of GAS-protein complexes (supershift) in MORC (Fig. 4A, compare Lanes 2 and 6) and COS (Fig. 4B, compare Lanes 2 and 6). Although the anti-STAT1-induced supershift was not as clearly detected in other cell lines, including HIB (Fig. 4C, Lane 4), HUO (Fig. 4C, Lane 8), and RAV (Fig. 4C, Lane 14), intensity of the GAS-STAT1 complexes was reduced in the presence of the antibody. In contrast, addition of an antibody unrelated to STAT1 had no effect on formation and migration of the STAT1-DNA complexes, as shown in Fig. 4A (Lane 7, MORC), Fig. 4B (Lane 7, COS), and Fig. 4C (Lane 15, RAV), indicating that this DNA-binding activity was specific of...
STAT1 in HMCLs extracts. The absent or reduced DNA-binding activity that was observed upon IFN-γ treatment in the cell lines FER (Fig. 4A, Lanes 8–13) and CORO (Fig. 4B, Lanes 8–12), respectively, suggested that STAT1 was not activated or not fully activated in these HMCLs.

**IFN-γ-induced IRF-1 Gene Expression Correlates with Anti-proliferative Effect in HMCLs.** Induction of the immediate early gene *IRF-1* expression by transcription factor STAT1 has been related to some of the downstream effects of IFN-γ, including inhibition of proliferation (8, 27–29). Therefore, we further assessed that STAT1 was functional by analyzing HMCLs for *IRF-1* gene expression and cellular proliferation in response to IFN-γ treatment (Fig. 5). Effect of IFN-γ on *IRF-1* RNA level was examined in HMCLs by Northern blot analysis on total RNA isolated after 6 h of treatment (Fig. 5, A and B). In addition, because the activity of IFN-γ on cell growth is detectable after a certain delay following addition of IFN-γ, we assessed DNA synthesis in cells treated for 72 h (Fig. 5C). Fig. 5C represents results of [3H]thymidine incorporation in IFN-γ-treated cultures expressed as a percentage of incorporation in untreated control cultures. Basal level of IRF-1 RNA was extremely low (not detected) in untreated cells (Fig. 5A). Following treatment with IFN-γ, accumulation of IRF-1 RNA occurred within 6 h in the cell lines HIB, HUO, and MORC (Fig. 5A) and was associated with a marked inhibition of DNA synthesis (Fig. 5C). [3H]Thymidine incorporation was only 4.8, 27.3, and 11.7% of control in HIB, HUO, and MORC, respectively, after 72 h of treatment with 500 units/ml IFN-γ. Conversely, in the cell line FER, in which the IFN-γ signaling pathway was altered, no induction of IRF-1 RNA was observed after 6 h of treatment, and DNA synthesis was unaffected after 72 h in the presence of IFN-γ. In other HMCLs, including COS, CORO, and RAV, induction of IRF-1 RNA expression was associated with a limited effect on proliferation (Fig. 5). In these cell lines, [3H]thymidine incorporation was 77.1, 77.7, and 79.0% of control, respectively, after 72 h of treatment. These results suggested that in HMCLs, inefficient growth control might originate from various levels, i.e., either from altered activation of STAT1 upstream IRF-1 (as in CORO and FER) or from defects present downstream IRF-1 (as in COS and RAV).

**IFN-γ Induces STAT1 Protein Expression in Growth-Inhibited HMCLs.** To further examine the functional differences between these HMCLs, we next examined the IFN-γ-mediated induction of STAT1 protein expression as an additional indicator of the activation of one of the IFN-γ-responsive genes (19, 29). Immunoprecipitation assays (Fig. 3) and Western blot analysis of total cell lysates (data not shown) indicated that all of the HMCLs expressed constitutive amounts of STAT1, despite differences in expression levels. As shown in Fig. 6A for HIB, IFN-γ treatment induced accumulation of STAT1 protein, which was detectable in HMCLs as early as 9 h after addition of the cytokine. In responsive HMCLs, STAT1 protein expression was dramatically enhanced after 24 h and remained elevated (6–15-fold above basal level in untreated cells) at 72 h (Fig. 6B, left, and Table 2). In contrast, STAT1 protein levels was unchanged in the resistant cell line FER as compared to untreated cells (Fig. 6B, right). Notably, a moderate increase (less than 2.5-fold) was detected in the poorly responsive HMCLs (see Table 2), which was consistent with the limited growth inhibition measured in the corresponding thymidine assay (see Fig. 5C).

**DISCUSSION**

Use of IFN-γ in clinical trials has led to encouraging results with DMM patients (3, 4, 14). To determine whether IFN-γ might operate through a direct mechanism to reduce tumor growth in DMM patients, we have investigated the activation of the JAK/STAT signaling pathway following IFN-γ treatment of HMCLs that were differentially growth inhibited. As summarized in Table 2, we showed that the IFN-γ-signaling pathway was rapidly activated upon treatment in all the HMCLs in which IFN-γ inhibited cell proliferation, i.e., HIB, HUO, and MORC. IFN-γR activation occurred within 2 min and was followed by phosphorylation of JAK2 and STAT1 on tyrosine residues in most cell lines. Activation of the IFN-γ-signaling pathway was also demonstrated by the capacity of transcription factor STAT1 to bind to a consensus DNA sequence using EMSAs and by induction of the immediate early gene *IRF-1* RNA expression using Northern blot analysis. In these cells, DNA synthesis was inhibited by 72.7–95.2%. Conversely, in cells that were responding poorly to growth inhibition, we observed impaired activation of key components of the cellular signaling pathway, such as JAK2 and STAT1, or of other downstream transcription factors. For example, absence or low level of JAK2 in one cell line (FER) and inefficient activation of STAT1 in another (CORO) were associated with absent (FER) or limited (CORO) STAT1-DNA binding activity and IRF-1 induction, and these alterations were consistent with the lack of growth-inhibitory response. In other HMCLs, the moderate growth inhibition was associated with limited induction of STAT1 protein following IFN-γ.
Fig. 4. Analysis of GAS-binding activity of STAT1 in EMSA. Whole cell extracts were prepared from HMCLs that were left untreated (−) or treated with 500 units/ml IFN-γ (+) for 15 min. Extracts (10–15 µg) were incubated on ice for 30 min with [α-32P]dCTP end-labeled GAS oligonucleotide corresponding to the sequence present in the high affinity Fcγ receptor gene promoter, and GAS complexes were separated on 5% non-denaturing polyacrylamide gels, as described in “Materials and Methods.” GAS, competition with 100-fold molar excess of unlabeled oligonucleotide. Where indicated, cell extracts were incubated for 1 h at 4°C with either 1 µg of STAT1 antiserum (anti-STAT1) or 1 µg of antibody unrelated to STAT1 (Ab), prior to GAS-binding reaction. NS, migration of a nonspecific DNA-protein complex.
treatment, despite normal upstream activation of JAK2 and STAT1 proteins.

Our results are in agreement with previous studies which have demonstrated the need for functional JAKs and STAT in IFN-γ-mediated responses (8, 23). Mutational experiments have indicated that defects in the signal transduction pathway could result in altered response (17, 20, 22, 24, 26). More specifically, lack of STAT1 expression has been associated with impaired growth-inhibitory response in mutagenized cell lines (24, 26). We now show that alteration of expression and/or activation of JAK2 or STAT1 was associated with altered growth response to IFN-γ in tumor cell lines isolated from human mesothelioma, and these results are of particular interest in the context of mesothelioma therapy.

In one cell line, FER, no tyrosine phosphorylation was detected, and immunoblotting of JAK2 immunoprecipitates indicated that this cell line was probably deficient in JAK2. In addition, although the IFN-γR α chain was expressed in FER, it was not phosphorylated upon IFN-γ treatment. This contrasted with the rapid phosphorylation of the IFN-γR that occurred in responsive HMCLs. These observations are consistent with the sequence of events that have been described previously in various cell types treated with IFN-γ (23, 30, 31). That is, following IFN-γ binding to its receptor, the presence of a functional JAK2 is necessary for subsequent activation of JAK1-mediated phosphorylation of the IFN-γR α chain and recruitment and phosphorylation of STAT1. For example, JAK1 or JAK2 kinase-deficient cell lines displayed partial or absent response to IFN-γ treatment. Conversely, complementation with JAK2 tyrosine kinase reversed the phenotype of IFN-γ-unresponsive mutant cell lines (18). The requirement of JAK2 for phosphorylation of the receptor, as well as of JAK1 and STAT1, has also been recently demonstrated for the growth hormone signaling pathway in JAK2−/− mutant cells (32). In addition, specific inhibition of JAK2 tyrosine kinase activity by a tyrophostin derivative resulted in inhibition of DNA synthesis in lymphoblastic leukemia cell lines (33). Therefore, reduced or missing expression of JAK2 in the tumor cell line FER is likely to contribute to the interruption of IFN-γ-signaling pathway, thereby preventing IFN-γ-mediated responses. In FER, defect in JAK2 correlated with the absence of induction in class II MHC expression (16), as well as in indoleamine 2,3-dioxygenase (16) and (2′, 5′)-oligoadenylate synthetase activities (data not shown), in addition to the lack of growth-suppressive effect in response to IFN-γ treatment. Altogether, these results confirmed that the IFN-γ-signaling pathway was blocked at a proximal step in this tumor cell line and suggested that this defect was likely to participate in uncontrolled growth.

We showed that the sequence of events triggered by IFN-γ treatment was followed by inhibition of growth in responsive HMCLs. Conversely, the absence of JAK2 prevented IFN-γ-mediated growth control in the resistant cell line FER. However, in three of the HMCLs, COS, CORO, and RAV, inhibition of proliferation was moderate (21–23%, as compared to untreated cells) despite phosphorylation of JAK2 and STAT1. Although lower in the cell line CORO, the EMSA analysis ascertained that the capacity of phosphorylated STAT1 to recognize the GAS sequence was not impaired in these cells (Fig. 4). Similarly, induction of IRF-1 RNA expression by IFN-γ in all three cell lines reflected the functionality of the signaling pathway, despite differences between cell lines (Fig. 5). However, it is noteworthy that the limited IFN-γ-induced growth inhibition in the group of poorly responsive cell lines was associated with a lower increase of the STAT1 protein levels (2.5-fold or less after 72 h of treatment: see Table 2), as compared to responsive HMCLs. It has been suggested that STAT1 autoregulates the STAT1 gene (29). Thus, the moderate induction of STAT1 protein expression could be considered a good indication that STAT1 was not fully transcriptionally active in response to IFN-γ treatment in the three poorly responsive HMCLs. In this regard, there is increasing evidence that the efficacy of STAT1 transcriptional activity depends on additional phosphorylation on one or more serine residues (34–36), as well as cooperative binding of STAT1 dimers to GAS sites (37). In a recent study, expression of STAT1 protein bearing a Ser-727-to-Ala-727 mutation resulted in complete resistance to IFN-γ-antiproliferative activity (26). Similarly, it is possible that moderate growth response to IFN-γ in some HMCLs might be related to altered phosphorylation on serine residues on STAT1 or to endogenous
could affect JAK2 because of the possible involvement of this molecule in various other signaling pathways. For instance, JAK2 becomes phosphorylated in response to other members of the cytokine family, such as IL-6, IL-3, erythropoietin, granulocyte macrophage colony-stimulating factor, growth hormone, and prolactin (19, 32, 38). Similarly, expression and activity of STAT1 and IRF-1 proteins are regulated by various cytokines and growth factors (19, 38), and they are both part of the mechanisms of growth regulation (27-29). These data suggest that HMCLs with missing or inactive signaling components might also exhibit dysregulated growth control by other molecules. Conversely, it is also possible that the moderate response to IFN-γ in some HMCLs might be the result of contradictory influences of growth regulatory molecules. Indeed, it has been previously shown that HMCLs secreted and responded to numerous cytokines and growth factors that stimulate proliferation of mesothelioma cells (39, 40). Thus, it will be of interest to study further the interactions between IFN-γ and these other cytokines and growth factors for activation of STAT1 and IRF-1 in our series of HMCLs. It remains to be determined which factor(s) are responsible for the differences in the activation of STAT1 and IRF-1 and the resulting altered growth response in HMCLs. These observations will be of particular interest with regard to development of new therapeutic strategies for DMM.

To our knowledge, this is the first study that examines activation of JAK2 and STAT1 in response to IFN-γ treatment in human tumor-derived cell lines. In summary, we showed that activation of the IFN-γ-signaling pathway was connected to growth inhibition in mesothelioma tumor cells and, conversely, that the lack of responsiveness to IFN-γ-antiproliferative effect was related to alterations in the signaling pathway. Furthermore, exploration of this pathway tends to suggest that resistance of HMCLs to antiproliferative effects of IFN-γ is not commonly related to intrinsic molecular defects of signaling components (with the exception of one cell line) but rather to modulation of STAT1 activation by a mechanism that remains to be clarified. Taken together, our data provide further evidence that efficient activation of both JAK2 and STAT1 is required to achieve optimal IFN-γ-antiproliferative effect in HMCLs. The consistent IFN-γ-mediated modifications we observed in HMCLs with regard to IRF-1 gene expression, STAT1 protein levels, and DNA synthesis suggested that these effects are directly coupled to the activation of the JAK/STAT pathway in these cells. Hence, our data also strongly support the idea that IFN-γ acts directly on mesothelioma cell growth and, thereby, substantiate the use of IFN-γ for mesothelioma therapy. Further characterization of both the responsive and unresponsive HMCLs will be of interest to elucidate the mechanisms of action of IFN-γ in DMM, and it is predictable that enhancing IFN-γ sensitivity in

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**Table 2** Summary of IFN-γ-mediated activation of the JAK/STAT signaling pathway in HMCLs

<table>
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<tr>
<th>Cell line</th>
<th>IFN-γ-induced phosphorylation</th>
<th>STAT1 activity (GAS binding)</th>
<th>IRF-1 RNA expression (RNA increase)</th>
<th>STAT1 protein expression (fold induction)</th>
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<td>2.5</td>
</tr>
<tr>
<td>RAV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.6</td>
</tr>
<tr>
<td>FER</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Phosphorylation was detected by immunoprecipitation with antibodies to either anti-JAK2 or anti-STAT1, followed by Western blot analysis with an antiphosphotyrosine antibody.

* STAT1 activity was assessed by EMSA analysis using a [32P]dCTP-labeled oligonucleotide corresponding to the GAS sequence in the high-affinity Fcγ receptor gene promoter.

* IRF-1 gene expression was determined by Northern blot analysis in HMCLs treated for 6 h with IFN-γ.

* Accumulation of STAT1 protein in HMCLs treated for 72 h with IFN-γ was analyzed by Western blotting on whole cell lysates with an antibody to STAT1. Results are expressed as fold induction over expression in untreated cells, as determined by densitometry scanning.

* ND, not determined.
the poorly responsive HMCLs will be beneficial to clinical treat-

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